MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review

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Discovery, biogenesis and mechanisms of action

Approximately 20 years ago, investigators first determined that components of the genome traditionally considered nonfunctional had, in fact, gene regulatory capacity. MicroRNAs, initially discovered in 1993, when a small RNA encoded by the lin-4 locus was associated to the developmental timing of the nematode Caenorhabditis elegans by modulating the protein lin-14 (Lee et al, 1993), have then been revealed as essential part of the uncoding genome, playing a crucial role through a complicated gene regulation in all the most important processes and in different species, including vertebrates (Lagos-Quintana et al, 2001; a list of miRNA databases is reported in Table 1).

MicroRNAs are transcribed for the most part by RNA polymerase II as long primary transcripts characterized by hairpin structures (pri-miRNAs), and processed into the nucleus by RNAse III Drosha into 70–100 nts long pre-microRNAs (Lee et al, 2004). Drosha is a highly conserved 160 kDa protein containing two RNAse III domains and one double-strand RNA-binding domain. Drosha forms a huge complex, 500 kDa in Drosophila melanogaster (D. melanogaster) and 650 kDa in Homo sapiens, called Microprocessor and containing the co-factor Di George syndrome critical region 8 (DGCR8), also known as Pasha in D. melanogaster and C. elegans.

An alternative miRNA biogenesis pathway, called miRtron pathway, has been discovered among diverse mammals, drosophila and nematodes (Berezikov et al, 2007; Okamura et al, 2007; Ruby et al, 2007): miRtrons are regulatory RNAs, which get processed to form pre-miRs using the splicing machinery without Drosha-mediated cleavage.

The originated precursor molecules are exported by an Exportin 5-mediated mechanism to the cytoplasm (Yi et al, 2003), where an additional step mediated by the RNAse III Dicer, which acts in complex with the transactivating response RNA-binding protein (TRBP), generates a dsRNA approximately 22 nts long, named miRNA/miRNA*, including the mature miRNA guide, and the complementary passenger strand, the miRNA* (star miRNA; many publications refer to the two strand pair as...
miR-3p/miR-5p, referring to the direction of the functional miRNA). Whereas one of the two strands is selected as guide strand according to thermodynamic properties, the complementary one is usually subjected to degradation. The so-called miRNA* was initially thought to be the strand subjected to degradation, instead more recent evidence suggests that it does not simply represent a non-functional bioproduct of miRNA biogenesis, but it can be selected as a functional strand and play significant biological roles (Bhayani et al., 2011).

Dicer are very large enzymes (~200kDa) conserved among the species and containing different domains: a double strand RNA-binding domain (dsRBD), two RNase III catalytic domains, one PAZ domain, which binds the 3'-end of small RNAs, and other domains with ATPasic and RNA-helicasic activity. Dicer recognizes the double strand region of the pre-miRNA in association with different proteins: RDE-4 (RNA interference; RNAi defective 4) in C. elegans, R2D2 e FMR1 (fragile X mental retardation syndrome 1 homolog) in D. melanogaster and members of the Argonaut family in other species. In particular these proteins are not needed for the endonucleasic activity of Dicer but they play a role in stabilizing the complex Dicer-miRNA. In mammalians the Argonaut 2 (AGO2) protein complex, characterized by RNASp H activity, cooperates in the Dicer-mediated processing of some pre-miRNAs, yielding to another intermediate processing product, called AGO2-cleaved precursor miRNA (ac-pre-miRNA; Krol et al., 2010).

Completed the processing steps, the mature single stranded miRNA product is then incorporated in the complex known as

### Glossary

**AntagomiR**
Class of chemically engineered oligonucleotides able to silence endogenous miRNAs. This definition was used for the first time by Krutzfeldt et al. (2005), who developed a pharmacological approach for silencing miRNAs in vivo, designing chemically modified, cholesterol-conjugated single-stranded RNA analogues, complementary to miR-122.

**Biomarker**
Any parameter, molecule or protein, which can be used as indicator of a particular physiological or diseased state, disease progression, outcome and response to therapies.

**Dicer**
Endoribonuclease belonging to the RNase III family that cleaves double-stranded RNA (dsRNA) and pre-microRNA (miRNA) into short dsRNA fragments.

**Drosha**
RNase III enzyme responsible for initiating the processing of microRNA into the nucleus, where long primary transcripts characterized by hairpin structures (pri-miRNAs) are cleaved to generate 70–100 nts long pre-miRNAs.

**Epigenetic regulation**
Regulation of gene expression or cellular phenotypes caused by mechanisms that do not involve chromosomal aberrations or changes in the DNA sequence hence the name epi- (Greek: επί- over, above and outer) genetics.

**LNA-anti-miR**
Antisense RNA oligonucleotide modified to increase stability and specificity. In details, the ribose moiety of an LNA nucleotide is modified with an extra bridge connecting the 2'-oxygen and 4'-carbon, which 'locks' the ribose in the 3'-endo conformation. LNA nucleotides can be mixed with DNA or RNA residues in the oligonucleotide whenever desired.

**miRNAm**
The miRNA profiling describing the expression of all miRNAs in a specific biological context.

**miR-mask**
Single-stranded 2'-O-methyl-modified (or other chemically modified) antisense oligonucleotide fully complementary to predicted miRNA binding sites in the 3'-UTR of a specific target mRNA. The miR-mask is thus able to cover up the access of the miRNA to its binding site on the target mRNA, so as to impair its inhibitory function.

**miR-Risc**
Ribonucleoproteic complex (RNA-induced silencing complex) where the mature single stranded miRNA product is incorporated and which guides its inhibitory function on the target mRNA.

**miRNA sponge**
miRNA inhibitory transgene expressing an mRNA containing multiple tandem binding sites for an endogenous miRNA, and thus able to stably interact with the corresponding miRNA and prevent the association with its endogenous targets.

**MetastmiR**
MicroRNA playing a crucial role in the metastatic process.

**Oncogene**
Gene causing or contributing to tumour occurrence or progression. Oncogenes are generally mutated forms of normal cellular genes (proto-oncogenes). A gene capable, when activated, of transforming a cell.

**OncomiRNA**
MicroRNA playing an oncogenic role by targeting oncosuppressor molecules.

**Tumour suppressor gene**
Tumour suppressor gene (also called anti-oncogene) is a gene able to protect a cell from uncontrolled proliferation and other aberrant events leading to cancer development. When this gene is mutated to cause a loss or reduction in its function, the cell can progress to cancer, usually in combination with other genetic changes.

**Tumour suppressor miRNA**
MicroRNA playing an oncosuppressive role by targeting oncogenes.
miRNA-containing ribonucleoprotein complex (miRNP), miRNA-containing RNA-induced silencing complex (miRgonaut or miR-RISC), a ribonucleoprotein complex containing Argonaut proteins, of which AGO1 and 2 have been the most extensively studied, the two miRNA strands and several additional factors, including the trans-activator RNA binding protein (TRBP).

As a part of this complex, the mature miRNA is able to regulate gene expression at post-transcriptional level, binding for the most part through partial complementarity to target mRNAs, and mainly leading to mRNA degradation or translation inhibition.

More in detail, guided by the base pairing between the non-coding RNA and the target mRNA, miRNA-RISC-mediated gene inhibition can be split into three processes: (i) site-specific cleavage; (ii) enhanced mRNA degradation; and (iii) translational inhibition. The initial process, commonly defined as RNAi and restricted to miRNAs with a perfect or near-perfect match to the target RNA, is a very rare event in mammals, exclusively Ago2 dependent. Instead, the other two processes are more commonly associated with mismatched miRNA/target sequences that are the most likely scenario in mammals. The combination of these two processes is commonly defined as a non-cleavage repression, and can be carried out by any of the four mammalian Ago proteins (Su et al, 2009). Interestingly, recent genetic (Brodersen et al, 2008) and biochemical (Filipowicz et al, 2008) studies have also established that plant miRNAs can translationally repress their target mRNA, despite the near-perfect base pairing between the microRNA and target mRNA sequence.

However, the exact mechanism through which miRNAs can impair translation is still debated.

Moreover, even though it is known that microRNAs mainly recognize complementary sequences in the 3'-untranslated regions (UTRs) of their target mRNAs, more recent studies have reported that they can also bind to the 5'-UTR or the ORF (Lytle et al, 2007; Moretti et al, 2010; Ørom et al, 2008; Qin et al, 2010) and, even more surprisingly, they can upregulate translation upon growth arrest conditions (Vasudevan et al, 2007).

Finally, whereas the 5'-end of the microRNA (the so-called ‘seed site’) has always been considered the most important for the binding to the mRNA, recently the target sites have been further divided into three main classes, according to grade and localization of the complementarity (Brennecke et al, 2005): the dominant seed site targets (5' seed-only), the 5' dominant canonical seed site targets (5' dominant) and the 3' complementary seed site targets (3' canonical).

Considering the different rules regulating the interaction between a microRNA and its target mRNA, it is not surprising that each miRNA has the potential to target a large number of genes (Betel et al, 2008; Friedman et al, 2009; Krek et al, 2005; Lewis et al, 2005). Equally, roughly 60% of the mRNAs share one or more sequences that are evolutionarily conserved and predicted to interact with miRNAs. Bioinformatics analysis predicts that 3'-UTRs of single genes are often targeted by several different miRNAs (Lewis et al, 2005). Many of these predictions have been validated experimentally, suggesting that miRNAs might cooperate to regulate gene expression (a list of computational tools for miRNA target prediction is reported in Table 2).

To complicate the already intricate scenario, it has been recently reported that miRNAs can bind to ribonucleoproteins in a seed sequence and a RISC-independent manner and then interfere with their RNA binding functions (decoy activity; Beitzinger & Meister, 2010; Fig 1). Three studies have reported that miRNAs can also regulate gene expression at the transcriptional level by direct binding to the DNA (Gonzalez et al, 2008; Khraiwesh et al, 2010; Kim et al, 2008).

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

### MicroRNA expression in human cancer: diagnostic applications

The first evidence of the involvement of microRNAs in human cancer derived from studies on chronic lymphocytic leukemia (CLL), particularly in an attempt to identify tumour suppressors at chromosome 13q14, frequently deleted in CLL. Dr. Croce’s group reported that rather than containing a protein coding tumour suppressor gene, this critical region contains in fact two microRNA genes, miR-15a and miR-16-1, expressed in the same polycistronic RNA. This result provided the first evidence that microRNAs could be involved in the pathogenesis of human cancer as the deletion of chromosome 13q14 caused the loss of these two microRNAs (Calin et al, 2002). Indeed, study of a large collection of CLLs showed knock down or knock out of miR-15a and miR-16-1 in approximately 69% of CLLs.

Following these initial observations, the same group mapped all the known microRNA genes and found many of them located

| Name            | Website                                      | Reference       |
|-----------------|----------------------------------------------|-----------------|
| TargetsScan     | 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://www.microrna.org/microrna/home.do     | 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/       | Maragakis et al (2009) |
| miRecords       | http://mirecords.biolead.org/                | Xiao et al (2009) |
| Starbase        | http://starbase.sysu.edu.cn/                 | Yang et al (2011) |
in chromosomal loci prone to deletions or amplifications, as was found in many different human tumours (Calin et al, 2004). Indeed, chromosomal regions encompassing microRNAs involved in the negative regulation of a transcript encoding a known tumour suppressor gene can be amplified in cancer development. This amplification would result in the increased expression of the microRNA and consequent silencing of the tumour suppressor gene. Conversely, microRNAs repressing oncogenes are often located in fragile loci, where deletions or mutations can occur and result in reduced microRNA levels and overexpression of the target oncogene (Fig 2).

Therefore, alterations of microRNA expression are not exceptional but rather the rule in human cancer. Next, as an attempt to establish whether microRNA profiling could be used for tumour classification, diagnosis and prognosis, different platforms to assess the global expression of microRNA genes in normal and diseased tissues were developed (Calin & Croce, 2006): after an extensive use of custom-made (Liu et al, 2004) and then commercial miRNA microarrays, and bead-based flow cytometric miRNA analysis methods (Lu et al, 2005), the last generation of large-scale profiling method is represented by the high-throughput deep sequencing (Creighton et al, 2009; Farazi et al, 2011).

Genome-wide profiling showed that miRNA expression signatures (miRNome) allowed different types of cancer to be discriminated with high accuracy (Lu et al, 2005; Volinia et al, 2006) and the tissue of origin of poorly differentiated tumours to be identified. By contrast, mRNA profiles were highly inaccurate indicators of tissue or cancer type.

Indeed, miRNA mis-expression patterns are more accurate in identifying the origin of tumours that are otherwise difficult to be determined as the tumour spread to multiple metastatic sites, suggesting that tumours more clearly maintain a unique ‘tissue miRNA expression profile’. One study, for example, developed a classifier of 48 miRNAs from a sample of 336 primary and metastatic tumours, and was able to use this classifier to accurately predict the tissue origin in 86% of a blind test set, including 77% of the metastatic tumours (Rosenfeld et al, 2008). Given that cancers of undefined origin account for approximately 4% of all malignancies and are associated with poor prognosis (Oien & Evans, 2008), the continued development of miRNA classifiers has foreseeable benefits in aiding clinical diagnosis and subsequent treatment.

Another major issue in clinics is clearly represented by the need of biomarkers for an early diagnosis, extremely important considering that survival and prognosis of patients depends on the stage of the tumour at the time of detection, with an early diagnosis usually been associated with the best prognosis. MicroRNAs have revealed a great potential as new potential early diagnosis biomarkers: overexpression of miR-205 and miR-21 in ductal adenocarcinoma, for example has been reported to precede phenotypic changes in the ducts, thus suggesting the possibility to use them for an early detection of this neoplasm (du Rieu et al, 2010).

Furthermore, miRNAs are certainly more stable due to their small size as compared to long mRNAs, allowing expression profiling from fixed tissues or other biological material, and thus supporting their possible use as novel, minimally invasive and robust biomarkers. Certainly, miRNAs can be reliably extracted and detected from frozen and paraffin-embedded tissues, from blood (either total blood, plasma or serum; Mitchell et al, 2008; Schwarzenbach et al, 2011), circulating exosomes (Taylor & Gercel-Taylor, 2008), and from different biologic fluids like urine (Hanke et al, 2010), saliva (Michael et al, 2010; Park et al, 2009) and even sputum (Xie et al, 2010; Yu et al, 2010). Moreover, the profile of circulating miRNAs from individuals affected by different neoplasias was shown to reflect the pattern observed in the tumour tissues, suggesting the attractive possibility of using circulating miRNAs as easily detectable tumour biomarkers (Lawrie et al, 2008), especially for early diagnosis (Heneghan et al, 2010; Huang et al, 2010; Xing et al, 2010). More recently, by analysing plasma samples of lung cancer patients collected 1–2 years before the onset of disease,
Sozzi’s group (Boeri et al, 2011) has found microRNA signatures with strong predictive diagnostic and prognostic potentials. miRNA profiles can distinguish not only between normal and cancerous tissue and identify tissues of origin, but they can also discriminate different subtypes of a particular cancer, or even specific oncogenic abnormalities. Gene expression profiling has already demonstrated its effectiveness at subtyping various cancers, however, miRNA profiles are equally discriminatory and can even be more informative, as expression changes can provide insights into the multitude of gene permutations observed in various cancer subtypes: links have indeed been made between misregulated miRNAs and the target genes that are affected, thus unraveling some of the unique gene networks involved (O’Day & Lal, 2010). miRNAs, for example, are differentially expressed between basal and luminal breast cancer subtypes (Blenkiron et al, 2007; Sempere et al, 2007), and can specifically classify estrogen receptor (ER), progesterone receptor (PR) and HER2/neu receptor status (Iorio et al, 2005; Lowery et al, 2009; Mattie et al, 2006). Some of the miRNAs associated with luminal or basal subtype, respectively, reflect their epithelial and myoepithelial origins. For instance, miR-200 family associates with the luminal subtype, and it is not surprising since they directly target the EMT regulators ZEB1 and ZEB2 (Gregory et al, 2008), whereas miR-145 and miR-205, preferentially expressed in normal myoepithelial cells, are dramatically reduced in basal-like triple negative tumours (ER−/PR−/HER2−), suggesting that this expression change might be a consequence of disease progression in this subtype (Sempere et al, 2007). Other examples are the differential expression of microRNAs according to specific histotypes of ovarian carcinoma (Iorio et al, 2007) and the ability of miR-205 expression to discriminate squamous from non-squamous non-small cell lung carcinoma (Lebanony et al, 2009).

Beside the expression profile studies based on microarray platforms, many other methods for detecting microRNAs have been developed, as quantitative real-time polymerase chain reaction (RT-qPCR; Chen et al, 2005; Raymond et al, 2005), in situ hybridization (de Planell-Saguer et al, 2010; Nuovo et al,
MicroRNAs as prognostic and predictive biomarkers

Being able to discriminate tumour origins, subtypes, oncogenic mutations and cancer predisposition, and regulating the most important cellular processes, it is logical to hypothesize that miRNAs might be able to predict also cancer prognosis and/or response to specific therapies. Recently, several groups have reported success in utilizing miRNAs as prognostic markers to predict cancer outcome, thereby addressing this issue. After the first evidence in CLL, where a unique microRNA signature was associated with prognostic factors and disease progression in CLL (Caliń et al., 2005) and lung cancer, where miR-155 overexpression and let-7a downregulation were able to predict poor disease outcome (Yanaihara et al., 2006), several other reports have supported the significance of microRNAs as prognostic biomarkers. For example in gastric cancer a robust 7-miRNA signature can predict overall survival and relapse-free survival (Li et al., 2010). Similarly, low miR-191 and high miR-193a levels were associated with a significantly shorter survival time as measured by Kaplan–Meier curves in melanomas (Caramuta et al., 2010).

Although predicting survival might be important in a more general sense, the prediction of response to specific therapies is of far greater clinical value. For instance, low levels of miR-26 are an independent predictor of poor survival in patients suffering from hepatocellular carcinoma (HCC); notably, however, patients with low miR-26 responded well to interferon-α treatment, resulting in improved survival (Ji et al., 2009). Therefore, miR-26 expression might be a useful biomarker to discriminate patients who could benefit most from an interferon-α therapy. The other way around is also true, as several miRNAs have been correlated with a poor response to specific treatments. In various cancers, increased miR-21 expression is an indicator of poor outcome (Dillhoff et al., 2008; Rossi et al., 2010; Schetter et al., 2008) and it is also sufficient to predict poor response to adjuvant chemotherapy in adenocarcinomas (Schetter et al., 2008).

High levels of miR-125b in breast cancer predict poor response to taxol-based treatments in vitro (Zhou et al., 2010), and a similar finding has been reported for miR-21 in pancreatic cancer patients treated with gemcitabine (Giovannetti et al., 2010). Beside the use as predictive biomarkers, the correlation between microRNA expression and response to specific therapies has also suggested their promising potential as therapeutic adjuvant, even though this hypothesis mostly derives from in vitro studies of gain or loss of function, where candidate miRNAs are initially identified in tumour cell lines with different degrees of resistance to specific therapeutic drugs and then targeted in order to overcome drug resistance. One of the first reports describing the involvement of microRNAs in chemoresistance was performed in cholangiocarcinoma cell lines (Meng et al., 2006), where inhibition of miR-21 and miR-200b increased sensitivity to gemcitabine. This first evidence was followed by many other studies (Chen et al., 2010a; Kovalchuk et al., 2008; Xia et al., 2008). Beside chemotherapy, microRNAs can also improve the responsiveness to targeted therapies: overexpression of miR-221 and miR-222 is responsible for resistance to anti-estrogenic therapies, as Tamoxifen (Miller et al., 2008; Zhao et al., 2008), and Fulvestran (Rao et al., 2011), whereas ectopic expression of oncosuppressor miR-205 is able to improve the responsiveness to tyrosin kinase inhibitors through direct targeting of HER3 (Iorio et al., 2009).

These associations highlight not only the importance of microRNAs as predictive biomarkers, but also the possibility to use them as an alternative approach for tackling drug resistance.
Mechanisms of miRNAs deregulation: from genetic abnormalities to epigenetic regulation

Regulation of microRNA expression can be exerted through several mechanisms, which result to be altered in human diseases, including cancer: chromosomal abnormalities, as suggested by the evidence that microRNAs are frequently located in regions of the genome involving in alterations in cancer (Calin et al, 2004), and confirmed by several studies (Calin et al, 2002; Tagawa & Seto, 2005; Zhang et al, 2006); mutations, as the inherited mutations in the primary transcripts of miR-15a and miR-16-1 responsible for reduced expression of the two microRNAs in vitro and in vivo in CLL (Raveche et al, 2007); polymorphisms (SNPs), as described in lung cancer (Hu et al, 2008).

In addition to structural genetic alterations, microRNA expression can be also modulated as a consequence of defects in the microRNA biogenesis machinery, as supported by changes in microRNA levels consequent an altered Drosha or Dicer activity in different tumour types (Merritt et al, 2008; Nakamura et al, 2007; Thomson et al, 2006). Moreover, it has been reported that also deregulation of different cofactors can affect miRNA expression with important biological implications: DGR8 knock out mice arrest early in development and have defects in ES cell proliferation and differentiation (Wang et al, 2007); Lin-28, originally discovered as a heterochronic gene regulating developmental timing in worms, is able to block let-7 biogenesis and it is activated in many human tumours (15%), being particularly associated with less differentiated cancers (Viswanathan et al, 2008).

The deregulated microRNA expression in cancer can also be due to epigenetic changes, as altered DNA methylation. An extensive analysis of genomic sequences of miRNA genes have shown that approximately half of them are associated with CpG islands, suggesting that they could be subjected to this mechanism of regulation (Weber et al, 2007). Several reports have indeed shown that aberrant methylation can result in aberrant microRNA expression in cancer: by treating T24 bladder cancer cells and human fibroblasts with DNMT inhibitor 5-Aza-2'-deoxycytidine, Saito and colleagues (Saito et al, 2006) reported a strong upregulation of miR-127, a microRNA characterized by a CpG island promoter able to target the proto-oncogene BCL-6, found silenced in several cancer cells. With the same approach of unmasking epigenetically repressed microRNAs inducing chromatin-remodelling by drug treatment, hypermethylation and down-modulation of miR-9-1 has been described in breast cancer (Lehmann et al, 2008), as well as the clustered miR-34b and miR-34c in colon cancer (Toyota et al, 2008).

Conversely, upregulation of putative oncogenic microRNAs can result from DNA hypomethylation, as shown in lung adenocarcinoma for let-7a-3 (Brueckner et al, 2007) or epithelial ovarian cancer for miR-21 (iorio et al, 2007).

A differential methylation approach was used to identify epigenetically regulated microRNAs by profiling DNMT1- and DNMT3b-deficient colorectal cancer cells: out of 18 upmodulated microRNAs in comparison to WT cells, the only unmethylated in normal tissue but hypermethylated and silenced in tumour, was miR-124a, which embedded in a large CpG island and is able to target cyclin D kinase 6, thus mediating the phosphorylation of RB tumour suppressor gene (Lujambio et al, 2007).

Methylation is not the only epigenetic modification that can affect microRNAs expression: Scott et al (Scott et al, 2006) showed that in SKBR3 breast carcinoma cells, an extensive and rapid alteration of microRNA levels followed histone deacetylase inhibition.

Epigenetic drugs, such as DNA demethylating agents and histone deacetylase inhibitors, are able to reverse an aberrant methylation or acetylation status, thereby raising the intriguing possibility of also restoring the expression of tumour suppressor microRNAs, and reverting a tumoural phenotype.

To complicate the scenario, however, microRNAs themselves can regulate the expression of components of the epigenetic machinery, creating a highly controlled feedback mechanism: miR-29 family can directly target the de novo DNA methyltransferases DNMT-3A and -3B, while indirectly, through regulation of the transactivator Sp1, maintain the DNA methyltransferase DNMT1. Interestingly, introduction of miR-29s into lung cancers and AMLs was shown to result in reactivation of silenced tumour suppressors and inhibition of tumourigenesis (Fabbrì et al, 2007; Garzon et al, 2009). Loss of miR-290 cluster in Dicer-deficient mouse embryonic stem cells leads to the upmodulation of the RBL-2, which repressed DNMT3a, DNMT3b and DNMT1 (Benetti et al, 2008; Sinkkonen et al, 2008); miR-1, involved in myogenesis and related diseases, directly targets HDAC4 (Chen et al, 2008).

Finally, miRNA dysregulation can result from increased or decreased transcription activity of a transcription factor at the promoter. The miR-34a, miR-34b and miR-34c family of miRNAs, for instance, was shown to be directly induced by the tumour suppressor p53 and to be partially responsible of the phenotype induced by this oncossuppressor (Chang et al, 2007; He et al, 2007).

Vice versa, oncogenes can also affect microRNA expression, and a clear example is represented by the oncoprotein MYC, which is able to both induce oncogenic microRNAs, as the miR-17-92 cluster, and negatively regulate transcription of tumour suppressor miRNAs, such as let-7 (Chang et al, 2009) and miR-29 family members (Mott et al, 2010).

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

MicroRNAs as oncogenes or oncosuppressor genes: functional evidence

Over time, cancers have developed sophisticated networks of biological activities allowing them to develop and, in some cases, evade treatment. This complex program relies on the communication between multiple cell types, including the primary tumour as well as the stromal cells. Hanahan and
Weinberg described six essential features of cancer progression: self-sufficiency in growth signals, insensitivity to anti-growth signals, apoptosis evasion, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (Hanahan & Weinberg, 2000). Dysregulated miRNAs may function as either tumour suppressors or oncogenes in cancer by targeting each one of these features (Fig 3).

Gain- and loss-of-function experiments, together with target prediction analyses, have provided insights into the role of miRNAs in carcinogenesis.

Computational algorithms for target identification (Table 2), mainly based on the free binding energy between a miRNA and a putative target mRNA sequence, are by definition prediction tools, which need an experimental validation. The validation method most commonly used is represented by a reporter assay, where the co-transfection of the miRNA of interest and the tested 3’-UTR cloned downstream a luciferase gene reduces the reporter activity, inhibitory effect reverted by mutating the miRNA binding sequence of the target mRNA. However, even though this method suggests a physical and functional interaction between a miRNA and its target, it does not prove it directly. To this aim, more rigorous pull-down assays have been designed, as immunoprecipitation of labelled miRNA/mRNA complexes and consequent target identification by RT-PCR and sequencing (Hsu et al, 2009), or immunoprecipitation with Ago2 antibody, thus isolating the ternary, presumably functional, miRNA/mRNA/Ago2 complex (Chi et al, 2009).

However, the rules of miRNA/target mRNA regulation are even more complicated than expected. A very recent report by Pandolfi’s group (Salmena et al, 2011) has introduced the revolutionary concept that miRNA effect on mRNA containing common miRNA recognition elements (MREs) can be affected by ceRNAs (competing endogenous RNAs): RNA transcripts, both protein coding and non-coding, can compete for miRNA binding, thus co-regulating each other.

Gain-of-function approaches have shown that miRNAs acting as tumour suppressors target oncoproteins with crucial roles in various cancer pathways, such BCL2 (targeted by miR-15a–miR-16-1; Cimmino et al, 2005), RAS (regulated by let-7; Johnson et al, 2005), myeloid cell leukaemia sequence 1 (BCl-2-related, MCL1, and targeted by miR-29; Garzon et al, 2009; Mott et al, 2007) and MYC (targeted by let-7; Sampson et al, 2007). Vice versa, to assess the biological effects of oncogenic miRNAs, often overexpressed in cancer cells, in vitro silencing was carried out using antisense oligonucleotides. For example miR-21 expression has been reported at high levels in breast (Iorio et al, 2005), glioblastomas (Ciafre et al, 2005), pancreas (Bloomston et al, 2007) and colon cancer (Schetter et al, 2008) among others. Chan and colleagues blocked miR-21 expression in glioblastoma cell lines and reported an increased activation of

**Figure 3.** MicroRNAs targeting the hallmarks of cancer.
caspases and apoptosis (Chan et al, 2005). Additional studies showed that miR-21 anti-apoptotic effects occurs by targeting the tumour suppressors phosphatase and tensin homologue (PTEN) and programmed cell death 4 (PDCD4) (Frankel et al, 2008; Meng et al, 2007). More recently, Slack’s group (Medina et al, 2010) has shown that mice conditionally expressing miR-21 develop a pre-B malignant lymphoid-like phenotype, thus demonstrating that miR-21 is a genuine oncogene.

miR-17-92 cluster and miR-155, both discovered to be overexpressed in lymphoproliferative disorders, including lymphomas and leukaemia (Garzon et al, 2008; He et al, 2005), were the first examples of miRNAs with oncogenic activity validated in engineered animal models. Infection of murine haematopoietic stem cells with a retrovirus carrying the miR-17-92 cluster accelerated the development of lymphomas in Myc transgenic mice (He et al, 2005). Transgenic mice overexpressing miR-17-92 cluster in B cells were discovered to develop lymphoproliferative disease and autoimmunity (Xiao et al, 2008). Lymphocytes higher rate of proliferation and lower rate of activation-induced cell death in these mice were partially accredited to the direct targeting of the anti-apoptotic genes Bim and Pten by miR-17-92 cluster. Moreover, Ventura and colleagues showed that mice deficient for miR-17-92 cluster die shortly after birth with lung hypoplasia and a ventricular septal defect (Ventura et al, 2008). Finally, Mu and colleagues showed that deletion of the complete miR-17-92 cluster slows down Myc-induced oncogenesis (Mu et al, 2009).

In contrast, miR-155 overexpression in the lymphoid compartment was sufficient to cause cancer without any other cooperative mutation or Myc expression. miR-155 transgenic mice developed polyclonal lymphoid proliferation followed by acute lymphocytic lymphoma or leukaemia (Costinean et al, 2006). These data were the first to report that the dysregulation of a single miRNA can lead to malignancy. Further, it was one of the first, and still of the few, microRNA engineered animal models, which, through knock out or transgene introduction, provide the genetic demonstration of the causative involvement of a specific microRNA in a biological phenomenon.

In addition to classical tumour suppressor or oncogene functions, miRNAs have been implicated also in cell migration and metastasis. The highly expressed miR-10b in metastatic breast cancer, positively regulates cell migration and invasion (Ma et al, 2007). Elegant experiments confirmed that overexpression of miR-10b in non-metastatic breast cancer cells initiates invasion and metastasis. The authors showed that these effects are mediated by direct targeting of HOXD10 by miR-10b, facilitating the overexpression of the well-known pro-metastatic gene RHOC. Besides, recently, the same lab reported that silencing miR-10b can inhibit metastasis in a breast cancer mouse model, thereby highlighting the therapeutic potential of targeting metastasis-associated miRNAs (Ma et al, 2010). Another study showed that miR-126 and miR-335 act as negative regulators of tumour invasion and metastasis in human breast and lung cancer (Tavazoie et al, 2008).

MiR-34a, lost in several tumour types and involved into the network mediated by the well known ‘genome guardian’ p53 (He et al, 2007), inhibits migration and invasion by down-regulation of MET expression in human HCC cells (Li et al, 2009).

Because the ‘epithelial–mesenchymal transition’ (EMT) is thought to promote malignant tumour progression, several groups have recently reported that microRNAs are involved in this process. Indeed, members of miR-200 family of microRNAs and miR-205 have been shown to reduce cell migration and invasiveness targeting ZEB transcription factors, known inducers of EMT (Gregory et al, 2008) and PKCζ in prostate cancer (Gandellini et al, 2009). The oncogenic miR-21 stimulates invasion, extravasation and metastasis in different tumour types, included colorectal cancer (Asangani et al, 2008) and breast cancer (Zhu et al, 2008), while oncosuppressor miR-205 has opposite effects, reducing invasion in vitro and suppressing lung metastasis in vivo (Wu et al, 2009).

Interestingly, it has been observed that primary tumours and metastasis from the same tissue show a similar pattern of microRNAs expression (Rosenfeld et al, 2008). microRNA profiling is a more accurate classifier than mRNA profiling, and thus has the potential to solve one of the most challenging issues in cancer diagnostic: the origin of metastasis of unknown primary tumours.

During metastatic process, neo-angiogenesis is the crucial step allowing cells to reach and disseminate through the systemic circulation. MicroRNAs can control tumour progression at this level, either promoting or inhibiting the proliferation of endothelial cells. In endothelial cells, miR-221 and miR-222 repress proliferative and angiogenic properties of c-Kit, while hypoxic signals reduce expressions of miR-16, miR-15b, miR-20a and miR-20b, directly targeting VEGF, and supporting the angiogenic process (Poliseno et al, 2006). Angiogenesis can be also promoted by miR-210, activated by hypoxia and directly repressing endothelial ligand Ephrin A3 (Pulkkinen et al, 2008). miR-17-92 cluster sustains MYC angiogenic properties by repressing connective tissue growth factor (CTGF) and anti-angiogenic adhesive glycoprotein thrombospondin 1 (TSP1; Dews et al, 2006), also targeted by miR-27b and let-7i (Kuehbacher et al, 2007).

It is unlikely that miRNAs will be found responsible for a specific phenotype by aiming at a specific target. Instead, it is largely accepted that miRNAs engage in complex interactions with the machinery that controls the transcriptome and concurrently target multiple miRNAs. This is probably the most intriguing rationale supporting the idea of using microRNAs as anticancer drugs.

**MicroRNAs as therapeutic targets or tools**

One of the most appealing properties of miRNAs as therapeutic agents, and probably the most important advantage in comparison with approaches targeting single genes, is their ability to target multiple molecules, frequently in the context of a network, making them extremely efficient in regulating distinct biological cell processes relevant to normal and malignant cell biology.
One example was clearly reported by Chen and colleagues, who demonstrated that miR-181 has a critical role in the regulation of T cell receptor sensitivity targeting multiple phosphatases, effect that was not obtained silencing single component of the pathway by RNAi (Li et al, 2007). Moreover, O’Day and colleagues (O’Day & Lal, 2010) underlines how microRNAs altered in breast cancer regulate a network of interconnected molecules, where the central node is represented by Myc. The same group had already provided evidence that miR-24 inhibits proliferation through direct targeting of c-Myc, EZF1 and a number of related molecules (Lal et al, 2009).

The first indications of the feasibility and the efficacy of a microRNA-based therapy in cancer, using these small molecules as both targets and tools, came from preclinical models aimed to understand the biological role of a specific miRNA. Reintroduction of miR-15a/16-1, for example induces apoptosis in both targets and tools, came from preclinical models aimed to understand the biological role of a specific miRNA. Reintroduction of miR-15a/16-1, for example induces apoptosis in under the response to targeted therapies or chemotherapies, thus suggesting their possible use also as adjuvant tools.

In summary, there are two main strategies to target miRNA expression in cancer. Direct strategies involve the use of oligonucleotides or virus-based constructs to either block the expression of an oncogenic miRNA or to reintroduce a tumour suppressor miRNA lost in cancer. Indirect strategies involve the use of drugs to modulate miRNA expression by targeting their transcription and their processing.

Nevertheless, stability and effective delivery into target tissues remain a major hurdle for direct microRNA-based therapy. Indeed, the first challenging aspect in delivering a therapeutic RNA is represented by its exiting the circulatory system, transiting the cell membrane and escaping from endosomal vesicles into the cytoplasm. Moreover, the size of an unconjugated therapeutic RNA is 7–20 kDa, and molecules less than 50 kDa are filtered by the kidney and excreted. Finally, systemic delivery into the bloodstream is challenged by phagocytic immune cells, such as macrophages and monocytes, which remove complexed RNAs from the body. To overcome these issues, several modifications, some of which already developed for siRNAs, can be applied to miRNAs.

For reduction in mature microRNA levels (due to mutation or any other mechanisms like defects in processing machinery or aberrant transcription), the therapeutic approach could be to exogenously deliver synthetic double-stranded hairpin by complexing with lipids or delivery proteins. As reported by Tazawa et al (Tazawa et al, 2007), miR-34a transiently inhibits human colon cancer tumour progression when administered subcutaneously in complexes with atelocollagen, which was recently shown to be a very powerful system for efficient delivery of small interfering RNA molecules into tumours. Chen and colleagues have developed a LPH (liposome–polycation–hyaluronic acid) nanoparticle formulation modified with tumour-targeting single chain antibody fragment (scFv) for systemic delivery of miR-34a to lung metastasis of murine melanoma cells (Chen et al, 2010b).

Unmodified dsRNAs are vulnerable to nucleases in vivo, which limits the use of this class of compound to privileged local environments where local administration is possible. Using a conditional mouse lung cancer model, where conditional expression of oncogenic K-ras could be activated, Esquela-Kerscher et al showed that the intranasal administration of an adenovirus expressing let-7a RNA hairpin reduced tumour formation in vivo (Esquela-Kerscher et al, 2008). In 2009, Kota et al (Kota et al, 2009) used a systemic administration of miR-26a in a mouse model of HCC using adenovirus-associated virus (AVV) to inhibit cancer cell proliferation and induction of tumour-specific apoptosis. These results are consistent with previous findings made by that same group, which demonstrated that MYC-induced liver tumours result in concomitant downregulation of various microRNAs (Chang et al, 2008).

In short term experiments of cardiac hypertrophy, conducted by Carè et al (Care et al, 2007), overexpression of miR-133 by adenovirus delivery significantly reduced the expression of foetal genes and resulted in reduced size of left ventricular cardiac myocytes. For stable miRNA reintroduction, the expression can be enforced by a viral vector with Pol III promoter, including U6, H1 and rRNA (Kawasaki & Taira, 2003; Tiscornia et al, 2003; Xia et al, 2004), upstream an artificial short hairpin RNA (shRNA), which bypasses Drosha processing but is caved and loaded into miRISC by Dicer. The advantage of Pol III promoters is to provide high expression of miRNAs from well-defined transcription start and termination sites; however, these promoters have no cell specificity. In addition, exceedingly high levels of shRNA expression increase the probability of off-target silencing, elicit non-specific effects such as interferon response and can also saturate Exportin5 pathway of endogenous miRNAs with fatal consequences (Grimm et al, 2006).

Alternatively, the pri-miRNA can be expressed from an RNA Pol II promoter, leaving open the possibility for tissue-specificity or induced ectopic miRNA expression. Furthermore, miRNAs are known to be downstream Pol II promoters, within known protein coding genes and expressed by Pol II activity (Lee et al, 2004). Therefore, strategies using Pol II-directed synthesis of shRNA that mimic the natural miRNA synthesis could be an efficient therapeutic approach.

However, it is important to underline the possible hazards in reintroducing a microRNA with a viral system. Depending on the nature of the system, the delivered material can be integrated into the host DNA or remain episomal: retroviral and lentiviral vectors integrate their DNA into the host genome, whereas adenoviral vector replicates as autonomous unit. Because the site of integration is unpredictable, there is always a risk of insertional mutagenesis and activation of proto-oncogenes. Another drawback of retroviral vectors is that their use is limited to actively dividing cells. Adenoviral vectors, which could be used in principle for both dividing and quiescent cells, are in fact mainly recommended for stable expression in non-dividing cells, to prevent dilution of the adenoviral genome (which
remains not integrated into the host genome). Another limitation of using adenoviruses is the strong immunological response that they potentially induce, even though new species are being tested to reduce these effects (reviewed in Nayak & Herzog, 2010).

To achieve miRNA loss-of-function, chemically modified antimiR oligonucleotides (AMOs) have been developed (Weiler et al., 2006). The most important property of these oligonucleotides is their specificity and high binding affinity to RNA and a number of them has been pursued in clinical studies. miRNA down-regulation has been achieved using 2-O-methyl oligonucleotides (Hutvagner et al., 2004; Meister et al., 2004). miR-122 inhibition was obtained by treating mice with AMOs containing 2-O-methoxethyl groups resulted in lower plasma cholesterol (Esau et al., 2006). Intravenous administration of cholesterol-conjugated AMOs against miR-16, miR-122, miR-192 and miR-194 resulted in a significant reduction of corresponding miRNA levels in liver, lung, kidney, heart, intestine, fat, skin, bone marrow, muscle, ovaries and adrenals (Krutzfeldt et al., 2005; Weiler et al., 2006).

Very interestingly, Ma and colleagues, after demonstratig the crucial role of miR-10b as metastmiR in breast cancer, have exploited a possible therapeutic application, reporting that systemic treatment of tumour-bearing mice with miR-10b antagonirs suppresses breast cancer metastasis (Ma et al., 2010).

Other modified AMOs are represented by locked nucleic acid (LNA)-oligonucleotides, able to inhibit exogenously introduced miRNAs with high specificity (Ørom et al., 2006). The first clinical trial in human of LNA-anti-miR (a placebo-controlled, double-blind, randomized, single dose, dose-escalating safety study of SPC3649 in a total of 64 healthy male volunteers) has been conducted by Santaris to study SPC3649 (LNA-antimiR-122; ClinicalTrials.gov, Identifier: NCT00688012). miR-122 is an abundant miRNA in the liver. The hepatitis C virus (HCV) genome harbours two closely spaced miR-122 target sites in the 5′ non-coding region required for HCV replication. Kauppinen and colleagues showed that administration of LNA-anti-miR into mice resulted in a dose-dependent depletion of mature miR-122 (Elmen et al., 2008b). An efficacy study conducted by Elmen et al on non-human primates (Elmen et al., 2008a), showed a dose-dependent sequestration of mature miR-122 and a long lasting decrease of total plasma cholesterol.

Nevertheless, the same limitations encountered with the application of synthetic miRNA duplexes are encountered in the applications of antagonirs, namely their effective delivery into target tissues.

An interesting approach to overcome these problems is to target miRNA by saturating them with target miRNAs. Ebert et al (Ebert et al., 2007) developed miRNA inhibitory transgenes, called ‘miRNA sponges’, expressing an mRNA containing multiple tandem binding sites for an endogenous miRNA and thus able to stably interact with the corresponding miRNA and prevent the association with its endogenous targets. Both designed polymerase Pol II- and Pol III-driven miRNA sponges showed more efficiency for miRNA inhibition compared to standard 2′-O-Me antagonirs.

Lentiviral vectors have proven to be effective tool to ectopically express miRNAs using suitable transcriptional control units. It has been reported by Gentner et al (Gentner et al., 2009) that stable miRNA-223 knock down can be achieved in vivo by transducing bone marrow stem and progenitor cells with multiple target sequences from strong promoters and transplanting them into lethally irradiated congenic recipients. They showed that overexpressing miR-targets specifically affects the targeted miRNA rather than saturating the effector pathway. However, the need for strong promoters and multiple vector integrations to obtain a high miR-target expression could increase the risk of insertional mutagenesis in target cells, potentially confounding the identification of miRNA knock down phenotypes, and thus representing a potential limitation of this strategy.

miRNA-masking antisense oligonucleotides technology (miR-mask) is another strategy developed by Choi and colleagues (Choi et al., 2007). In contrast to miRNA sponges, miR-masks consist of single-stranded 2′-O-methyl-modified antisense oligonucleotides fully complementary to predicted miRNA binding sites in the 3′-UTR of a specific target miRNA. Although unwanted effects or off-target effects can be dramatically reduced with this approach, this may be a disadvantage for cancer therapy for which the targeting of multiple pathways might be desirable.

Su et al (Su et al., 2011) have applied a nanotechnologic approach to the use of anti-miRNAs: systemic delivery of a chemically stabilized anti-miR-122 complexed with interfering nanoparticles (iNOPs) effectively silences the liver-expressed miR-122 in mice, resulting in lowering of plasma cholesterol.

Beside targeted therapies and chemotherapies, microRNAs could also alter the sensitivity to radiotherapy, as recently reported by Slack’s group (Weidhaas et al., 2007): a potential therapeutic use for anti-miR-34 as a radiosensitizing agent in p53-mutant breast cancer could be envisaged; in lung cancer cells, let-7 family can suppress the resistance to radiotherapy, likely through RAS regulation.

Evidence described up to date provides the experimental bases for the use of microRNAs as both targets and tools in anticancer therapy, but there are at least two primary issues to address to translate these fundamental research advances into medical practice: development of engineered animal models to mimic the complexity of human cancer, and effective delivery vehicles for the use of synthetic microRNAs.

Moreover, all the strategies developed so far to modulate miRNA expression are designed to only modify one miRNA or a family of miRNAs. As miRNAs coordinate in cancer pathogenesis and the phenotypical effects result from multiple interactions between miRNAs and the transcriptome, it is reasonable to search for strategies that aim at re-programming aberrant miRNA networks in cancer.

A possible approach might be represented by the modulation of microRNA expression by targeting components of the biogenesis machinery, or elements of the regulatory networks, as the epigenetic program. Demethylating agents as Decitabine and 5-azacytidine, for example, are currently approved for the treatment of myelodysplastic syndrome, although they have shown activity in many other malignancies, including AML (Galm et al., 2006). These drugs are known to inhibit DNA methyltransferases, resulting in tumour suppressor gene re-expression. As previously described, miRNAs have also
been shown to be actively re-expressed after treatment with these drugs and to largely contribute to the therapeutic effects of these compounds. Even though it is tempting to suggest that many of the biological effects of these drugs may be mediated by the re-expression of non-coding RNAs, this still needs to be verified.

Moreover, it may sound surprising that reprogramming of the whole ‘microRNAome’, including both oncomiRNAs and tumour suppressor miRNAs, can lead to a specific anti-tumoural effect: how is the balance shifted in favour of a specific effect? This might be due to the possibility that most microRNAs seem to exert a role as oncosuppressors, and consequently are mostly downregulated in human neoplasia (Lu et al, 2005). Even though still debated and controversial, this hypothesis is supported by the evidence that Dicer or Drosha silencing promotes cellular transformation and tumourigenesis in vivo: conditional loss of Dicer1 in the lung tissues of mice enhances the development of lung tumours in a K-ras mouse model (Kumar et al, 2009). Finally, loss of Dicer and/or Drosha has also been inversely correlated with outcome in lung cancer (Karube et al, 2005), cancers of the ovarian epithelium (Merritt et al, 2008), and more recently in other tumour types as nasopharyngeal carcinoma (Guo et al, 2012), neuroblastoma (Lin et al, 2010) and breast (Grelier et al, 2009).

A few reports, however, describing a positive correlation between Dicer expression and poor outcome in colorectal cancer (Faber et al, 2011) and in prostate cancer (Chiosea et al, 2006), or the overexpression of Drosha in cervical cancer (Muralidhar et al, 2011), raise the important issue to validate this still debated question, and verify whether the effect of targeting the microRNA machinery might be tissue-related.

To make it even more complicated, recently Piccolo’s group (Martello et al, 2010) described a microRNA family, miR-103-107, able to empower the metastatic potential targeting Dicer and thus attenuating the global microRNA biosynthesis, with a particular effect mediated by the downregulation of miR-200 family, and consequent switch to a more mesenchymal and aggressive phenotype.

Conclusions and future perspectives

The past decade has witnessed an explosion of research focused on small non-coding RNAs. From viruses to plants, to humans, these small RNA regulators of gene expression have been demonstrated to be involved in every biological system.

Cancer is defined by abnormal and uncontrolled cell division, a phenotype that arises from the misregulation of several genes. miRNAs are major regulators of gene expression, with roles in nearly every area of cell behaviour, development and survival; therefore, it is not surprising that miRNAs are actively altered in all types of cancers (Croce, 2009), acting as oncogenes or tumour suppressor genes.

However, although significant advances have been made for the future role of miRNAs in diagnostics, there have been far

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### Pending issues

- A complete understanding of the complex mechanisms regulating the interaction between microRNAs and their targets, to unravel the networks regulated by these small molecules and exploit the possibility to modulate their activity with therapeutic purposes, is mandatory. To this aim, important issues to take into account are the potential tissue specificity, the possible side or off target effects and the existence of several other players able to affect miRNA function on a specific miRNA, as the competition for miRNA binding exerted by other coding and non-coding RNAs.

- To have a clear, genetic demonstration of the involvement of a specific miRNA in the occurrence or progression of a specific tumour, engineered mouse models, as reported in few cases to date, need to be developed.

- To use miRNAs as reliable diagnostic, predictive or prognostic biomarkers, the development or optimization of efficient, sensitive and reproducible detection methods are of primary importance. Indeed, reported lack of consistency between different studies certainly gives rise to some concern, and may arise from sample selection or preparation, experimental design and/or data analysis, suggesting the need of a more comprehensive validation.

- Despite the increasing and encouraging body of evidence suggesting a possible use of microRNAs as therapeutic agents, either as tools or targets, there are several issues that need to be clarified and overcome to translate the success obtained in preclinical settings to the bedside. Indeed, whereas several chemical modifications have been developed to prevent degradation and increase the stability of a therapeutic RNA, either a miRNA or an antisense, and different methods have been exploited to increase the delivery efficiency, several aspects need to be further and carefully evaluated, as: an efficient and specific delivery, through systemic administration, to less accessible organs; the evaluation of possible side effects; the careful analysis of potential hazards by using viral particles.

- The potential of microRNA expression to predict response to different therapies needs to be further investigated and validated: either the possibility to select subgroups of patients that most likely will present a better response to a specific treatment, and the possible use of microRNAs as therapeutic adjuvant tools to improve the response and overcome resistance, are both critically important aspects in clinics. Indeed, microRNAs involved in specific networks, as the apoptotic, proliferation or receptor-driven pathways, could likely influence the response to targeted therapies or to chemotherapy, but unfortunately most part of the current reports relies on preclinical, generally in vitro, experiments, thus indicating that this represents still an open question.
fewer reported successes in the development of miRNAs therapeutic strategies: the main issues that need to be addressed are certainly the discovery of the miRNAs playing a crucial role in the biology of a specific tumour type by altering a whole network of target proteins; the validation of the targets and the accurate prevision of the putative unwanted off target effects; the development of efficient methods of a specific drug delivery.

Referring to the use of miRNAs as the targeted therapy of the future is probably too optimistic and premature at this point; however, the number of discoveries, increasing so fast in the last few years, is certainly encouraging and promising.

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