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Shielding the messenger (RNA): microRNA-based anticancer therapies

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Abstract

It has been a decade since scientists realized that microRNAs (miRNAs) are not an oddity invented by worms to regulate gene expression at post-transcriptional levels. Rather, many of these 21–22-nucleotide-short RNAs exist in invertebrates and vertebrates alike and some of them are in fact highly conserved. miRNAs are now recognized as an important class of non-coding small RNAs that inhibit gene expression by targeting mRNA stability and translation. In the last ten years, our knowledge of the miRNAs world was expanding at vertiginous speed, propelled by the development of computational engines for miRNA identification and target prediction, biochemical tools and techniques to modulate miRNA activity, and last but not least, the emergence of miRNA-centric animal models. One important conclusion that has emerged from this effort is that many microRNAs and their cognate targets are strongly implicated in cancer, either as oncogenes or tumor and metastasis suppressors. In this review we will discuss the diverse role that miRNAs play in cancer initiation and progression and also the tools with which miRNA expression could be corrected *in vivo*. While the idea of targeting microRNAs towards therapeutic ends is getting considerable traction, basic, translational, and clinical research done in the next few years will tell whether this promise is well-founded.

Keywords

miRNA; cancer; therapy; target prediction; chemosensitivity; adjuvant

1. Introduction

microRNAs (miRNAs or miRs) are an integral part of the RNA interference (RNAi) machinery which results in post-transcriptional gene silencing. In general, RNAi is triggered by double-stranded RNAs (dsRNAs) that are processed first into short RNA duplexes, and then into single-stranded small RNAs that will bind target cellular mRNAs or viral genomic RNAs (Fire et al. 1998). Depending on their origin, function and structure, there are three types of short RNAs that follow the RNAi pathway for gene silencing: siRNAs (small interfering RNAs), piRNAs (PiwiRNAs) and miRNAs [reviewed in (Liu & Paroo, 2010; Paroo et al. 2007; Rana, 2007)].

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miRNAs were first discovered in *C.elegans* by the laboratories of Ambros and Ruvkun. Lin-4 was identified in 1993 (Lee et al. 1993; Wightman et al. 1993) and let-7 - in 2000 (Reinhart et al. 2000; Slack et al. 2000). A year later, the Ambros and Bartel labs demonstrated that they represent an extensive class of small RNAs (Lau et al. 2001; Lee & Ambros, 2001). Importantly, in the companion paper from the Tuschl lab the existence of many microRNAs was confirmed in vertebrates, including humans (Lagos-Quintana et al. 2001). In subsequent years, microRNAs have been shown to be involved in many biological processes: signaling, differentiation, and possibly cell fate determination, as hinted at by the existence of tissue-specific microRNAs (e.g., miR-1b-2 and miR-99b in the brain, miR-1 in the muscle, and miR-122 in the liver).

Germanely to this review, miRNAs play a role in tumor pathogenesis and progression. This is because miRNAs can regulate the expression and activity of canonical, protein-coding tumor suppressor genes and oncogenes. Furthermore, several miRNAs themselves have been reported to function as tumor suppressor (TSmiRs) or oncogenes (oncomiRs) (see section 4). In addition, miRNA expression profiling and generation of transgenic mice have been of great utility in identifying miRNAs which attenuate or enhance the development of specific types of tumors or are involved in the acquisition of invasive capabilities.

2. miRNA Biogenesis

miRNA-encoding genes are localized in either intronic or exonic regions of protein-coding genes, or alternatively in intergenic regions of non-coding DNA. They are transcribed mainly by the RNA-polymerase II into primary miRNA transcripts (primiRNA). Consequently, pri-miRNAs contain cap structures as well as poly(A) tails and their synthesis is inhibited by α-amanitin (Lee et al. 2004). The initial steps of pri-miRNA processing depend on whether the miRNA gene is located within a intragenic (protein-coding) or an intergenic region (Fig. 1). miRNA encoded by intronic regions of protein-coding DNA (“mirtrons”) in flies and worms (Okamura et al. 2007; Ruby et al. 2007) as well as in mammals (Berezikov et al. 2007) skip the initial processing by Drosha (see below), but the biogenesis pathway in the cytoplasm is identical to other miRNAs processed from pri-miRNAs.

Approximately half of all human miRNAs are encoded by genomic clusters that are transcribed as single polycistronic units (Lee et al. 2002; Lau et al. 2001; Lagos-Quintana et al. 2001; Mourelatos et al. 2002). An extreme example of this grouping is the *C19MC* locus, which encodes the largest human microRNA gene cluster consisting of 40+ tandemly repeated, primate-specific pre-miRNAs (Bentwich et al. 2005). Each specific miRNA from this and other clusters will be processed individually, and mature miRNAs from the same cluster can exhibit different patterns of expression.

Pri-miRNAs then fold into imperfect hairpins, which serve as substrates for Drosha (Lee et al. 2003). A member of the RNase III family of enzymes, Drosha functions as part of a protein assembly called the *microprocessor complex*, which also includes the dsRNA binding protein DGCR8 (called Pasha in flies) (Denli et al. 2004; Gregory et al. 2004). Drosha and DGCR8 will cleave and transform a pri-miRNA into a pre-miRNA, a stem-loop structure of ~70 nucleotides and a two-nucleotide 3′-overhang. Generally, pre-miRNAs will be exported to the cytoplasm by Exportin-5 and Ran-GTP (Lund et al. 2004; Yi et al. 2003; Bohnsack et al. 2004). However, it is important to highlight that there are some variations in the cellular distribution of mature miRNAs and, for instance, miR-29b, miR-320 and miR-373 have been shown to function and accumulate primarily in the nuclear compartment (Liao et al. 2010; Hwang et al. 2007; Kim et al. 2008; Place et al. 2008).

Once in the cytoplasm, pre-miRNA will be cleaved for the second time, this time by Dicer, another endoribonuclease from the RNAse III family (Hutvagner et al. 2001; Grishok et al. 2001). Because Dicer is also involved in processing siRNA, it represents a common node in RNAi pathways - as discovered by the Hannon and Plasterck labs (Bernstein et al. 2001; Ketting et al. 2001)

Before Dicer can cleave pre-miRNA, it has to form a complex with TRBP (a dsRNA binding protein) (Chendrimada et al. 2005), and perhaps auxiliary, non-essential proteins such as PACT (protein activator of PKR) (Lee et al. 2006). Dicer cleaves the loop off the pre-miRNA stem-loop structure generating a 22–25 nucleotides long dsRNA also known as miRNA duplex. This duplex consists of the functional, or guide, miRNA strand and its complementary non-functional, or passenger, strand (miRNA*). In principle, both strands (miRNA and miRNA*) have the ability to be incorporated into the miRISC (miRNA-Induced Silencing Complex) and become mature miRNA - or get degraded. Thermodynamics usually is the deciding factor, and the strand with the less stable 5′ end is loaded into the RISC while the strand with more stable terminal base pairing is degraded (Schwarz et al. 2003; Khvorova et al. 2003).

In order to function, miRNA-loaded RISC complexes recruit one of the 4 Argonaute proteins (Ago1-4), with no apparent preference; however only Ago2 has endonuclease activity and can cleave target mRNA (Meister et al. 2004; Liu et al. 2004). Neither TRBP nor PACT are necessary for processing activity itself, but to the extent that they participate in the recruitment of Argonaute-2 (Ago-2) their depletion reduces the efficiency of posttranscriptional gene silencing (Lee et al. 2006; Chendrimada et al. 2005; Macrae et al. 2008).

Once the miRISC is formed, it will bind to the 3′-UTR (untranslated region) of its mRNA target. This binding occurs, most frequently (but not always) through the miRNA seed sequence, a region of 7–8 nucleotides located at the miRNA 5′-terminus (Lewis et al. 2005; Lewis et al. 2003). Generally speaking, a perfect mRNA/miRNA complementarity has destabilizing effects (Davis et al. 2005; Davis et al. 2005; Yekta et al. 2004; Shin et al. 2010). At least three independent mechanism of miRNA-mediated mRNA silencing have been described: target cleavage, translational inhibition and mRNA decay. Typically, the seed sequence extends from nucleotides 2 to 7, and when the miRNA/mRNA pairing extends to positions 10 and 11, Ago2 catalyzes the cleavage of the mRNA target (Yekta et al. 2004; Elbashir et al. 2001; Hutvagner & Zamore, 2002). Very recently, it has been shown that higher concentrations of magnesium allow for a more permissive cleavage of mRNA/miRNA duplexes, requiring less complementarity between the two molecules (Shin et al. 2010).

Once the miRISC binds the target mRNA it inhibits its translation either by a cap-dependent mechanism (Humphreys et al. 2005; Pillai et al. 2005) whereby Ago proteins prevent cap recognition by the initiation factor eIF4E (Kiriakidou et al. 2007) or by directly recruiting ribosome inhibitory proteins, like eIF6 (Chendrimada et al. 2007). This, however, is usually accompanied by mRNA deadenylation (Wu & Belasco, 2005; Giraldez et al. 2006), and consequently both protein and mRNA levels are usually affected (Baek et al. 2008; Selbach et al. 2008). Additionally, microRNAs have been reported to promote relocation of mRNAs to sites of RNA storage named *P-bodies*, which are devoid of ribosomes (Liu et al. 2005; Liu et al. 2005). Overall, there is no doubt that microRNAs exert profound effects on gene expression. However, identification of key target genes proved difficult and wrought with uncertainties.

3. Target Prediction and Validation

It wasn't until 2004 that the basis of miRNA target prediction was established by Doench and Sharp, who found that the efficiency and specificity with which miRNAs repress target mRNAs are mostly determined by the free energy of binding of the first eight nucleotides in the 5′ region of a miRNA (the "seed" sequence) (Doench & Sharp, 2004). They also uncovered that one mRNA can be simultaneously inhibited by more than one miRNA species. To complicate the matters, over-expression of a certain miRNA can result in "forced" non-physiological inhibition of mRNA targets through competition with other mRNAs or miRNAs (Doench & Sharp, 2004). Subsequent work confirmed the bias towards 5′-end-driven recognition (although not necessarily nucleotides 2–8) (Miranda et al. 2006) but also the existence of non-seed-based recognition mechanisms (Tay et al. 2008; Lal et al. 2009).

There are currently several computational algorithms to predict putative targets of given miRNAs, as well as complete databases where newly discovered or putative miRNA sequences are annotated (Table 1). Prediction tools must be used cautiously and taken for what they are: predictors necessitating the use of experimental validation techniques, which can be laborious and challenging.

Confirmation of predicted targets is generally achieved using luciferase sensor assays where the tested 3′UTR is attached to the luciferase reporter gene and then introduced into cells expressing the miRNA of interest (Doench et al. 2003; Stark et al. 2003). Simultaneously, mutated versions of the targeted 3′UTR must be included as controls to ensure that downregulation of the luciferase activity upon exposure to a certain miRNA is mediated by base-pairing between the miRNA's seed sequence and the predicted site, and is not due to secondary indirect effects.

While luciferase sensor assays can suggest physical interaction between miRNA and mRNA, they don't prove it directly. For this purpose, more rigorous pull-down assays can be used. In this case, a labeled miRNA is mixed with cellular extracts and immunoprecipitated with an antiserum against the labeling moiety or passed through affinity columns. Once labeled-miRNA/mRNA complexes have been isolated, cDNA is obtained using reverse transcriptase-PCR (RT-PCR) and sequenced (Hsu et al. 2009). Additionally, to assert that the mRNA/miRNA interaction results in effective gene silencing, Darnell's lab has very recently developed a method of high-throughput sequencing (HITS) of RNAs isolated by crosslinking and immunoprecipitation (CLIP) with Ago2 antibodies. Using this Ago-HITS-CLIP assay, it is possible to detect not just the binary mRNA/miRNA complex, but the ternary (and presumably functional) miRNA/mRNA/Ago2 complex (Chi et al. 2009). This technique provides a precise map of miRNA/mRNA interactions and suggests specific sequences for targeting clinically relevant miRNA/mRNA interactions. What exactly these interactions are is still being aggressively researched, but there is a preponderance of newly emerging evidence that some microRNAs and their cognate targets are implicated in cancer.

4. miRNA and Cancer

Some initial evidence that miRNAs are linked to cancer was the transcriptional regulation of miRNAs by oncogenes and tumor suppressor genes. In parallel, more hard genetic evidence has emerged showing that miRNA genes could be amplified, deleted, or epigenetically regulated in cancer in the same way that canonical cancer genes are affected by somatic or germline mutations. Many of these putative cancer miRNAs have also been validated in mouse models. Finally, consistent overexpression/ downregulation of certain miRNAs in

advanced versus early-stage cancers lead to the idea that some microRNA might confer metastatic properties and function as metastamiRs (Hurst et al. 2009)

4.a) Transcriptional deregulation of miRNAs in cancer cells

Depending on the genomic localization of the miRNA gene, it will be under the control of the host gene within which it is transcribed, or its own dedicated promoter. In the first case, the microRNA pattern of expression will parallel that of the host gene, leading to some confusion as to which of the two plays a role in cancer. This was recently highlighted in a study where intronic miR-211- and not its host gene melastatin - was found to function as a tumor suppressor in malignant melanoma (Levy et al. 2010). The analysis is somewhat simpler when a miRNA gene has its own promoter. These dedicated miRNA transcripts were shown in the last 5 years to be affected by such well-known oncogenic and tumor suppressive transcription factors such as Myc and p53.

The first evidence emerged in 2005 from the Mendell and Dang laboratories and demonstrated that Myc, via direct DNA binding, activates transcription of the miR-17~92 cluster composed of seven mature miRNAs: miR-17-5p, miR-18a, miR-19a, miR-19b-1, miR-20a and miR-92a-1 (O'Donnell et al. 2005). Subsequently, through work from Mendell's and our own laboratories, it became apparent that Myc also down-regulates many microRNAs, by mechanisms that range from direct DNA binding/transcriptional repression (Chang et al. 2008) to the deregulation of the RNA-binding protein Lin28b (Chang et al. 2009). Importantly, down-regulation of miRNA expression by Myc not merely accompanies Myc-induced B-lymphomagenesis, but in many cases (e.g., miR- 15a/16) is required for efficient expansion of neoplastic B-cells in vivo (Chang et al. 2008; Chung et al. 2008)

On the opposite side of the cancer gene spectrum, the tumor suppressor *par excellence* p53 also counts miRNAs among its transcriptional targets. A very complex network of cross-regulation between p53 and miRNAs is being untangled (Fabbri et al. 2011; Neveu et al. 2010; Welch et al. 2007). Its centerpiece is miR-34a, a TSmiR (see section 4.b) which has been identified as one of the main effectors of p53-induced apoptosis and cell cycle arrest (Tarasov et al. 2007; Chang et al. 2007; Raver-Shapira et al. 2007; Bommer et al. 2007; He et al. 2007; Corney et al. 2007). This occurs via targeting of key proteins like BCL-2, CDK4, CDK6, or G1-Cyclins (Sun et al. 2008; Ji et al. 2008) and, simultaneously, via enhancing p53 activity through inhibition of the SIRT-1 deacetylase (Yamakuchi et al. 2008; Fujita et al. 2008). To make the p53/miRNA network even more intricate, miR-125b, miR-504, miR-30d and miR-25 all have been shown to directly target p53 (Le et al. 2009; Hu et al. 2010; Kumar et al. 2010).

In addition to regulation by transcription factors, epigenetic regulation by methylation of CpG island and histone modifications at miRNA regulatory regions have also been described for ~5% of all human miRNAs (Lujambio et al. 2007; Saito et al. 2006; Scott et al. 2006). Conversely, some miRNAs are known to regulate key components of the epigenetic machinery, including histone deacetylases and DNA methyltransferases (Fabbri et al. 2007; Noonan et al. 2009; Tuddenham et al. 2006; Sinkkonen et al. 2008; Benetti et al. 2008).

A good example of this complexity is the epigenetic regulation of miR-127 in bladder cancer (Saito et al. 2006). In normal cells, miR-127 is expressed as part of the miRNA cluster, but its expression is shut down in cancer cells. When bladder cancer cells are treated with a DNA demethylating agent (5-aza) and an inhibitor of histone deacetylation, 17 out of 313 human miRNAs increase their expression levels more than 3-fold. Of note, miR-127 is upregulated ~50 fold, resulting in the inhibition of the transcription factor and proto-oncogene BCL6, one of its direct targets. Interestingly, the polycistronic region encoding

miR-127 also includes miR-136, miR-432 and miR-433, but miR-127 is the only member of the cluster upregulated upon inhibition of methylation and deacetylation.

4.b) Tumor Suppressor miRNAs

This category includes miRNAs whose loss promotes tumor formation. The genetic mechanisms involved in down-regulation of a TSmiR gene in cancer are the same as those described for protein-coding genes: biallelic deletion, loss of heterozygosity, somatic mutations, altered processing, or epigenetic modification. The parallelism between miR-15a/16 and Rb is the case in point.

Deletions of the chromosomal region 13q were initially described in retinoblastoma (Vogel, 1979) and later in hematological malignances (Cheng et al. 1990). Once the retinoblastoma gene was mapped to the 13q14.14 region, it was assumed that RB1 deletion is the sole cause of leukemias. However, as techniques for gene mapping were developed and refined, it became apparent that the 13q locus deleted in B-cell chronic lymphocytic leukemia (CLL) and multiple myeloma (MM) did not involve the RB1 gene (Brown et al. 1993; Migliazza et al. 2001), but instead overlapped a minimal deletion region called MDR. However, it wasn't until 2001 that DLEU2 was identified as the putative key transcript of the MDR (Migliazza et al. 2001). One year later, DLEU2 was found to host miR-15a/16-1, whose allelic loss is responsible for CLL pathogenesis (Calin et al. 2002). Soon thereafter, the miR-15a/16-1 role as a tumor suppressor was confirmed using a megakaryocytic leukemia cell line, where its expression caused apoptosis and downregulation of BCL-2 (Cimmino et al. 2005). Furthermore, miR-15a/16 induces growth arrest in multiple myeloma cells (Roccaro et al. 2009).

Even more definitive genetic evidence of the tumor suppressive effects of miR-15a/16-1 came recently from the Dalla-Favera lab, where *MDR* and *miR-15a/16-1* knockout mice were generated. Mice of both genotypes initially developed peripheral blood clonal lymphoproliferative disease similar to human monoclonal B cell lymphocytosis (MBL). By 18 months of age, 42% of $MDR^{-/-}$ and 26% or $miR15a/16\text{-}1^{-/-}$ mice developed MBL, CLL and non-Hodgkin lymphoma (Klein et al. 2010). The increase in B cells proliferation detected in both mouse models was due to elevated levels of cell cycle-related proteins that drive exit from quiescence. The authors demonstrated that miR-15a/16-1 target several proteins involved in the G0/G1 to S phase transition: Cyclin D3, Cyclin E, CDK6, CHK1 and MCM5. Interestingly, $MDR^{-/-}$ mice displayed a more aggressive phenotype, suggesting that DLEU2 itself may have important regulatory functions, besides serving as the primary transcript for miR15a/16-1. Alternatively, additional genetic elements within the MDR locus could contribute to its tumor suppressive functions.

Another TSmiR frequently lost in cancer is miR-34a located in the chromosomal region 1p36. This region is recurrently deleted in neuroblastoma, breast, hepatic, pancreatic and colon carcinomas [reviewed in (Bagchi & Mills, 2008)]. Initial evidence suggested that tumor suppressive effects of miR-34a stem from its essential role in the p53 network. As discussed above, miR-34a is transcriptionaly activated by p53 and also enhances its function by targeting the p53 deacetylase SIRT-1, thus creating a positive feed-back-loop [reviewed in (Hermeking, 2010)]. More recently, it has been shown that over-expression of miR-34a induces cell cycle arrest in glioblastoma cell lines by targeting, besides SIRT-1, multiple oncogenes such as Notch-1, Notch-2 and CDK6 (Li et al. 2009). Also, we and others have shown that miR-34a, together with miR-34b and miR34c, target Myc by recognizing one, and potentially more, non-canonical seed sequences in its 3′UTR (Christoffersen et al. 2010; Kong et al. 2008; Sotillo et al, 2011).

As mentioned above, loss of expression of a TSmiR also can be due to epigenetic modifications, such as methylation. miR-34b and miR-34c have been shown to be under epigenetic regulation in gastric carcinoma. In one study, 13 out of 13 gastric carcinoma cell lines tested showed miR-34b and miR-34c epigenetically silenced by hypermethylation of the neighboring CpG island. The same chromosomal region was not methylated in normal gastric mucosa from *Helicobacter pylori*-negative healthy individuals (Suzuki et al. 2010).

Methylation of miRNA-associated CpG islands is not limited to the miR-34 loci. It frequently occurs, for example, at the miR-137 promoter in colorectal carcinoma where it inversely correlates with miR-137 expression. In a set of 409 tissue samples obtained from cancer patients, 82.3% adenomas and 81.4% colorectal carcinomas showed silencing of miR-137 by methylation, versus only 14.4% of non-cancerous samples from the same patients or 4.7% of samples from healthy individuals (Balaguer et al. 2010). Other miRNAs shown to be silenced by hypermethylation in tumor samples or tumor cell lines are: miR-193-b in prostate cancer (Rauhala et al. 2010), miR-124 in cervical cancer (Wilting et al. 2010), miR-107 in pancreatic cancer (Lee et al. 2009), miR-199b-5 in medulloblastoma (Garzia et al. 2009), miR-1-1 in hepatocellular carcinoma (Datta et al. 2008) or miR-203 in Philadelphia chromosome-positive human leukemias (Bueno et al. 2008). Finally, epigenetic modifications are largely responsible for the silencing of miR-200 family members [reviewed in (Mongroo & Rustgi, 2010)], whose importance in cancer will be described below.

Many other miRNAs have been shown to inhibit tumor growth when expressed ectopically. Examples include miR-26, miR-29, miR-30, miR-145, miR-146a, miR-150, miR-181, miR-192, miR-194, miR-215 and the let-7 family of miRNAs (Chang et al. 2008; Shin et al, 2010; Chen et al. 2010; Fu et al. 2010; Pichiorri et al. 2010; Ma et al, 2010; Mott et al. 2007; Pekarsky et al. 2006; Xiong et al. 2010; Gebeshuber et al. 2009; Xu et al. 2009; Lan et al. 2011; Trang et al. 2010; Mayr et al. 2007; Lee & Dutta, 2007; Johnson et al. 2007; Chang et al. 2009; Esquela-Kerscher et al. 2008; Sachdeva et al. 2009). However, the genetic evidence for their bona fide tumor suppressor roles is still lacking. One possible exception is the let-7 family. While its members are too numerous to be eliminated by somatic or germ-line mutations, there exists indirect but intriguing evidence for tumor suppressive effects of let-7. It turns out that a single nucleotide polymorphism at the let-7-complementary site in the KRAS 3′ UTR increases the risk of developing non-small cell lung and ovarian cancers (Chin et al. 2008; Ratner et al. 2010).

4.c) Oncomirs

The term “oncomiRs” is reserved for miRNAs whose constitutive over-expression by genetic means initiates or accelerates cancer formation or progression. Although there seems to be a general trend towards down-regulation of cellular miRNAs expression throughout tumor formation (Lu et al. 2005; Kumar et al. 2007), some miRNAs follow the opposite pattern. The first identified oncomiR (oncomir-1) was the miR17~92 cluster discussed in section 4a. In addition to being a direct Myc target (O’Donnell et al. 2005), miR-17~92 acts as an oncogene in its own right, as evidenced by two key pieces of data. One is that this polycistronic gene (previously known as c13orf25) is located in the chromosomal locus 13q31-q32, which is frequently amplified in solid and hematopoietic tumors (Ota et al. 2004). Not surprisingly, Lowe’s, Hannon’s and Hammond’s labs have found that oncomir-1 accelerates Myc–induced B-cell lymphoma in the Eμ-myc mouse model (He et al. 2005). Interestingly, E2F1, an oncogenic and pro-apoptotic transcriptional target of c-Myc, was found to be repressed by miR-17-5p and miR-20a, two members of the oncomir-1 cluster (O’Donnell et al. 2005). Since then, the list of targets inhibited by oncomir-1 has grown and it now includes, among others, inhibitors of proliferation (CDKN1A) (Inomata et al. 2009; Ivanovska et al. 2008), transcription factors of the E2F family (O’Donnell et al. 2005;

Sylvestre et al. 2007), regulators of angiogenesis (CTGF, Tsp1) (Dews et al. 2006; Ernst et al. 2010), various members of the TGF-β pathway such as type II TGF-β receptor and SMADs (Volinia et al. 2006; Dews et al. 2010; Mestdagh et al. 2010), and direct regulators of apoptosis (Bim, PTEN) (Olive et al. 2009; Xiao et al. 2008; Koralov et al. 2008; Mu et al. 2009). Over-expression of *miR-17~92* gene has also been documented in several solid tumors of breast, lung, colon, stomach, pancreas, and prostate (Volinia et al. 2006; Petrocca et al. 2008).

Another miRNA with oncomiR aspirations is miR-21. Although there is no genetic evidence for its involvement in tumorigenesis, miR-21 over-expression is common in most cancers, its expression is linked to increased cell growth in cancer cells, and data obtained from knock-down of miR-21 in cell lines supports its role as an oncomiR [reviewed in (Selcuklu et al. 2009; O'Day & Lal, 2010; Gabriely et al. 2008)]. Consistent with this idea, among miR-21- targeted genes are several well-recognized tumor suppressors, such as PTEN, PDCD4, TPM-1, Tap63, SPRY2, and hMSH2 depending on the cell type (Meng et al. 2006; Zhu et al. 2008; Papagiannakopoulos et al. 2008; Sayed et al. 2008; Valeri et al. 2010). For example, microarray profiling performed on MCF7 cells showed that knock-down of miR-21 alters expression of 737 genes: 45% of genes were down-regulated and 55% up-regulated. As expected, the set of up-regulated genes was enriched with those with putative target sequences for miR-21 in their 3′UTRs. Of all those putative targets, the authors pinpoint the tumor suppressor Programmed Cell Death 4 (PDCD4) gene as a miR-21 target central to its oncogenic functions (Frankel et al. 2008). Not surprisingly, over-expression of miR-21 in primary breast cancer samples is associated with advance clinical stage, lymph node metastasis and poor prognosis (Yan et al. 2008),

It is important to stress that the oncogenic or tumor suppressor effects of a certain miRNA occur in the context of a given disease. For instance, in prostate cancer, knockdown of miR-21 does not affect cell proliferation or invasiveness. Nor does it modulate PTEN or Pdcd4 (Folini et al. 2010), indicating that in prostate cancer a miR-21 inhibitor has no therapeutic potential by itself. One obvious way the changing cellular context can render miRNA ineffective is by altering the repertoire of its target mRNAs. For example, we have shown that ectopic expression of miR-34a in the context of overexpressed Myc [i.e., in Burkitt's lymphoma cells] does not enhance p53 function via down-regulation of SIRT-1. Instead, it efficiently targets Myc which sustains p53 levels via the Arf-Mdm-2 pathway. As a net result, levels of p53 in miR-34-overexpressing lymphoblasts decrease, as do p53-dependend responses (Sotillo et al, 2011).

4.d) MetastamiRs

From the therapeutic point of view, the ability to prevent tumor cells dissemination outside their primary sites determines how favorable the prognosis will be. As mentioned above, miRNAs involved in the acquisition of invasive abilities are called *metastamiRs*. These miRNAs have been implicated in the maintenance of different components of the metastatic cascade: loss of cellular adhesion, entry into and exit from the vascular system and survival in the vascular system and at distant sites [reviewed in (Yilmaz & Christofori, 2010)]. How does this occur? At the cellular level, there is solid evidence that the epithelial-to-mesenchymal transition (EMT) tightly correlates with the invasive and metastatic phenotype (Yang et al. 2004; Thiery, 2003). During EMT, neoplastic epithelial cells lose their cell-cell adhesion and assume a mesenchymal phenotype by becoming more motile and invasive (Figure 2). EMT is a reversible process (counterbalanced by mesenchymal-to-epithelial transition) and as such, it is regulated by numerous signaling pathways, including Wnt, Notch, TGF-β and Hedgehog. These pathways in turn regulate several transcriptions factors, such as Twist, Snail and ZEB (Comijn et al. 2001; Cano et al. 2000; Thiery, 2003; Yang et

al. 2004; Zhou et al. 2004; Batlle et al. 2000), which directly control cell adhesion and motility genes.

Numerous miRNAs have been shown to be positively or negatively regulated during EMT. Moreover, some of them are known to target many genes that are critical for the regulation of EMT, thus becoming desirable therapeutic targets in metastatic cancers (Zavadil et al. 2007; Wang et al. 2010). Some of these miRNAs are miR-192/215 which target E-cadherin repressors ZEB-1 and ZEB-2 (Wang et al. 2010), miR-10b which is induced by the transcription factor Twist (Ma et al. 2007), miR-9 which directly inhibits E-cadherin (Ma et al. 2010), miR-30, miR-200, and miR-382 which are regulated by the TGFβ pathway (Kriegel et al. 2010; Braun et al. 2010), miR-155 and miR-31 which regulate RhoA (O'Day & Lal, 2010; Kong et al. 2008; Valastyan et al. 2009), miR-181 which inhibits the tumor suppressor metalloprotease inhibitor 3 (TIMP3) (Wang et al. 2009), and miR-205 which targets Erb3 and VEGF-A (Wu et al. 2009). Many other examples were revealed in the recent study by (Castilla et al. 2011).

One of the better studied examples of miRNA involvement in EMT is the miR-200 family, which directly targets ZEB-1 and ZEB-2, potent repressors of E-cadherin (Park et al. 2008; Korpal et al. 2008; Bracken et al. 2008; Gregory et al. 2008). It has been shown that during EMT miR-200 is silenced by methylation of its promoter, leading to upregulation of ZEB-1 and-2, which in turn repress E-cadherin (Vrba et al. 2010; Wiklund et al. 2010). Interestingly, Zeb-1 has been shown to repress transcription of two miR-200 family members, miR-200c and miR-141, resulting in a negative feed-back loop (Burk et al. 2008).

After undergoing EMT, tumor cells still must be able to spread to distant sites, and miRNAs have been strongly implicated in the acquisition of the metastatic behavior. Valuable data explaining how this might occur were recently obtained in Massagué's lab. This group analyzed human lung (CN34-LM1) and bone (CN34-BoM1) metastatic lesions obtained after injection of malignant cells from a metastatic breast cancer patient (CN34) into mice (Tavazoie et al. 2008). They discovered that both CN34-LM1 and CN34-BoN1 cells had lost expression of miR-355, miR-126 and miR-206, relative to parental CN34 cells. Furthermore, exogenous expression of any of the three miRNAs significantly reduced the ability of CN34-LM1 and CN34-BoM1 to metastasize to lung and bone, respectively. Interestingly, these miRNAs were suppressing metastasis by different mechanisms. While miR-126 was inhibiting cell proliferation, miR-355 and miR-206 were targeting cell migration, without affecting the overall mesenchymal phenotype as defined by vimentin expression. Inhibition of migration was due in part to the targeting by miR-355 of the transcription factor SOX4 and the extracellular matrix component tenascin C, both important mediators of metastasis *in vivo.*

Based on these and similar data, a concept has emerged that inhibition of oncomiRs and metastamiRs as well as restoration of TSmiRs hold considerable promise for the treatment of cancer.

5 miRNA-Based Therapies

Given the importance of miRNAs in cancer, several strategies to manipulate miRNA activity and expression are being pursued. Due to the way miRNAs function, they can be targeted at two different levels: reducing/increasing their levels and/or interfering with the miRNA/mRNA interaction. When the levels of an active miRNA are altered, it affects all of its downstream targets, while by modulating mRNA/miRNA interaction higher degrees of specificity could be obtained. Still, the challenges are daunting. In this section we will discuss and evaluate commonly used methods to deliver microRNAs or their inhibitors *in*

vitro and *in vivo*, as well as the strategies to interfere, either directly or indirectly, with miRNA function (Fig.3).

5.a) Delivery Strategies

There are two basic ways to deliver microRNAs into target cells: either in their prêt-à-porter, already-functional version, or as genetic precursors. Both alternatives have their pros and cons, and the choice between the two is usually determined by specific experimental conditions and desired biological scenarios.

Delivery of chemically synthesized oligonucleotides—The main advantage of this method is that the compound is already functional and doesn't need to be further processed. On the other hand, therapeutic oligonucleotides must successfully avoid degradation in the host body, penetrate tumor microenvironment, access the neoplastic compartment, negotiate the intracellular trafficking, localize at the same subcellular compartment as their intended targets, and have specific activity while avoiding off-target effects. The main challenge is that unmodified nucleotides generally have difficulty permeating through the plasma membrane, and once within the cells they are very unstable and prone to degradation. Nevertheless, these two weaknesses could be overcome. The first problem can be solved by using one of the several types of conjugation and/or encapsulation systems to enhance cellular intake and to efficiently deliver oligonucleotides inside the cells. The second solution is to engineer chemically modified oligonucleotides to prevent rapid degradation by nucleolytic enzymes.

One well-known example of these extensively engineered oligonucleotides are **antagomirs** (Krutzfeldt et al. 2005). Antagomirs are 21–23 long ribonucleotide chains wherein 2′-hydroxyl on the ribose is replaced with the 2′-O-methyl group. The backbone is also modified by replacing some of the phosphodiester linkages by phosphothioate ones. These two modifications greatly improve the biostability yielding oligonucleotides more resistant to degradation. To enhance cell penetration, the single-stranded modified RNA is conjugated to a molecule of cholesterol. As an additional clever trick, antagomirs sequences are not perfectly complementary to their targeted miRNA; instead they have base modifications and mispairings that inhibit cleavage by Ago proteins. This results in a near-irreversible binding between the miRNA and the antagomir, which prevents recognition by miRNA of its cognate mRNA target.

The first *in vivo* delivery of antagomirs was achieved by Stoffel's group at the Rockefeller University. Via intravenous injection they successfully delivered antagomirs against miR-16, miR-122, miR-192 and miR-194 and attained long-lasting reduction of these miRNAs in several organs (Krutzfeldt et al. 2005). They further focused on inhibition of miR-122, the most abundant miRNA in the liver and a key regulator of cholesterol metabolism. Not surprisingly, Stoffel and co-authors found that cholesterol biosynthesis was affected and that mice with impaired miR-122 function had lower levels of cholesterol in plasma.

Yet for this to occur milligram amounts of antagomirs had to be delivered (80 mg per kg body weight) rising a question about the feasibility of antagomir use in human subjects – and also about possible off-target effects, especially in organs other than the liver (Morrisey, 2010). The latter problem is illustrated by studies where inhibition of cardiac hypertrophy and fibrosis had been observed in mice using a miR-21 antagomir (Thum et al. 2008) but subsequent genetic ablation of the miR-21 gene failed to elicit a similar cardiac phenotype (Patrick et al. 2010).

Another example of chemically modified oligonucleotides are **Morpholinos.** These are oligonucleotides which contain six-member morpholine rings in place of five-member ribose or deoxyribose residues and are fully resistant to the action of nucleases. Oligomers of 25 morpholinos units are very stable inside the cell and they do not trigger immune responses (Summerton, 1999). To enhance cellular intake, morpholinos can be covalently conjugated with cell-penetrating peptides. These conjugated morphorlinos, called *PPMOs* (*P*eptide-conjugated *P*hosphorodiamidate *M*orpholino *O*ligomers) have been tested in various animal models of Duchenne muscular dystrophy (DMD) [reviewed in (Moulton & Moulton, 2010)] where the antisense oligonucleotide is designed to modify the splicing of the aberrant dystrophin mRNA by skipping the mutated exon (Aartsma-Rus et al. 2004; Alter et al. 2006; McClorey et al. 2006). Furthermore, AVI-4658, a PMO-based drug that targets exon 50 and is delivered by intramuscular injection, is currently in clinical trial for systemic treatment of DMD (NCT00844597) (Kinali et al. 2009).

Locked Nucleid Acids (LNAs) are another version of chemically modified ribonucleotides. In this case, the ribose moiety has an extra bridge connecting the 2′ oxygen and 4′ carbon, resulting in a “locked” conformation. LNAs can be mixed with regular RNA and DNA nucleotides yielding more stable oligomers (Kaur et al. 2006). Currently, a LNA-based miR-122 inhibitor developed by Santaris Pharma is in phase II clinical trial for the treatment of hepatitis C (Lanford et al. 2010).

Another approach to improve cellular intake of oligonucleotides is the use of **liposomal nanoparticles**. Systemic delivery of miR-34a in a lipid-based vehicle by intravenous injection was enough to block proliferation of engrafted subcutaneous human lung cancer cells, inducing tumor cells death without triggering toxicity in liver, kidney or heart, as shown in a recent study from Mirna Therapeutics, Inc. Furthermore, the formulated miR-34a didn’t trigger an immune response (Wiggins et al. 2010).

In another study, direct vaginal and intrarectal administration of siRNA delivered using liposomes (called lipoplexes) was used to target endogenous mRNAs expressed in vaginal and intrarectal mucosae, respectively. The gene silencing effects of the siRNA in the mucosae persisted for up to 7 days, although maximal inhibition was maintained over the first 4 days, with 86% reduction in expression. At day 10 after delivery, expression levels of targeted mRNA were back to normal (Zhang et al. 2006). This promising method has not yet been used to target – or enhance - miRNA expression, but there is no obvious reason why it couldn’t be applied to the microRNA field.

Delivery of genetic precursors—While chemically synthesized oligonucleotides can only provide transient overexpression, the use of genetic precursors could in principle allow for stable continuous expression of the encoded oligonucleotide. Viral and non-viral expression cassettes have been used for decades for the introduction of foreign genetic material into host cells, but there are lingering concerns about their safety and their susceptibility to elimination by the immune system. Furthermore, the designing of expression cassettes is not a straightforward process. The choice of promoter, the length of the genetic precursor and the nature of the targeted cell or tissue must be taken into consideration.

Depending on the nature of the viral vector, the delivered material will be integrated into the host DNA or remain in the episomal state [reviewed in (Verma & Weitzman, 2005)]. Retroviral and lentiviral vectors integrate their DNAs into host genomes, while adenoviral vectors replicate as autonomous units. Because the site of integration is unpredictable, there is always the risk of insertional mutagenesis and activation of proto-oncogenes. Apparently, retroviruses are more likely than lentiviruses to integrate in places that potentially cause

cancer (Cattoglio et al. 2007; Montini et al. 2006; Montini et al. 2009). A classical example of unintended consequences of retroviral gene therapy is the development of T-cell lymphomas in patients with X-linked SCID. This happened as a result of retroviral transfer of the γ-chain gene into autologous bone marrow-derived CD34+ cells (Howe et al. 2008; Hacein-Bey-Abina et al. 2003; Hacein-Bey-Abina et al. 2008). In those cases, supposedly beneficial retroviral vector was found integrated next to promoter regions of one of several proto-oncogenes: *LMO2, BIM1 and CCDN2*, leading to their aberrant activation. This scenario could also unfold in case of microRNA-encoding retroviruses. Another drawback of retroviral vectors is that their use is limited to actively dividing target cells..

Lentiviral and adenoviral particles can in principal be used for both dividing and quiescent cells. Nevertheless, because adenoviral DNA is neither integrated nor replicated with each cell division, they are recommended mainly for stable expression in non-dividing cells, to prevent dilution of the adenoviral genomes. Another caveat of using adenoviruses is that they induce strong immunological responses, although new adenoviral species are being tested that do not unnecessarily provoke the immune system [reviewed in (Nayak & Herzog, 2010)].

The use of viral expression vectors also limits the size of the DNA that can be inserted. Thus, it is important to determine the minimal amount of genetic material required to drive expression of mature miRNA mimics or inhibitors. At first approximation, this issue was resolved in the study from Cullen's lab where a 70-nucleotide sequence corresponding to the miR-30 precursor was cloned into a plasmid under the control of the CMV promoter (Zeng et al. 2002). This plasmid was transiently transfected into 293T cells, resulting in the production of mature miR-30. This exogenously encoded miR-30 was able to induce degradation of an artificial mRNA target in 293T, HeLa and NIH3T3 cells. In contrast when the same plasmid was engineered to encode just the mature 22-nucleotide RNA sequence, no functional miR-30 was expressed, which is consistent with our current knowledge of miRNA biogenesis. However, it remains to be determined whether clusters of microRNAs could be expressed in a way that faithfully reproduces processing of the primary transcript.

The introduction of miRNA-encoding genes into retroviral cassettes was pioneered by Bartel's lab. Similarly to what Zeng and collaborators had observed, Bartel and coauthors established that for successful miRNA expression, they needed the entire pre-miRNA-coding DNA plus 125 nucleotides of genomic sequence flanking each side of the miRNA for proper processing and maturation (Chen et al. 2004). Armed with this knowledge, they used retroviral constructs expressing precursors of miR-181, miR-223, miR-142 or miR-30 to infect hematopoietic progenitor cells from mouse bone marrow to study the effects of ectopic expression of these miRNAs on hematopoietic lineages in vitro. They also transplanted these cells into lethally irradiated mice, resulting in reconstitution of all blood lineages but with different ratios of each depending on the manipulated miRNA (Chen et al. 2004). Subsequently, bone marrow transplantation of hematopoietic cells with manipulated miRNA levels was widely used to study the role of miRNAs in normal hematopoiesis (Starczynowski et al, 2010; O'Connell et al. 2010) and in hematological malignancies (Olive et al. 2009; Chang et al. 2008; Mu et al. 2009). The latter studies built on the original observation that bone marrow transplantation of hematopoietic progenitor cells from Eμ-myc mice overexpressing the miR17~92 cluster resulted in accelerated Myc-induced B-lymphomagenesis (He et al. 2005).

5.b) Strategies to alter miRNA function

As stated above, to rescue expression levels of a TS-miRNA, either dsRNA identical to the mature miRNA duplex (miRNA mimics) or cDNA encoding the miRNA of interest could be used. Similarly, oncomiR or metastamiR activities can be targeted by either directly

inhibiting their expression using antisense oligonucleotides or by interfering with the miRNA/mRNA interaction using miRNA decoys and target protectors. All these strategies enjoyed some measure of success in the last few years, albeit in preclinical settings.

i) Delivery of miRNA mimics—In vivo small RNA delivery techniques used to be challenging but are clearly improving. In a very recent high-profile paper, Liu and coauthors observed that systemic delivery of miR-34a inhibited prostate cancer metastasis and extended survival of tumor-bearing mice, at least in part by targeting CD44 (Liu et al. 2011). The delivery was achieved using intratumoral and, notably, intravenous injection and newly emerging delivery reagents such RNALancerII (BIOO Scieintific) and siPORT™ amine (Ambion).

ii) Delivery of miRNA genetic precursors—In a study from Slack's laboratory, adenoviral particles expressing miRNAs were delivered intranasally using a mouse model of lung cancer (Esquela-Kerscher et al. 2008). Specifically, mice with mutant *K-ras* were infected with an adenovirus expressing let-7a pre-miRNA (Ad-let-7) along with the β-galactosidase marker to track adenoviral expression. The authors detected β-galactosidase staining within 72 hours after intranasal infection in cells surrounding the bronchial tree, and the signal persisted in the lungs for as long as 2 weeks. What is more important is that the size of K-ras-induced lung tumors was reduced by 66–90% in mice treated with Ad-let-7.

In parallel, Mendell's group demonstrated that systemic delivery of a miR-26a expressing adenoviral vector by intravenous injection resulted in impaired Myc-induced hepatic cancer progression by inducing tumor-specific cell cycle arrest and apoptosis (Kota et al. 2009). Undoubtedly such approaches will be rigorously tested in other pre-clinical models as well. However, their utility is probably limited to miRs with proven tumor suppressor qualities, that is to say 5 to 10 microRNAs (see above). On the other hand, inhibition of microRNA function could probably be performed on a larger repertoire, as detailed below.

iii) Antisense oligonucleotides—Cell culture experiments using antagomirs helped identified numerous miRNAs involved in tumor proliferation or resistance to drugs, as well as their cognate target genes. For instance, antagomirs against miR-221 and miR-222 impair proliferation of cancer cells lines by targeting of p27 (le Sage C. et al. 2007), while antagomirs against miR-27a and miR-451, which regulate MDR1/P-glycoprotein, increase cancer cell sensitivity to therapeutic drugs, such as vinblastine, that are pumped out by P-glycoprotein (Zhu et al. 2008).

Antagomirs are also beginning to prove their worth in xenograft models. For example, Fontana *et al.* showed that tumors generated from MYCN-amplified LAN-5 neuroblastoma cells completely regress in 30% of the cases upon injection of the antagomir against miR-17-5p (Fontana et al. 2008). Using a more complicated tumor progression model, the Massagué laboratory showed that poorly metastatic breast cancer cells treated with antagomir against miR-355 and reinjected into mice are more capable of colonizing lungs that control antagomir-treated cells (Tavazoie et al. 2008).

To be sure, making tumors more metastatic is not a therapeutic goal; however approaches like this are useful for determining what microRNAs drive tumor dissemination. Indeed, in a recent paper from Weinberg's lab, systemic administration of miR-10b antagomirs to mice bearing highly metastatic breast cancer cells had no effect on primary tumors but markedly suppresses formation of lung metastases (Ma et al. 2010). This work was based on the previous identification of miR-10b as a metastamiR [(Ma et al. 2007), see above]. Likewise, the above-mentioned miR-34a delivery study was based on the initial observation that

antagomirs against miR-34a, when introduced into prostate cancer cells, promoted tumor development and metastasis (Liu et al. 2011).

iv) miRNA Decoys—One notable disadvantage of antisense inhibitors is that they cannot be stably expressed or target multiple members of the same miRNA family. This lead to the idea of competitive inhibitors of miRNA function, or "sponges", developed by Sharp's group. Instead of inhibiting miRNAs via direct binding, sponges were designed to inhibit miRNA activity by providing target sites that will function as decoys, namely by attracting multiple miRNA family members and letting the cognate mRNA target escape inhibition (Ebert et al. 2007). An miRNA sponge consists of a strong promoter driving expression of an artificial transcript that carry numerous tandem repeats complementary to the heptameric seed sequence of an specific miRNA (or family of miRNAs.) The efficiency of a sponge depends on the affinity of its binding sites and on the miRNA:sponge ratio. It has been shown that sponges with bulged sites that are unpaired at miRNA positions 9–12 are better baits. A very recent review on sponges by Ebert and Sharp summarizes currently available miRNA sponge constructs and their applications, together with information about the composition of the expression-cassettes (Ebert & Sharp, 2010). Of note, sponge cassettes can be readily delivered by viral vectors. These viral vectors typically have 4 to 10 miRNA-targeted sites separated by several nucleotides, and they often include selection markers, such as fluorescent tags or antibiotic resistance genes. This makes possible the isolation, follow-up and subsequent analysis of sponge-expressing cells.

One recent example of the use of sponges came from Weinberg's lab, where the researchers targeted miR-31 proving its role in the development of breast cancer metastasis (Valastyan et al. 2009). Specifically, the authors cloned a miR-31 sponge into a retroviral vector and infected non-metastatic MCF7-Ras cells. This procedure yielded a stable sponge-expressing cell line (MCF7-Ras-sp31) with 4.5-fold less miR-31 activity than control cells. When these cells were orthotopically implanted into mice, there was no difference in primary tumor growth or proliferation but MCF7-Ras-sp31cells were more invasive and metastasized to the lungs at 10-times the frequency of control cells.

A more recent example of the use of sponges helped confirm the function of miR-15/16 as tumor suppressors and validated some of their targets in multiple myeloma. Carrasco's lab showed that *in vitro* transduction of MM cells with a lentiviral vector targeting the miR-15/16 seed sequence was sufficient to increase cell proliferation 1.5–2 fold and invasiveness in matrigel experiments 3-fold (Gatt et al, 2010). Not surprisingly, intravenous injection of miR-16 sponge-transduced cells into sublethally-irradiated mice accelerated disease progression and enhanced metastatic potential. The inhibition of the miR-16 family also induced an increase in cell resistance to conventional MM therapies: adriamycin and melphalan, confirming the therapeutic importance of the miR15a/16-1 cluster.

v) Target protection—Although morpholinos were initially designed to block mRNA translation (Taylor et al. 1996; Wienholds et al. 2003) they are currently being directed towards different aspects of the miRNA activity. First, they can bind directly to the miRNA functional guide strand incorporated into the miRISC complex, thus inhibiting binding to target mRNA. Second, they can bind pri-miRNA hairpin or pre-miRNA stem-loop, inhibiting processing by Drosha or Dicer, respectively. Third, morpholinos can be used as "target protectors", designed to bind and protect the target sequence on the mRNA in such a way that the miRNA cannot access it (Choi et al. 2007). The great advantage of target protection is its specificity, leading to inhibition of miRNA activity towards a single mRNA target, while all other miRNA loss-of-function methods affect all downstream targets indiscriminately.

So far, target protectors have been used mostly in zebrafish developmental biology to elucidate the role of microRNAs in patterning and cell fate determinaiton (Choi et al. 2007; Leucht et al. 2008; Li et al. 2008; Zeng et al. 2009). Most recently, the Lodish and Lim laboratories used morpholinos against miR-125b in zebrafish to demonstrate its role in regulation of p53 *in vivo* (Le et al. 2009). They first showed that ectopically expressed miR-125b represses endogenous p53 expression and p53-induced apoptosis in human cells. However, the analysis of zebrafish development also allowed the authors to observe an inverse correlation between endogenous miR-125b expression and p53 activity during zebrafish embryogenesis. The injection of morpholinos (125bMO) against the mature guide strand or against the three alternative miR-125b precursors almost completely blocked the expression of mature miR-125b. By performing injections at the one-to-four-cells stage (18-14 hours post fertilization), they observed increased apoptosis in the embryonic brain, along with higher levels of p53 protein expression. Furthermore, injection of miR-125b mimics into 125bMO embryos rescued the phenotype by restoring the normal levels of p53. Finally, inhibition of p53 activation and expression either by injection of miR-125b mimics or morpholinos against p53, prevented apoptosis when embryos were γ-irradiated or treated with camptothecin. In an important follow-up study, the effects of miR-125b on p53 were reproduced in a prostate cancer xenograft model, where the levels of this microRNA correlated with neoplastic growth (Shi et al. 2011)

Also relevantly to the field of cancer therapeutics, a recent version called *vivomorpholinos* has been developed to be used in adult animals including vertebrates (Morcos et al. 2008). Vivo-morpholinos can be delivered systemically via intravenous injection or to specific organs by localized injection. So far, vivo-morpholinos have been successfully used in xenopus (Matsuda & Shi, 2010), newt (Maki et al. 2010), zebrafish (Carrillo et al. 2010; Guo et al. 2010), and finally mice (Parra et al, 2010; Kang et al. 2010; Azoitei et al. 2010). Although none of these studies involves targeting of miRNA activity or expression, custom-made morpholinos are now available for research purposes from Gene Tools, LLC.

6. The promise of miRNAs as Therapeutic Adjuvants

Contrary to what occurs in other genetic diseases where a single mutation is responsible for a given phenotype, most human cancers arise from several genetic mutations that accumulate over time. In fact, human tumorigenesis is a dynamic biological process where tumor cells evolve to overcome their limitations and escape therapeutic interventions (Merlo et al. 2006).

Having considered this natural selection process, it is hard to imagine cures based on inhibition or enhancement of a single miRNA, even after repeated treatments. Moreover, it has been shown that long-term exposure to short dsRNAs results in the saturation of the endogenous RNAi machinery. This saturation leads to a shortage of proteins required for the processing of miRNAs, such as exportin-5, resulting in high organ toxicity and, in some cases, death (Grimm et al. 2006).

This leads us to believe that miRNAs would have more therapeutic value as acute, rather than chronic adjuvant targets. This utility is supported by numerous studies showing that miRNAs affect sensitivity to different chemotherapeutic agents in multiple tumor types. Admittedly, most of these are *in vitro* gain- or loss-of-function experiments, where candidate miRNAs are first identified in tumor cell lines with different degrees of resistance to specific chemotherapeutic drugs and subsequently targeted in order to overcome drug resistance. Although the *in vitro* studies discussed below still await *in vivo* validation, the chemosensitization effects appear to be very robust.

One of the first studies linking miRNA levels and chemoresistance was performed in human cholangiocarcinoma cell lines (Meng et al. 2006). In this study, levels of expression of several miRNAs were compared in malignant and non-malignant human cholangiocytes. Not only the authors identified miR-21, miR-141 and miR-200b as highly over-expressed in malignant cells, but they also discovered that while inhibition of miR-141 primarily decreases cell growth, inhibition of miR-21 and miR-200b increase sensitivity to gemcitabine. Another example of miRNAs involvement in chemoresistance is the work by Kovalchuk and collaborators (Kovalchuk et al. 2008). First, by microarray analysis, the authors compared the miRNA profiles of MCF-7 breast adenocarcinoma cells resistant or sensitive to doxorubicin. They identified 137 differentially regulated miRNAs (75 down-regulated, 63 upregulated). Of these, miR-451 silencing was critical for the acquisition of drug resistance, due to its targeting of the P-glycoprotein (Mdr1) gene. Many more studies on the same subject came out in the last two years (Table 2).

We have recently shown that inhibition of miR-34a in Myc-transformed B-cells enhances apoptotic responses to the protease inhibitor bortezomib (Sotillo et al, 2011). This is because in cells with the functional Myc-Arf-Mdm2 axis miR-34a downregulates steady-state levels of p53, which is a key determinant of bortezomib sensitivity (Yu et al. 2007). In contrast, ectopic expression of miR-34a in colorectal cancer cells resistant to treatment with 5-fluorouracile (5-FU), inhibited cell proliferation but also sensitized cells to 5-FU treatment (Akao et al. 2011). Thus, once again, the involvement of microRNAs in chemoresistance is context dependent, and close attention needs to be paid to the repertoire of relevant microRNA targets in a given cancer or cell type.

7. Conclusions and Perspectives

There is no doubt that miRNAs play critical roles in cancer initiation and progression. The proof of this principle came from numerous animal models as well as human cancer genetics, in particular the studies on hematological malignancies. In these neoplasms often addicted to a single genetic lesion or pathway, manipulating microRNA levels is proving to be a viable therapeutic option, especially in light of recent advances in the field of small RNA delivery. However, there is a concern that in genetically complex solid cancers these single-pronged attacks might ultimately prove unsuccessful. Thus, the idea that targeting of microRNAs should be considered as an adjuvant therapy is getting considerable traction. Basic, translational, and clinical research done in the next few years will tell whether this promise is well-founded.

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Abbreviations

Ago	Argonaute
dsRNA	double stranded RNA
EMT	Epithelial to Mesenchimal Transition
mRNA	messenger RNA
miRNA	microRNA

RISC RNA Induced Silencing Complex

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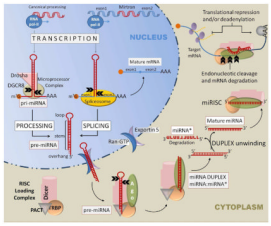

Figure 1. miRNA Biogenesis

Canonical miRNA and mirtron processing pathways are depicted. See text for more explanations. Double black arrows indicate cleavage sites.

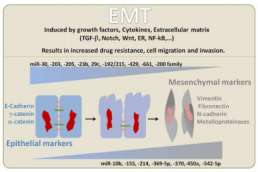

Figure 2. EMT and miRNAs

Some of the miRNAs regulated during and implicated in EMT.

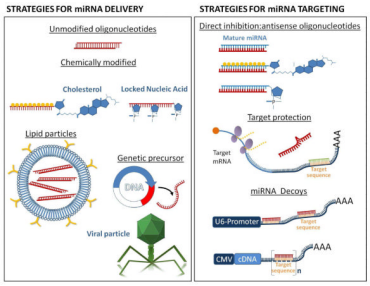

Figure 3.
Strategies for miRNA delivery and targeting.

Table 1

Computational tools for miRNA target prediction and miRNA databases.

Computational Tools for miRNA Target Prediction (excluding miRNAs from plants)		**References**
TargetScan	http://www.targetscan.org	(Lewis et al. 2005)
PicTar	http://www.pictar.org	(Krek et al. 2005)
RNA22	http://cbcsrv.watson.ibm.com/rna22.html	(Miranda et al. 2006)
TarBase	http://diana.cslab.ece.ntua.gr/tarbase/	(Sethupathy et al. 2006)
PITA	http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html	(Kertesz et al. 2007)
microRNA	http://www.microrna.org/microrna/home.do	(Betel et al. 2008)
Diana-microT	http://diana.cslab.ece.ntua.gr/microT/	(Maragkakis et al. 2009)
miRecords	http://mirecords.biolead.org/	(Xiao et al. 2009)
Starbase	http://starbase.sysu.edu.cn/	(Yang et al. 2010)
miRNAs Databases (excluding miRNAs from plants)		**References**
miRNAMap	http://mirnamap.mbc.nctu.edu.tw/	(Hsu et al. 2006)
miRBASE	http://www.mirbase.org/	(Griffiths-Jones et al. 2008)
microRNA	http://www.microrna.org/microrna/home.do	(Betel et al. 2008)
coGemiR	http://cogemir.tigem.it/	(Maselli et al. 2008)
miRGEN	http://www.diana.pcbi.upenn.edu/miRGen.html	(Alexiou et al. 2010)
deepBase	http://deepbase.sysu.edu.cn/	(Yang et al. 2010)

Table 2

miRNAs linked to chemoresistance or chemosensitivity to chemotherapeutic agents in human cancer.

Cancer Type	miRNA	Drug	Targeted gene	Reference
miRNAs whose expression increased sensitivity to chemotherapeutic drugs				
Lung	miR-1	Doxorubicine	MET, Pim-1	(Nasser et al. 2008)
	miR-16	NSC236613, NSC670550		(Blower et al. 2008)
	miR-21	NSC63878, NSC265450		(Blower et al. 2008)
Multidrug-resistant gastric cancer	miR-15b	Vincristine, adriamycin, cisplatin, ateposide	BCL2	(Xia et al. 2008)
	miR-16			
Prostate Cancer	miR-34a	Camtothecin	SIRT-1	(Fujita et al. 2008)
Gastric cancer (p53mutant, high BCL2)	miR34a miR43b miR34c	Doxorubicine, cisplatin, gemcitabine, docetaxel	Bcl-2, Notch, HMGA2	(Ji et al. 2008)
Gastric cancer/Cisplatin^{res}*	miR-200c	Cisplatin, Ψ5-FU, paclitaxel, and adriamycin	E-cadherin	(Chen et al. 2010)
Hepatocellular carcinoma	miR-101	Etoposide, curcumin, doxorubicine	Mcl-1	(Su et al. 2009)
	miR-199a-3p	Doxorubicine	mTOR, c-Met	(Fornari et al. 2010)
	miR-122		Cyclin G1	(Fornari et al. 2009)
Breast cancer/doxorubibine^{res}	miR-451	Doxorubicine	Mdr1	(Kovalchuk et al. 2008)
Breast cancer	miR-200b		Ferritin	(Shpyleva et al, 2010)
Breast cancer/VP-16res	miR-326	Doxorubicine, VP-16	MRP-1	(Liang et al. 2010)
Glioblastoma	miR-451	Imitanib	SMAD3, SMAD4	(Gal et al. 2008)
Leukemia/doxorubicine^{res}	miR-331-5p miR27a	Doxorubcin	P-glycoprotein	(Feng et al. 2010)
Leukemia/VCR**	miR-138	Vincristine, adriamycin, cisplatin, 5-FU	P-glycoprotein, MDR1, BCL2	(Zhao et al. 2010)
Small cell lung carcinoma	miR-134 miR-379 miR-495	Cisplatin, Etoposide and Doxorubicin	MRP1/ABCC1	(Guo et al. 2010)
miRNAs whose inhibition increased sensitivity to chemotherapeutic drugs				
Lung	Let-7i	NSC670550		(Blower et al. 2008)
	miR-21	NSC621888, NSC622700, NSC670550, NSC122750		(Blower et al. 2008)
Ovarian Cancer	miR-214	Cisplatin	PTEN	(Yang et al. 2006)
Breast Cancer	miR-221	Tamoxifen	ER-γ	(Zhao et al. 2008)
	miR-222			

Cancer Type	miRNA	Drug	Targeted gene	Reference
	miR-21	Topotecan	PTEN	(Meng et al. 2006)
Neuroblastoma	miR-17-5p	Doxycline	p21, BIM	(Fontana et al. 2008)
Malignant Cholangiocytes	miR-21	Gemcitabine	PTEN	(Meng et al. 2006)
	miR-200b			
Malignant Cholangiocytes/IL6	Let-7a	Gemcitabine, 5-FU, Campothecin		
Glioblatoma	miR-21	NSC670550, NSC122750		(Blower et al. 2008)
Burkitt's Lymphoma	miR-34a	Bortezomib	c-Myc	(Sotillo et al, 2011)
Leukemia	miR-21	Arabinosylcytosine	PDCD4	(Li et al. 2010)
Pancreatic adenocarcinoma	miR-21	5-FU		(Hwang et al. 2010)
Multiple Myeloma	miR-15a/16-1	adriamycin and melphalan	FGFR1, PI3KCa, MDM4, VEGFa	(Gatt et al, 2010)
miRNAs whose inhibition decreased sensitivity to chemotherapeutic drugs				
Ovarian Cancer	miR-21	NSC265450		(Blower et al. 2008)
	Let-7i	Cisplatin	H-RAS, HMGA2	(Yang et al. 2008)
Glioblastoma	miR-21	NSC123127		(Blower et al. 2008)
miRNAs whose expression decreased sensitivity to chemotherapeutic drugs				
HNSCC†	miR-98	Cislatin, doxorubicine	HMGA2	(Hebert et al. 2007)
SCC, HCC §	Let-7a	Doxorubicine, paclitaxel, IF-γ	RAS, HMGA2	(Tsang & Kwok, 2008)
Hepatocellular carcinoma	miR-188b	Doxorubicine	metalloprotease 3	(Wang et al. 2009)
Breast cancer	miR-221 miR-222	Tamoxifen	P27	(Miller et al. 2008)

* res: Resistant;

** VCR: Vincristine-induced multidrug resistance;

† HNSCC: Head and Neck Squamous Cell Carcinoma;

§ SCC, HCC: Squamous cell carcinoma, Hepatocellular Carcinoma.

ψ 5-FU: 5-Fluorouracil.