Targeting microRNAs in cancer: rationale, strategies and challenges

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Abstract | MicroRNAs (miRNAs) are evolutionarily conserved small non-coding RNAs that regulate gene expression. Early studies have shown that miRNA expression is deregulated in cancer and experimental data indicate that cancer phenotypes can be modified by targeting miRNA expression. Based on these observations, miRNA-based anticancer therapies are being developed, either alone or in combination with current targeted therapies, with the goal to improve disease response and increase cure rates. The advantage of using miRNA approaches is based on its ability to concurrently target multiple effectors of pathways involved in cell differentiation, proliferation and survival. In this Review, we describe the role of miRNAs in tumorigenesis and critically discuss the rationale, the strategies and the challenges for the therapeutic targeting of miRNAs in cancer.

Uncovered from the forgotten landscape of 'dark genomic matter', microRNAs (miRNAs) have become rising stars in cancer genetics. miRNAs are small evolutionarily conserved non-coding RNAs of 18–25 nucleotides in length that act as expression regulators of genes involved in fundamental cell processes, such as development, differentiation, proliferation, survival and death[1].

miRNAs are mostly transcribed from intragenic or intergenic regions by RNA polymerase II into primary transcripts of variable length (usually between 1 kb and 3 kb), called pri-miRNAs[2,3] (FIG. 1). The primary transcripts undergo further processing by the ribonucleases Drosha and DiGeorge syndrome critical region gene 8 (DGCR8) complex in the nucleus, thereby resulting in a hairpin intermediate of about 70–100 nucleotides, called pre-miRNA[4,5]. The pre-miRNA is then transported out of the nucleus to the cytoplasm by exportin 5 (REF. 6). In the cytoplasm, the pre-miRNA is processed by another ribonuclease, Dicer, into a mature double-stranded miRNA of variable length (~18–25 nucleotides)[7]. After strand separation, the guide strand or mature miRNA is incorporated into an RNA-induced silencing complex (RISC), whereas the passenger strand, denoted with a star (miRNA*) is commonly degraded[8–10]. The RISC is the effector complex of the miRNA pathway and is comprised of miRNA, argonaute proteins (argonaute 1– argonaute 4) and other protein factors[8–10]. Argonaute proteins have a crucial role in miRNA biogenesis, maturation and miRNA effector functions[8–10]. The mature strand is important for target recognition and for the incorporation of specific target mRNAs into the RISC[8–10] (FIG. 1).

The specificity of miRNA targeting is defined by Watson–Crick complementarities between positions 2 to 8 from the 5′ miRNA (also known as the seed), with the 3′ untranslated region (UTR) of their target mRNAs[10]. When miRNA and its target mRNA sequence show perfect complementarities, the RISC induces mRNA degradation. Should an imperfect miRNA–mRNA target pairing occur, translation into a protein is blocked[10]. Regardless of which of these two events occur, the net result is a decrease in the amount of the proteins encoded by the mRNA targets.

Each miRNA has the potential to target a large number of genes (on average about 500 for each miRNA family)[11–14]. Conversely, an estimated 60% of the mRNAs have one or more evolutionarily conserved sequences that are predicted to interact with miRNAs[11–14]. Bioinformatical analysis predicts that the 3′ UTR of a single gene is frequently targeted by several different miRNAs[11,12]. Many of these predictions have been validated experimentally, suggesting that miRNAs might cooperate to regulate gene expression[15].

Besides the aforementioned canonical mechanisms of miRNA gene regulation through 3′ UTR interactions, other 'non-canonical' miRNA-mediated mechanisms of mRNA expression modulation are emerging[16–21]. Some miRNAs have been shown to bind to the open reading frame or to the 5′ UTR of the target genes and, in some cases, they have been shown to activate rather than to inhibit gene expression[16,17]. Our group has recently reported that miRNAs can bind to ribonucleoproteins in a seed sequence and a RISC-independent manner and

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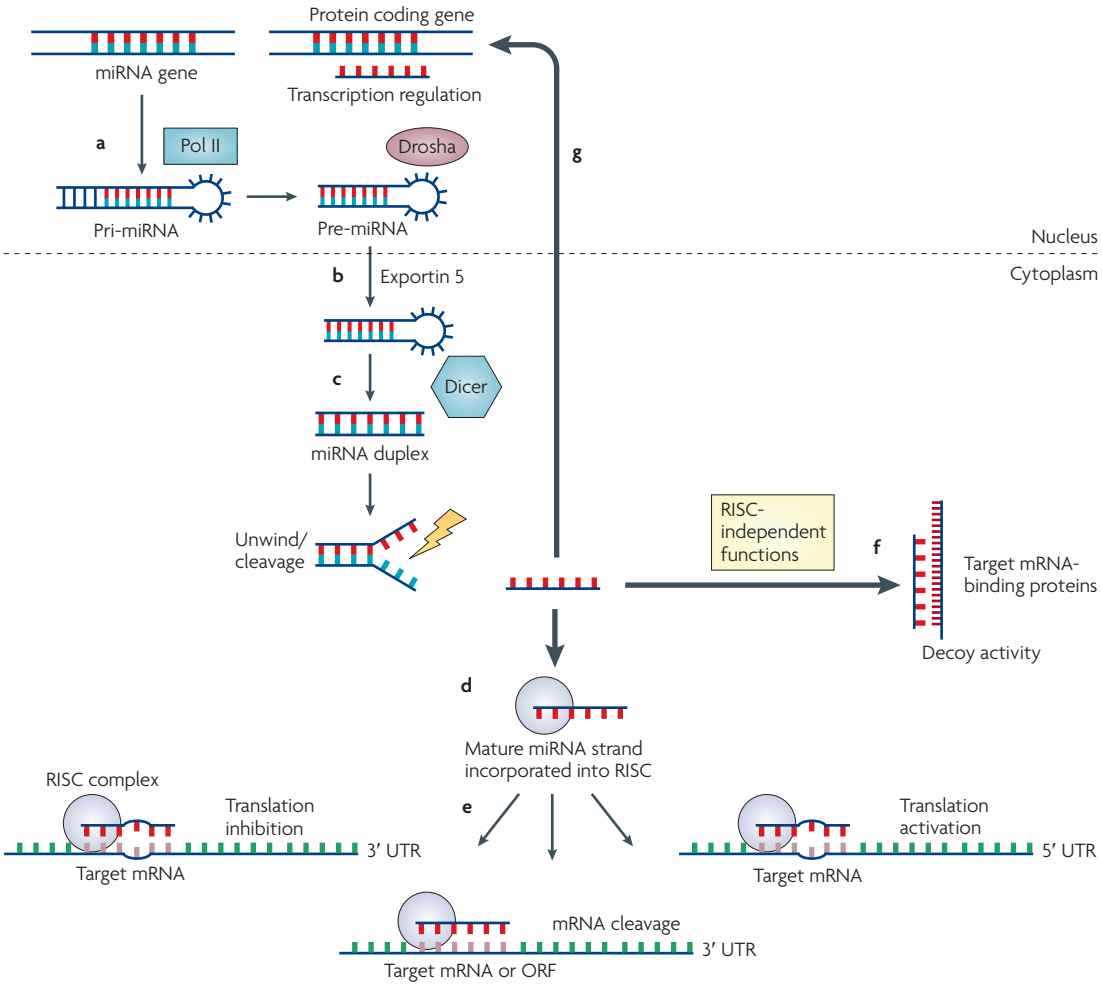

Figure 1 | **MicroRNA biogenesis and effector pathways. a** | MicroRNAs (miRNAs) are transcribed by RNA polymerase II (Pol II) into long primary miRNA transcripts of variable size (pri-miRNA), which are recognized and cleaved in the nucleus by the RNase III enzyme Drosha, resulting in a hairpin precursor form called pre-miRNA[1–3]. **b** | Pre-miRNA is exported from the nucleus to the cytoplasm by exportin 5 and is further processed by another RNase enzyme called Dicer. **c** | Dicer produces a transient 19–24 nucleotide duplex[4–7]. **d** | Only one strand of the miRNA duplex (mature miRNA) is incorporated into a large protein complex called RNA-induced silencing complex (RISC)[8–10]. **e** | The mature miRNA leads RISC to cleave the mRNA or induce translational repression, depending on the degree of complementarity between the miRNA and its target[8–10]. Although the most frequent site of interaction is the 3′ untranslated region (UTR) of the target mRNA, miRNAs have been described that bind to the open reading frame (ORF) sequences, as well as to the 5′ UTR[16–17]. This final interaction has been associated with activation rather than repression[17]. **f** | miRNAs can also bind directly to proteins, in particular RNA-binding proteins, in a sequence dependent manner and prevent these proteins from binding to their RNA targets. These decoy activities of miRNAs are RISC independent[18]. **g** | miRNAs can also regulate gene transcription by binding directly or by modulating methylation patterns at the target gene promoter level[20–22].

then interfere with their RNA binding functions (decoy activity)[18,19]. Three studies have reported that miRNAs can also regulate gene expression at the transcriptional level by binding directly to the DNA[20–22] (FIG. 1). Overall, these data show the complexity and widespread regulation of gene expression by miRNAs that should be taken into consideration when developing miRNA-based therapies.

miRNAs in cancer: a paradigm shift

Following our initial demonstration that the *mir-15a–mir-16-1* cluster is deleted or downregulated in B cells of patients with chronic lymphocytic leukaemia (CLL)[23], other studies established that malignant tissues in patients with cancer exhibited distinctive miRNA expression signatures[24,25]. Genome-wide profiling showed that these miRNA expression signatures allowed different types of cancer to be discriminated with high accuracy[24,25] 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[24].

Supporting the mechanistic involvement of miRNAs in specific cancers, it was reported that selected groups of distinct miRNAs were commonly and concurrently upregulated or downregulated in distinct types of human neoplasia and were often associated with distinct cytogenetic abnormalities[25]. miR-17 and miR-21

Table 1 | **MicroRNAs involved in cancer**

MicroRNA	Genomic location	Expression in patients	Deregulation mechanism	Function	Targets	Experimental data	Therapeutic strategy
miR-15a–miR-16-1	13q31	Down in CLL[24], prostate cancer[44] and pituitary adenomas[45]	Genomic loss[24]; mutations[30]; positive regulation by p53 (REFS 58–59)	Tumour suppressor	*BCL-2* (REF. 61), *MCL1* (REF. 61)	*In vitro* overexpression induces apoptosis in CLL and prostate cancer cells[24,44]; *in vivo* silencing causes CLL in mice[62]	Mimics; vector-based (viral); drugs
Let-7a-2	11q24	Down in lung[46], colon[33] breast[31], ovarian[50] and stomach cancer[27]	Negative regulation by MYC[70]	Tumour suppressor	*KRAS*, *NRAS*[46], *CDK6*, *CDC25A*[124], *HMGA2* (REF. 124), *MYC*[64]	*In vitro* overexpression reduces cell growth in lung, breast and colon cancer cells[46,48–49]; *In vivo* overexpression reduces breast and lung tumour burden in mice[48,124]	Mimics; vector-based (viral); drugs
miR-29b-1–miR-29a miR-29b-2–miR-29c	7q32 1q30	Down in NPM1 wild-type AML[39], CLL[30], lung[28] and breast cancer[31], cholangiocarcinoma[41], lymphoma[43], hepatocarcinoma[42] and rhabdomyosarcoma[40]	Genomic loss[63]; negative regulation by MYC[60]; positive regulation by p53 (REF. 59)	Tumour suppressor	*MCL1*, *CDK6* (REFS 41,63) *TCL1*, *DNMT1* (REFS 118–125), *DNMT3α*, *DNMT3β*[119]	*In vitro* overexpression induces apoptosis, inhibits cell proliferation and induces DNA hypomethylation in several cancers[41,63,118,119]; *in vivo* overexpression inhibits tumorigenicity in AML, liver and lung cancer in mice[42,63,119]	Mimics; vector-based (viral); drugs
miR-34a miR-34b and miR-34c	1p36 11q23	Down in colon, lung, breast, kidney and bladder cancer, neuroblastoma[34] and melanoma cell lines[126]	Methylation regulation[65,126]; positive regulation by p53 (REFS 58–59); deletion	Tumour suppressor	*CDK4*, *CDK6*, (REFS 65,127) *CCNE2*, *CCND1* (REFS 127–128), *MET*, *MYC*[127,129], *CREB*, *E2F3* (REFS 130,131), *BCL-2* (REF. 131)	*In vitro* overexpression induces cell cycle arrest, apoptosis and inhibits cell proliferation[61–62]	Mimics; vector-based (viral); drugs
miR-26a	3p22	Down in liver cancer[93]	Negative regulation by MYC[60]	Tumour suppressor	*CCND2*, *CCNE2* (REF. 93)	Restoration of miR-26 inhibits MYC-induced liver cancer[93]	Vector-based (viral)
miR-155	21q21	Up in high risk CLL[30], AML[32,39], lung[28], colon[33], breast cancer[31] and in lymphomas[37–38]	Positive regulation by NF-κB[118]	Oncogene	*SHIP1*, *CEBPB*[71,73]	Overexpression in HSC-induced myeloid proliferation and blocks erythropoiesis in mice[72]; *in vivo* overexpression in lymphocytes induces pre-B lymphoma and leukaemia in mice[71]	Antisense oligonucleotides; miR-mask; miRNA sponges; drugs
miR-17-92	13q22	Up in lung[28], breast[31], colon[33] and stomach cancer[27], myeloma[36] and t(11q23) AML[132]	Amplification[23]; positive regulation by E2F and MYC[133]	Oncogene	*BIM*, *PTEN*[27,70], *CDKN1A*[27]	Cooperates with MYC to induce lymphoma[73]; *in vivo* overexpression in lymphocytes induces lymphoid proliferation and autoimmunity in mice[72]	Antisense oligonucleotides; miR-mask; miRNA sponges; drugs
miR-21	17q23	Up in pancreas[33], breast[31], lung[28], prostate and stomach cancer[23], CLL[30], AML[32], myeloma[39] and gliobastoma[34]	Positive regulation by IL-6 and GF1α[134–135]	Oncogene	*PDCD4*, *PTEN*[67–68], *TPM1* (REF. 136)	*In vitro* silencing enhances apoptosis in glioblastoma, lung, breast and hepatocarcinoma cell lines[66–70]	Antisense oligonucleotides; miR-mask; miRNA sponges; drugs
miR-372 miR-373	19q13	Up in testicular germ cell tumours and in breast cancer[31,130]	Unknown	Oncogene	*LATS2* (REF. 137)	Neutralizes the p53 pathway *in vitro*[137]; *in vivo* overexpression stimulated cancer cell invasion[130]	Antisense oligonucleotides; miR-mask; miRNA sponges; drugs

AML, acute myeloid leukaemia; BCL-2, B-cell lymphoma protein-2; CCN, cyclin; CDC, cell division cycle; CDKN1A, cyclin-dependent kinase inhibitor 1A; CEBPB, CCAAT/enhancer binding protein β; CLL, chronic lymphocytic leukaemia; CREB, cAMP response element-binding protein; DNMT, DNA methyltransferase; HMGA2, high mobility group AT-hook 2; HSC, haematopoietic stem cells; IL-6, interleukin-6; KRAS, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue; LATS2, LATS, large tumour suppressor, homologue 2; MCL1, myeloid cell leukaemia sequence 1 (BCL-2-related); NF-κB, nuclear factor-κB; NPM1, nucleophosmin (nucleolar phosphoprotein B23, numatrin); NRAS, neuroblastoma RAS viral (v-ras) oncogene homologue; PDCD4, programmed cell death 4; PTEN, phosphatase and tensin homologue; SHIP1, Src homology 2 domain-containing inositol 5-phosphatase 1; TPM1, tropomyosin 1.

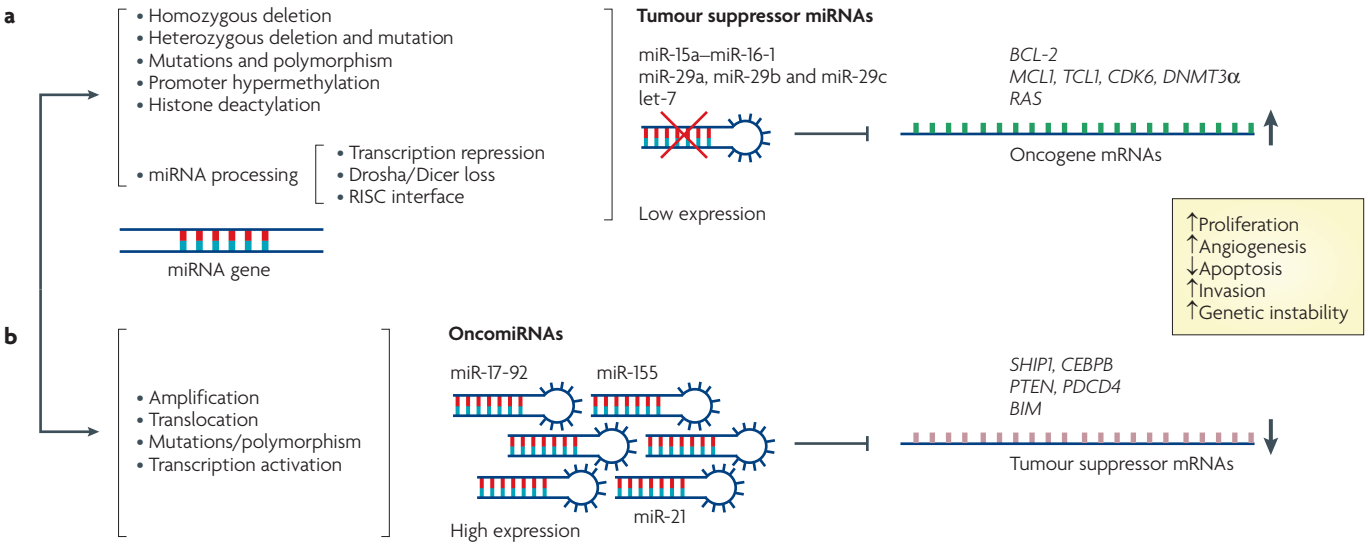

Figure 2 | **MicroRNAs as oncogenes and tumour suppressors. a** | In this model, we propose that a microRNA (miRNA) that normally downregulates the expression of an oncogene can be defined as a tumour suppressor and is often lost in tumour cells. The loss of function of this miRNA by mutation, deletion, promoter methylation or any abnormalities in the miRNA biogenesis might result in an abnormal expression of the target oncogene, which subsequently contributes to tumour formation by inducing cell proliferation, invasion, angiogenesis and decreased cell death. Some of the proposed mechanisms for the inactivation of miRNAs in cancer are experimentally proved, such as the downregulation of miR-15a–miR-16-1 expression in patients with chronic lymphocytic leukaemia that harbour homozygous and heterozygous deletions at 13q14.3, in which the *mir-15a–mir-16-1* cluster is located[23], and the loss of the *mir-29b-1–mir-29a* cluster in patients with acute myeloid leukaemia, located in 7q32 (REF. 63). In addition, germline mutations were identified in the *mir-15a–mir-16-1* precursor, which resulted in lower miR-15a and miR-16-1 expression levels. Overall, the loss of both the *mir-15a–mir-16-1* and the *mir-29b-1–mir-29a* cluster results in the upregulation of target oncoproteins, such as B-cell lymphoma protein-2 (BCL-2), myeloid cell leukaemia sequence 1 (BCL-2-related) (MCL1), TCL1, CDK6 and DNA methyltransferase 3α (DNMT3α)[61,41,63,119,123]. **b** | The amplification or overexpression of a miRNA that downregulates a tumour suppressor or other important genes involved in differentiation might contribute to tumour formation by stimulating proliferation, angiogenesis and invasion and by preventing apoptosis and increasing genetic instability. For example, amplifications of the oncogenic miRNAs, *mir-17-92* cluster, *mir-21* and *mir-155*, have been clearly associated with tumour initiation and progression by repressing the expression of tumour suppressor genes, such as phosphatase and tensin homologue (*PTEN*), *BIM* and programmed cell death 4 (*PDCD4*)[27,67–68,70]. The effect of the aberrant miRNA expression on the transcriptome and proteome will result in increased cell proliferation, angiogenesis, invasion, anti-apoptosis and genomic instability, which in turn will damage the genome further, perpetuating a dangerous cycle. For example, increased genomic instability may predispose for more mutations that may induce cancer progression or refractoriness to treatment. CEBPB, CCAAT/enhancer binding protein β; PDCD4, programmed cell death 4; RISC, RNA-induced silencing complex; SHIP1, Src homology 2 domain-containing inositol 5-phosphatase 1.

were identified to be consistently upregulated in colon, lung, stomach, prostate and pancreatic tumours and miR-155 was discovered to be upregulated in breast, lung and colon cancer[25]. These results have been validated over time in different cohorts of patients and similar results were reported in other types of cancer as well[26–39] (TABLE 1). By contrast, miR-29 was reported to be downregulated in CLL, acute myeloid leukaemia (AML), rhabdomyosarcoma, cholangiocarcinoma, mantle cell lymphoma and in lung, breast and liver cancer[28,30–32,40–43]. In addition miR-15a–miR16-1 was found to be downregulated in CLL, prostate and pituitary adenomas[23,44,45] and members of the let-7 family were identified to be downregulated in lung, colon, breast, ovarian and stomach cancer[28,33,31,46–51].

These commonalities in miRNA expression patterns suggested that deregulation of these miRNAs were unlikely to be a random event in cancer. This led to the hypothesis that upregulated miRNAs may act as oncogenes and downregulated miRNAs may act as tumour suppressors (FIG. 2). Consequently, similar to the coding genes involved in cancer, it was postulated that genes encoding miRNAs can be subject to genomic alterations leading to expression upregulation, for example, translocations or amplification, or loss of function, for example, deletions, insertions or mutations (FIG. 2).

Consistent with this hypothesis, we discovered that the genes encoding miRNAs are indeed frequently located inside or close to fragile sites and in minimal regions of loss of heterozygosity, in minimal regions of amplification and in common breakpoints associated with cancer[52]. The *mir-17-92* cluster, which comprises six miRNAs (*mir-17*, *mir-18a*, *mir-19a*, *mir-19b-1*, *mir-20a* and *mir-92-1*), is located in an 800 base-pair region of the non-coding gene *C13orf25*, a genomic region commonly reported to be amplified in lymphomas. The miR-17-92 cluster has frequently been

identified to be overexpressed in solid tumours or in haematological malignancies[26–46]. By contrast, the *mir-15a–mir-16-1* cluster, which is located in the chromosome 13q14 region (between exon 2 and exon 5 of the non-coding gene *LEU2*), is frequently downregulated in patients with CLL because of genomic deletion of this region[23,30] (FIG. 2).

In addition to structural genetic alterations, the silencing of structurally normal miRNA genes by DNA promoter hypermethylation and/or histone hypoacetylation has been described in solid tumours and in haematological malignancies[53–55]. Saito and colleagues first showed that miR-127 is downregulated owing to promoter hypermethylation in human bladder cancer. It is re-expressed in response to treatment with hypomethylating agents, correlating with a downregulation of the oncogene B-cell lymphoma protein-6 (*BCL-6*), which is a *bona fide* target of miR-127 (REF. 53).

Aberrant miRNA expression in cancer may also result from downstream miRNA processing (FIG. 2). Kumar *et al.* showed that global repression of miRNA expression can be induced by short hairpin RNAs against Dicer and Drosha, the two ribonucleases involved in miRNA processing. They also showed that this treatment promotes cellular transformation and tumorigenesis *in vivo*[56]. Furthermore, the conditional loss of *Dicer1* in the lung tissues of mice enhances the development of lung tumours in a *Kras* mouse model[56]. The loss of Dicer and Drosha has also been inversely correlated with outcome in cancers of the ovarian epithelium [57].

Finally, a deregulation of miRNA expression can be a result of increased or decreased transcription from their respective miRNA genes by aberrant transcription factor activity. For example, the miR-34a, miR-34b and miR-34c family of miRNAs was shown to be directly induced by the tumour suppressor p53 and it was suggested that some of the p53 effects could be mediated through the transcriptional activation of miRNAs[58,59]. Using different models, the authors compared miRNA expression in cells with high or low p53 expression and discovered that miR-34 expression is increased in cells with high p53 levels[58,59]. Chromatin immunoprecipitation experiments showed that p53 binds to the promoter of miR-34 (REFS 58,59).

Recent work also suggests that the oncoprotein MYC negatively regulates transcription of tumour suppressor miRNAs, such as *let-7* (*let-7a*, *let-7c*, *let-7d*, *let-7f1* and *let-7g*) and *mir-29* family members (*mir-29a*, *mir-29b* and *mir-29c*)[60]. Chromatin immunoprecipitation experiments showed that MYC binds to conserved sequences of the miRNA promoter that it represses. Functionally, it was shown that MYC-induced repression of miRNAs contributes to lymphomagenesis because the restoration of the silenced miRNAs decreases the tumorigenic potential of the lymphoma cells[60].

Nevertheless, despite the advances in our understanding of the mechanisms causing miRNA deregulation, the daunting task is to elucidate the biological role of miRNAs in the initiation and in the development of cancer.

Functional investigations of miRNAs in cancer. Gain-of-function and loss-of-function experiments, in combination with target prediction analyses, have provided insights into the role of miRNAs in carcinogenesis. For example, experiments in which miRNAs that are frequently lost in cancer, such as miR-15a and miR-16 in CLL[23], were ectopically expressed in leukaemic cells showed that the miR-15a–miR-16-1 cluster overexpression resulted in apoptosis of the leukaemic cells[61]. Target prediction programmes identified *BCL-2,* a known anti-apoptotic gene, which is upregulated in a subset of patients with CLL, albeit by unknown mechanisms, as the target of miR-15a–miR-16-1 (REF. 61). Additional work by our group showed that miR-15a–miR-16-1 directly interact with the *BCL-2* 3′ UTR and inhibit its protein translation[61]. A negative correlation was also identified between miR-15a–miR-16-1 and BCL-2 protein expression in patients with CLL, supporting this proposed interaction. Thus, the loss of the *mir-15a–mir-16-1* cluster in patients with CLL by genomic deletion and mutations results in the unblocking of *BCL-2* transcription in CLL cells[61] (FIG. 2). It was recently reported that *mir-15a–mir-16-1* cluster knockout mice developed a CLL-like disease and lymphomas, further supporting a tumour suppressor role of these miRNAs in CLL[62]. Other examples of miRNAs that act as tumour suppressors are listed in TABLE 1. These target oncoproteins that have crucial roles in various cancer pathways, such RAS (let-7)[46], myeloid cell leukaemia sequence 1 (BCL-2-related) (MCL1) (miR-29)[41,63] and MYC (let-7 and miR-34)[64,65] (FIG. 2; TABLE 1).

To assess the biological effects of miRNAs discovered to be overexpressed in cancer cells, *in vitro* experiments were carried out to block their expression using antisense oligonucleotides. For example, miR-21 expression has been reported at high levels in glioblastomas[34], pancreas[35], breast[31] and colon cancer[33] among others (TABLE 1). Chan and colleagues blocked miR-21 expression in glioblastoma cell lines and reported an increased activation of caspases and of apoptosis[66]. Additional studies showed that miR-21 exerts its anti-apoptotic effects by targeting the tumour suppressors phosphatase and tensin homologue (*PTEN*) and programmed cell death 4 (REFS 67–68).

The oncogenic activity of the miR-17-92 cluster and of miR-155, both discovered to be overexpressed in lymphoproliferative disorders, including lymphomas and leukaemia[32,69], were reported in animal models (FIG. 2; TABLE 1). Infection of murine haematopoietic stem cells with a retrovirus carrying the *mir-17-92* cluster accelerated the development of lymphomas in *Myc* transgenic mice[69]. Transgenic mice that overexpressed the *mir-17-92* cluster in B cells were discovered to develop lymphoproliferative disease and autoimmunity[70]. The higher rate of proliferation and the lower rate of activation-induced cell death of lymphocytes in these mice were partially attributed to the direct targeting of the anti-apoptotic genes *Bim* and *Pten* by the miR-17-92 cluster[70]. Ventura and colleagues showed that mice deficient for *mir-17-92* die shortly after birth with lung hypoplasia and a ventricular septal defect[71]. The miR-17-92 cluster is also essential

Antisense
The term antisense is generally used for nucleic acid-based approaches that interferes, in a sequence-selective way, with the processing of RNA from its transcription via mRNA to protein or with the effects of other forms of functional RNA.

for B cell development as the loss of the *mir-17-92* cluster results in the inhibition of B cell development at the pro-B cell to pre-B cell transition[71].

The role of each of the six miRNAs encoded by the *mir-17-92* cluster in oncogenesis was investigated by two different groups. Mu and colleagues determined that deletion of the complete *mir-17-92* cluster slows Myc-induced oncogenesis[72]. This phenotype was rescued by the reintroduction of the full cluster, but not by the cluster lacking miR-19a and miR-19b, thereby suggesting miR-19 is the most important miRNA of the cluster[72]. Using a different approach, Olive and colleagues overexpressed individual miRNAs in the *Eμ-Myc* mouse model. The authors discovered that overexpressing the whole cluster, the cluster without *mir-92*, but not the cluster lacking *mir-19a* or *mir-19b*, promotes oncogenesis[73]. Additional studies by both groups identified *Pten* as the major target for miR-19 (REFS 72,73). Altogether, both studies indicate that miR-19 is important for the oncogenic activities of this cluster.

By contrast to the *mir-17-92* cluster, overexpression of mir-155 alone in the lymphoid compartment was sufficient to cause cancer and did not require any other cooperative mutation or the expression of Myc. *mir-155* transgenic mice developed polyclonal lymphoid proliferation followed by acute lymphocytic lymphoma or leukaemia[74] (FIG. 2; TABLE 1). To our knowledge, this was the first report that the dysregulation of a single miRNA can lead to malignancy. Another group reported that the ectopic overexpression of miR-155 in haematopoietic stem cells through infection with retroviral constructs caused a myeloproliferative disorder[75].

Recently, the mechanisms for these effects were discovered. Elegant experiments performed independently by two groups have shown that the Src homology 2 domain-containing inositol 5-phosphatase 1 (*SHIP1*) is the target of miR-155 (REFS 76,77). SHIP1 is expressed in the haematopoietic system and by blocking the AKT pathway it has an important role in the differentiation of macrophages and lymphocytes[78]. *Ship1* deficient mice develop a myeloproliferative disorder characterized by increased granulocyte–monocyte populations and decreased B lymphocyte numbers, similar to the phenotype observed for the miR-155 overexpressing mice[76]. Thus, *SHIP1* repression by miR-155 seems to be one of the important events for miR-155-induced leukaemogenesis.

In addition to classical tumour suppressor or oncogene functions, miRNAs have recently also been implicated in cell migration and metastasis. It was determined that miR-10a, which is highly expressed in metastatic breast cancer, positively regulates cell migration and invasion[79]. Elegant experiments confirmed that overexpressing miR-10a in non-metastatic breast cancer cells initiates invasion and metastasis[79]. The authors showed that these effects are mediated by the direct targeting of *HOXD10* by miR-10a, facilitating the overexpression of the well known pro-metastatic gene *RHOC*[79]. Furthermore, a recent study reported that silencing *mir-10b* inhibits metastasis in a mouse breast tumour model, thereby highlighting the therapeutic potential of targeting metastasis-associated miRNAs[80]. Another study showed that miR-126 and miR-335 act as negative regulators of tumour invasion and metastasis in human breast and lung cancer[81].

In FIG. 2, we present a simplified model of miRNAs acting as oncogenes and tumour suppressors. It should be stressed that the function of miRNAs depends on the expression of their crucial targets. Some miRNAs could function as oncogenes in some cell types and as suppressors in others. Thus the definition of miRNAs as oncogenes or as tumour suppressor genes requires an indication of the type of cells in which they act. It is anticipated that this model will need to be refined in the near future as other potentially key aspects of miRNA biology are uncovered. It is unlikely that miRNAs will be responsible for a specific phenotype by aiming at a single target. Instead, it is thought that miRNAs engage in complex interactions with the machinery that controls the transcriptome and concurrently target multiple mRNAs.

Establishing the rationale for targeting miRNAs

The rationale for using miRNAs as anticancer drugs is based on two major findings: that miRNA expression is deregulated in cancer compared with normal tissues and that the cancer phenotype can be changed by targeting miRNA expression[23–40,47–51]. The potential advantages of miRNA-based therapeutic approaches over other strategies, such as targeting protein expression, is discussed below.

Unus pro omnibus: 'one for all'. One of the most appealing properties of miRNAs as therapeutic agents is their ability to target multiple genes, frequently in the context of a network, making them extremely efficient in regulating distinct biological cell processes relevant to normal and malignant cell homeostasis[59,63,70,82–84] (FIG. 3). This concept was elegantly shown by the Chen group in their study of T cell receptor biology[84]. It is known that T cell receptor signalling and antigen recognition are controlled by sequential phosphorylation and dephosphorylation events by more than 40 different kinases and phosphatases[84]. miR-181 was shown to have a critical role in the regulation of T cell receptor sensitivity and signalling strength at the post-transcriptional level by targeting multiple phosphatases. More importantly, the authors showed that this task can be carried out efficiently by miR-181a alone, but not by RNA interference a single short hairpin RNA (shRNA), which is designed to target individual genes[84]. The ability of miRNAs to regulate multiple genes in a molecular pathway makes them excellent candidates for novel molecular-targeting treatments.

Cancer networks are connected by miRNA. In cancer, as a result of multiple genetic and epigenetic events, perturbations of important gene and protein networks occur, resulting in malignant transformation. Apoptosis, cell cycle, cell adhesion, chromosome stability and DNA repair networks are frequently affected in carcinogenesis[85] (FIG. 3a). As miRNAs regulate many different pathways and

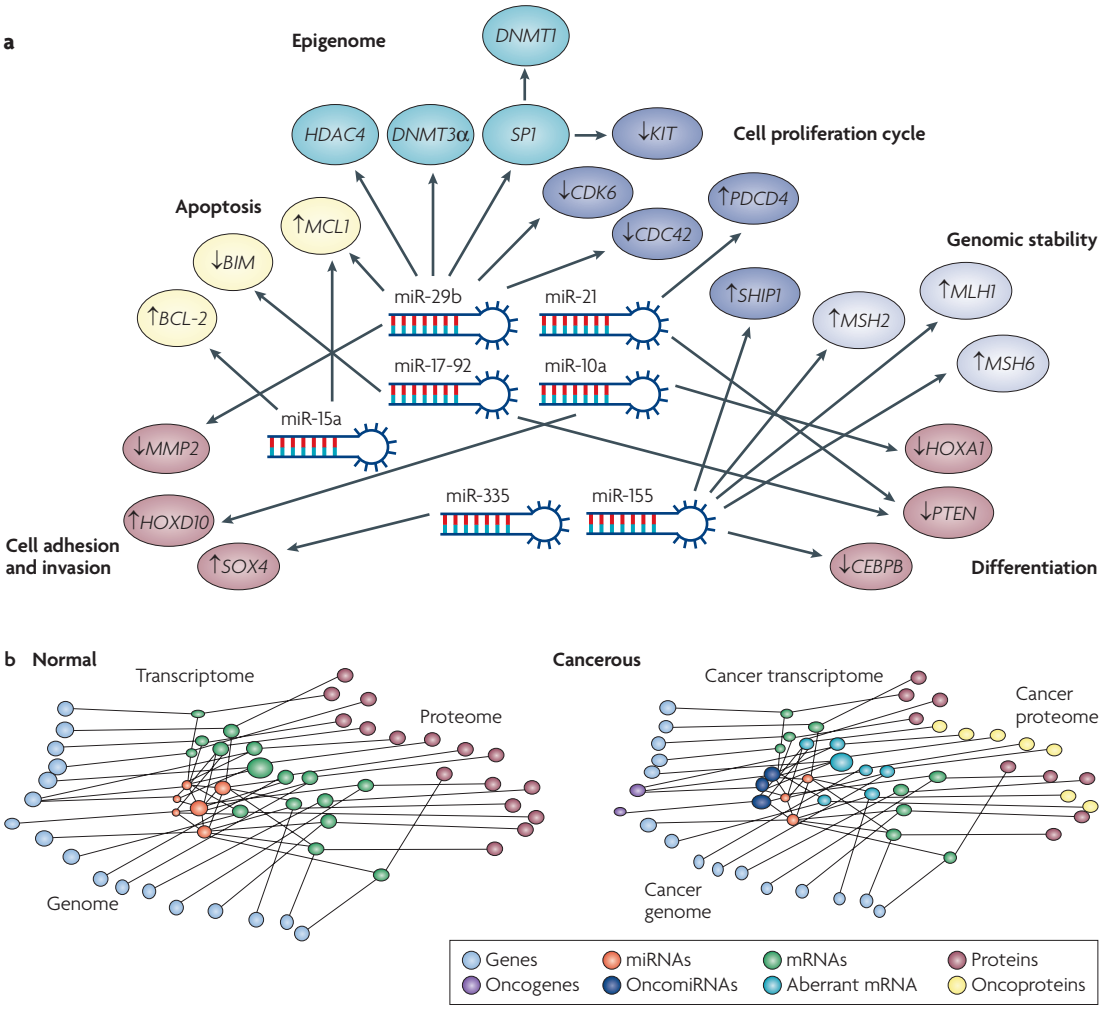

Figure 3 | **Transcriptome–microRNA networks in cancer. a** | In this figure we graphically represent the relationship between vital oncogenic transcriptome networks and the miRNAome. Target mRNAs for each major pathway are represented by circles with a unique colour. MicroRNAs (miRNAs) are represented as hairpin structures in the centre. The arrows connecting miRNAs and mRNAs indicate validated mRNA–miRNA interactions. The small arrow in the circles indicates the biological effects on the pathway by the miRNA acting on its target. For example, miR-15a induces apoptosis by targeting B-cell lymphoma protein-2 (*BCL-2*) or miR-29b suppresses cell proliferation by blocking *CDK6* (REFS 61,63). Some miRNAs, for example miR-29b, coordinately regulate multiple targets in different pathways[63]. As shown in the figure, miR-29b modulates target mRNAs involved in apoptosis, cell proliferation, DNA methylation, histone acetylation and cell adhesion[63,118–119]. **b** | In this figure we graphically represent a gene–protein network in normal tissues and in cancer. miRNAs are transcribed from miRNA non-coding genes, which have their own transcriptional unit, or from introns of protein-coding genes. In general, one gene is transcribed to one mRNA and translated to one protein. By contrast, miRNAs are transcribed from one or in certain cases from two genes[63,118]. For example, *mir-29b1* is transcribed from chromosome 7 and *mir-29b-2* is transcribed from chromosome 1, which are both coding for the same mature sequence. They coordinately regulate multiple mRNAs (shown as a net of connections), thus affecting the output of many proteins. miRNAs have a crucial role in keeping the gene–protein network interconnected. Proteins subsequently regulate gene transcription by directly binding to the gene promoters. In cancer, as a result of mutations, deletion or epigenetic alterations in miRNA genes or in protein-coding genes, aberrant miRNA and mRNA expression occurs, resulting in the expression of oncogenic proteins that cause a certain cancer phenotype. As miRNAs coordinate responses in a network by targeting multiple genes, the perturbation of the miRNA network has a vital function during carcinogenesis causing aberrations in the transcription of large numbers of genes. The miRNA network is 'hijacked' to promote malignancy. By modifying the miRNA network it may be possible to restore homeostasis in cancer. CDC42, cell division cycle 42; CEBPB, CCAAT/enhancer binding protein β; DNMT, DNA methyltransferase; HDAC4, histone deacetylase 4; HOXA1, homeobox A 1; HOXD10, homeobox D 10; KIT, v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homologue; MCL1, myeloid cell leukaemia sequence 1 (BCL-2-related); MLH1 mutL homologue 1, colon cancer, nonpolyposis type 2; MMP2, matrix metalloproteinase 2; MSH, mutS homologue, colon cancer, nonpolyposis type 1; PDCD4, programmed cell death 4; PTEN, phosphatase and tensin homologue; SHIP1, Src homology 2 domain-containing inositol 5-phosphatase 1; SOX4, SRY (sex determining region Y)-box 4; SP1, Sp1 transcription factor.

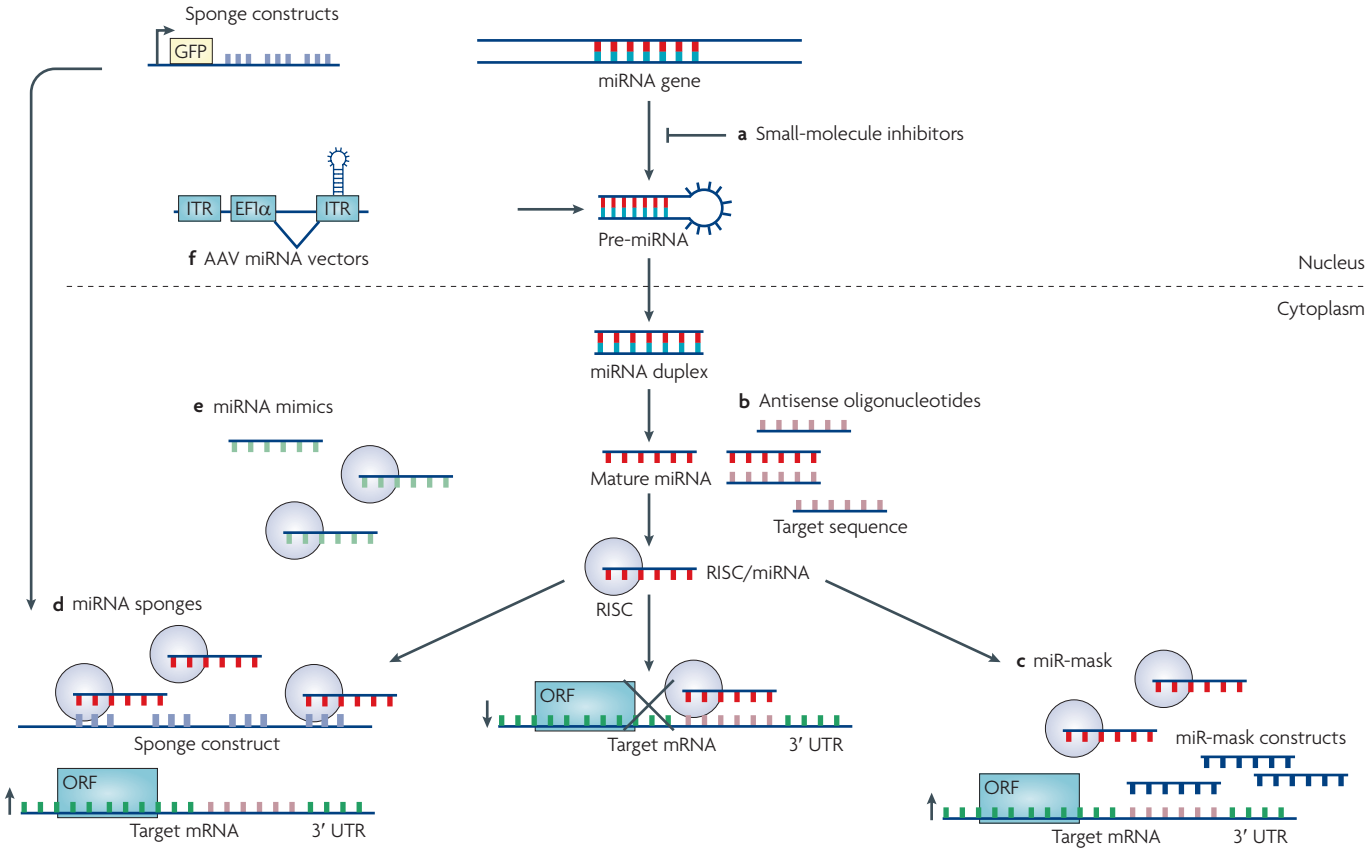

Figure 4 | **Strategies for microRNA-based therapies.** Blocking oncogenic microRNAs (miRNAs) can be achieved by the use of antisense oligonucleotides, miRNA sponges, miR-mask and small RNA inhibitors[91–99]. **a** | Small-molecule miRNA inhibitors can regulate miRNA expression at the transcriptional level[99]. **b** | Antisense oligonucleotides can bind to the target miRNAs following the Watson–Crick complementarities and induce either degradation or duplex formation[101,107]. **c** | The miR-mask oligonucleotides are synthetic oligonucleotides complementary to the 3′ untranslated region (UTR) target mRNA that compete with endogenous miRNAs for its target[98]. Therefore, the miR-mask is able to block oncogenic miRNA deleterious functions at the target level and activate translation of target mRNAs. **d** | The miRNA sponges are oligonucleotide constructs with multiple complementary miRNA binding sites (in tandem) to the target miRNA[97]. When introduced to the cell, sponges will 'soak up' endogenous miRNAs, decreasing the expression levels of an oncogenic miRNA. **e** | Restoring downregulated miRNA expression could be achieved by using synthetic miRNAs (miRNA mimics). **f** | Restoring downregulated miRNA expression could also be achieved by inserting genes coding for miRNAs into viral constructs, such as the adenovirus-associated vectors (AAV)[100–101]. EF1α, elongation factor 1 α; GFP, green fluorescent protein; ITR, inverted terminal repeats; ORF, open reading frame; RISC, RNA-induced silencing complex.

orchestrate integrated responses in normal healthy cells and tissues, it is reasonable to think that they also have key roles in coordinating cancerous networks. One can envision miRNAs as a 'power grid' that keeps all these genes and protein networks connected (FIG. 3b). The degree of miRNA perturbation in cancer could be measured and compared with normal tissue patterns. This way, it might be possible to obtain a miRNA snapshot map, the 'core of the cancer connectivity grid'. Restoring normal miRNA programmes in the cancer cell may rewire the cell connectivity map and reverse cancer phenotypes. Developing therapeutic strategies to restore homeostasis by modifying miRNA expression may prove to be more comprehensive and successful than targeting individual genes or proteins, as there are only some miRNAs deregulated in cancer, compared with the large perturbations of the transcriptome and proteome in cancer cells.

Strategies for miRNA-based therapeutics

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 substitute for the loss of expression of a tumour suppressor miRNA. The indirect strategy involves the use of drugs to modulate miRNA expression by targeting their transcription and their processing (see also FIG. 4 and TABLE 1).

Blocking oncogenic miRNAs using antisense oligonucleotides. The demonstration that oncogenic miRNAs are upregulated in cancer (TABLE 1) provided a rationale to investigate the use of antisense oligonucleotides to block their expression. Antisense oligonucleotides work as competitive inhibitors of miRNAs, presumably

by annealing to the mature miRNA guide strand and inducing degradation or stoichiometric duplex formation. Although this mechanism was illustrated successfully by injecting complementary double-strand sequences to miRNAs into *Drosophila melanogaster* embryos[86], further experiments using naked or unmodified antisense DNA oligonucleotides were ineffective in *Caenorhabditis elegans*[87]. Researchers overcame these difficulties by introducing modifications to the chemical structure of the oligonucleotides to increase stability, binding affinity and specificity[88] (FIG. 5). Among these modifications, the introduction of 2′-*O*-methyl groups contributes to nuclease resistance and improved binding affinities to RNA[87]. Oligonucleotides with 2′-*O*-methyl groups have proved to be effective inhibitors of miRNA expression in several cancer cell lines[39–41,61,63]. Other modifications, such as the addition of 2′-*O*-methoxyethyl groups, increases the affinity and the specificity to RNA compared with the 2′-*O*-methyl analogues[87].

As a proof of principle, Krutzfeldt *et al.* developed 2′-*O*-methyl-modified cholesterol-conjugated single-stranded RNA analogues, with phosphorothioate linkages, named 'antagomirs', complementary to miR-122, which is abundant in the liver. These antagomirs were injected into the tail vein of mice and specific targeting of miR-122 in the liver was observed after 24 hours[89]. The silencing of endogenous miRNAs by this novel method was specific, efficient and long lasting and the effects were still observed 23 days after injection[89]. Gene expression and bioinformatic analysis of the whole transcriptome (mRNA) from antagomir-treated animals showed that the 3′ UTRs of upregulated transcripts were strongly enriched in miR-122 recognition motifs, whereas downregulated genes were depleted of these motifs. Using an antagomir against the ubiquitously expressed miR-16, the authors also investigated the bioavailability and the silencing activity of antagomirs in different tissues. In mice treated with this antagomir, miR-16 was efficiently silenced in all the tissues tested except brain tissue[89].

Locked nucleic acid (LNA) constructs. LNA nucleosides are a class of nucleic acid analogues in which the ribose ring is 'locked' by a methylene bridge connecting the 2′-*O* atom and the 4′-*C* atom (FIG. 5). By locking the molecule with the methylene bridge, LNA oligonucleotides display unprecedented hybridization affinity towards complementary single-stranded RNA and complementary single-stranded or double-stranded DNA[90]. In addition, they display excellent mismatch discrimination and high aqueous solubility. So-called 'LNA anti-miR' constructs have been used successfully in several *in vitro* studies to knock down specific miRNA expression[41,64,65].

Studies in mice using LNA anti-miR have shown the feasibility and the high efficiency of this approach. Recently, Elmen and colleagues examined whether combining LNA anti-miR with phosphorothioate modifications could improve delivery of the compounds and silence miR-122 in mice without requiring additional chemical modifications[91]. The authors chose to target miR-122 based on previous data that indicated that miR-122 binds to the hepatitis C virus (HCV) and stimulates its replication[92]. In a mouse model, intravenous injections of about 1 mg per kg to 25 mg per kg of such LNA anti-miR showed markedly improved efficiency in antagonizing miR-122 compared with cholesterol-conjugated antagomir that targets miR-122 (REF. 91). A previous report indicated that three doses of 80 mg per kg of a cholesterol-conjugated oligonucleotide were needed to silence miR-122 in mice[89]. These data suggest that LNA anti-miRs are able to effectively silence their targets at much lower doses than cholesterol-based oligonucleotides.

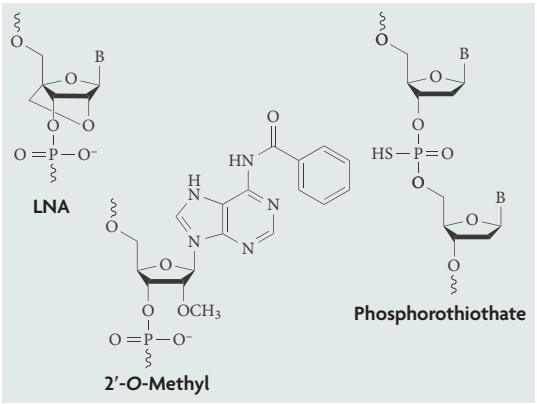

Figure 5 | **Common oligonucleotide modification structures.** The three most common oligonucleotide modification structures are shown; locked nucleic acid (LNA), 2′-*O*-methyl and phosphorothiolate[101,107]. B, base.

The simple systemic delivery of an unconjugated LNA anti-miR-122 (SPC3649, developed by Santaris Pharma) has also been shown to effectively antagonize liver-expressed miR-122 in non-human primates[93]. Using three intravenous doses of 10 mg per kg in African green monkeys, the authors observed an effective depletion of miR-122 in the liver without any evidence of LNA-associated toxicities or histopathological changes in the animals. LNA-mediated antagonism of miR-122 in primates was effective and long lasting[93].

The same group recently investigated the potential of miR-122 antagonism by LNA anti-miR-122 as a new anti-HCV therapy in a chimpanzee model system of chronic infection[94]. The animals were treated at two dose levels (5 mg per kg and 1 mg per kg) by intravenous injections of LNA anti-miR-122, on a weekly basis for 12 weeks, followed by a treatment-free period of 17 weeks. Although a considerable decline in HCV RNA was observed in the serum after 3 weeks of treatment with the higher dose of LNA anti-miR-122, high variability was observed at the lower dose levels[94]. Measurements of miR-122 expression showed substantial and durable silencing in the liver. Furthermore, although there was no evidence of viral resistance or side effects in the treated animals, transcriptome and histological analyses of liver biopsies showed derepression of target mRNAs with miR-122 seed sites, downregulation of interferon-regulated genes and improvement of HCV-induced liver pathology[94].

Phosphorothioate
An oligonucleotide in which the oxygen atom normally linking two consecutive nucleotides has been replaced with sulphur, which resists degradation by cellular enzymes. The phosphorothioate backbone, although reducing affinity to the target RNA, confers considerable stability to nuclease degradation and is essential for *in vivo* delivery of antisense oligonucleotides to tissues, as the phosphorothioate promotes protein binding and delays plasma clearance.

Overall, these studies not only support the rationale for targeting miR-122 as a new treatment for HCV infection to prevent cirrhosis and associated liver cancer, but also provide proof of principle for antagomir and LNA anti-miR therapies in inflammatory, degenerative or neoplastic human diseases characterized by aberrant upregulation of a specific miRNA family. Indeed, based on these encouraging results, Santaris Pharma is carrying out early clinical studies using SPC3649 in healthy human subjects in Denmark. A clinical Phase I trial, which is currently ongoing, will provide valuable information about pharmacokinetics and safety profiles.

miRNA sponges. miRNA sponges are transcripts that contain multiple tandem-binding sites to a miRNA of interest and are transcribed from mammalian expression vectors. Ebert and colleagues recently reported the use of miRNA sponges in mammalian cells[95]. The authors reasoned that miRNA target sequences expressed at high levels could compete with *bona fide* targets in a cell for miRNA binding. To increase the affinity of these decoy transcripts, the authors introduced not only multiple miRNA binding sites, but also a bulge at the position normally cleaved by argonaute 2, therefore facilitating the stable association of miRNA sponges with ribonucleoprotein complexes loaded with the corresponding miRNA. Using these constructs, a derepression of miRNA targets was observed and indicated effective *in vitro* silencing of miRNAs[96]. Theses effects were comparable with those obtained with 2′-*O*-methyl-modified oligonucleotides or LNA antisense oligonucleotides. Furthermore, sponges that contained only the heptameric seed were shown to effectively repress an entire miRNA family that shares by definition the same seed sequence[96]. In a recent study, Loya and colleagues applied miRNA sponges to inhibit miRNA activity in transgenic *Drosophila in vivo*[97].

The art of disguise: miR-mask. miRNA-masking antisense oligonucleotides technology (miR-mask) is another strategy developed by Xiao *et al.*[98]. In contrast to miRNA sponges, miR-masks consist of single-stranded 2′-*O*-methyl-modified antisense oligonucleotides that are fully complementary to predicted miRNA binding sites in the 3′ UTR of the target mRNA[98]. In this way, the miR-mask covers up the miRNA-binding site to derepress its target mRNA, thereby its effects are gene specific. This technology has been applied successfully in a zebrafish model to prevent the repressive actions of miR-430 in transforming growth factor-β signalling pathways[98]. Although unwanted effects or off-target effects can be dramatically reduced with this approach, this may be a disadvantage for cancer therapy in which the targeting of multiple pathways might be desirable.

Small-molecule inhibitors. Several drugs may have the ability to modulate the expression of miRNAs by targeting signalling pathways that ultimately converge on the activation of transcription factors that regulate miRNA encoding genes. Furthermore, it is possible to modulate the machinery that contributes to the miRNA maturation and degradation processes. The identification of these compounds, however, is not straightforward and requires the efficient screening of chemical libraries. Recently, Gumireddy and colleagues identified a method to screen for small-molecule inhibitors of miRNAs[99]. As a proof of principle for this approach, the authors selected miR-21 because this miRNA is frequently upregulated in cancer (TABLE 1). Complementary sequences to miR-21 were cloned into a luciferase reporter gene, which was then used as a sensor to detect the presence of specific mature miRNA molecules. The construct was transfected into HeLa cells, which express high miR-21 levels, resulting in low luciferase activity. Subsequently, a primary screen of more than 1,000 small-molecule compounds was done and an initial hit compound, diazobenzene 1, produced a 250% increase in the intensity of the luciferase signal relative to the untreated cells[99]. Additional characterization showed that this compound affects the transcription of *mir-21* (REF. 99). This strategy could be applied to the screening of small molecules as inhibitors for other distinct oncogenic miRNAs. These could be used with conventional cancer therapeutics to develop novel combinatorial approaches for cancer treatment.

Restoring tumour-suppressor miRNA expression. The loss or downregulation of a tumour-suppressor miRNA could be overcome by introducing synthetic oligonucleotides that are identical to the selected miRNA, known as miRNA mimics. Introduction of synthetic miRNA mimics with tumour-suppressor function in cancer cells have been shown to induce cell death and block proliferation in several studies[40–42,44,49,63]. For example, using mimics of miR-15a and miR-29 in prostate and AML cell lines, respectively, induced apoptosis[44,63]. These miRNA mimics are small, usually double stranded and chemically modified (2′-*O*-methyl with phosphorothioate modifications). Some of these include longer sequences such as the miRNA precursor (for example, a pre-miRNA developed by Ambion). It has been reported that intratumoural injections of miR-29 mimics are effective in decreasing tumorigenicity in human rhabdomyosarcoma, liver and AML xenograft murine models[40,42,63]. However, there is no *in vivo* data using miRNA mimics delivered by intravenous injection.

Another strategy to increase the expression of a tumour-suppressor miRNA in cancer uses adenovirus-associated vectors (AAV). These vectors do not integrate into the genome and are eliminated efficiently with minimal toxicity, as shown in Phase I and Phase II clinical trials of about 200 patients[100,101]. Another advantage of AAV vectors is the efficient transduction of target cells[100]. The development of self-complementary genome and non-human primate AAV serotypes allow more than 90% transduction efficiency of hepatocytes and long-term gene expression without toxicity, following a single systemic administration of recombinant virus[100]. Kota and colleagues recently showed that miR-26 expression was lost in human liver cancers, although it was expressed at high levels in normal tissue[102]. Ectopic expression of this miRNA in liver cancer cell lines was shown to induce cell-cycle arrest. The authors further cloned *mir-26* into an AAV vector and viral particles were tested in an

established Myc-dependent liver cancer mouse model[102]. Intravenous injection of this miRNA resulted in the suppression of tumorigenicity by inducing tumour apoptosis and by repressing cell growth, without signs of toxicity. Interestingly, considerable anticancer effects were shown even when proteins other than the initiating oncoprotein (MYC) were targeted, for example, cyclin D2 and cyclin E2. This work is the first evidence that restoring the expression of a tumour suppressor miRNA blocks cancer progression *in vivo*[102]. This strategy could be viable for the treatment of liver cancer, as it is easily targeted by both viral and non-viral gene and small-molecule delivery systems[101]. However, the efficacy of this system for other types of tumours and in different locations is unknown. As there are multiple AAV serotypes available that allow efficient targeting of many tissues of interest, it is possible to target cancers that arise from different tissues. For example, muscle targeting could be desirable for the treatment of soft tissue sarcoma.

Reprogramming cancer cells: turning around a bad network. So far, all the strategies to modulate miRNA expression are designed to modify only one miRNA or a family of miRNAs. As it is likely that 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 to reprogramme aberrant miRNA networks in cancer. Reprogramming could be achieved by modulating several of the key miRNAs in a network using antisense oligonucleotides or mimics. However, targeting multiple miRNAs using antisense oligonucleotides or mimics may be technically challenging.

Another strategy to rewire miRNA expression is the use of chemotherapeutic drugs. Several groups reported miRNA expression changes in response to drug treatment *in vitro* and *in vivo*[53,54,103,104], suggesting that such changes may be responsible, at least in part, for the anticancer effects. In acute promyelocytic leukaemia, a subtype of AML characterized by maturation arrest at the promyelocytic stage and caused by the promyelocytic leukaemia–retinoic acid receptor-α fusion protein, pharmacological doses of all-*trans*-retinoic acid have been shown to reverse the dominant–negative effect of promyelocytic leukaemia–retinoic acid receptor-α fusion and induce granulocytic differentiation of the AML blasts and induce apoptosis[103]. Our group reported that the apoptotic effect observed after all-*trans*-retinoic acid treatment of acute promyelocytic leukaemia cells and of patients is partially explained by all-*trans*-retinoic acid-induced activation of *mir-15a–mir-16-1* cluster expression, which is known to target the expression of the anti-apoptotic protein BCL-2 (REF. 103).

Decitabine (Dacogen; Pharmachemie BV) and 5-azacytidine (Mylosar; Upjohn) are two well-known hypomethylating agents currently approved for the treatment of myelodysplastic syndrome, although they have shown activity in many other malignancies including AML[105]. It has long been known that these drugs work by DNA methyltransferase inhibition, resulting in tumour suppressor gene re-expression, mediated by promoter hypomethylation[105]. More recently, miRNAs have been shown to be actively re-expressed after treatment with these drugs and have been shown to have important roles for the therapeutic effects of these compounds. It is tempting to suggest that many of the biological effects of decitabine and 5-azacytidine may be mediated by the re-expression of non-coding RNAs[53–55].

Once a cancer miRNA network is identified, one can envision the use of drugs or other agents to modify the expression of such miRNAs and thereby restore normal patterns of miRNA expression. In a few years, we may be able to generate cell-specific miRNA expression profiles after drug treatment that may facilitate the discovery of functional connections between drugs, genes and diseases, similar to that of the connectivity map — a collection of genome-wide transcriptional expression data from cultured human cells treated with biologically active small molecules[106]. As miRNAs are fewer in number compared with mRNA, it could be assumed that there will be less noise and background in high-throughput-based experiments for miRNAs, such as microarrays, than the ones performed using mRNA[106]. These miRNA-drug maps could then be used to discover novel drug applications and establish drug combination treatments.

Challenges of miRNA-based therapies

The challenges for developing miRNA-based therapeutics are the same as the challenges for small interfering RNA therapeutics and include issues of delivery, potential off-target effects and safety (BOX 1; TABLE 2). One of the major problems for the use of miRNA therapeutics *in vivo* relates to tissue-specific delivery and to cellular uptake of sufficient amounts of synthetic oligonucleotides to achieve sustained target inhibition[101,107]. The first obstacle to overcome is the biological instability of these compounds in bodily fluids or tissues, as unmodified 'naked' oligonucleotides are rapidly degraded by cellular and serum nucleases[101,107]. The second obstacle is the poor cellular uptake of oligonucleotides owing to their size and negative charge, which could prevent them from crossing through cell membranes[101,107].

To overcome these delivery hurdles, viral and non-viral strategies have been developed (BOX 1). Various chemical modifications in oligonucleotides have been investigated, such as morpholinos, peptide nucleic acids, cholesterol conjugation (see antisense section) and phosphorothioate backbone modifications[108–110] (BOX 1; FIG. 5). Although chemical modifications have improved the delivery of oligonucleotides to tissues, this is often associated with impaired biological activity and increased toxicity, in particular when cholesterol-conjugated oligonucleotides are used[108–111] (TABLE 2). Other strategies, such as the use of cationic lipids, polymers and nanoparticles, have recently become popular, in an attempt to enhance the cellular uptake and the pharmacological effectiveness of antisense oligonucleotides *in vivo*[108,111–113]. Whereas cationic lipids are too toxic to the cell and elicit hypersensitive reactions *in vivo*, polymers and nanoparticle strategies are promising because they provide improved delivery and stability with minimal *in vivo* toxicity[108,111–113] (BOX 1; TABLE 2).

Box 1 | **Delivering synthetic microRNAs**

To overcome small RNA oligonucelotide delivery hurdles, non-viral and viral strategies have been developed[101,107]. The non-viral strategies include oligonucleotides with chemical modifications, liposomes, polymers, hydrogels and nanoparticles[101,107,109–113]. The most widely studied oligonucleotide modification is the replacement of each non-bridging oxygen in the backbone with a sulphur atom, thereby forming a phosphorothioate linkage[101,107] (FIG. 5). Phosphorothioate oligonucleotides exhibit a dramatically improved *in vivo* half-life compared with naked oligonucleotides[101,107]. However, these therapies have generally been administered by continuous intravenous injection and have been associated with several toxicities[101,107]. The efficacy data from Phase II studies show tumour regression; however, major responses are rare[101,107].

Liposomes are composed of a phospholipid bilayer with an enclosed aqueous compartment. They interact with oligonucleotides to form complexes stabilized by electrostatic interactions[107,109]. Cationic liposomes protect oligonucleotides from degradation by nucleases and increase circulating half-time and cellular uptake[107,109]. However, they are toxic to the cell and elicit hypersensitivity reactions *in vivo*[109,110]. Several efforts are underway to make liposomes safer, such as improving their formulation by adding chemical additives to reduce cell toxicity[101,107]. Another limitation of liposomes is that they tend to accumulate preferentially in the reticuloendothelial system, leading to a short half-life in the serum and reduced access to other tissues[101,107].

As chemically modified oligonucleotides alone or in combination with liposomes exhibit a short half-life and require either continuous infusion or frequent administration, a possible approach to overcome this problem was to develop sustained-release polymer formulations[112]. Polymers are biodegradable compounds that protect RNA from degradation and facilitate sustained delivery to the tissues[112]. There are many different types of polymers that vary in size, chemistry and pharmacological properties[101,112]. *In vitro* and *in vivo* studies have shown that biodegradable polymer antisense oligonucleotide combinations achieve sustained delivery and improved tissue biodistribution[112]. More research is still needed to guide the polymer architecture and the chemical structure that are most suited for oligonucleotide delivery and cell and tissue targeting.

Nanoparticles, microspheres and hydrogels have also been developed as gene delivery vehicles. These strategies are promising because they provide improved oligonucleotide delivery and stability with minimal toxicity in animal models[107,113].

Target-specific delivery could also be achieved by direct injection of the synthetic oligonucleotides into solid tumours. This may be a feasible strategy for mesothelioma (intrapleural injections), ocular tumours, brain tumours or sarcomas and should reduce or eliminate off-target effects. This could also be achieved by tagging nanoparticle–miRNA oligonucleotide complexes with antibodies that bind the desired target cell[101,107]. For example, one could envisage the development of miR15a–miR-16-1 oligonucleotide nanoparticles coated with CD20-specific antibodies to treat chronic lymphocytic leukaemia. This could be a potential strategy to overcome off-targets effects in haematological cancers.

Finally, because miRNAs regulate many genes, the potential off-target effects of miRNA therapeutics are a major concern, as they may cause toxic phenotypes[101,108]. As discussed above, *in vitro* and *in vivo* data for several types of cancer support the use of miR-29 oligonucleotide mimics as anticancer drugs[40–43,63]. Although miR-29 targets several oncogenic pathways, such as apoptosis (*MCL1*), proliferation (*CDK6*) and methylation (DNA methyltransferase 1 (*DNMT1*), *DNMT3a* and *DNMT3b*), it also modulates other processes, including bone development[114], immune function (T helper 1 cell responses)[115] and granulocytic differentiation[63]. Systemic overexpression of miR-29 using a synthetic mimic could target genes, in particular in non-cancerous tissues, and cause unwanted side-effects such as autoimmunity or myeloid hyperproliferation. These problems could be solved by engineering effective systems that deliver the synthetic miRNA oligonucleotides specifically to the diseased tissue and to cancer cells (BOX 1).

Early clinical trials using DNA antisense technologies showed that severe side-effects, such as cytokine-release syndrome, haematological toxicity (thrombocytopaenia) and liver damage may occur[108,111]. In some cases, these side effects were mainly owing to problems with formulation, for example liposomes being directly toxic or inducing hypersensitive reactions[111] (TABLE 2). In other circumstances they could be related to non-specific immunological activities triggered by certain CpG motifs in the oligonucleotides that activated mechanisms of innate immunity, mediated by Toll-like cell receptors and other inflammasome effectors, leading to interferon and other cytokine responses[108,116] (BOX 1). However, no data hitherto suggest that exogenous miRNAs may elicit cytokine responses in mammalian organisms. So far the use of LNA antimiR has proved safe when tested in non-human primates[93,94]. The ongoing Phase I clinical trial in humans using SPC3649 will be important to assess the safety of this approach (TABLE 2).

More concerning is a recent report suggesting that toxicity is closely linked to the small RNA concentration. Grim and colleagues elegantly showed that sustained high expression of short hairpin RNAs by AAV vectors induced severe dose-dependent liver damage, owing to interference with endogenous miRNA processing in the liver and resulting in liver-specific miRNA downregulation and injury[117]. As both shRNAs and miRNAs used the same processing pathways[7], these effects could be explained by the saturation of the processing machinery by exogenous shRNA, leading to loss of miRNA function. This work underscores the challenge for using vector-based therapies to overexpress miRNAs. Similar problems could arise using synthetic mature oligonucleotides, as they may also saturate RISC and compete and displace other endogenous miRNAs.

miRNA therapeutic applications

What are the potential types of cancer that could be amenable for miRNA-based therapy? Certain miRNAs, such as miR-155, miR-21, miR-17-92 and miR-29, are consistently deregulated in many cancers[25,27–32] (TABLE 1). Therefore, developing anticancer treatments targeting these miRNAs may be applicable to multiple malignancies. Silencing miR-155 and miR-21 expression in cancer cells would unblock the expression of vital tumour suppressor targets, such as the phosphatases *SHIP1* and *PTEN*, respectively, restoring normal patterns of cell differentiation and proliferation and inducing cancer cell death[73,67]. As miRNAs, in particular the miR-29 family, have been shown to downregulate DNA methyltransferases (*DNMT1*, *DNMT3a* and *DNMT3b*) and to induce global DNA hypomethylation and tumour-suppressor gene re-expression[118,119], restoring miR-29 expression could be used as an epigenetic hypomethylating strategy in malignancies. For example, certain subsets of AML have been shown to have low miR-29 expression[63] caused by aberrant epigenetic modifications[105]. In addition, owing to their hypomethylating effects, miR-29s are also negative regulators of apoptosis and cell proliferation by targeting the pro-apoptotic *MCL1* and the cell cycle regulator

Table 2 | **Limitations and advantages of direct microRNA-based therapeutic approaches**

Strategy	Limitations	Advantages	Experimental data	Solutions and future directions
2′-*O*-methyl phosphorothiolate oligonucleotides	Delivery; short serum half-life; poor cellular uptake; off-target effects; limited biological effects	Safe; improved stability; nuclease resistance; increased binding affinity	*In vitro* and *in vivo* data; animal models; Phase I, Phase II and Phase III clinical trials[39–41,61,63]	Improve delivery
2′-*O*-methyl phosphorothiolate oligonucleotides with cholesterol backbone	Toxicity; requires high doses	Good bioavailability	*In vitro* and *in vivo* (animals)[89]	Improve safety profile
Locked nucleic acid	Off-targets effects; potential dose toxicity effects	Safe; good biodistribution; effective	*In vitro* and *in vivo* (mice and chimpanzees); human trials ongoing[41,64,65,91–94]	Detailed pharmacokinetic, pharmacodynamic and toxicity studies in humans; develop tissue-specific delivery
Liposome–oligonucleotide complexes	Toxicity; hypersensitivity; potential dose toxicity effects	Improved stability and delivery	*In vitro* and *in vivo* (animals)[108,111–113]	Develop better formulations
Polymer–nanoparticle oligonucleotide complexes	Off-target effects; potential dose toxicity effects	Improved stability and delivery; minimal toxicity	*In vitro* and *in vivo* (animals)[101,112]	Develop tissue-specific delivery (antibody tagging)
miR-mask	Limited scope (one target); delivery	Effects are gene-specific; no off-target effects	*In vitro* studies[98]	Achieve delivery *in vivo*; assess activity *in vivo*
miRNA sponge	Delivery; off-targets effects	Able to silence a family of miRNAs	*In vitro* studies[95–97]	Achieve delivery *in vivo*; assess activity *in vivo*
Adenovirus-associated vectors coding for miRNAs	Potential dose toxicity effects; off-target effects	Safe, efficient transduction; long-term expression	*In vitro* and *in vivo* (animals); human trials for small interfering RNA; Phase I, Phase II and Phase III trials[101,102]	More extensive animal data is needed (in particular with other tumours)

CDK6, respectively[63]. Thus, therapeutic modulation of miR-29 would affect three pathways deregulated in cancer: epigenetics, apoptosis and cell proliferation or cell cycle (FIG. 3).

miRNAs could also be targeted for therapeutic applications other than cancer. For example, as miR-155 is not only involved in cancer[25,28] but also in inflammation and immunity[120,121], therapies targeting miR-155 could potentially be applicable to various autoimmune and inflammatory disorders.

Future directions

As the miRNA field continues to evolve, a better understanding of miRNA biogenesis and function will certainly affect the development of miRNA-based therapies. miRNA effects are currently largely interpreted as the result of miRNA–mRNA 3′ UTR interactions that cause target post-translational inhibition or degradation. However, focusing on this mechanism to design miRNA therapeutics is likely to prove too simplistic, owing to the emerging miRNA mechanisms, which include decoy activity and 5′ UTR and direct DNA regulatory activities[17–21].

Research efforts should focus on maximizing the benefit of target diversity, in addition to preventing off-target effects. Improving the chemical design of antisense and miRNA mimics, as well as developing new delivery methods, will be crucial to achieving this goal. Detailed pharmacokinetic and pharmacodynamic studies will be needed to ensure that the desired miRNA concentrations are achieved in tissues and the targets are downregulated. These pharmacokinetic and biological pharmacodynamic effects need to be correlated with the clinical outcome, including treatment responses.

As more miRNA profiling studies are performed after drug treatment in cell lines and in patients, distinctive drug-specific miRNA maps could be obtained. Based on these profiles, it might be possible to use drugs alone or in combination to reprogramme the miRNAome of patients with cancer. We also envisage that miRNA-targeted therapies could be used to enhance or to prevent resistance to standard chemotherapeutic agents or other biological agents. For example, miR-128 has been shown to modulate steroid refractoriness in acute lymphocytic leukaemia, therefore one strategy could be to use a synthetic miRNA in combination with chemotherapy to overcome this problem in the treatment of lymphocytic leukaemia[122].

However, challenges intrinsic to the oligonucleotide-based approaches remain to be overcome, including low bioavailability and poor cellular uptake resulting in suboptimal delivery, as well as off-target effects and long-term safety concerns in humans. New miRNA formulations, including nanoparticles and polymers, as well as virus-based approaches, could be used to overcome these problems. Overall, targeting miRNAs to reprogramme miRNA networks in cancer constitutes a reasonable and evidence-based strategy with a strong potential and chance for success. The enthusiasm for miRNA-based treatments is reflected by the large number of pharmaceutical companies pursuing this strategy.

1. Ambros, V. The functions of animal microRNAs. *Nature* **431**, 350–355 (2004).
2. Rodriguez, A., Griffiths-Jones, S., Ashurst, J. L. & Bradley, A. Identification of mammalian microRNA host genes and transcription units. *Genome Res.* **14**, 1902–1910 (2004).
3. Lee, Y. *et al.* MicroRNA genes are transcribed by RNA polymerase II. *EMBO J.* **23**, 4051–4060 (2004).
4. Lee, Y., Ahn, C., Han, J., Choi, H. & Kim, J. The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425**, 415–419 (2003).
5. Landthaler, M., Yalcin, A. & Tuschl, T. The human DiGeorge syndrome critical region gene 8 and its *D. melanogaster* homolog are required for miRNA biogenesis. *Curr. Biol.* **14**, 2162–2167 (2004).
6. Bohnsack, M. T., Czaplinski, K. & Gorlich, D. Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *RNA* **10**, 185–191 (2004).
7. Hammond, S., Bernstein, E., Beach, D. & Hannon, G. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* **404**, 293–229 (2000).
8. Thimmaiah, P. *et al.* TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature* **436**, 740–744 (2005).
9. Hutvanger, G. & Zamore, P. D. A microRNA in a multiple-turnover RNAi enxyme complex. *Science* **297**, 2056–2060 (2002).
10. Bartel, D. P. MicroRNAs: target recognition and regulatory functions. *Cell* **136**, 215–233 (2009).
11. Lewis, B., Shih, I., Jones-Rhoades, M., Bartel, D. & Burge, C. Prediction of mammalian microRNA targets. *Cell* **115**, 787–798 (2003).
12. Krek, D. *et al.* Combinatorial microRNA target predictions. *Nature Genet.* **37**, 495–500 (2005).
13. Betel, D., Wilson, M., Gabow, A., Marks, D. S. & Sander, C. The microRNA.org resource: targets and expression. *Nucleic Acids Res.* **36,** D149–D153 (2008).
14. Friedman, R. C., Farh, K. K., Burge, C. B. & Bartel, D. P. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res.* **19**, 92–105 (2009).
15. Ivanovska, I. & Cleary, M. A. Combinatorial microRNAs: working together to make a difference. *Cell Cycle* **20**, 3137–3142 (2008).
16. Stark, A. *et al.* Discovery of functional elements in 12 *Drosophila* genomes using evolutionary signatures. *Nature* **450**, 219–232 (2007).
17. Ørom, U. A., Nielsen, F. N. & Lund, A. H. MicroRNA-10a binds the 5′UTR of ribosomal protein mRNAs and enhances their translation. *Mol. Cell* **4**, 460–471 (2008).
18. Eiring, A. *et al.* miR-328 functions as an RNA decoy to modulate hnRNP E2 regulation of mRNA translation in leukemic blasts. *Cell* **140**, 652–665 (2010).
First evidence of miRNA decoy activity in gene regulation.
19. Beitzinger, M. & Meister, G. MicroRNAs: from decay to decoy. *Cell* **140**, 612–614 (2010).
20. Khraiwesh, B. *et al.* Transcriptional control of gene expression by microRNAs. *Cell* **140**, 112–122 (2010).
First report indicating transcriptional control of gene expression by miRNAs.
21. Gonzalez, S., Pisano, D. G. & Serrano, M. Mechanistic principles of chromatin remodeling guided by siRNAs and miRNAs. *Cell Cycle* **7**, 2601–2608 (2008).
22. Kim, D. H., Saetrom, P., Snøve, O. Jr & Rossi, J. J. MicroRNA directed transcriptional gene silencing in mammalian cells. *Proc. Natl Acad. Sci. USA* **105**, 16230–16235 (2008).
23. Calin, G. A. *et al.* Frequent deletions and downregulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc. Natl Acad. Sci. USA* **99**, 15524–15529 (2002).
First report of miRNA deregulation in cancer.
24. Lu, J. *et al.* MicroRNA expression profiles classify human cancers. *Nature* **435**, 834–838 (2005).
25. Volinia, S. *et al.* A microRNA expression signature in human solid tumors defines cancer targets. *Proc. Natl Acad. Sci. USA* **103**, 2257–2261 (2005).
Comprehensive analysis of miRNA deregulation in solid tumours.
26. Ota, A. *et al.* Identification and characterization of a novel gene, C13orf25, as a target for 13q31–q32 amplification in malignant lymphoma. *Cancer Res.* **64**, 3087–3095 (2004).
27. Petrocca, F. *et al.* E2F1-regulated microRNAs impair TGFβ-dependent cell-cycle arrest and apoptosis in gastric cancer. *Cancer Cell* **13**, 272–286 (2008).
28. Yanaihara, N. *et al.* Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell* **9**, 189–198 (2006).
29. Diosdado, B. *et al.* MiR-17-92 cluster is associated with 13q gain and c-myc expression during colorectal adenoma to adenocarcinoma progression. *Br. J. Cancer* **101**, 707–714 (2009).
30. Calin, G. A. *et al.* A microRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N. Engl. J. Med.* **353**, 1793–1801 (2005).
31. Iorio, M. V. *et al.* MicroRNA gene expression deregulation in human breast cancer. *Cancer Res.* **65**, 7065–7070 (2005).
32. Garzon, R. et *al.* MicroRNA signatures associated with cytogenetics and prognosis in acute myeloid leukemia. *Blood* **111**, 3183–3189 (2008).
33. Schetter, A. J. *et al.* MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA* **299**, 425–436 (2008).
34. Ciafre, S. A. *et al.* Extensive modulation of a set of microRNAs in primary glioblastoma. *Biochem. Biophys. Res. Commun.* **334**, 1351–1358 (2005).
35. Bloomston, M. *et al.* MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. *JAMA* **297**, 1901–1908 (2007).
36. Pichiorri, F. *et al.* MicroRNAs regulate critical genes associated with multiple myeloma pathogenesis. *Proc. Natl Acad. Sci. USA* **105**, 12885–12890 (2008).
37. Metzler, M. *et al.* High expression of precursor microRNA-155/BIC RNA in children with Burkitt lymphoma. *Genes Chromosomes Cancer* **39**, 167–169 (2004).
38. Kluiver, J. *et al.* BIC and miR-155 are highly expressed in Hodgkin, primary mediastinal and diffuse large B cell lymphomas. *J. Pathol.* **207**, 243–249 (2005).
39. Garzon, R. *et al.* Distinctive miRNA signature of acute myeloid leukemia bearing cytoplasmic mutated nucleophosmin. *Proc. Natl Acad. Sci. USA* **105**, 3945–3950 (2008).
40. Wang, H. *et al.* NF-κB–YY1–miR-29 regulatory circuitry in skeletal myogenesis and rhabdomyosarcoma. *Cancer Cell* **14**, 369–381 (2008).
41. Mott, J. L., Kobayashi, S., Bronk, S. F. & Gores, G. J. Mir-29 regulates Mcl-1 protein expression and apoptosis. *Oncogene* **26**, 6133–6140 (2007).
42. Xiong, Y. *et al.* Effects of microRNA-29 on apoptosis, tumorigenicity, and prognosis of hepatocellular carcinoma. *Hepatology* **51**, 836–845(2010).
43. Zhao, J. J. *et al.* MicroRNA expression profile and identification of miR-29 as a prognostic marker and pathogenetic factor by targeting CDK6 in mantle cell lymphoma. *Blood* **115**, 2630–2639 (2010).
44. Bonci, D. *et al.* The miR-15a–miR-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities. *Nature Med.* **14**, 1271–1277 (2008).
45. Bottoni, A. *et al.* MiR-15a and miR-16-1 down-regulation in pituitary adenomas. *J. Cell Physiol.* **204**, 280–285 (2005).
46. Johnson, S. M. *et al. RAS* is regulated by the *let-7* microRNA family. *Cell* **120**, 635–647 (2005).
47. Takamizawa, J. *et al.* Reduced expression of the *let-7* microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res.* **64**, 3753–3756 (2004).
48. Yu, F. *et al. let-7* regulates self renewal and tumorigenicity of breast cancer cells. *Cell* **131**, 1109–1123 (2007).
49. Akao, Y., Nakagawa, Y. & Naoe, T. *let-7* microRNA functions as a potential growth suppressor in human colon cancer cells. *Biol. Pharm. Bull.* **29**, 903–906 (2006).
50. Yang, N. *et al.* MicroRNA microarray identifies *let-7i* as a novel biomarker and therapeutic target in human epithelial ovarian cancer. *Cancer Res.* **68**, 10307–10314 (2008).
51. Motoyama, K. *et al.* Clinical significance of high mobility group A2 in human gastric cancer and its relationship to *let-7* microRNA family. *Clin. Cancer Res.* **14**, 2334–2340 (2008).
52. Calin, G. A. *et al.* Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc. Natl Acad. Sci. USA* **101**, 2999–3004 (2004).
A systematic analysis of miRNA localization and the fragile sites associated with cancer.
53. Saito, Y. *et al.* Specific activation of microRNAs-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. *Cancer Cell* **9**, 435–443 (2006).
First evidence that miRNAs are regulated by epigenetic mechanisms in cancer.
54. Lujambio, A. *et al.* Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. *Cancer Res.* **67**, 1424–1429 (2007).
55. Hackanson, B. *et al.* Epigenetic modification of CCAAT/enhancer binding protein α expression in acute myeloid leukemia. *Cancer Res.* **68**, 3142–3151 (2008).
56. Kumar, M. S. *et al. Dicer1* functions as a haploinsufficient tumor suppressor. *Genes Dev.* **23**, 2700–2704 (2009).
57. Merritt, WA. *et al.* Dicer, drosha, and outcomes in patients with ovarian cancer. *N. Engl. J. Med.* **359**, 2641–2650 (2008).
58. He, L. *et al.* A microRNA component of the p53 tumour suppressor network. *Nature* **447**, 1130–1134 (2007).
59. Chang, T. C. *et al.* Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Mol. Cell* **26**, 745–752 (2007).
References 58 and 59 report a link between p53 activity and miRNA expression.
60. Chang, T. S. *et al.* Widespread microRNA repression by Myc contributes to tumorigenesis. *Nature Genetics* **40**, 43–50 (2007).
Comprehensive analysis of MYC-regulated miRNAs in cancer cells.
61. Cimmino, A. *et al.* miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proc. Natl Acad. Sci. USA* **102**, 13944–13949 (2006).
62. Klein, U. *et al.* The *DLEU2/miR-15a/16-1* cluster controls B cell proliferation and its deletion leads to chronic lymphocytic leukemia. *Cancer Cell* **17**, 28–40 (2010).
63. Garzon, R. *et al.* MicroRNA-29 functions in AML. *Blood* **114**, 5331–5341(2009).
64. Sampson, V. B. *et al.* MicroRNA *let-7a* down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells. *Cancer Res.* **67**, 9762–9770 (2007).
65. Lujambio, A. *et al.* A microRNA DNA methylation signature for human cancer metastasis. *Proc. Natl Acad. Sci. USA* **105**, 13556–13561 (2008).
Comprehensive analysis of epigentically regulated miRNAs in cancer metastasis.
66. Chan, J. A., Krichevsky, A. M. & Kosik, K. S. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer Res.* **65**, 6029–6033 (2005).
67. Meng. F. *et al.* MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology* **133**, 647–658 (2007).
68. Frankel, LB. *et al.* Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. *J. Biol. Chem.* **283**, 1026–1033 (2008).
69. He, L. *et al.* A microRNA polycistron as a potential human oncogene. *Nature* **435**, 828–833 (2005).
70. Xiao, C. *et al.* Lymphoproliferative disease and autoimmunity in mice with elevated miR-17-92 expression in lymphocytes. *Nature Immunol.* **9**, 405–414 (2008).
References 69 and 70 established the role of the miR-17-92 cluster in cancer and immunity.
71. Ventura, A. *et al.* Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. *Cell* **132**, 875–886 (2008).
First report analysing the contribution of each miR-17-92 paralogues to the phenotype observed in mice with deletion of the whole miR-17-92 cluster.
72. Mu, P. *et al.* Genetic dissection of the *miR-17-92* cluster of microRNAs in Myc-induced B-cell lymphomas. *Genes Dev.* **23**, 2806–2811 (2009).
73. Olive, V. *et al.* miR-19 is a key oncogenic component of miR-17-92. *Genes Dev.* **23**, 2839–2849 (2009).
References 72 and 73 established the importance of miR-19 in the oncogenic role of the miR-17-92 cluster.
74. Costinean, S. *et al.* Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in Eμ-miR155 transgenic mice. *Proc. Natl Acad. Sci. USA* **103**, 7024–7029 (2006).
First report indicating that the overexpression of a single miRNA can cause cancer.

75. O'Connell, R. M. *et al.* Sustained expression of micro-RNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. *J. Exp. Med.* **205**, 585–594 (2008).
76. O'Connell, R. M., Chaudhuri, A, A., Rao, D. S. & Baltimore, D. Inositol phosphatase SHIP1 is a primary target of miR-155. *Proc. Natl Acad. Sci. USA* **106**, 7113–7118 (2009).
77. Costinean, S. et al. Src homology 2 domain-containing inositol-5-phosphatase and CCAAT enhancer-binding protein β are targeted by miR-155 in B cells of Eμ-*MiR-155* transgenic mice. *Blood* **114**, 1374–1382 (2009).
78. Liu, Q. *et al.* The inositol polyphosphate 5-phosphatase ship is a crucial negative regulator of B cell antigen receptor signaling. *J. Exp. Med.* **188**, 1333–1342 (1998).
79. Ma, L, Teruya-Feldstein, J. & Weinberg, R. A. Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. *Nature* **449**, 682–688 (2007).
First evidence that miRNAs are involved in tumour invasion and metastasis.
80. Ma, L. *et al.* Therapeutic silencing of miR-10b inhibits metastasis in a mouse mammary tumor model. *Nature Biotechnol.* **28**, 341–347 (2010).
81. Tavazoie, S. F. *et al.* Endogenous human microRNAs that suppress breast cancer metastasis. *Nature* **451**, 147–152 (2008).
82. Linsley, P. S. *et al.* Transcripts targeted by the microRNA-16 family cooperatively regulates cell cycle progression. *Mol. Cell Biol.* **27**, 2240–2252 (2007).
83. Calin, G. A. *et al.* MiR-15a and miR-16-1 cluster functions in human leukemia. *Proc. Natl Acad. Sci. USA* **105**, 5166–5171 (2008).
84. Li, Q. J. *et al.* miR-181a is an intrinsic modulator of T cell sensitivity and selection. *Cell* **129**, 147–161 (2007).
Elegant study showing the multitarget activity of a single mRNA in a pathway.
85. Croce, C. M. Oncogenes and cancer. *N. Engl. J. Med.* **358**, 502–511(2008).
86. Boutla, A., Delidakis, C. & Tabler, M. Developmental defects by antisense-mediated inactivation of micro-RNAs 2 and 13 in *Drosophila* and the identification of putative targets. *Nucleic Acids Res.* **31**, 4973–4980 (2003).
87. Hutzvanger, G., Simard, M. J., Mello, C. C. & Zamore, P. D. Sequence specific inhibition of small RNA function. *PLoS Biol.* **2**, E98 (2004).
88. Weiler, J., Hunziker, J. & Hall, J. Anti-miRNA oligonucleotides (AMOs); amunition to target miRNAs implicated in human disease? *Gene Therapy* **13**, 496–502 (2006).
89. Krutzfeldt, J. *et al.* Silencing of microRNAs *in vivo* with 'antagomirs'. *Nature* **438**, 685–689 (2005).
First *in vivo* report using antisense oligonucleotides against miRNAs with success.
90. Vester, B. & Wengel, J. LNA (locked nucleic acid): high affinity targeting of complementary RNA and DNA. *Biochemistry* **43**, 13233–13241 (2004).
91. Elmén, J. *et al.* Antagonism of microRNA-122 in mice by systemically administered LNA-antimiR leads to up-regulation of a large set of predicted target mRNAs in the liver. *Nucleic Acids Res.* **36**, 1153–1162 (2008).
92. Jopling, C. L., Yi, M., Lancaster, A. M., Lemon, S. M. & Sarnow, P. Modulation of hepatitis C virus RNA abundance by a liver-specific microRNA. *Science* **309**, 1577–1581 (2005).
93. Elmén, J. *et al.* LNA-mediated microRNA silencing in non-human primates. *Nature* **452**, 896–899 (2008).
94. Lanford, R. E. *et al.* Therapeutic silencing of microRNA-122 in primates with chronic hepatitis c virus infection. *Science* **327**, 198–201 (2010).
95. Ebert, M. S., Neilson, J. R. & Sharp, P. A. MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. *Nature Methods* **4**, 721–726 (2007).
96. Xiao, J. *et al.* Novel approaches for gene-specific interference via manipulating actions of micro-RNAs: examination on the pacemaker channel genes *HCN2* and *HCN4*. *J. Cell Physiol.* **212**, 285–292 (2007).
97. Loya, C. M., Lu, C. S., Van Vactor, D. & Fulga, T. A. Transgenic microRNA inhibition with spatiotemporal specificity in intact organisms. *Nature Methods* **6**, 897–903 (2009).
98. Choi, W. Y., Giraldez, A. J. & Schier, A. F. Target protectors reveal dampening and balancing of Nodal agonist and antagonist by miR-430. *Science* **318**, 271–274 (2007).
99. Gumireddy, K. *et al.* Small-molecule inhibitors of microrna miR-21 function. *Angew. Chem. Int. Ed. Engl.* **47**, 7482–7484 (2008).
100. Michelfelder, S. & Trepel, M. Adeno-associated viral vectors and their redirection to cell-type specific receptors. *Adv. Genet.* **67**, 29–60 (2009).
101. Aagaard, L. & Rossi, J. J. RNAi therapeutics: principles, prospects and challenges. *Adv. Drug Deliv. Rev.* **59**, 75–86 (2007).
102. Kota, J. *et al.* Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. *Cell* **137**, 1005–1017 (2009).
First report using adenovirus-associated vectors to deliver miRNAS to the liver.
103. Garzon, R. *et al.* MicroRNA gene expression during retinoic acid-induced differentiation of human acute promyelocytic leukemia. *Oncogene* **26**, 4148–4157 (2007).
104. Roccaro, A. *et al.* MicroRNA expression in the biology, prognosis, and therapy of Waldenström macroglobulinemia. *Blood* **113**, 4391–4402 (2009).
105. Galm, O., Herman, J. G. & Baylin, S. B. The fundamental role of epigenetics in hematopoietic malignancies. *Blood Rev.* **20**, 1–13 (2006).
106. Lamb, J. *et al.* The connectivity map: using gene-expression signatures to connect small molecules, genes, and disease. *Science* **313**, 1929–1935 (2006).
107. Zhao, X. *et al.* Controlled delivery of antisense oligonucleotides: a brief review of current strategies. *Exp. Opin. Drug Deliv.* **6**, 673–686 (2009).
108. Dias, N. & Stein, C. A. Antisense oligonucleotides: basic concepts and mechanisms. *Mol. Cancer Ther.* **1**, 347–355 (2002).
109. Malik, R. & Roy, L. Design and development of antisense drugs. *Exp. Opin. Drug Discov.* **3**, 1189–1207 (2008).
110. Chiarantini, L. *et al.* Comparison of a novel delivery systems for antisense peptide nucleic acids. *J. Control. Release* **109**, 24–36 (2005).
111. Zuhorn, I. S., Engberts, J. B. & Hoeckstra, D. Gene delivery by cationic lipid vectors: overcoming cellular barriers. *Eur. Biophys. J.* **36**; 349–362 (2007).
112. Chirila, T. V. *et al.* The use of synthethic polymers for delivery of therapeutic antisense oligodeoxynucleotides. *Biomaterials* **23**, 321–342 (2002).
113. Rosi, N. L. *et al.* Oligonucleotide-modified gold nanoparticles for intracellular gene regulation. *Science* **312**, 1027–1030 (2006).
114. Li, Z. *et al.* Biological functions of miR-29b contribute to positive regulation of osteoblast differentiation. *J. Biol. Chem.* **284**, 15676–15684 (2009).
115. Xu, H., Cheung, I. Y., Guo, H. F. & Cheung, N. K. MicroRNA miR-29 modulates expression of immunoinhibitory molecule B7-H3: potential implications for immune based therapy of human solid tumors. *Cancer Res.* **69**, 6275–6281 (2009).
116. Hornung, V. *et al.* Sequence-specific potent induction of IFN-α by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nature Med.* **11**, 263–270 (2005).
117. Grimm, D. *et al.* Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* **441**, 537–541 (2006).
118. Garzon, R. *et al.* MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly *DNMT3A* and *3B* and indirectly *DNMT1*. *Blood* **113**, 6411–6418 (2009).
This work established miR-29 oligonucleotides as hypomethylating agents in leukaemia.
119. Fabbri, M. *et al.* MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. *Proc. Natl Acad. Sci. USA* **104**, 15805–15810 (2007).
First report that a miRNA can target the DNA methyltransferase machinery.
120. Thai, T. H. *et al.* Regulation of the germinal center response by microRNA-155. *Science* **316**, 604–608 (2007).
121. Rodriguez, A. *et al.* Requirement of bic/microRNA-155 for normal immune function. *Science* **316**, 608–611 (2007).
References 120 and 121 established the role of miR-155 in the regulation of the immune system.
122. Kotani, Ai. *et al.* miR-128b is a potent glucocorticoid sensitizer in MLL-AF4 acute lymphocytic leukemia cells and exerts cooperative effects with miR-221. *Blood* **114**, 4169–4178 (2009).
123. Pekarsky, Y. *et al.* Tcl1 expression in CLL is regulated by *miR-29* and *miR-181*. *Cancer Res.* **66**, 1590–11593 (2006).
124. Trang, P. *et al.* Regression of murine lung tumors by the *let-7* microRNA. *Oncogene* **29**, 1580–1587 (2010).
125. Lee, Y. S. & Dutta, A. The tumor suppressor microRNA *let-7* represses the HMGA2 oncogene. *Genes Dev.* **21**, 1025–1030 (2007).
126. Lodygin, D. *et al.* Inactivation of *miR-34a* by aberrant CpG methylation in multiple types of cancer. *Cell Cycle* **7**, 2591–2600 (2008).
127. Toyota, M. *et al.* Epigenetic silencing of *microRNA-34b/c* and *B-cell translocation gene 4* is associated with CpG island methylation in colorectal cancer. *Cancer Res.* **68**, 4123–4132 (2008).
128. Sun, F. *et al.* Downregulation of CCND1 and CDK6 by miR-34a induces cell cycle arrest. *FEBS Lett.* **582**, 1564–1568 (2008).
129. Yamakuchi, M., Ferlito, M. & Lowenstein, C. J. miR-34a repression of SIRT1 regulates apoptosis. *Proc. Natl Acad. Sci. USA* **105**, 13421–13426 (2008).
130. Pigazzi, M., Manara, E., Baron, E. & Basso, G. miR-34b targets cyclic AMP-responsive element binding protein in acute myeloid leukemia. *Cancer Res.* **69**, 2471–2478 (2009).
131. Cole, K. A. *et al.* A functional screen identifies miR-34a as a candidate neuroblastoma tumor suppressor gene. *Mol. Cancer Res.* **6**, 735–742 (2008).
132. Li, Z. *et al.* Distinct microRNA expression profiles in acute myeloid leukemia with common translocations. *Proc. Natl Acad. Sci. USA* **105**, 15535–15540 (2008).
133. O'Donnell, K. A. *et al.* c-Myc-regulated microRNAs modulate E2F1 expression. *Nature* **435**, 839–843 (2005).
134. Velu, C. S., Baktula, A. M. & Grimes, H. L. Gfi1 regulates miR-21 and miR-196b to control myelopoiesis. *Blood* **113**, 4720–4728 (2009).
135. Löffler, D. *et al.* Interleukin-6 dependent survival of multiple myeloma cells involves the Stat3-mediated induction of microRNA-21 through a highly conserved enhancer. *Blood* **110**, 1330–1333 (2007).
136. Zhu, S., Si., M-L, Wu, H. & Mo, Y. Y. MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (*TPM1*). *J. Biol. Chem.* **282**, 14328–14336 (2007).
137. Voorhoeve, P. M. *et al.* A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. *Cell* **124**, 1169–1181 (2007).

Competing interests statement
The authors declare no competing financial interests.