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MicroRNAs in Cancer: Small Molecules With a Huge Impact

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ABSTRACT

Every cellular process is likely to be regulated by microRNAs, and an aberrant microRNA expression signature is a hallmark of several diseases, including cancer. MicroRNA expression profiling has indeed provided evidence of the association of these tiny molecules with tumor development and progression. An increasing number of studies have then demonstrated that microRNAs can function as potential oncogenes or oncosuppressor genes, depending on the cellular context and on the target genes they regulate. Here we review our current knowledge about the involvement of microRNAs in cancer and their potential as diagnostic, prognostic, and therapeutic tools.

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INTRODUCTION

After the initial discovery in 1993, when a small RNA encoded by the lin-4 locus was associated to the developmental timing of the nematode *Caenorhabditis elegans* by modulating the protein lin-14,[1] microRNAs have undergone a long period of silence. It took indeed several more years to realize that these small (19 to 22 nucleotides [nts]) RNA molecules are actually expressed in several organisms, including *Homo sapiens*, are highly conserved across different species, are highly specific for tissue and developmental stage, and play crucial functions in the regulation of important processes, such as development, proliferation, differentiation, apoptosis, and stress response. In the last few years, microRNAs have indeed took their place in the complex circuitry of cell biology, revealing a key role as regulators of gene expression.

MicroRNA genes represent approximately 1% of the genome of different species, and each of them has hundreds of different conserved or nonconserved targets: it has been estimated that approximately 30% of the genes are regulated by at least one microRNA.[2]

MicroRNAs are transcribed for the most part by RNA polymerase II as long primary transcripts characterized by hairpin structures (pri-microRNAs) and processed into the nucleus by RNAse III Drosha into 70- to 100-nts long premicroRNAs. These precursor molecules are exported by an Exportin 5-mediated mechanism to the cytoplasm, where an additional step mediated by the RNAse III Dicer generates a dsRNA of approximately 22 nts, named miR/miR*. The mature single-stranded microRNA product is then incorporated in the complex known as microRNA-containing ribonucleoprotein complex (miRNP), miRgonaute, or microRNA-containing RNA-induced silencing complex (miRISC), whereas the other strand is likely subjected to degradation. In this complex, the mature microRNA is able to regulate gene expression at the post-transcriptional level, binding through partial complementarity for the most part to the 3′UTR of target mRNAs, and leading at the same time to some degree of mRNA degradation and translation inhibition (Fig 1).

Our laboratory was involved in an attempt to identify tumor suppressors at chromosome 13q14, involved in the pathogenesis of chronic lymphocytic leukemia (CLL), the most common human leukemia in the Western world. Deletions of chromosome 13 at band q14 are detected by cytogenetic studies in approximatively 50% of CLLs, whereas loss of heterozygosity studies indicate deletions at 13q14 in approximatively 70% of CLLs. By taking advantage of chromosome translocations and small deletions, we found, however, that the critical region of 13q14 does not contain a protein-coding tumor suppressor gene, but rather two microRNA genes, *miR-15a* and *miR-16-1*, that are expressed in the same polycistronic RNA. This result indicated that the deletion of chromosome 13q14 caused the loss of these two microRNAs, representing the first evidence that microRNAs could be involved in the pathogenesis of human cancer.[3] Study of a large collection of CLLs showed knockdown or knockout of *miR-15a* and *miR-16-1* in approximatively 69%

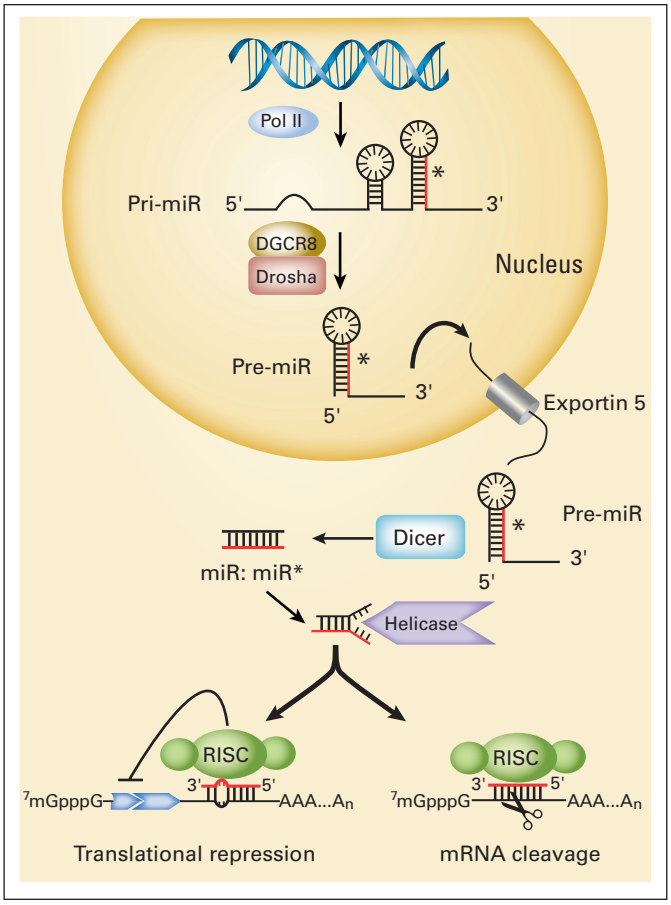

Fig 1. Biogenesis, processing, and maturation of microRNAs (miRs). miRs are transcribed mainly by RNA polymerase II as long primary transcripts characterized by hairpin structures (pri-miRs) and processed in the nucleus by RNAse III Drosha in a 70-nucleotide-long pre-miR. This precursor molecule is exported by the Exportin 5 to the cytoplasm, where RNAse III Dicer generates a dsRNA of approximately 22 nucleotides, named miR/miR*. The mature miR product is then incorporated in the complex known as miRISC, whereas the other strand is likely subjected to degradation. As part of this complex, the mature miR is able to regulate gene expression binding through partial homology the 3′UTR of target mRNAs and leading to mRNA degradation in case of perfect matching or translation inhibition. RISC, RNA-induced silencing complex.

of CLLs. Because such alteration is present in most indolent CLLs, we speculated that loss of miR-15a and miR-16-1 could be the initiating event or a very early event in the pathogenesis of the indolent form of this disease.[3]

Immediately after these initial observations, we mapped all the known microRNA genes and found that many of them are located in regions of the genome involved in chromosomal alterations, such as deletion or amplification, in many different human tumors, in which the presumed tumor suppressor genes or oncogenes, respectively, failed to be discovered after many years of investigation.[4]

Here we will show that alterations in microRNA expression are not isolated, but the rule, in human cancer. After these early studies indicating the role of microRNA genes in the pathogenesis of human cancer, we and others have developed platforms to assess the global expression of microRNA genes in normal and diseased tissues and have carried out profiling studies to assess microRNA dysregulation in human cancer. This was an attempt to establish whether microRNA profiling could be used for tumor classification, diagnosis, and prognosis.

MicroRNAs PROFILING IN CANCER DIAGNOSIS AND PROGNOSIS

Profiling of different cell types and tissues indicated that the pattern of expression of microRNAs is cell type and tissue specific, suggesting that the program of expression of microRNAs is exquisitely cell-type dependent and tightly associated with cell differentiation and development. MicroRNAs aberrantly expressed in tumors are listed in Table 1.

Leukemia/Lymphoma

CLL. As mentioned, the first evidence of alterations of microRNA genes in human cancer came from studies of CLL. In a large study of indolent versus aggressive CLL, Calin et al[6] discovered a signature of 13 microRNAs capable of distinguishing between indolent and aggressive CLL. Interestingly, it was found that miR-155, overexpressed in different lymphomas, including the activated B-cell–like type of diffuse large B-cell lymphoma, is also upregulated in aggressive CLLs, whereas members of the miR-29 family and miR-181 were found to be underexpressed and later demonstrated to directly regulate the TCL1 oncogene, which is overexpressed in the aggressive form of CLL.[5] Because of the "wait and watch" approach to the treatment of CLL, a signature able to distinguish between CLL with good and bad prognosis was also found. Sequencing of many microRNAs, including those in the signature, allowed the identification of germline and somatic mutations of microRNA genes, including *miR-15*, *miR-16-1*, and *miR-29* family members. Interestingly, mutations in the miR-15/16 precursor were also identified, affecting the processing of the pri-miR into the pre-miR. In two cases, the mutant was in homozygosity in the leukemic cells, whereas normal cells of the two patients were heterozygous for this abnormality, indicating a loss of the normal miR-15/16 allele in the leukemic cell.[6] Thus miR-15a and miR-16-1 behave like typical tumor suppressors in CLL. Interestingly, Raveche et al[36] have mapped a gene responsible for an indolent form of CLL in the New Zealand Black mouse strain on chromosome 14, in a region homologous to 13q14 in humans. Sequence analysis of this region showed a mutation in the precursor of miR-15/16 in the New Zealand Black mouse strain 6 nts 3′ to miR-16-1 (in the human cases, the mutation was 7 nts 3′ to miR-16-1), that also affected the processing of the miR-15/16 precursor. Thus germline mutation of miR-15/16 can cause the indolent form of CLL both in human and mouse. By using different algorithms to identify targets of miR-15a and miR-16-1, it was found that *BCL2*, an oncogene protecting cells from apoptosis, was a putative target of both miR-15a and miR-16-1. Knockdown experiments showed this to be the case.[7] Thus loss of miR-15a and miR-16-1 leads to high constitutive level of the oncogene *BCL2*, contributing to the development of an indolent B-cell leukemia. In follicular lymphoma, another common indolent B-cell malignancy, the *BCL2* gene becomes dysregulated as result of a t(14;18) chromosome translocation, because of its juxtaposition to immunoglobulin enhancers, indicating that constitutive overexpression of *BCL2* causes an indolent B-cell tumor. More recently, it was also found that loss of miR-15a and miR-16-1 causes, although indirectly, overexpression of *MCL1*,

Table 1. MicroRNAs Aberrantly Expressed in Tumors

Tumor Type	Upregulated MicroRNA	Downregulated MicroRNA	Target
CLL[5-7]		miR-29, miR-181	*TCL1*
	miR-155		
		miR-15a, miR-16-1	*BCL2*
AML[8]		miR-29	*MCL1*
			DNMT
Lymphoma[9-15]	miR-155		
	miR-17-92		*PTEN, BIM,E2F1*
	miR-106b-25		*E2F1*
MM[16,17]	miR-21		
	miR-19a, miR-19b		*SOCS1*
Breast cancer[18-27]	miR-21		*PTEN, PDCD4, TPM1*
		miR-125b	*HER2, HER3*
		miR-205	*HER3*
		miR-10b (associated with metastasis)	*HOXD10*
	miR-373		
		miR-200	*ZEB*
Lung cancer[28-32]		let-7	*RAS, HMGA2, C-MYC*
	miR-155		
HCC[28,34,35]		miR-122a	*Cyclin G1*
	miR-221		*p27*
		miR-34a	*MET*

Abbreviations: CLL, chronic lymphocytic leukemia; AML, acute myelocytic leukemia; MM, multiple myeloma; HCC, hepatocellular carcinoma.

another oncogene of the *BCL2* family of inhibitors of apoptosis.[37] Interestingly, a recent clinical trial of patients with CLL with ABT737, an inhibitor of *BCL2* developed by Abbott Laboratories (Abbott Park, IL), showed partial resistance of the leukemic cells to the drug, because ABT737 is specific for *BCL2* but not for *MCL1*. Thus treatment with either miR-15a or miR-16-1 may abrogate resistance to the drug and improve responsiveness. Additional experiments in vitro and in vivo also showed that miR-15a or miR-16-1 can be exploited to cause death of leukemic cells, suggesting the possibility of a microRNA-based therapeutic intervention.[37]

Acute myelocytic leukemia. Acute myelocytic leukemia (AML) is a heterogeneous disease that includes several entities with different genetic abnormalities and clinical features. Garzon et al[38] have reported unique microRNA profiles in the main molecular and cytogenetic subgroups of AML. In addition, a subset of these microRNAs was associated with overall and disease-free survival. The members of the miR-29 family are located in two clusters on two human chromosomes: *miR-29b1/29a* are located on chromosome 7q32, whereas *miR-29b2/c* are located on chromosome 1q23. Importantly, chromosome 7q is the region frequently deleted in myelodysplastic syndrome and therapy-related AML.[39] Members of the miR-29 family have been shown to be downregulated in aggressive CLL,[6] invasive breast cancer,[18] lung cancer,[40] and cholangiocarcinoma.[8] Transfection of miR-29b induces apoptosis in cholangiocarcinoma cell lines and reduces the tumorigenicity of lung cancer cells in nude mice. Very recently, it was shown that rhabdomyosarcoma loses miR-29 expression because of an elevation of NFkB and YY1 levels, and introduction of miR-29s into the tumor delays rhabdomyosarcoma progression in mice.[41] MiR-29s were also found to directly target *MCL1*,[8] an oncogene overexpressed in AMLs, and the de novo DNA methyltransferases DNMT-3A and -3B, although indirectly, through regulation of the transactivator Sp1, the maintenance DNA methyl transferase DNMT1.[40,42] Thus loss of miR-29 family members results in the constitutive overexpression of *MCL1* and of *DNMT*, causing epigenetic changes characteristic of AML. These recent results suggest that loss of miR-29s may be important, perhaps critical, for the pathogenesis of a major group of myelodysplastic syndromes and AMLs (Fig 2).

Lymphoma. Early studies have shown that miR-155 is upregulated in a subgroup of Burkitt's lymphoma, diffuse large B-cell lymphoma, primary mediastinal B-cell lymphoma, and Hodgkin's lymphoma.[9,10] This microRNA is encoded by the terminal portion of the *BIC* (B-cell integration cluster) gene, which was originally identified as a common retroviral integration site in avian leukosis virus–induced B-cell lymphomas.[43] Our group demonstrated that mice overexpressing miR-155 in B lymphocytes develop polyclonal preleukemic pre–B-cell proliferation followed by full-blown B-cell malignancy.[11] More recently, two knockout mice models have demonstrated a critical role of miR-155 in immunity by showing that *BIC*/miR-155−/− have defective dendritic cell functions, impaired cytokine secretion, and T_H cells intrinsically biased toward T_H2 differentiation.[44,45] Moreover, miR-155 could represent the connection between inflammation, immunity, and cancer, because its expression can be induced by mediators of inflammation and is involved in response to endotoxic shock.[46]

He et al[12] reported that miR-17-92 polycistron was upregulated in 65% of patients with B-cell lymphoma and demonstrated in a mouse model that this microRNA cluster cooperates with the oncogene *MYC* in accelerating tumor development. More recently, a different group observed that the overexpression of miR-17-92 in lymphocytes caused a lymphoproliferative disease, autoimmunity, and premature death.[13] The enhanced proliferation of the transgenic lymphocytes was mediated by direct regulation of pro-apoptotic phosphatase and tensin homolog gene (*PTEN*) and Bim. O'Donnell et

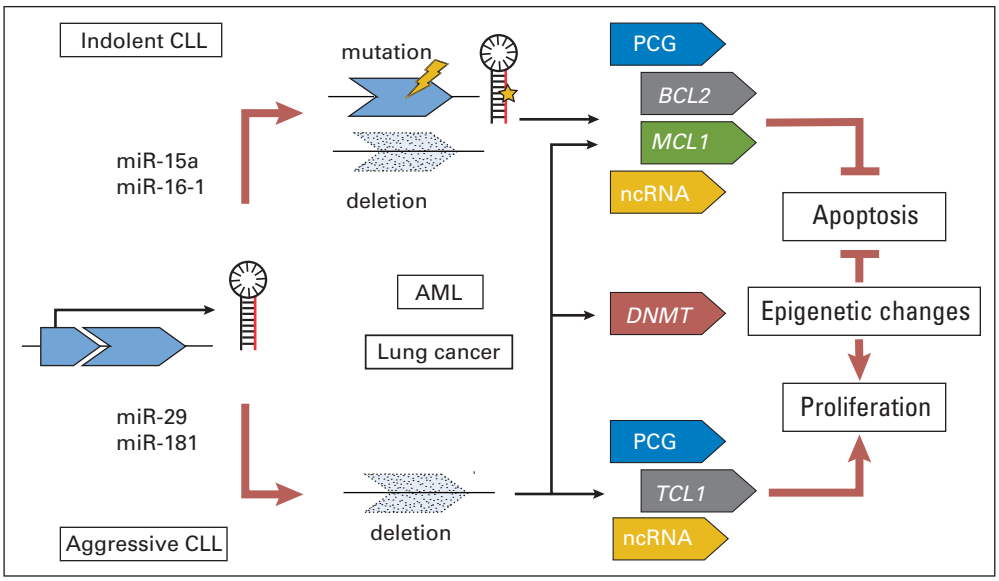

Fig 2. Molecular alterations in chronic lymphocytic leukemia (CLL) and acute myelocytic leukemia (AML). Deletion or downregulation of microRNA (miR)-15a/miR-16-1 cluster, located at chromosome 13q14.3 and directly involved in the regulation of *BCL2* and *MCL1* expression, represent an early event in the pathogenesis of CLL. During the evolution of malignant clones, other micro RNAs (miRs) can be deleted (such as miR-29) or overexpressed (such as miR-155), contributing to the aggressiveness of B-cell CLL. Such abnormalities can influence the expression of other protein-coding genes (PCGs), as *TCL1* oncogene, directly regulated by miR-29 and miR-181, or affect other noncoding RNAs (ncRNAs). The consequences of this steady accumulation of abnormalities are represented by the reduction of apoptosis and the induction of survival and proliferation of malignant B cells, leading to the evolution of more aggressive clones. Members of the miR-29 family, lost in AML and in other tumor types as lung cancer, have also been shown to directly target *MCL1* and *DNMT3A* and *B*.

al[14] investigated the regulation of miR-17-92 in lymphoma, demonstrating that the expression of this cluster is directly activated by the oncogene *c-Myc*. Moreover, miR-17-92 cluster, as well as its paralog, miR-106b-25,[15] establish with the transcription factor E2F1, a downstream target of *c-Myc*, a negative feedback loop: E2F1 indeed represents a direct target of the two microRNA clusters, but it also induces their expression. Thus *MYC* simultaneously activates E2F1 transcription and limits its expression, allowing a tightly controlled proliferative signal.

Multiple myeloma. Few recent evidences have linked microRNAs to this plasma cell malignancy, as the aberrant expression of miR-335, miR-342-3p, and miR-561 in comparison with normal plasma cells[47] or the Stat3-mediated activation of the oncogenic miR-21 in response to interleukin-6.[16] More recently, Pichiorri et al[17] described a microRNA signature characteristic of this neoplasia. They evaluated by both microarray analysis and real-time polymerase chain reaction the expression of microRNAs in multiple myeloma (MM) –derived cell lines, $CD138^+$ bone marrow peripheral cells from subjects with MM or monoclonal gammopathy of undetermined significance, and normal donors, identifying the oncogenic miR-21 and miR-181 among the microRNAs aberrantly expressed. Two miRNAs, miR-19a and 19b, part of the miR-17-92 cluster, were also shown to downregulate expression of *SOCS-1*, a gene frequently silenced in MM that plays a critical role in inhibiting interleukin-6 growth signaling. Moreover, xenograft studies using human MM cell lines treated with miR-19a and b precursors or miR-181a and b antagonists resulted in significant suppression of tumor growth in nude mice, confirming the involvement of these microRNAs in the development of MM and supporting the idea of a possible use of these molecules as a therapeutic tool.

MicroRNAs in Solid Malignancies

Breast cancer. One of the first solid tumors to be profiled for microRNA expression was, in 2005, breast cancer. Iorio et al[18] described the first microRNA signature characteristic of breast carcinoma, identifying 13 microRNAs able to discriminate tumors and normal tissues with an accuracy of 100%. Among the most significant microRNAs differentially expressed, some were extensively studied since their initial discovery and revealed an important role on the biology of breast cancer: miR-21, overexpressed in breast carcinoma, has been demonstrated to mediate cell survival and proliferation directly targeting the oncosuppressor genes *PTEN*, *PDCD4*, and *TPM1*, and it has been associated with advanced clinical stage, lymph node metastasis, and poor patient prognosis.[19,20] This microRNA, one of the first cancer microRNAs described, has been found overexpressed in a variety of other malignancies: glioblastoma,[48,49] ovary,[50] lung,[28,51] and more.[52] In colorectal cancer and pancreas endocrine and exocrine tumor, miR-21 overexpression is also associated with poor survival and poor therapeutic outcome.[53-55]

Conversely, downregulated microRNAs, as miR-125b and miR-205, regulate oncogenes as tyrosine kinase receptors HER-2 and HER-3, respectively.[21,22] Ectopic expression of miR-205 in a breast cancer cell line decreases proliferation and improves the responsiveness to tyrosine kinase inhibitors as gefitinib or lapatinib.[22] MicroRNA expression is also related to some histopathologic features of breast carcinoma, such as estrogen receptor (ER) and progesterone receptor expression, grade and stage, and presence of invasion.[18] Further studies have investigated the correlation between microRNA expression and the classification in different subtypes of breast cancer.[56] Very recently, few groups have reported experimental evidence supporting the correlation between microRNAs and ER status: miR-206 directly targets ERα, and miR-221 and -222 confer tamoxifen resistance regulating p27[57] and ERα.[58] Unpublished data from our laboratories [58a] describe a regulatory loop between ERα and miR-221 and miR-222: the two microRNAs are able to directly target ERα receptor, which in turn negatively regulates their expression, binding estrogen-responsive elements on their promoter region.

Lung cancer. Let-7, tumor suppressor microRNA initially discovered in *C. elegans*, where it induces cell cycle exit and terminal differentiation, has been described as a new regulator of self-renewal and tumorigenicity of breast cancer cells,[59] targeting molecules originally described in lung cancer: RAS[29] as well as the oncogene *HMGA2*,[29,30] and even *MYC* itself.[31] Overexpression of let-7 microRNA family can suppress tumor development in mouse models of

breast and lung cancer.[59,60] In the two most common forms of non–small-cell lung cancers (NSCLCs; adenocarcinomas and squamous cell carcinomas), high expression of miR-155 and low expression of oncosuppressor let-7 are correlated with poor prognosis.[28] The association of let-7a with survival was also confirmed by an independent study performed by Yu et al,[32] who identified an microRNA signature as an independent predictor of cancer relapse and survival of patients with NSCLC.

As in other tumor types, also in lung cancer microRNAs can represent accurate diagnostic markers: very recently, it has been described that squamous and nonsquamous NSCLCs can be distinguished according to the expression of miR-205.[61]

Hepatocellular carcinoma. In hepatocellular carcinoma (HCC), Murakami et al[62] reported that miR-222, miR-106a, and miR-17-92 clusters are associated with the degree of tumor differentiation, whereas high levels of the oncosuppressor miR-125b are correlated with good survival.[63] MiR-125b has also been shown to induce growth inhibition in vitro in a model of human thyroid anaplastic carcinoma.[64] Other studies focused on the identification of molecules targeted by microRNAs deregulated in HCC: miR-122a, downmodulated in HCC, directly regulates Cyclin G1,[33] and miR-221, upregulated in this neoplasia, directly targets p27,[34] as also shown in thyroid cancer,[64] glioblastoma,[65] prostate cancer,[66] and melanoma.[67] One of the first evidences proving miR alteration in human melanoma is a genomic study performed by Zhang et al,[68] who reported DNA copy abnormalities in microRNA genes also in two other epithelial tumors, breast and ovary. Interestingly, the results obtained by this genomic analysis were largely overlapping with the expression profiles on the same tumor types.[18,50]

MicroRNAs IN INVASION, ANGIOGENESIS, AND METASTASIS

MicroRNAs have been implicated not only in the development of primary tumors, but also in affecting progression and the metastatic phase of the disease. Indeed, several evidences show how microRNAs are involved in the regulation of biologic processes leading to the acquisition of metastatic potential, as adhesion, migration and invasion, and angiogenesis.

One of the first studies reporting a prometastatic role for a microRNA was published by Ma et al.[24] They observed that miR-10b was downmodulated in all the breast carcinomas from metastasis-free patients, as previously reported,[18] but surprisingly, 50% of metastasis-positive patients had elevated miR-10b levels in their primary tumors. Induced by transcription factor Twist, miR-10b inhibits the translation of mRNA encoding homeobox D10 (HOXD10), releasing the expression of the prometastatic gene *RHOC* and thus leading to tumor cell invasion and metastasis.

Through a functional screen aimed to discover microRNAs promoting cell migration in vitro, Huang et al[25] identified miR-373 and validated its metastatic potential in tumor transplantation experiments using breast cancer cells.

MiR-34a, lost in several tumor types and involved in the network mediated by the well-known "genome guardian" p53,[35] inhibits migration and invasion by downregulation of *MET* expression in human HCC cells.[69]

Being the epithelial-mesenchymal transition (EMT) thought to promote malignant tumor progression, several groups have recently investigated whether microRNAs are involved in this process, and a number of evidences support this hypothesis. Indeed, members of the miR-200 family of microRNAs and miR-205 have been shown to reduce cell migration and invasiveness targeting ZEB transcription factors, known inducers of EMT,[26,27] and PKCε, as recently demonstrated in prostate cancer.[70] The oncogenic miR-21 stimulates invasion, extravasation, and metastasis in different tumor types, included colorectal cancer[71] and breast cancer,[72] whereas oncosuppressor miR-205 has opposite effects, reducing invasion in vitro and suppressing lung metastasis in vivo.[23] With the same aim of searching for regulators of breast cancer metastasis, Tavazoie et al[73] identified miR-126 and miR-335 as metastasis suppressors: reduced levels of the two microRNAs are associated with poor metastasis-free survival of patients with breast cancer, whereas their re-expression inhibits metastasis in a cell transplantation model.

Interestingly, it has been recently observed that primary tumors and metastasis from the same tissue show a similar pattern of microRNA expression.[74] Being a more accurate classifier than mRNA expression studies, microRNA profiling has thus revealed the potential to solve one of the most demanding issues in cancer diagnostics: the origin of metastasis of unknown primary tumors.

In the metastatic process, neoangiogenesis is the crucial step allowing cells to reach and disseminate through the systemic circulation. microRNAs can also control tumor progression at this level, either promoting or inhibiting the proliferation of endothelial cells. miR-221 and miR-222 repress proliferative and angiogenic properties of c-Kit in endothelial cells,[75] whereas hypoxic reduction of miR-16, miR-15b, miR-20a, and miR-20b, directly targeting vascular endothelial growth factor, supports the angiogenic process.[76] On the other hand, vascular endothelial growth factor levels can be indirectly increased by miR-27b, through reduction of the zinc finger protein ZBTB10 and the consequent activation of Sp transcription factor,[77] and by miR-126, through repression of Sprouty-related protein Spred-1 and phosphoinositol 3-kinase regulatory subunit 2.[78] Angiogenesis can be also promoted by miR-210, activated by hypoxia and directly repressing endothelial ligand ephrin A3,[79] and by miR-17-92 cluster, which sustains *MYC* angiogenic properties through repression of connective tissue growth factor and the antiangiogenic adhesive glycoprotein thrombospondin 1,[80] also targeted by miR-27b and let-7f.[81]

MicroRNA AND EPIGENETICS

MiRNA expression can be altered by several mechanisms in human cancer (Fig 3): chromosomal abnormalities, as suggested by the evidence that microRNAs are frequently located in regions of the genome involved in alterations in cancer,[4] and recently confirmed by a genetic study in ovarian carcinoma, breast cancer, and melanoma[68]; 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[36]; and polymorphisms (SNPs), as described in lung cancer.[82] The deregulated microRNA expression in cancer can also be due to defects in the microRNA biogenesis machinery, as supported by the changes in microRNA levels consequent to altered Drosha or Dicer activity in different tumor types,[83-86] and epigenetic changes, as altered DNA methylation. An extensive analysis of genomic sequences of miRNA genes have

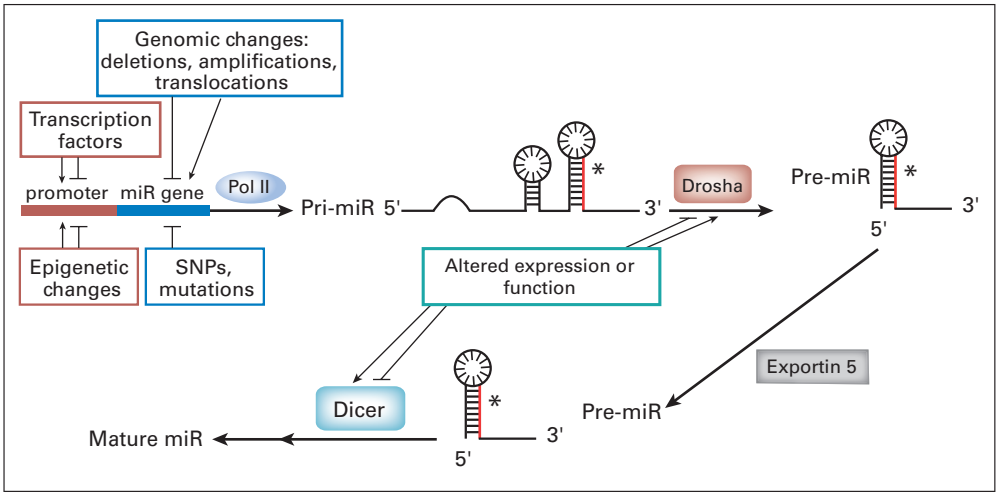

Fig 3. Mechanisms of microRNA (miR) regulation. The deregulated microRNA expression observed in cancer can be caused by chromosomal abnormalities, mutations, polymorphisms (SNPs), transcriptional deregulation, defects in the microRNA biogenesis machinery, and epigenetic changes.

shown that approximately half of them are associated with CpG islands, suggesting that they could be subjected to this mechanism of regulation.[87] Several evidences have indeed proved that an altered methylation status can be responsible for the deregulated expression of microRNAs in cancer, as the silencing of putative tumor suppressor microRNAs: treating T24 bladder cancer cells and human fibroblasts with DNMT inhibitor 5-Aza-2′-deoxycytidine, Saito et al[88] observed a strong upregulation of miR-127, microRNA characterized by a CpG island promoter, able to target the proto-oncogene *BCL-6*, and silenced in several cancer cells. With the same approach of unmasking epigenetically silenced microRNAs inducing chromatin-remodeling by drug treatment, it has been demonstrated that miR-9-1 is hypermethylated and consequently downmodulated in breast cancer,[89] as well as the clustered miR-34b and miR-34c in colon cancer.[90]

Conversely, the upmodulation of putative oncogenic microRNAs in cancer can be due to DNA hypomethylation, as shown in lung adenocarcinoma for let-7a-3[91] or in epithelial ovarian cancer for miR-21.[50]

A different approach to identifying epigenetically regulated microRNAs was represented by the microRNA profiling of DNMT1- and DNMT3b-deficient colorectal cancer cells: among the 18 microRNAs upmodulated in comparison with wild-type cells, the only one resulting unmethylated in normal tissue but hypermethylated, and thus silenced, in tumor was miR-124a, embedded in a large CpG island and able to target cyclin D kinase 6, which mediates the phosphorylation of the RB tumor suppressor gene.[92]

Methylation is not the only epigenetic mechanism that can affect microRNA expression: Scott et al[93] showed that in SKBR3 breast carcinoma cells, histone deacetylase inhibition is followed by the extensive and rapid alteration of microRNA levels.

The existence of epigenetic drugs, such as DNA demethylating agents and histone deacetylase inhibitors, able to reverse

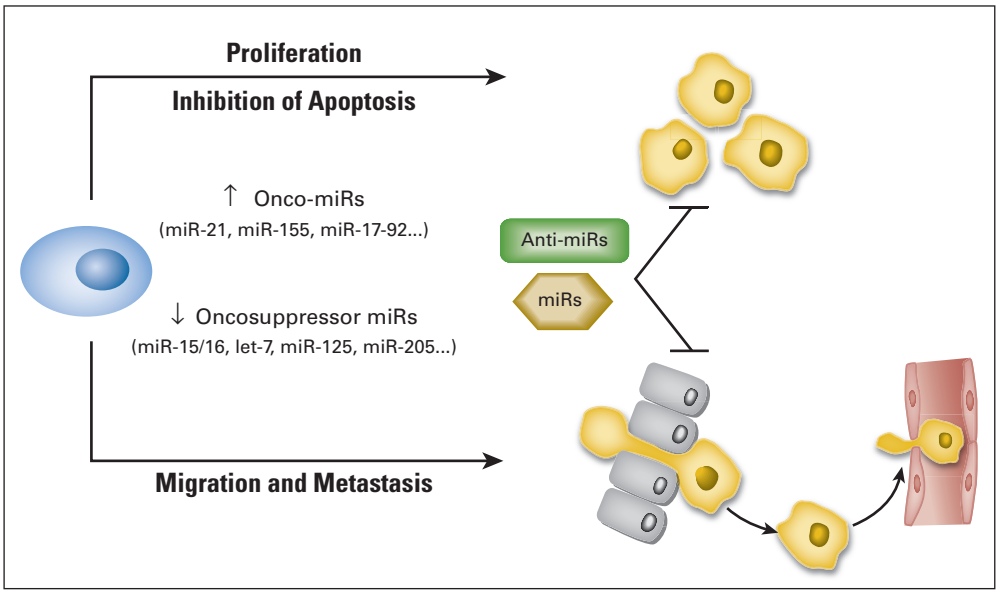

Fig 4. MicroRNAs (miRs) as therapeutic tools. The reintroduction by transfection of synthetic miRs lost during cancer development or progression or the inhibition of oncogenic miRs by using anti-miR oligonucleotides could contribute to counteract tumor proliferation, extended survival, and the acquisition of a metastatic potential, thus representing potential therapeutic tools.

an aberrant methylation or acetylation status, raises the intriguing possibility of regulating microRNA levels, for example, to restore the expression of tumor suppressor microRNAs, thus reverting a tumoral phenotype.

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: the miR-29 family directly targets the de novo DNA methyltransferases DNMT-3A and -3B, although 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.[40,42] Loss of miR-290 cluster in Dicer-deficient mouse embryonic stem cells leads to the downregulation of DNMT3a, DNMT3b, and DNMT1 through upmodulation of their repressor, RBL-2, proven target of miR-290[94,95]; miR-1, involved in myogenesis and related diseases, directly targets HDAC4.[96]

MicroRNA/Anti-microRNAs IN CANCER TREATMENT

The evidences collected to date demonstrate how microRNAs could represent valid diagnostic, prognostic, and predictive markers in cancer. Indeed, the aberrant microRNA expression is correlated with specific biopathologic features, disease outcome, and response to specific therapies in different tumor types.

Considering the importance of microRNAs in development, progression, and treatment of cancer, the potential usefulness of a microRNA-based therapy in cancer is now being exploited, with the attempt to modulate their expression, reintroducing micro RNAs lost in cancer, or inhibiting oncogenic microRNAs by using anti-microRNA oligonucleotides (Fig 4). For example, transfection of miR-15a/16-1 induces apoptosis in leukemic MEG01 cells and inhibits tumor growth in vivo in a xenograft model,[37] whereas the inhibition of miR-21 with antisense oligonucleotides generates a proapoptotic and antiproliferative response in vitro in different cellular models and reduces tumor development and metastatic potential in vivo.[97]

Moreover, microRNAs involved in specific networks, as the apoptotic, proliferation, or receptor-driven pathways, could likely influence the response to targeted therapies or to chemotherapy: inhibition of miR-21 and miR-200b enhances sensitivity to gemcitabine in cholangiocytes, probably by modulation of *CLOCK*, *PTEN*, and *PTPN12*,[98] whereas reintroduction of miR-205 in breast cancer cells can improve the responsiveness to tyrosine kinase inhibitors through HER-3 silencing.[22]

Beside targeted therapies and chemotherapy, microRNAs could also alter the sensitivity to radiotherapy, as recently reported by Slack et al[99]: in lung cancer cells, the let-7 family of microRNAs can suppress the resistance to anticancer radiation therapy, probably through RAS regulation.

Evidence described to date represents the experimental bases for the use of microRNAs as both targets and tools in anticancer therapy, but there are at least two primary issues to address to translate these fundamental research advances into medical practice: the development of engineered animal models to study cancer-associated microRNAs and the improvement of the efficiency of miRNAs/anti-microRNAs delivery in vivo. To this aim, modified microRNA molecules with longer half-lives and efficiency have been developed, such as anti-microRNA oligonucleotides,[100] locked nucleic acid–modified oligonucleotides,[101] and cholesterol-conjugated antagomirs.[102] Interestingly, Ebert et al[103] have recently described a new approach to inhibit microRNAs function: synthetic mRNAs containing multiple binding site for a specific microRNA, called microRNA sponges, are able to bind up the microRNA, preventing its association with endogenous targets.

To improve the in vivo delivery of either microRNAs or anti-microRNAs, the methods that have been tested in preclinical studies over the last decades for short-interfering RNAs (siRNA) or short heteroduplex RNA (shRNA)[104] could be applied also to micro RNAs. Moreover, the advantage of microRNAs over siRNA/shRNA is their ability to affect multiple targets with a single hit, thus regulating a whole network of interacting molecules.

In conclusion, 15 years ago, when microRNAs seemed just a peculiar discovery in *C elegans*, the scientific world probably did not even imagine that those small noncoding molecules would have a large impact on our understanding of cellular biology and gene regulation.

MicroRNAs contribute to maintaining the balance among genes regulating cells' fate, and their deregulation, a frequent hallmark in different human malignancies, can destabilize this equilibrium, thus contributing to cancer development and/or progression, from initiation to metastatic disease. However, despite the increasing and encouraging evidences linking microRNAs to cancer biology, many important questions remain to be addressed; in fact, although the identification and validation of microRNA targets greatly improved in the last few years, we still know very little about the cellular and molecular circuits where they are involved. The scenario is surely complicated by the ability of microRNAs to target multiple molecules, sometimes belonging to related pathways, and at the same time by the redundancy existing among microRNAs. This gives rise to a complex regulatory network in which biologic effects and properties of a particular microRNA do not always allow a linear explanation.

Improvement of computation programs of microRNA target prediction and experimental methods of validation will certainly contribute to elucidating their mechanisms of action, and genetically modified murine models will likely help in determining the oncogenic and tumor suppressor potential of individual microRNAs.

Data available to date clearly support the involvement of microRNA in cancer etiology and strongly suggest a possible use of these molecules as markers of diagnosis and prognosis and, eventually, as new targets or tools for a specific therapy. Stepping from the bench to clinical applications would be the next great challenge in cancer research.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

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