Oncomirs: Fxrom Tumor Biology to Molecularly Targeted Anticancer Strategies

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Abstract: Deregulation of microRNA (miRNA) promotes carcinogenesis, as these molecules can act as oncogenes or tumor suppressor genes. Here we provide an overview of miRNA biology, discuss the most recent findings on miRNA and cancer development/progression, and report on how tumor-related miRNAs (oncomirs) are being used to develop novel cancer specific therapeutic approaches.

Key Words: MicroRNA, miRNA, oncomirs, cancer biology, molecular targets, anticancer therapeutics.

INTRODUCTION

The discovery in viruses, plants and animals of small non-coding RNA molecules called microRNA (miRNA) that mediates RNA interference has opened a new era in our understanding of the control of gene expression [1, 2]. The latest version of miRBase (release 11.0, April 2008 [3]) has annotated 678 miRNA sequences in the human genome and this number is expected to double as more miRNAs are awaiting experimental validation. Based on their sequences, miRNAs are predicted to negatively target up to one-third of human messenger RNA (mRNA) [4]. Because base-pairing with the mRNA 3' untranslated region (3' UTR) is generally imperfect, a single miRNA may target over 200 transcripts simultaneously. Therefore, regulation of miRNA itself (e.g., at the epigenetic level) may well be a potent, albeit indirect, way to control simultaneously numerous genes [5]. In the present work, we briefly describe the biogenesis and function of miRNAs, review their role in tumor biology and report on how these molecules are being exploited as molecular targets for novel therapeutic approaches to fight cancer.

miRNA BIOGENESIS AND FUNCTION

miRNAs are endogenously produced RNA molecules of about 18-25 nucleotides (nt) in length. With the exception of those within the Alu repeats transcribed by polymerase III (Pol III) [6], most miRNA genes are derived from primary miRNA transcripts (pri-miRNA) produced by Pol II and containing a 5' cap and a poly(A) tail [7]. The pri-miRNA is cleaved within the nucleus by a multiprotein complex called Microprocessor, which is composed of the RNAse III enzyme Drosha and the double-stranded RNA-binding domain (dsRBD) protein DGCR8/Pasha into an about 70-nt long hairpin precursor known as pre-miRNA (Fig. **1**). Next, the pre-miRNA is exported into the cytoplasm by Exportin-5 *via* a Ran-GTP-dependent mechanism. The pre-miRNA is further cleaved into the mature approximately 22-nt long miRNA:miRNA* duplex by an RNAse III enzyme, Dicer, in association with its partners, TRBP/Loquacious and PACT in human cells. Subsequently, an RNA-induced silencing complex called RISC is assembled with Argonaute-2 (Ago2, a component with RNase catalytic function) and other not fully characterized proteins [8]. The miRNA strand is then selectively incorporated into RISC and guides the complex specifically to its mRNA targets through base-pairing interaction. miRNAs downregulate the expression of their target genes in two ways [2, 4, 5] depending upon the complementarity between them: A) most miRNAs bind imperfectly to their target sequence and inhibit protein translation; B) by contrast miRNAs with perfect complementarity to the target sequence induce the cleavage and degradation of the transcript. In particular, efficient miRNA-guided translational repression requires an m^7G-cap as well as a poly(A) tail [9]. Recently, the mechanism by which miRNA ribonucleoprotein complexes (miRNP) that are bound to the 3' UTR of a target mRNA interfere with translational initiation has been discovered by some investigators who identified a motif (MC) within the Mid domain of Ago proteins, which bears significant similarity to the m^7G cap-binding domain of eIF4E, an essential translation initiation factor: in this experimental model, the Ago proteins compete with eIF4E for cap binding and thus repress the translational initiation [10].

miRNAs AND CANCER BIOLOGY

Cancer is a multigenic disease characterized by uncontrolled cell proliferation, resistance to apoptosis, loss of differentiation and ability to invade tissues and metastasize. Cells possess many safeguard mechanisms to ensure that all molecular mechanisms potentially underlying malignant behavior are under control both during development and in the adult body. These regulatory pathways/circuits are based on the switch on or switch off of genes coding for proteins that directly mediates the physiological functions. Dysregulation of these genes, which are referred to as tumor-suppressor genes and oncogenes, is believed to represent the "primum movens" in tumor development and progression and is the main focus of preclinical cancer research [11]. Until few years ago, tumor suppressor genes and oncogenes were believed to function only by being first transcribed

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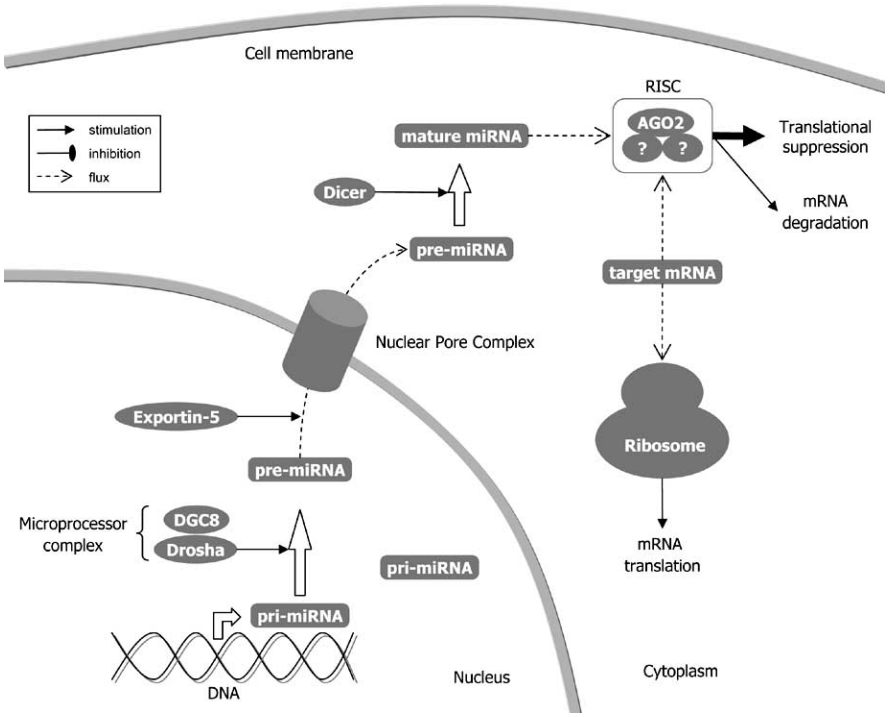

Fig. (1). Biogenesis of microRNA (see text for details).

from DNA into RNA, and are then translated into protein to exert their effects. Recent evidence indicates that small non-protein-coding miRNAs also play a key role in cancer biology by acting as tumor suppressors and oncogenes [12]: these cancer-related miRNAs have been named oncomirs [13-15] (although some Authors prefer to use this name to indicate oncogenic miRNA only).

Despite the fact that the biology of tumor related miRNAs has only begun to be investigated, a relatively large body of data is already available on this subject. Although a comprehensive list of tumor related miRNAs and their biological effects is beyond the scope of this review (for such a list, visit www.mmmp.org [16]), we will describe some of the most significant examples to underscore the relevance of these molecules as cancer targets (Table **1**).

Tumor Suppressor miRNAs

The first indication of miRNAs as tumor suppressors came from the observation that patients diagnosed with B-cell chronic lymphocytic leukemia have frequent deletions or downregulation of mir-15a and mir-16-1 located at chromosome 13q14.3 [17]. A follow-up study demonstrated that miR-15a and miR-16-1 negatively regulate the anti-apoptotic protein BCL2 [18], thus potentially acting as tumor suppressor genes. Some other miRNAs have also been shown to function as tumor suppressor genes. Among them, the let-7 family (which includes let-7a-1, let-7a-2, let-7a-3, let-7b, let-7c, let-7d, let-7e, let-7f-1, let-7f-2, let-7g, and let-7i) is downregulated in ovarian carcinoma [19], lung cancer [20] and breast carcinoma stem cells [21]; this miRNA family has been found to negatively regulate tumor-promoting factors such as RAS [22], IMP-1 [23] and HMGA2 [23, 24]. Similarly, mir-143 and mir-145, which exhibit decreased abundance in colorectal carcinoma [25] cervical cancer [26] and B-cell malignancies [27], inhibit cell proliferation [28], likely by controlling the expression of ERK5 [27, 29]. However, recent evidence suggests that miRNA physiology can be highly complex and members of a miRNA family can behave very differently. For instance, while the other let-7 family members have been so far associated with tumor suppressive functions, in ovarian carcinoma let-7a-3 gene hypermethylation (and thus silencing) is associated with better prognosis and lower insulin-like growth factor-2 (IGF2) expression [30]. Moreover, the let-7a-3 gene is heavily methylated in normal human tissues but hypomethylated in some lung adenocarcinomas and in human lung cancer cells let-7a-3 gene hypomethylation increases let-7a-3 expression and results in enhanced tumor phenotypes and oncogenic changes in transcription profiles [31].

Oncogenic miRNAs

Other miRNAs can function as oncogenes. mir-155 is remarkably overexpressed and linked to tumorigenesis in lymphomas [32-35] and breast cancer [36], likely in cooperation with MYC, while mir-372 and mir-373 have been implicated as oncogenes in testicular germ cell tumors [37]. Upregulation of mir-21 has been reported in glioblastomas [38] and breast cancer [36], where it exerts an anti-apoptotic function [38, 39]. Moreover, mir-21 inhibits the expression of tumor suppressor genes such as tropomyosin-1 (TPM1) [40] and phosphatase and tensin homolog (PTEN) [41]. The mir-17-92 cluster (mir-17-5p, mir-17-3p, mir-18a, mir-19a, mir-20a, mir-19b-1 and mir-92-1) has been found upregulated in 65% of B-cell lymphomas and its overexpression expedited the development of malignant lymphomas in a transplantation mouse model [42]. As regards the mechanism of action, preclinical models have demonstrated that miRNAs from this cluster function cooperatively as onco-

Table 1. Selected Cancer-Related microRNA and their Proven mRNA Targets

microRNA	Target	Tumor	Ref.
let-7 family	RAS	Lung cancer	[22]
	RAS	Colon cancer	[106]
	RAS	Breast cancer	[21]
	HMGA2	Breast cancer	[21]
	C-MYC	Colon cancer	[106]
let-7a	Integrin beta (3)	Melanoma	[107]
	C-MYC	Burkitt lymphoma	[82]
let-7a-3	IGF2	Ovarian cancer	[30]
let-7b	CDK4	Melanoma	[83]
	Cyclin D1	Melanoma	[83]
let-7g	K-RAS	Lung cancer	[84]
mir-7	EGFR, AKT	Glioblastoma	[108]
mir-10b	HOXD10	Brest cancer	[109]
mir-15a	Bcl2	Chronic lymphocytic leukemia	[110]
mir-16-1	Bcl2	Chronic lymphocytic leukemia	[110]
mir-17-92 cluster	Tsp1, CTGF	Colon cancer	[43]
	E2F1	HeLa cells, human fibroblasts	[44]
mir-21	PDCD4	Breast cancer	[111]
	TPM1	Breast cancer	[40]
	Maspin	Breast cancer	[112]
	NFIB	HL-60 cells	[113]
	Bcl2	Breast cancer	[39]
	RECK, TIMP3	Glioma	[114]
	PTEN	Hepatocellular cancer	[41]
mir-25	E2F, p21, BIM	Gastric cancer	[115]
mir-27a	ZBTB10	Breast cancer	[116]
	RYBP/DEDAF	Breast cancer	[56]
mir-29a	FHIT, WWOX	Lung cancer	[117]
mir-29b	FHIT, WWOX	Lung cancer	[117]
	MCL1	Cholangiocarcinoma	[118]
mir-34a	E2F3	Neuroblastoma	[119]
	Bcl2, MYCN	Neuroblastoma	[86]
mir-93	E2F, p21, BIM	Gastric cancer	[115]
mir-106b	p21/CDKN1A	Brest, colon kidney, gastric, lung cancer	[120]
	E2F, p21, BIM	Gastric cancer	[115]
mir-122a	cyclin G1	Hepatocellular carcinoma	[121]

(Table 1. Contd….)

microRNA	Target	Tumor	Ref.
mir-125a	ERK1/2, AKT, ERBB2/3	Breast cancer	[122]
mir-125b	ERK1/2, AKT, ERB2/3	Breast cancer	[122]
	Bak1	Prostate cancer	[123]
	Akt	Hepatocellular carcinoma	[124]
mir-126	Crk	Lung cancer	[85]
mir-127	BCL6	Bladder cancer, colon cancer, embryonic carcinoma, cervical cancer, pancreatic cancer, lymphomas, breast cancer	[93]
mir-137	MITF	Melanoma	[125]
mir-143	ERK5	B-cell malignacies	[27]
	ERK5	Colon cancer	[29]
mir-145	ERK5	B-cell malignacies	[27]
mir-146	NfKb	Breast cancer	[88]
mir-155	TP53INP1	Pancreatic cancer	[126]
mir-200c	E-cadherin	Pancreatic, colorectal, breast cancer	[127]
mir-203	ABL1	Hematopoietic malignacies	[95]
mir-210	E2F3	Ovarian cancer	[128]
mir-214	PTEN	Ovarian cancer	[129]
mir-221	p27, p57	Hepatocellular carcinoma	[130]
	p27	Thyroid carcinoma	[131]
	p27, c-KIT	Melanoma	[79]
mir-222	p27, c-KIT	Melanoma	[79]
	p27	Prostate carcinoma	[132]
	p27	Thyroid carcinoma	[133]
mir-372	LATS2	Testicular germ cell tumors	[37]
mir-373	LATS2	Testicular germ cell tumors	[37]
mir-378	SUFU, FUS-1	Glioma	[134]
mir-451	MDR/ABCB1	Breast cancer	[89]
mir-520c	CD44	Breast cancer	[135]

genes, possibly by targeting pro-apoptotic factors that are activated in response to MYC overexpression: when the brakes of the apoptotic pathway are removed, MYC can induce cells to proliferate uncontrollably, which results in cancer. The oncogenic function of the mir-17-92 cluster is further supported by the fact that its members can promote tumor angiogenesis (by inhibiting the expression of anti-angiogenic factors thrombospondin-1 [Tsp1] and connective tissue growth factor [CTGF]) [43] and are upregulated in both hematological and solid tumors. Nevertheless, as above mentioned for tumor suppressor miRNAs, the relationship between putatively oncogenic miRNAs and cancer biology does not always appears univocal. As a matter of fact, in a study conducted on a human B-cell line that overexpresses MYC [44], investigators showed that MYC induces the expression of the mir-17-92 cluster and that, in turn, this cluster inhibits the expression of E2F1 (without affecting mRNA abundance, which is a hallmark of miRNA-mediated gene repression), a MYC target that controls the transition from G1 to S phase of the cell cycle by regulating genes that are involved in DNA replication, cell division and apoptosis [45]. Therefore, a model is proposed in which MYC-

mediated cell growth is tightly regulated by the mir-17-92 cluster: in the presence of MYC, miRNAs of the mir-17-92 cluster limit the activity of E2F1 and dampen the proliferative effects of MYC by breaking the positive feedback loop between MYC and E2F1. In this context the mir-17-92 cluster would function as a tumour suppressor, which is in contrast to the above reported findings. However, it has also been reported that although E2F1 drives cellular proliferation, when E2F1 expression levels cross a certain threshold, excessive levels of this protein induce apoptosis [45]. Therefore, the negative regulation of E2F1 by miRNAs of the mir-17-92 cluster might function to block the apoptotic activity of E2F1 and stimulate MYC-mediated cell proliferation, a model that would explain the apparently conflicting findings.

Mechanisms of miRNA Dysregulation in Cancer

The causes of miRNA dysregulation in human cancers are just beginning to be elucidated. First of all, it is interesting to remember that about 50% of annotated human miRNAs are located in particular areas of the genome called "fragile sites" [46], which have been associated with the genome instability proper of malignant cells [47].

Second, using a high-resolution array-based comparative genomic hybridization (aCGH) approach, some investigators found that a large proportion of miRNA gene-containing genomic loci exhibit DNA copy number alterations in ovarian cancer (37.1%), breast cancer (72.8%) and melanoma (85.9%), suggesting that miRNA deregulation stemmed at the genomic level may be frequent [48].

Third, abnormalities of the protein machinery involved in miRNA biogenesis can affect the global miRNA expression and/ or processing and appear to affect cancer biology. For instance, in a study on non-small cell lung cancer (NSCLC), lower Dicer1 expression levels were significantly associated with poor tumor differentiation and shortened postoperative survival [49], suggesting that impaired miRNA processing (and thus globally decreased miRNA function) favors tumor aggressiveness/progression. Recently, other investigators performed a large-scale profiling of mammalian miRNAs in 334 tumor samples and found a global decrease of mature miRNA expression in human cancers [50]. More recent evidence suggests that other proteins involved in miRNA processing might play a role in cancer biology. For instance, Lin28, a developmentally regulated RNA binding protein, selectively blocks the processing of pri-let-7 miRNA in embryonic cells: in particular, Lin28 was found to be necessary and sufficient for blocking Microprocessor-mediated cleavage of pri-let-7 miRNA, which identifies Lin28 as a negative regulator of miRNA biogenesis and suggests that Lin28 may play a central role in blocking miRNA-mediated differentiation in stem cells and in certain cancers [51].

Also mutations of tumor related miRNAs have been implicated in tumor biology: in fact, both somatic and germline miRNA mutations can be found in tumor specimens, are cancer-specific and can alter miRNA expression or function. For instance, in chronic lymphocytic leukemia a germ-line mutation of the mir-16-1-mir-15a primary precursor causes low levels of miRNA expression *in vitro* and *in vivo* and is associated with deletion of the normal allele; in the same study, germ-line or somatic mutations were found in 5 of 42 sequenced microRNAs in 11 of 75 patients, while no such mutations were found in 160 subjects without cancer [52]. Moreover, the single nucleotide polymorphism (SNP) rs11614913 of mir-196a2 is associated with survival in individuals with lung cancer: specifically, survival is significantly decreased in individuals who are homozygous CC at SNP rs11614913; the same study revealed that the rs11614913 SNP can affect binding of mature mir-196a2-3p to its target mRNA [53].

Finally, the epigenetic regulation of miRNA expression has been recently discovered: in particular, both promoter methylation [54, 55] and histone acetylation [56] have been demonstrated to regulate cancer related miRNA expression. Of interest, the mechanisms of tumor suppressor miRNA silencing might be exploited for therapeutic purposes, as below discussed.

TUMOR RELATED miRNAs AND ANTICANCER THERAPY

RNA interference is being intensively investigated as a tool to fight cancer by selectively inhibiting the expression of single known oncogenes [57, 58]. As with molecularly targeted drugs, the main limitation of this approach is that tumors can rely on multiple, redundant pathways for the maintenance of their survival/aggressiveness/chemoresistance, which likely accounts for most failures of current therapeutic regimens. The discovery of miRNAs acting as oncogenes or tumor suppressor genes has opened an unprecedented avenue in the targeted approach to cancer treatment [59-62]. In fact, once the biology of tumor related miRNAs is better defined, the use of these miRNAs as cancer targets or as anticancer bullets might enable researchers/clinicians to simultaneously target tens if not hundreds of tumor-related genes at a time, hopefully interrupting many pathological pathways with a single hit and thus leaving malignant cells with no way out of therapeutically induced death.

Depending on their role, different strategies have been devised to either inhibit oncogenic miRNAs or use tumor suppressive miRNAs for anticancer gene therapy.

Oncogenic miRNAs as Therapeutic Targets

Like "conventional" protein-coding oncogenes [11], oncogenic miRNAs are being studied as potential therapeutic targets whose inhibition might contribute to kill malignant cells. Although other approaches have been described (e.g. miRNA "sponges" [RNA molecules expressed in cells from transgenes containing multiple, tandem binding sites to a microRNA of interest] [63], and interfering RNA [against the loop region of a given micro-RNA precursor] [64]), inhibition of miRNAs is usually obtained with antisense oligonucleotides (ASO), also called anti-miRNA oligonucleotides (AMO) [61, 65, 66]. AMO molecules have complementary sequences to miRNA and contain chemical modifications to achieve two main goals: strong binding to the miRNA and stability in physiological conditions that allows to prolong their half-life particularly when administered *in vivo* with therapeutic intent. To this aim, two types of modified oligonucleotides have been developed: 2'-O-methylation of RNA nucleotides (unlike double stranded small interfering

RNA [siRNA], they are single stranded RNA) [67] and locked nucleic acid (LNA) DNA nucleotides [68].

In human cell cultures, 2-O-methyl oligoribonucleotides (2-O-Me-RNA) - but not 2'-deoxyoligonucleotides - can specifically downregulate miRNAs [69]. As for RNA interference mediated by siRNA, transfection of 2-O-Me-RNA requires lipophilic agents (e.g. cationic lipids such as Lipofectamine (R) or Oligofectamine (R)) to get the oligonucleotide inside the cells. In order to increase the cell-permeability of 2-O-Me-RNA, cholesterol-linked AMO called "antagomirs" have been successfully developed to downregulate several mouse miRNAs in a number of mouse tissues following intravenous injection *in vivo* [70].

Inhibition of oncogenic miRNAs has been shown to have therapeutic potential in many tumor models, not only *in vitro* but also *in vivo* [71-75]. For instance, inhibition of mir-17-5p and mir-20a (but not other members of the mir-17-92 cluster) induces apoptosis selectively in lung cancer cells overexpressing mir-17-92, suggesting the possibility of an "addiction" of malignant cells to these miRNAs (at least in a subset of lung cancers) that might be exploited for therapeutic purposes [76]. Downreglation of mir-21 in cultured human hepatocellular carcinoma (HCC) cells increases the expression of the PTEN tumor suppressor and decreases tumor cell proliferation, migration, and invasion [41]. Moreover, inhibition of mir-21 (and mir-200b) sensitizes cholangiocarcinoma cells to the cytotoxic activity of the conventional chemotherapeutic gemcitabine [77]. Even more importantly, in a human breast cancer xenograft model, downmodulation of mir-21 inhibits tumor growth, likely by inducing re-expression of tumor suppressor gene TPM1 [40]. Similarly, miRNA-21 knockdown disrupts human glioma growth *in vivo* and displays synergistic effects with cytotoxic agent tumor necrosis factor-related apoptosis inducing ligand (TRAIL) [78].

As a further example, inhibition of mir-221 and mir-222 increases p27/Kip1/CDKN1B in PC3 prostate carcinoma cells and strongly reduces their clonogenicity *in vitro*. Consistently, mir-221 and mir-222 knockdown is associated with re-expression of p27/Kip1/CDKN1B and c-KIT, which promotes cell cycle arrest and tumor differentiation and ultimately inhibits human melanoma growth both *in vitro* and *in vivo* [79].

Tumor Suppressor miRNA Gene Therapy

Until few years ago, the rational anticancer gene therapy hinged upon the substitution of the lost function of a tumor suppressor gene encoding a specific oncoprotein [80]. After the discovery of miRNAs with tumor suppressing activity, the arsenal of anticancer gene therapy has gained a new weapon capable of targeting multiple genes potentially involved in cancer biology by means of a single shot [59]. Tumor suppressor miRNAs can be transfected in human cells using either modified oligonucleotides (as above reported) or viral vectors (lentivirus is the preferred vector for stable, sustained transgenic expression).

Several examples are already available supporting the therapeutic potential of forcing the expression of tumor suppressor miRNAs. In this regard, the most studied miRNAs are the let-7 family members. Ectopic expression of let-7 reduces HMGA2 expression and inhibits the proliferation of lung cancer cells [24, 81]. Reduced proliferation is also observed in Burkitt lymphoma cells transfected with let-7a, which is accompanied by decreased c-MYC protein expression [82]. Forced expression of let-7b in melanoma cells *in vitro* downregulates the expression of cyclin-D1, cyclin-D3 and cyclin-A, as well as cyclin-dependent kinase-4 (CDK4), all of which had been described to play a role in melanoma development: in line with the downmodulation of cell cycle regulators, let-7b inhibits cell cycle progression and anchorage-independent growth of melanoma cells [83].

Remarkably, in a human breast carcinoma preclinical model, let-7 coding lentivirus reduces proliferation, mammosphere formation, and the proportion of undifferentiated cells *in vitro* and tumor formation and metastasis in nude mice *in vivo* [21]. Analogously, in an autochthonous model of murine lung carcinoma, let-7g expression substantially reduces the tumor burden [84]. Although in its infancy, miRNA-based gene therapy has been proven effective in many other preclinical models. For instance, forced overexpression of mir-126 leads to decrease in adhesion, migration and invasion in a lung cancer cell line, which is associated with a decrease in the protein levels of the CRK proto-oncogene [85]. Synthetic mir-143 and mir-145 precursors transiently transfected into HeLa cells suppress the growth of this immortal cell line derived from cervical carcinoma [28]. Yet, the mir-34a (1p36) and mir-34c (11q23) precursor mimics induce dramatic growth inhibition in neuroblastoma cell lines with 1p36 hemizygous deletion: interestingly, Bcl-2 and MYCN have been identified as mir-34a targets and likely mediators of the tumor suppressor phenotypic effect [86, 87]. Lentiviral-mediated expression of mir-146a/mir-146b significantly downregulates nuclear factor kappa B (NFkB) expression and activity in breast cancer cells, which is associated with impaired tumor invasion and migration capacity relative to control cells [88]. Finally, forced expression of mir-451, which regulates the expression of multidrug resistance 1 gene (MDR/ABCB1), increases the sensitivity of breast cancer cells to doxorubicin [89], which opens a new avenue in the development of chemotherapy sensitizers.

Tumor Suppressor miRNAs and Epigenetic Therapy

Re-expression of protein-coding tumor suppressor genes is a recently developed anticancer therapeutic strategy that has reached in some cases the clinical phase of experimentation [90]. Two main drug classes can be used to reactivate tumor suppressive genes and cause malignant cell death: demethylating agents (mainly represented by cytosine analogues 5-azacytosine [azacytidine] and 2'-deoxy-5-azacytidine [decitabine]) [91] and histone deacetylase inhibitors (e.g. trichostatin-A, SAHA, LAQ-824, depsipeptide, sodium butyrate, MS-275, CI-994) [92].

As regards tumor suppressor miRNAs, their epigenetic regulation has been shown by means of two main experimental models. In the first model, investigators analyze the expression profile of miRNAs in malignant cells untreated or exposed to chromatin-modifying drugs. For instance, in bladder cancer cells 17 of 313 human miRNAs are upregulated by simultaneous treatment with the DNA-demethylating agent, 5-aza-20-deoxycytidine (5-Aza-CdR) and the

histone deacetylase inhibitor 4-phenylbutyric acid (PBA) [93]. Interestingly, these upregulated miRNAs are quite different as compared to those upregulated in LD419 human normal fibroblasts, indicating that DNA methylation status and chromatin structure around miRNA genes are different between cancer and normal cells, although tissue-specific expression may not be completely ruled out. Among these miRNAs, mir-127 was focused on because it was greatly upregulated (49-fold) in malignant cells following the 5-Aza-CdR and PBA treatment [93]: the gene encoding mir-127 is embedded within a CpG island and is silenced in various cancer cells while it is expressed in normal fibroblasts; a candidate target of mir-127, the proto-oncogene BCL6, is translationally suppressed after mir-127 upregulation by 5-Aza-CdR and PBA treatment, suggesting that DNA demethylation and histone deacetylase inhibition may activate expression of miRNAs acting as tumor suppressors.

Using the second experimental model, other investigators have confirmed the effect of methylation on the expression of tumor related miRNAs. As an example, using DNMT1 and DNMT3b knockout HCT116 colorectal cancer cells, some authors performed microarray profiling of 320 human miRNAs and found that 18 miRNAs are upregulated by >3-fold in the DNMT1/DNMT3b double knockout HCT116 cells [54]. In that study, one of the main targets resulted mir-124a, which undergoes transcriptional inactivation by CpG island hypermethylation in human tumors from different cell types. Interestingly, the same investigators also functionally linked the epigenetic loss of mir-124a with the activation of cyclin D kinase 6 (CDK6), a bona fide oncogenic factor that phosphorylates and thus inhibits the retinoblastoma protein (pRB), a tumor suppressor factor: accordingly, CpG island hypermethylation of tumor suppressor miRNAs has been proposed as an important mechanism in tumorigenesis [54].

Following the demonstration that tumor suppressor miRNAs can be epigenetically silenced [73], the use of chromatin remodeling drugs to interfere with miRNA expression in a therapeutic perspective is just beginning. For instance, analysis of patient's primary leukemia blasts revealed that those carrying the t(8;21) generating AML1/ETO, the most common acute myeloid leukemia-associated fusion protein, display low levels of mir-223, a regulator of myelopoiesis [94]. In the same study, mir-223 was found to be a direct transcriptional target of AML1/ETO: in particular, by recruiting chromatin remodeling enzymes at an AML1-binding site on the pre-mir-223 gene, AML1/ETO induces heterochromatic silencing of mir-223. Importantly, demethylating treatment enhances mir-223 levels and restores cell differentiation [94], which represents a key therapeutic objective in acute leukemia.

As a further example in the field of hematological malignancies, it is well known that ABL1 is specifically activated in chronic myeloid leukemia as a BCR-ABL1 fusion protein (Philadelphia chromosome): mir-203 has been recently demonstrated to target the ABL1 gene and is downmodulated in this type of tumor [95]. In line with the oncogenic activity of ABL1, re-expression of mir-203 by a combination of epigenetic drugs (i.e. 5'-azacytidine plus 4-phenylbutyrate) reduces both ABL1 and BCR-ABL1 fusion protein levels and ultimately inhibits tumor cell proliferation in an ABL1-dependent manner: thus, mir-203 functions as a tumor suppressor, and re-expression of this microRNA might have therapeutic benefits in specific hematopoietic malignancies [95].

FUTURE CHALLENGES

Although the therapeutic potential of cancer related miRNAs is only beginning to be explored, enough preclinical evidence has already accumulated to reasonably foresee that in the next few years it will be possible to test the anticancer activity of this molecularly targeted approach in the clinical setting [60, 62, 71, 74, 96]. In this regard, the announcement of the first clinical trial of SPC3649 (a LNA-based antisense molecule against miR-122) for the treatment of hepatitis-C [97] is a tangible sign of the rapid progresses being made in the field of miRNA-based therapeutics development. Nevertheless, we must remember that this promising research avenue is constellated by numerous biological and technical challenges.

As regards the former type of challenges, the complex biology of microRNA must be better elucidated before clinical trials can be safely carried out to demonstrate the therapeutic potential of the miRNA-based approach in humans. In fact, the ever growing knowledge on the physiological roles played by miRNAs warrants great caution in interfering with their activity; in addition, as above mentioned, some tumor related miRNAs appear to function as both tumor suppressor gene and oncogenes, which highlights our incomplete knowledge of the network these molecules belong to. Moreover, the theoretical advantage of tumor suppressor miRNAs (i.e. the ability of each miRNA to target tens of oncogenes, as compared to the single gene specificity of other antisense strategies), is counterbalanced by safety issues raised by the so called "off target" effects, a phenomenon first recognized as a relatively infrequent lack of specificity of small interfering RNA (siRNA) molecules [98, 99]. In addition, for each miRNA multiple targets (tens to hundreds) are usually expected, which makes highly challenging to predict the side effects of therapeutic interference with the activity of tumor related miRNAs. These simple observations warrant the creation of systematic, comprehensive and continuously updated biomaps depicting the upstream and downstream regulatory circuits oncomirs belong to, a recently started online project to which any researcher can contribute [16].

As regards the latter type of challenges, the delivery of miRNA-targeting therapeutics represents the major technical obstacle to their implementation in the clinical setting [59, 100, 101]. In fact both molecules inhibiting oncogenic miRNAs (e.g. AMO) and tumor suppressor miRNAs are instable in the serum and their delivery across the cell membrane is highly inefficient (as above mentioned). The delivery of AMO within human cells depends upon the combination with lipophilic compounds, which are characterized by an intrinsic toxicity: to solve this problem new carriers are being developed [102], but they are still under preclinical testing and their clinical safety is to be demonstrated. On the other hand, tumor suppressor miRNAs are mainly delivered by means of viral vectors that bring the mutagenic risk linked to gene therapy in general [103]. In addition, some investigators have recently shown that overexpression of

miRNA mimics from adeno-associated viral vectors in mice could saturate the miRNA pathway and cause an otherwise unexpected severe liver toxicity [104].

Finally, the "redundancy" of miRNAs might make the task even more difficult: for instance, would targeting mir-17 be sufficient or would all miRNAs of the mir-17-92 cluster have to be targeted? Although this issue can be at least in part addressed by functional studies performed in the pre-clinical setting, the effects observed *in vivo* in humans might be quite different, as recently underscored for genetic interactions [105]; as a consequence, it might be necessary to test multiple combinations before the therapeutic effect of the miRNA-based anticancer approach is demonstrated in the clinical setting.

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Received: 15 August, 2008 **Revised: 12 September, 2008** **Accepted: 19 September, 2008**