

MicroRNAs en route to the clinic: progress in validating and targeting microRNAs for cancer therapy

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Abstract | In normal cells multiple microRNAs (miRNAs) converge to maintain a proper balance of various processes, including proliferation, differentiation and cell death. miRNA dysregulation can have profound cellular consequences, especially because individual miRNAs can bind to and regulate multiple mRNAs. In cancer, the loss of tumour-suppressive miRNAs enhances the expression of target oncogenes, whereas increased expression of oncogenic miRNAs (known as oncomirs) can repress target tumour suppressor genes. This realization has resulted in a quest to understand the pathways that are regulated by these miRNAs using *in vivo* model systems, and to comprehend the feasibility of targeting oncogenic miRNAs and restoring tumour-suppressive miRNAs for cancer therapy. Here we discuss progress in using mouse models to understand the roles of miRNAs in cancer and the potential for manipulating miRNAs for cancer therapy as these molecules make their way towards clinical trials.

miR-17 ~ 92

This is a cluster of miRNAs containing miR-17, miR-20a, miR-18a, miR-19a, miR-19b and miR-92a. Clusters are expressed as a polycistronic message from a single promoter; this is indicated in the text by a tilde (~).

Paralogues

Genes that are derived from the same ancestral gene but that reside in different locations in the same genome.

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MicroRNAs (miRNAs) are a class of small, non-coding RNAs that post-transcriptionally control the translation and stability of mRNAs. The first miRNAs were identified through detailed forward genetic screens, which enabled the placing of these miRNAs into defined genetic pathways, thus providing a great deal of information regarding the biological roles of miRNAs in stem cell development^{1–5}. More recent identification of miRNAs has been accomplished through enormous, high-throughput biochemical screens that unveiled a plethora of over 1,000 human miRNAs⁶. Interestingly, hundreds of these miRNAs map to regions of the human genome that are known to be altered in cancer⁷, and a similar number are aberrantly expressed in cancerous tissues, and/or bodily fluids or waste products from cancer patients (reviewed in REF. 8). This new wealth of knowledge points to miRNAs as being novel cancer genes and biomarkers. For example, miRNA expression profiles are now used to classify tumours based on the tissue type and stage of disease^{8–10}. Unfortunately, the lack of high-throughput techniques to study miRNA functions has resulted in a pipeline of miRNAs that are ‘cancer related’, without having clearly defined molecular roles. Although hundreds of miRNAs are known to have dysregulated expression in cancer, key studies evaluating their biological and molecular roles, and their potential

therapeutic applications, are still rare. Yet understanding the functions of miRNAs is crucial if we hope to uncover the roles of this form of gene regulation in cancer and to harness this knowledge for therapeutic benefit.

In this Review we focus on mouse models in which specific miRNAs are overexpressed or knocked out in order to understand the biological and molecular roles of miRNAs in cancer and metastasis. We also review the recent literature regarding the transition of these master regulators into clinical settings both as direct cancer therapeutics and as tools to sensitize tumours to traditional chemotherapeutics.

Uncovering miRNA functions using mouse models

Although individual miRNAs are dysregulated in various diseases, clear, causal evidence of their role in cancer has only recently come to light. Specifically, several strains of mice lacking or overexpressing cancer-associated miRNAs have been developed and characterized. These include germline transgenic or knockout mice for: miR-155; miR-21; miR-17 ~ 92 and its paralogues; miR-15 and miR-16; miR-146; and miR-29. Additional mouse models are the LIN-28-overexpressing strain (which begins to evaluate the *in vivo* loss of mature *let-7*) and the multiple conditional DICER knockout models (TABLE 1). Interestingly, most of these mouse models for miRNA

At a glance

- Recently generated germline transgenic and knockout mice have provided a detailed view of the implication of the gain or loss of individual microRNAs (miRNAs), miRNA clusters and the miRNA processing machinery in cancer. These have been classified as oncogenic (such as miR-155, miR-21 and miR-17~92), tumour-suppressive (such as miR-15~16, LIN28, DICER) or context-dependent (such as miR-146 and miR-29).
- miRNAs and the miRNA processing machinery are involved in all stages of metastatic disease. Some — such as miR-200, the LIN28–let-7 interaction and DICER — contribute to both metastasis and primary tumour development, whereas others, such as miR-31 and miR-10b, are unique to metastasis.
- Studies uncovering miRNA function have led to their therapeutic application. Delivering tumour-suppressive miRNAs and silencing oncogenic miRNAs with antagomirs has been successful in various mouse models. Many of these studies began with overexpression and knockdown strategies and have since progressed to delivering miRNA-based molecules intranasally, intratumorally or systemically.
- Expanding on their therapeutic application, miRNAs are also being evaluated for their ability to sensitize cancers to chemotherapeutics. Much of this work is being accomplished in cell culture, with the hope that it will soon transition into preclinical model systems.

DICER

An endoribonuclease that cleaves precursor microRNAs (pre-miRNAs) into 20–25 nucleotide double-stranded RNAs.

Adaptive immune response

The adaptive immune response — also known as specific or acquired immunity — is mediated by antigen-specific lymphocytes and antibodies.

Innate immune response

The innate immune system provides an immediate, non-specific defence against pathogens until an adaptive, pathogen-specific immune response is able to develop.

Nestin promoter

This promoter drives the expression of genes in the central and peripheral nervous system and in myogenic and other tissues.

DMBA–TPA model

A two-stage chemical skin carcinogenesis model using a single dose of the genotoxic carcinogen, 7,12-dimethylbenz(a)anthracene (DMBA), followed by multiple doses of a non-genotoxic tumour-promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA).

Papillomas

Benign tumours of epithelial origin that grow outwards.

dysregulation present with defects in the immune system, and many of these models progress to haematopoietic cancers and, in some cases, solid tumours.

In discussing these mouse models and the supporting cell culture work and human tissue analysis, we have subdivided the following sections according to whether the miRNAs have strong data to support their role as either an oncogene or a tumour suppressor at this time. We follow with discussions of miRNAs for which there is evidence for context-dependent effects, and then we provide an overview of miRNAs that are involved in metastasis.

Oncogenic miRNAs

miR-155. The independent generation of transgenic, *mir-155*-overexpressing mice and *mir-155*-knockout mice demonstrated that this gene has a crucial role in the immune system^{11–14}; wild-type levels of miR-155 are essential for preserving normal immune system function, including the maintenance of both major classes of cells (B and T lymphocytes) of the adaptive immune response and dendritic cells, which are involved in the innate immune response^{13,14}. Although *mir-155*-knockout mice are immunocompromised owing to defects in these cell lineages, overexpression of miR-155 specifically in the B cell lineage results in pre-leukaemic pre-B cell proliferation in the spleen and bone marrow, followed later in life by B cell malignancy¹¹. The delay to malignancy is probably explained by the time that is required to accumulate the necessary secondary mutations, as miR-155 has recently been shown to repress genes encoding DNA damage response proteins¹⁵. In this model, miR-155 overexpression represents the ‘first hit’, thus establishing a pre-cancerous environment, which may be pushed towards malignancy by further mutations.

miR-21. One of the first oncogenic miRNAs identified was miR-21. Because of its elevated levels in many different cancers^{16–18}, three groups generated *mir-21*-knockout or *mir-21*-overexpressing mice^{19–21}. Forced 15–30-fold inducible overexpression of miR-21 under the control

of the nestin promoter resulted in severe pre-B-cell lymphoma²¹. In concordance, similar if not higher levels of miR-21 are reported in the serum and tumours of patients with cancer^{16,22}. Upon returning miR-21 to endogenous levels the mouse tumours disappeared. Notably, this was the first report indicating the addition of tumours to a single oncogenic miRNA (termed ‘oncomir addiction’). A miR-21 effect was also observed in a separate series of models¹⁹. Ubiquitous expression of miR-21, fourfold to sixfold over endogenous levels, resulted in no obvious phenotypes; however, miR-21 overexpression could potentiate the phenotype of mice with a latent *Kras*^{G12D} allele (*Kras*^{LA2}), a constitutively activated version of the KRAS proto-oncoprotein. Doubly transgenic animals had an increased lung tumour burden relative to the *Kras*^{LA2} mice, but no increase in the rate of conversion from adenoma to adenocarcinoma. By contrast, the lung tumour burden was decreased in *Kras*^{LA2}; *mir-21*^{-/-} animals, relative to the *Kras*^{LA2} controls. Unlike the previous study²¹, here miR-21 is involved in the later stages of tumorigenesis and not in tumour promotion, as it has no effect on tumorigenesis in the absence of oncogenic KRAS.

A third study reported an oncogenic role for miR-21 in skin carcinogenesis²⁰. In this DMBA–TPA model, wild-type animals developed early skin papillomas that progressed into invasive carcinomas. In identically treated *mir-21*^{-/-} animals, papilloma multiplicity and incidence were reduced. The molecular explanation probably involves increased expression of the pro-apoptotic miR-21 target genes sprouty homologue 1 (*SPRY1*), *PTEN* and programmed cell death protein 4 (*PDCD4*), which negatively regulate the RAF, PI3K and RAL guanine-nucleotide dissociation stimulator (RALGDS) pro-survival signalling pathways, respectively. In accordance, *mir-21* loss was associated with enhanced cellular apoptosis and a moderate reduction in proliferation.

These studies, combined with human tissue data and cell culture experiments, confirm that miR-21 is an oncogene and provide a rationale for the therapeutic inhibition of miR-21.

miR-17~92. Often miRNAs are found in large clusters that are expressed polyclonally; in these cases, it is often of value to evaluate the function of the cluster and of the individual miRNAs within the cluster. The *mir-17~92* cluster and its paralogues, *mir-106b~25* and *mir-106a~363*, are several such regions that have been extensively examined^{23,24}. The *mir-17~92* cluster, contained within a fragile site in the genome⁷, is amplified in both solid tumours and haematopoietic malignancies^{25–29}. As expected, mice overexpressing the miR-17~92 cluster in lymphocytes develop lymphoproliferative disease and autoimmunity, and they die prematurely²⁴. Note that even in the absence of genomic amplification, miR-17~92 expression can be directly induced through transactivation by MYC or MYCN³⁰. The interplay between MYC and miR-17~92 was demonstrated in a mouse model of B cell lymphoma. Tumour development was accelerated in animals reconstituted with haematopoietic stem cells expressing a truncated version

Table 1 | Germline overexpression and knockout models to evaluate *in vivo* miRNA functions

Gene product	Expression in the study	Observed phenotypes	Refs
Oncogenes			
miR-155	Overexpressed in the B cell lineage	Late-onset B cell malignancy	11
	Overexpressed in mature B cells	Increased fraction of germinal centre B cells	14
	Deleted in B cells	Reduction in the number of germinal centre B cells	14
	Ubiquitously deleted	Lung airway remodelling; enteric inflammation; impaired protective immunity owing to diminished B and T cell responses and impaired dendritic cell integrity	13
	Ubiquitously deleted	Loss of antigen- and tissue-specific inflammation	12
miR-21	Overexpressed in nestin-expressing cells	Pre-B-cell lymphoma	21
	Ubiquitously overexpressed	No phenotype alone; potentiated <i>Kras</i> ^{G12D} -induced lung tumorigenesis	19
	Ubiquitously deleted	Attenuated <i>Kras</i> ^{G12D} -induced lung tumourigenesis	19
	Ubiquitously deleted	Reduction in DMBA-TPA-induced skin papillomas	20
miR-29 [†]	Overexpressed in immature and mature B cells	Indolent B-CLL	59
miR-17~92	Overexpressed in lymphocytes	Lymphoproliferative disease and autoimmunity	24
	Ubiquitously deleted	Post-embryonic premature death, lung hypoplasia and ventricular septal defect; inhibited pro-B to pre-B transition; in combination with miR-106b~25 deletion, led to death at midgestation	23
miR-106a~363 [§]	Ubiquitously deleted	No phenotype	23
miR-106b~25 [§]	Ubiquitously deleted	No phenotype alone; death at midgestation when in combination with miR-17~92 deletion	23
	Overexpressed in prostate epithelium (in combination with its host gene, <i>MCM7</i>)	Prostatic intraepithelial neoplasia	31
LIN28	Overexpressed	Enhanced growth and delayed puberty	60
Tumour suppressors			
miR-15~16	Point mutation 3' to the stem-loop structure of precursor (pre)- <i>mir-16-1</i>	Autoimmune and B cell lymphoproliferative disease; B-CLL	36, 37
	Deletion of 13q14	Indolent B cell-autonomous, clonal lymphoproliferative disorders (monoclonal B cell lymphocytosis, CLL and non-Hodgkin lymphoma); disorders slightly more aggressive than <i>mir-15a-</i> and <i>mir-16-1</i> -null animals	38
	Deletion of <i>mir-15a~16-1</i>	Indolent B cell-autonomous, clonal lymphoproliferative disorders (monoclonal B cell lymphocytosis, CLL and non-Hodgkin lymphoma)	38
miR-146a	Ubiquitously deleted	Myeloproliferation and haematolymphoid tumours (myeloid sarcomas and lymphomas); autoimmune disorders (splenomegaly, lymphadenopathy, multi-organ inflammation)	45, 47
DICER	Conditionally deleted in lung	Enhanced lung tumour development in <i>Kras</i> ^{LSL-G12D} mice (<i>Kras</i> ^{LSL-G12D} ; <i>Dicer1</i> ^{+/-} and <i>Kras</i> ^{LSL-G12D} ; <i>Dicer1</i> ^{-/-}); reduced survival of <i>Kras</i> ^{LSL-G12D} ; <i>Dicer1</i> ^{+/-} mice when transgenes were induced in the lung	64, 160
	Conditionally deleted in lung or muscle	Haploinsufficient tumour suppressor; decreased survival in <i>Kras</i> ^{LSL-G12D} ; <i>Trp53</i> ^{R1/1} ; <i>Dicer1</i> ^{+/-} (relative to <i>Dicer1</i> ^{+/-} or <i>Dicer1</i> ^{-/-} triple transgenics)	64
	Conditionally deleted in retinoblasts	Haploinsufficient tumour suppressor in a retinoblastoma-sensitized background	65

[†] Additional data support a role for miR-29a as a tumour suppressor. [§]Paralogue of miR17~92. ^{||}*In vitro* data support an alternative role for miR-146a as an oncogene. B-CLL, B cell chronic lymphocytic leukaemia; DMBA, 7,12-dimethylbenz(a)anthracene; *MCM7*, mini-chromosome maintenance protein 7; miRNA, microRNA; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

Polycistronic
A single transcript that carries the information of several genes.

Fragile site
A site in a chromosome that is susceptible to chromosome breakage and fusion with other chromosomes.

of the cluster, *mir-17~19b-1*, and *E_μ*-driven *Myc*, probably through anti-apoptotic mechanisms²⁷ that might include the downregulation of the miR-17~92 targets PTEN and BCL-2-like protein 11 (BCL2L11; also known as BIM)²⁴. Genetic deletion of *mir-17~92* confirmed its importance for B cell development, whereas deletion of the paralogues, *mir-106b~25* or *mir-106a~363*, had no obvious phenotypes²³. By contrast, overexpression of the *mir-106b~25* cluster cooperated with overexpression of

its host gene, mini-chromosome maintenance protein 7 (*Mcm7*) to induce prostatic intraepithelial neoplasia. This provides an example of overexpression of a single genetic locus contributing to two 'oncogenic hits': elevated MCM7 levels and an increased expression of oncogenic miR-106b~25 (REF. 31).

Although the roles for these clusters in oncogenesis have been documented, the causal role for individual miRNAs within the clusters is just beginning to be defined. There

Host gene

A gene containing another gene (such as a microRNA within an intron of a protein-coding gene).

Seed region

Nucleotides 2–7 of the microRNA, typically having 100% complementarity with the target gene.

Xenografts

Grafts of cells or tissues that are transplanted from one species to another.

are six individual mature miRNAs within the miR-17~92 cluster, subdivided into three distinct families (miR-17, miR-20a and miR-18a; miR-19a and miR-19b; and miR-92a) based on sequence homology within the seed region. Of the three, the miR-19 family has been confirmed by two groups to contain the key oncomirs^{32,33}. Overexpression of the miR-17~92 cluster lacking both miR-19 family members in *E_μ-Myc* B cell lymphoma cells increased the latency of lymphoma when these cells were injected into a cohort of nude mice, relative to cells with the intact cluster³². In addition, the overexpression of individual miRNAs within the cluster, or inactivating mutations in both *mir-19a* and *mir-19b*, confirmed that miR-19 was both sufficient and necessary for promoting MYC-induced lymphomagenesis³³. Furthermore, multiple members of the miR-17~92 cluster — miR-19b, miR-20a and miR-92 — are capable of individually promoting NOTCH1-induced T cell acute lymphoblastic leukaemia (T-ALL) in a mouse model³⁴. Contrary to the above experiments that propose miR-19 as the major oncomir in the cluster, miR-20b and miR-92 had similar if not enhanced ability to reduce disease latency. These miRNAs reduce the expression of the tumour suppressors PTEN and BIM, which are frequently downregulated in T-ALL.

Tumour-suppressive miRNAs

miR-15~16. Similarly to the overexpression of miR-17~92, a B cell lymphoproliferative disorder is observed in mice that are deficient for miR-16 and/or miR-15a, which are the first miRNAs that were implicated in cancer³⁵. These two miRNAs constitute a small miRNA cluster that is located in 13q14, a fragile site in the genome that is deleted in >50% of cases of B cell chronic lymphocytic leukaemia (B-CLL). A naturally occurring mouse model, the New Zealand black (NZB) mouse, develops symptoms that are similar to human B-CLL, and a comprehensive genetic study identified a causative point mutation in the 3' flanking region of *mir-16* that reduced miR-16 expression³⁶. Reintroducing miR-15a~16 into cells that were derived from NZB mice restored cell cycle control and increased apoptosis, concurrent with reduced levels of the cell cycle regulator and miR-15 and miR-16 target, cyclin D1 (REF. 37). To further validate that the causal gene within the 13q14 deleted region is the *mir-15a~16* cluster, Dalla-Favera and colleagues³⁸ generated two strains of transgenic mice. The first strain lacks the 13q14 minimal deleted region (MDR); this deletion removes the *mir-15a~16* cluster and its host gene, deleted in leukaemia 2 (*Dleu2*). The second strain lacks only the *mir-15a~16* cluster. In both strains, a clonal population of B cells became evident as the mice aged. Furthermore, the disease was determined to be B cell autonomous; similar pathologies were obtained whether the deletions were ubiquitous or strictly confined to the B cell lineage. Although mice lacking the MDR had a more aggressive disease course, suggesting that an additional genetic element within the MDR locus contributes to the tumour-suppressive function, restoration of miR-15a~16, but not *DLEU2*, decreased cellular proliferation *in vitro*.

These models suggest a crucial function of the *DLEU2/mir-15a~16* locus in B cells; however, deletion of this region also occurs in other cancers, such as multiple myeloma and prostate cancer, which indicates its importance in other cellular contexts^{39,40}. For example, miR-15a and miR-16 are often downregulated in the stroma of prostate cancers, implicating a non-cell-autonomous mechanism for the cluster³⁹. Increased miR-15a and miR-16 expression in supporting fibroblasts impaired the expansion of prostate cancer xenografts through direct downregulation of fibroblast growth factor 2 (FGF2) and FGF receptor 1 (FGFR1)³⁹; this pathway promotes cell survival through activation of the RAS–MEK–ERK and PI3K–AKT pathways. (Note that aberrant expression of FGFs and FGFRs are found in multiple cancers, including prostate cancer⁴¹.)

These models support a tumour-suppressive role for the *mir-15a~16* cluster *in vivo*. Therefore, miR-15 and miR-16 replacement therapy should be considered for tumours that have lost miR-15a and miR-16 expression or that show elevated expression of cognate target genes.

Context-dependent miRNAs

miR-146. Some miRNAs, such as miR-146, have what seem to be opposing roles in tumorigenesis that depend on the cellular context. Expression levels of miR-146 family members, *mir-146a* and *mir-146b-5p*, which are encoded by separate genes, are elevated in breast, prostate, endocrine pancreatic, cervical, thyroid and ovarian carcinomas^{18,42–44}. Whether this elevation drives tumorigenesis or is a cellular safeguard that is put in place to prevent it is not entirely clear. Although most data support a tumour-suppressive function for miR-146 (see below)^{45–47}, a recent report showed that miR-146 can reduce the expression of the DNA repair enzyme BRCA1 (REF. 48) (FIG. 1). Although this remains to be confirmed in miR-146-overexpressing mice, it is suggestive of an oncogenic role in the breast cancer lines in which it was tested.

Albeit not a germline transgenic, overexpression of miR-146a in transplanted bone marrow cells provides evidence in support of a tumour-suppressive role⁴⁶. miR-146 overexpression reduced the survival and engraftment of haematopoietic stem cells in recipient mice, as shown by decreased erythropoiesis and impaired lymphopoiesis⁴⁶. Likewise, studies from *mir-146*-knockout mice further imply that miR-146 functions as a tumour suppressor in cells of haematopoietic origin. *mir-146a*-knockout mice develop massive myeloproliferation followed by tumours of haematolymphoid origin, including myeloid sarcomas and lymphomas^{45,47}. The myeloproliferative phenotype correlates with enhanced nuclear factor κB (NF-κB) signalling, which is most pronounced in the spleen and bone marrow⁴⁷. Indeed, miR-146a suppresses the NF-κB activators interleukin 1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6)^{45,49} (FIG. 1).

Based on the reported literature, miR-146 function may depend on the cellular context. Note that NF-κB signalling upregulates miR-146, which indirectly represses NF-κB⁴⁹ (FIG. 1). Thus, in cells in which

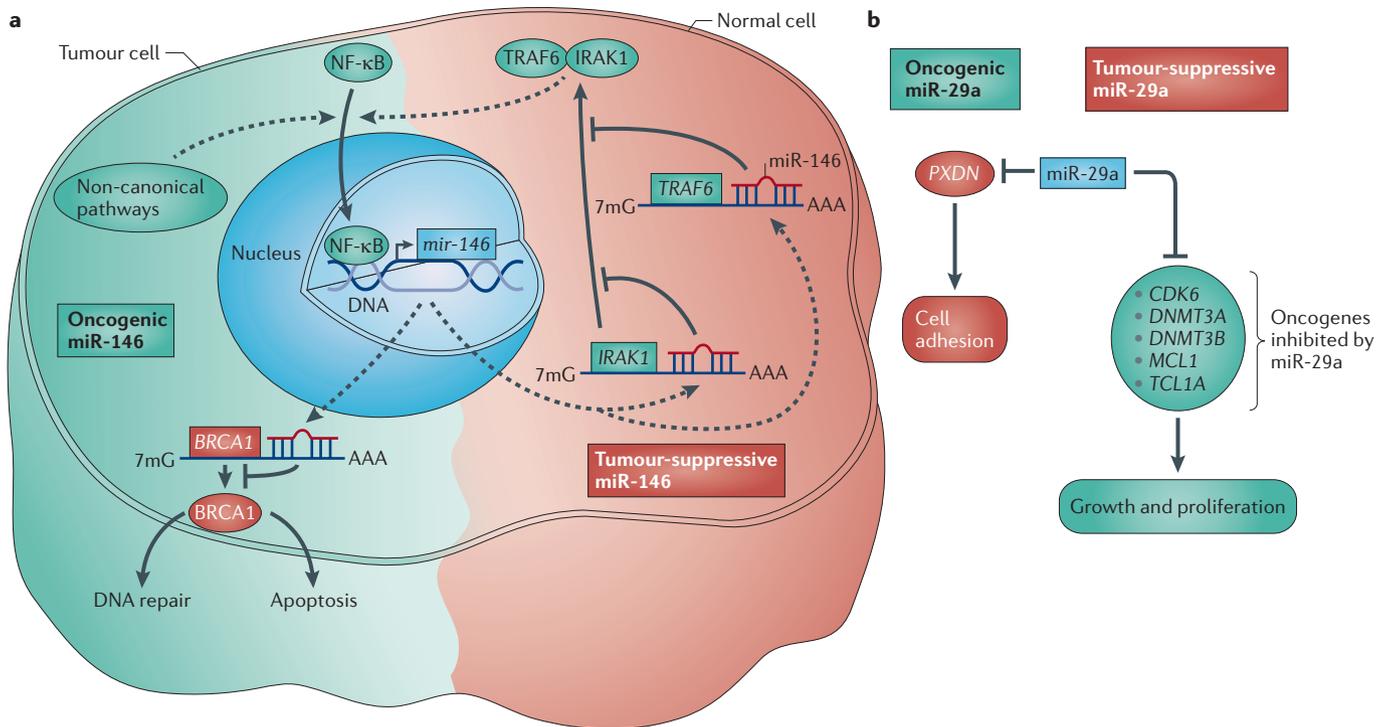


Figure 1 | Opposing roles of miRNAs in cancer. a | Oncogenic (shown in green) versus tumour-suppressive (shown in red) functions of miR-146 can be explained based on upstream nuclear factor κ B (NF- κ B) signals. In cells that are dependent on interleukin 1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) for NF- κ B signalling, miR-146 expression prevents NF- κ B activation, resulting in a tumour-suppressive phenotype. However, if an alternative mechanism for NF- κ B activation is present (other than through IRAK1 and TRAF6), activated NF- κ B would transactivate miR-146. In addition to targeting IRAK1 and TRAF6, miR-146 also targets BRCA1, thus preventing the pro-apoptotic effects of BRCA1 and resulting in a pro-survival response. **b** | Tumour-suppressive miR-29a targets multiple oncogenes, such as cyclin-dependent kinase 6 (CDK6), DNA (cytosine-5)-methyltransferase 3A (DNMT3A), DNMT3B, myeloid cell leukaemia sequence 1 (MCL1) and T-cell leukaemia/lymphoma 1A (TCL1A). Targeting inhibits growth and proliferation and, in the case of B cell chronic lymphocytic leukaemia (B-CLL), aggressive disease. Oncogenic miR-29a prevents cell adhesion through repressing peroxidase homologue (PDXN). 7mG, 7-methylguanine; miRNA, microRNA.

the primary driver of NF- κ B is IRAK1 and TRAF6, miR-146 will probably have tumour-suppressive effects through the negative regulation of NF- κ B (and hence negative autoregulation). By contrast, NF- κ B can still be activated in IRAK1- or TRAF6-deficient cells^{50,51} through other pathways; in these cells negative feedback through IRAK1 or TRAF6 will not have dramatic effects. Therefore, because NF- κ B induces *mir-146* transcription, the potential downstream oncogenic effects of this miRNA may be brought to the fore in these contexts. Therefore, perhaps the difference between the tumour-suppressive and oncogenic roles of miR-146 in haematopoietic cancers and solid tumours is the degree to which NF- κ B is regulated by IRAK1 or TRAF6.

Additionally, the complexity involved in miRNA regulation cannot be ruled out. Some factors that fluctuate greatly between cell types include miRNA expression, processing and nuclear export (FIG. 2) and the relative expression and subset of target genes (FIG. 3). Additionally, alternative polyadenylation, splicing and single nucleotide polymorphisms in either the mRNA or miRNA (BOX 1) can amend the miRNA binding sites (FIG. 3). Many of these anomalies are common in

oncogenic transcripts in cancer cells, rendering them less responsive to miRNA-dependent regulation. Regardless, more studies are needed to evaluate miR-146 and other miRNAs whose roles in tumorigenesis are ambiguous.

miR-29. The miR-29 family has several seemingly opposing functions in tumorigenesis. miR-29a and miR-29b, which are downregulated in mantle cell lymphoma, are suggested to be tumour suppressors that target multiple cell cycle regulators and oncogenes⁵²⁻⁵⁵ (FIG. 1). Consistent with a tumour-suppressive role, overexpression of miR-29 induces apoptosis and suppresses lung cancer and acute myeloid leukaemia (AML) cell tumorigenicity in xenografts^{52,56}. Furthermore, in indolent B-CLL, which has an average survival in humans of 25 years, miR-29 is proposed to target oncogenic *TCL1A*, potentially preventing fully malignant disease progression⁵⁴ (reviewed in REF. 57).

By contrast, the observation of miR-29 overexpression in AML and B-CLL, and *in vivo* miR-29 overexpression studies, imply that *mir-29* is an oncogene. In one experiment, lethally irradiated mice were transplanted with miR-29-transduced haematopoietic stem and progenitor cells, resulting in symptoms that were consistent

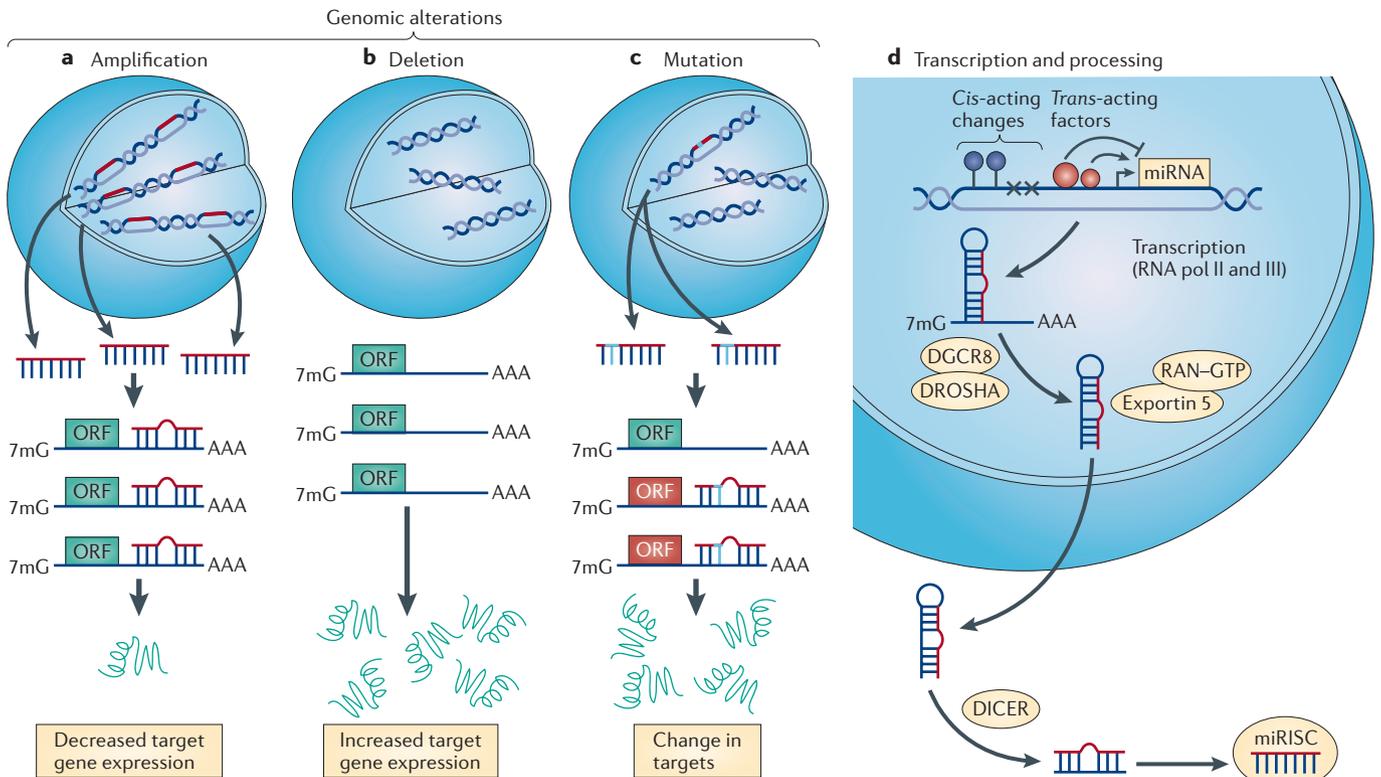


Figure 2 | RNAs are subject to genomic, transcriptional and post-transcriptional modes of regulation. **a** | Allelic amplification (which is typical of oncogenic microRNAs (miRNAs)) results in decreased expression of target genes, including those targets with less miRNA affinity that may not normally be repressed. **b** | Genomic deletion (which is typical of tumour-suppressive miRNAs) enhances target gene expression. **c** | Single nucleotide polymorphisms (SNPs) in the miRNA can either create or destroy miRNA binding sites (BOX 1). This alteration in the miRNA changes the ability of the miRNA to bind to and repress target genes. In cancer, SNPs can alter the miRNA such that it now targets tumour-suppressive genes while losing its ability to target oncogenes. **d** | At the transcriptional level, cis-acting changes to the promoter, including epigenetic regulation (such as promoter methylation, which is depicted by the blue circles) or genomic mutation (which is depicted as 'x') and the availability of trans-acting factors change the expression profile of miRNAs in a cell. Finally, miRNA processing defects can change the population of mature miRNAs in a cell. These processing steps include: primary miRNA (pri-miRNA) to precursor miRNA (pre-miRNA) cleavage by the DROSHA–DGCR8 complex; nuclear export by the RAN GTPase and exportin 5; a final cleavage event by DICER; and loading of the mature miRNA into the RNA-induced silencing complex (RISC). 7mG, 7-methylguanine; ORF, open reading frame.

with myeloproliferative disease and a latent progression to AML⁵⁸. A second system specifically evaluated the contribution of overexpressed miR-29a to B-CLL. When miR-29a was expressed in immature and mature B cells, there was an expansion of CD5⁺ B cells, mice presented with enlarged spleens, and roughly 20% of animals developed overt leukaemia and died late in life, suggesting that in this case miR-29a can predispose cells to a cancerous state⁵⁹. The molecular mechanism probably involved direct translational silencing of the tumour-suppressive cell-adhesion molecule peroxidase homologue (PXDN) by miR-29a (FIG. 1).

These data suggest that miR-29a functions as a tumour suppressor or oncogene, depending on the cellular context.

miRNA processing machinery

LIN28. Models in which the miRNA processing machinery is perturbed give insight into the function

of particular miRNA families, as observed in the LIN28-overexpressing strain. LIN28 is an RNA-binding protein that suppresses the maturation of *let-7* family miRNA precursors. In this way, a mouse model overexpressing *Lin28* overcomes the technical challenges associated with knocking out all 14 *let-7* family members^{60,61}. Because *let-7* is a bona fide tumour suppressor⁶², loss of *let-7* at the organismal level is predicted to predispose animals to cancer. These studies, which focused on the role of LIN28 in development⁶⁰ and glucose metabolism⁶¹, did not report any cancerous phenotypes. Whether overexpressing LIN28 at later timepoints can induce tumorigenesis or potentiate tumour-prone mice, such as those that overexpress activated *Kras* or *Myc* or that lack *Trp53*, remains to be determined.

DICER. In cancer, miRNA expression is frequently decreased⁶³; defects in DICER or other miRNA processing machinery could elicit such an effect (FIG. 2).

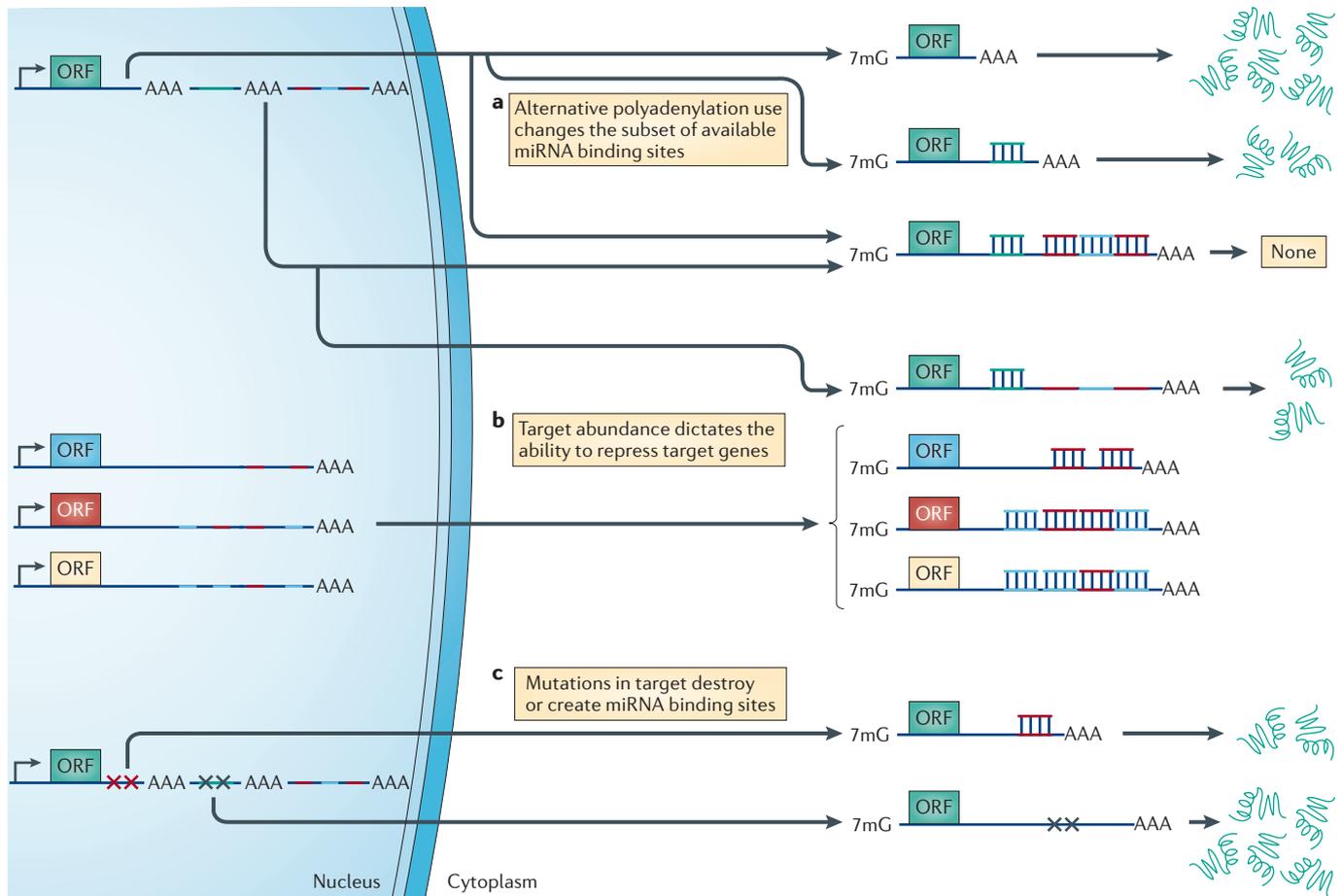


Figure 3 | RNA expression patterns dictate miRNA repressibility in cells. **a** | Alternative polyadenylation signals give an mRNA transcript the ability to evade regulation by microRNAs (miRNAs). Longer 3' untranslated regions (UTRs) usually contain more miRNA binding sites and are therefore more sensitive to repression by miRNAs. **b** | An increased abundance of additional target genes can 'soak-up' the miRNA pools, leading to target gene derepression. **c** | Mutations in the target gene can create or destroy miRNA binding sites. 7mG, 7-methylguanine; ORF, open reading frame.

Indeed, deletion of *Dicer1* reduces the overall levels of mature miRNAs⁶⁴. Perhaps surprisingly, animals with a single copy of *Dicer1* were tumour-prone and succumbed to death earlier than homozygous deleted animals⁶⁴. Moreover, tumours harvested from *Dicer1*^{fl/fl} mice, which in the presence of the CRE recombinase should lead to loss of both *Dicer1* alleles, retained one intact *Dicer1* allele suggesting that DICER functions as a haploinsufficient tumour suppressor^{64,65}. Overexpression of miRNAs or reductions in transcription factors that specifically regulate DICER (discussed below)^{66,67} recapitulates the phenotypes of *Dicer1* heterozygote mice, suggesting that the regulation of DICER in cancer is a multifactorial process. Importantly, human tumours also frequently present with hemizygous deletion of *DICER1* and overexpression of miRNAs that target DICER expression⁶⁶.

Interestingly, a phenotype similar to that of *Dicer1*^{fl/fl} mice was observed in *Tap63*^{-/-} mice⁶⁷. Multiple different isoforms exist for p63: the p63ΔN isoform lacks the transactivation domain and acts in a dominant-negative fashion against the tumour suppressor p53. By contrast, the full-length isoform, Tap63, which contains the transactivation domain, acts in concordance with p53.

Recent data suggest that Tap63 functions as a haploinsufficient tumour suppressor, similar to that of DICER⁶⁷. In *Tap63*^{-/-} animals, DICER was expressed at very low levels suggesting a genetic link between them; indeed, Tap63 binds to the *Dicer1* promoter and transactivates *Dicer1* expression. Accordingly, restoration of DICER expression in *Tap63*^{+/-} cells reduced their invasive potential.

miRNAs involved in metastasis

miRNAs have been identified as key players in several stages of metastasis (FIG. 4). For this Review we focus on miR-200, the LIN28–let-7 interaction and DICER — which are all involved in primary tumour development and early metastatic disease — and miR-31 and miR-10b, which have roles that are specifically constrained to metastatic progression without affecting the primary tumour. Because this was recently reviewed⁶⁸ we focus here on recent developments.

miR-200. Screens for miRNAs that are downregulated during the epithelial to mesenchymal transition (EMT) have identified five members of the miR-200

Haploinsufficient

A phenotype that arises in diploid organisms owing to the loss of only one allele.

Hemizygous

A state of having a single copy of a gene (in a diploid organism).

Epithelial to mesenchymal transition

(EMT). The conversion of polarized, immotile epithelial cells to motile mesenchymal cells. It is characterized by the loss of adhesion, the repression of E-cadherin, the expression of mesenchymal markers and increased cell motility.

Adjuvant

Treatment given in addition to the primary therapy. For cancer treatment, this typically refers to the therapy given after the surgical resection of a tumour.

family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429)^{69,70}. miR-200 loss is common in aggressive lung, prostate and pancreatic cancers^{71–73}. These studies suggest an association between EMT and the loss of miR-200; however, whether miR-200 loss is capable of inducing metastasis or is just a read-out of disease progression was only recently evaluated. In the highly aggressive *Kras*^{LA1}; *Trp53*^{R172HΔG} mouse lung cancer model, in which *Kras*^{G12D} is activated somatically through a latent allele and wild-type p53 function is perturbed, attenuation of miR-200 was required for metastasis⁷¹. Ectopic miR-200 expression prevented the invasion and metastasis of cells from these mice⁷¹ by repressing the mesenchymal markers zinc finger E-box-binding homeobox 1 (ZEB1) and ZEB2, thus restoring E-cadherin expression^{69,70,74} (FIG. 4). These studies support the use of miR-200 restoration therapy for aggressive cancers.

LIN28 and *let-7*. LIN28, which is involved in stem cell maintenance^{75–77} and is also a marker of cancer stem cells, is often activated in advanced stage and high-grade tumours⁷⁸. In concordance with this, *let-7* and LIN28 have recently been placed in a metastasis-signalling cascade involving the RAF kinase inhibitory protein (RKIP)⁷⁸. RKIP negatively regulates metastasis by inhibiting RAF kinase signalling and downstream MYC activation. Because MYC transcriptionally induces LIN28, RKIP indirectly suppresses LIN28 leading to enhanced *let-7* function (including the suppression of oncogenic *HMG2A* and RAS family members) (FIG. 4). Indeed, overexpressing LIN28 *in vivo* restores metastasis in RKIP-overexpressing tumours.

DICER. *In vivo* evidence supports the regulation of DICER expression by the miR-103/107 family, including a role for these miRNAs in metastasis. Piccolo and colleagues⁶⁶ screened *DICER1* for potential miRNA binding sites owing to its unusually long 3' untranslated region (UTR), and confirmed direct negative regulation by miR-103 and miR-107. miR-103 and miR-107 levels are inversely correlated with DICER levels in human breast cancer samples, and miR-103 and miR-107 upregulation is associated with metastasis^{66,79}. *In vivo* silencing of miR-103 and miR-107 inhibited metastasis, whereas overexpression induced metastasis of otherwise non-metastatic cells⁶⁶. Interestingly, upregulation of miR-103 or miR-107 in human samples occurs more often (37%)

than copy number variations of *DICER1* (18%), suggesting that reducing DICER levels by miRNAs is preferred to allelic loss⁶⁶.

The regulation of DICER by the miR-103/107 family was genetically linked to reduced miR-200 levels during EMT⁶⁶ (FIG. 4). Because miR-103 and miR-107 reduce DICER expression, most miRNAs, including miR-200, are downregulated when miR-103 and miR-107 are overexpressed. (This is also the case when *let-7* is overexpressed because it also targets DICER expression⁸⁰.) Bridging these pathways together, silencing of miR-103 and miR-107 restored miR-200 expression and prevented EMT, whereas miR-200 overexpression reverted EMT that was induced by miR-103 and miR-107.

miR-31 and miR-10b. Only a few miRNAs have been shown to have a clear role in metastasis without affecting the other steps of carcinogenesis; these include miR-31 and miR-10b. miR-31 expression is decreased in metastatic human breast cancer samples and has been confirmed to have an important role in metastasis^{81–83}. miR-31 overexpression causes the regression of metastases with no effect on the primary tumour⁸². Inducing miR-31 expression at various times following the transplantation of metastatic MDA-MB-231 human breast cancer cells did not alter primary tumour growth. However, miR-31 expression reverted the primary tumour from an invasive to a non-invasive phenotype through targeting prometastatic genes^{81,82} (FIG. 4). Similarly to treatment with traditional chemotherapies, miR-31 overexpression at later timepoints had no effect on the metastatic lesions. However, following primary tumour resection, miR-31 maintained its antimetastatic effects, thus pointing to the adjuvant use of miR-31. A similar scenario was observed for miR-10b, which is upregulated in — and seems to be restricted to — metastatic lesions (as discussed below in the 'miRNA-based therapeutics' section)⁸⁴.

Other miRNAs. Additional miRNAs that are involved in metastasis (in particular in breast cancer) include miR-373, miR-520c, miR-126 and miR-335 (REFS 85,86). In a genetic screen using a non-metastatic human breast tumour cell line that was transduced with a miRNA expression library, miR-373 and miR520c emerged as positive metastatic regulators⁸⁵. Overexpression of miR-373 and miR520c stimulated migration and invasion *in vitro* and *in vivo*, presumably through suppressing CD44, which is a cell surface marker of breast and prostate cancer stem cells. Conversely, loss of tumour-suppressive miRNAs miR-126, miR-335 and miR-206 was identified in tumours from patients with metastatic relapsing breast cancer⁸⁶, and forced overexpression of miR-126 and miR-335 in malignant breast cancer cells suppressed metastasis to the lung and bone in mice. Although miR-126 reduced overall tumour growth and proliferation, miR-335 specifically inhibited metastatic invasion by targeting the expression of proteins that are involved in cell migration, such as the cell fate determinant SOX4 and the extracellular matrix component tenascin C (TNC) (FIG. 4).

Box 1 | SNPs can destroy and/or create miRNA binding sites

Single nucleotide polymorphisms (SNPs) in either the mRNA¹⁵⁶ or the microRNA (miRNA) can alter the ability of a miRNA to regulate its target genes. The first mRNA SNP that was identified as affecting a potential miRNA binding site was in a *let-7* complementary site in *KRAS* (REF. 157). SNPs in oncogenes often destroy miRNA binding sites, thus allowing the gene to be expressed at higher levels. Conversely, SNPs in tumour suppressor genes generate novel binding sites, ultimately resulting in decreased expression of the tumour suppressor. As is the case for miRNAs, a single SNP can destroy and/or create a target site. In the case of cancer this would result in the destruction of oncogene repression and the creation of novel tumour suppressor gene repression. SNPs in miRNA promoters or precursors can also lead to expression¹⁵⁸ or processing¹⁵⁹ defects that alter mature miRNA levels (for a detailed review see REF. 8).

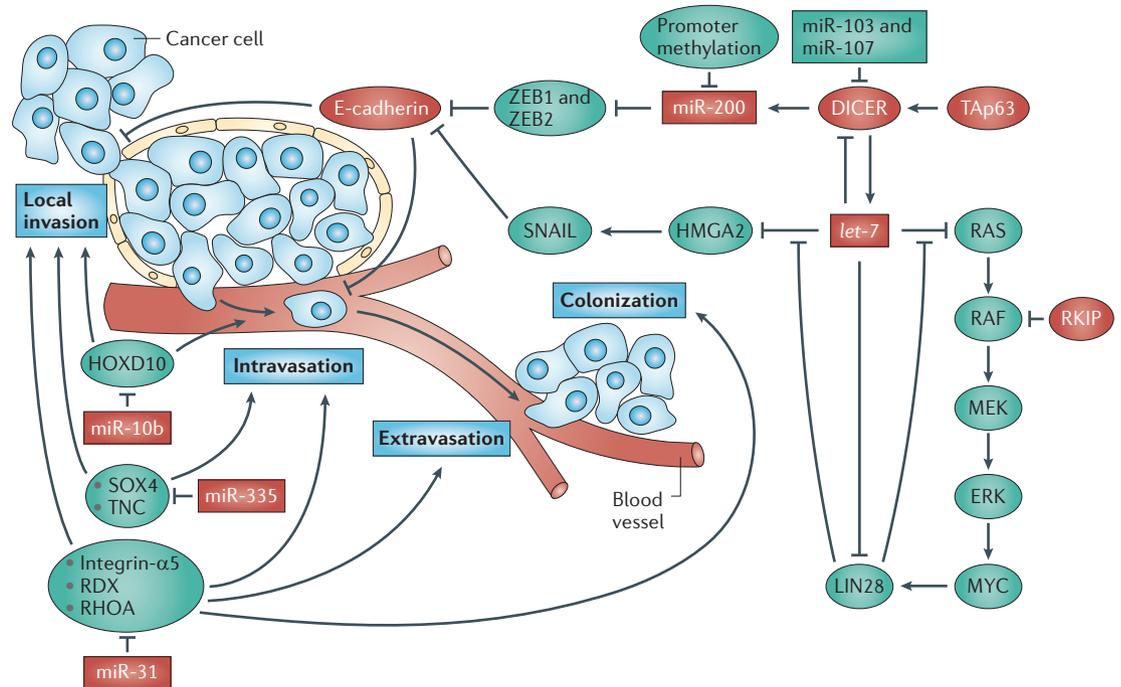


Figure 4 | miRNAs that contribute to metastasis. Metastasis occurs through a series of stages: local invasion, intravasation, extravasation and colonization (as indicated by the blue boxes). Protein-coding genes and microRNAs (miRNAs) that promote (shown in green) or prevent (shown in red) metastasis are involved at each step (the figure covers only those pathways that are discussed in the main text). miR-200 directly represses the expression of the mesenchymal markers zinc finger E-box-binding homeobox 1 (ZEB1) and ZEB2 in quiescent cells. As such, during the epithelial to mesenchymal transition (EMT) when miR-200 levels are reduced, the expression of ZEB1 and ZEB2 becomes elevated. ZEB1 and ZEB2 transcriptionally repress E-cadherin, a cell adhesion molecule that is lost in aggressive and metastatic tumours; thus, cells missing miR-200 are more able to disseminate and invade surrounding tissue. Furthermore, the inhibition between miR-200, and ZEB1 and ZEB2 is mutual, leading to a potentially bistable system: either high miR-200 and low ZEB1 and ZEB2 levels, or high ZEB1 and ZEB2 and low miR-200 levels. This latter state would seem to be particularly dangerous and could lead to aggressive tumours. Based on its miRNA-processing ability, DICER is positioned upstream of miR-200. *DICER1* itself is positively regulated at the transcriptional level by the full-length isoform of p63 (TAp63), and is negatively regulated by miR-103, miR-107 and *let-7*. In addition to suppressing DICER expression, *let-7* also inhibits the oncogenic RAS–RAF–MEK pathway and HMG2A2, an upstream activator of SNAIL. SNAIL inhibits E-cadherin, leading to local invasion and intravasation. Antimetastatic miRNAs that are involved outside of the E-cadherin pathway include miR-10b, which suppresses the expression of HOXD10; miR-335, which impairs SOX4 and tenascin C (TNC) signalling; and miR-31, which prevents all steps of metastasis through inhibiting the expression of integrin- α 5, radixin (RDX) and RHOA. RKIP, RAF kinase inhibitory protein.

miRNA-based therapeutics

It is well accepted that aberrant miRNA expression is linked to cancer, and the emerging mouse models described above provide evidence that miRNAs have a causal role in cancer. This implies that these molecules (in the case of tumour suppressors) or their antagomirs (in the case of oncomirs) might serve as effective therapeutics. Below we review some of the most promising individual miRNA-based therapeutic studies performed *in vivo*, which are summarized in TABLE 2.

As the therapeutic potential of individual miRNAs is explored, we note that combinatorial miRNA therapeutics will probably follow. Targeting an individual gene or a subset of genes with multiple tumour-suppressive miRNAs should enhance the therapeutic effect by reducing resistance. For example, a single mutation in the 3' UTR of an oncogene could disrupt the binding of a particular miRNA; however, combining two or three miRNAs that target the same gene would decrease the

likelihood of mutation-induced resistance (FIG. 3). In addition, simultaneous targeting of upregulated miRNAs with antagomir technology and replacement of lost tumour suppressor miRNAs may prove beneficial.

Replacing tumour suppressor miRNAs

let-7. Re-expressing tumour-suppressive miRNAs has great promise for cancer therapy. A prime example is the miRNA *let-7*, which is downregulated in multiple cancers (reviewed in REF. 87). Genomic loss of *let-7* and impaired *let-7* processing are two factors that reduce mature *let-7* levels^{7,75,88,89}. Therefore, restoring *let-7* may be beneficial in cancers that have genomic instability, abundant inhibitors of *let-7* processing or aberrantly expressed *let-7* targets (for example, *KRAS*, *HMG2A*, *MYC*, *LIN28*, cyclin-dependent kinase 6 (*CDK6*), *CDC25A* and cyclin D2 (*CCND2*)). Indeed, in the inducible *Kras*^{LSL-G12D/+} autochthonous model of non-small-cell lung cancer (NSCLC), delivering *let-7a* (REF. 88) or *let-7g*

Antagomirs
Chemically engineered, cholesterol-conjugated single-stranded RNA oligonucleotides that are complementary to microRNAs.

Autochthonous
Formed from endogenous tissue in the correct anatomical location.

Table 2 | Therapeutic use of miRNAs and antagomirs in vivo

miRNA [†]	Delivery method	Model used	Phenotypes	Refs
let-7	Intranasal delivery of viral particles	<i>Kras</i> ^{G12D/+} autochthonous NSCLC mouse	Suppression of lung tumour initiation when delivered at the same time as transgene activation	145, 146
	Intratumoral injection of lipid-based mimetic	Subcutaneous H460 NSCLC xenografts	Interfered with further tumour growth	62
	Intranasal delivery of viral particles	<i>Kras</i> ^{G12D/+} autochthonous NSCLC mouse	Reduced burden of tumours that were allowed to preform 10 weeks before let-7 therapy	62
	Systemic delivery of lipid-based mimetic	<i>Kras</i> ^{G12D/+} autochthonous NSCLC mouse	Reduced burden of tumours that were allowed to preform 10 weeks before let-7 therapy	90
	Transfected into cells before transplantation	Subcutaneous human U251 or U87 glioblastoma cells	Reduced tumour formation	91
	Transduced into cells before transplantation	Chemotherapy-resistant breast tumour initiating cells	Reduced tumour formation and metastasis	92
miR-143 and miR-145	Transduced into cells before transplantation	Subcutaneous MiaPaCa2 and Panc1 PDAC xenografts	Unable to form tumours	94
	Systemic delivery of lipid-based expression vectors	Subcutaneous MiaPaCa2 PDAC xenografts	Inhibited growth	96
	Systemic delivery of lipid-based expression vectors	Orthotopic MiaPaCa2 PDAC xenografts	Inhibited growth	96
miR-143	Systemic delivery of anti-miR-143	<i>p21-HBx</i> HCC mouse	Inhibited primary tumour and local and distant metastatic growth	100
miR-34	Intratumoral delivery of lipid-based mimetic	Subcutaneous H460 NSCLC xenografts	Inhibited growth	106
	Systemic delivery of lipid-based mimetic	Subcutaneous H460 and A549 NSCLC xenografts	Inhibited growth	106
	Systemic delivery of lipid-based mimetic	<i>Kras</i> ^{G12D/+} autochthonous NSCLC mouse	Reduced burden of tumours that were allowed to preform 10 weeks before miR-34 therapy	90
	Transfected oligonucleotides into cells before transplantation	Subcutaneous prostate cancer xenografts (multiple cell lines)	Reduced tumour incidence	107
	Transduced cells with virus encoding precursor (pre)-miR-34 before transplantation	Subcutaneous prostate cancer xenografts (multiple cell lines)	Reduced tumour incidence	107
	Intratumoral injection of lipid-based mimetic	Subcutaneous PPC1 prostate cancer xenografts	Inhibited growth	107
	Systemic delivery of lipid-based mimetic	Orthotopic PC3 prostate cancer xenografts	Reduced tumour burden	107
	Systemic delivery of lipid-based mimetic	Orthotopic LAPC9 prostate cancer xenografts	Reduced lung metastasis without affecting primary tumour growth; extended survival	107
	Systemic delivery of lipid-based expression vectors	Subcutaneous and orthotopic MiaPaCa2 PDAC xenografts	Inhibited growth	96
miR-122	Transduced into cells before transplantation	Orthotopic SKHEP1 HCC xenografts	Reduced tumorigenesis, angiogenesis and intrahepatic metastasis	111
	Systemic delivery of nucleic acid antagomir-miR-122	HCV-infected non-human primates	Suppression of HCV viraemia and improved liver pathology	112
	Systemic delivery of lysine-lipid nanoparticle antagomir-miR-122	C57BL/6J mice	Decreased plasma cholesterol levels	113
	Systemic delivery of antagomir-miR-122	C57BL/6J mice	Decreased plasma cholesterol levels	118
miR-26a	Systemic delivery of adeno-associated virus	HCC liver cancer model: MYC driven by the liver activator promoter	Inhibition of proliferation; induction of apoptosis; disease protection	114
miR-10b	Systemic delivery of antagomir-miR-10b	Implantation of 4T1 breast cancer cells into the mammary fat pad	Prevented metastasis with no effect on the primary tumour	84

[†]miRNAs are presented in the order in which they are discussed in the main text. miRNAs for which there is therapeutic evidence in endogenously occurring tumours or orthotopic implants are included. In some instances xenograft models are included but only as supporting evidence. HCC, hepatocellular carcinoma; HCV, hepatitis C virus; miRNA, microRNA; NSCLC, non-small-cell lung cancer; PDAC, pancreatic ductal adenocarcinoma.

Orthotopic

Transplanted into the correct anatomical location.

Silent information regulator 1

(SIRT1). A class-III histone deacetylase that regulates apoptosis in response to genotoxic and oxidative stress.

(REF. 89) to the lungs at the same time as *Kras*^{G12D} expression reduced tumour burden by as much as 66%. The burden of preformed xenografts was reduced by intratumoral delivery of *let-7b* (REF. 62), as was the burden of *Kras*^{G12D/+} lung tumours by the intranasal delivery of *let-7a* by lentiviral particles⁶² or by the systemic delivery of *let-7b* using a neutral lipid-based delivery system^{62,90}. Other xenograft studies further support the therapeutic potential of *let-7* replacement^{91,92}.

miR-143 and miR-145. Additional miRNAs also target oncogenic *KRAS*^{93–95}. Constitutively active *KRAS* activates RAS-responsive element binding protein 1 (RREB1), which directly inhibits the transcription of the miRNA cluster encoding miR-143 and miR-145. In a feed-forward mechanism, miR-143 and miR-145 repress the expression of RREB1 and *KRAS*, respectively⁹⁴; forced expression of miR-143 and miR-145 through cellular transduction⁹⁴ or lipid-based systemic delivery⁹⁶ in subcutaneous and orthotopic xenografts downregulated both *KRAS* and RREB1. A fine-tuned balance between the expression levels of the miR-143~145 cluster and *KRAS* is needed in quiescent cells. Loss of this cluster, as occurs in pancreatic, bladder, lung and colorectal carcinomas,^{94,96–99} or elevations in *KRAS* expression can perturb this balance, thus leading to carcinogenesis.

As with some other miRNAs, the role of miR-143 and miR-145 depends on cellular context. In hepatocellular carcinoma (HCC), the expression of miR-143 is elevated and its suppression through systemic delivery of antagomirs prevented local and distant metastasis¹⁰⁰. Similarly, the expression of miR-143 was significantly elevated in breast cancer tissue from individuals who relapsed¹⁰¹. However, a detailed analysis of 744 cancer samples identified miR-143~145 as a commonly deleted cluster, suggesting that the oncogenic roles of this cluster may be the exception rather than the norm¹⁰². Clearly, individualized profiles need to be performed before using many of these contradictory miRNAs as therapeutics.

miR-34. miRNAs of the p53 pathway have also been evaluated for therapeutic intervention. Transcriptionally induced by p53, miR-34 stimulates apoptosis or cellular senescence, induces G1 arrest and prevents migration. These effects are achieved through inhibiting the expression of silent information regulator 1 (SIRT1), BCL-2, CD44, various cyclins and CDKs and the proto-oncoproteins MYC and MYCN (reviewed in REFS 103,104). Similarly to *let-7*, miR-34 is repressed by multiple mechanisms, including epigenetic silencing owing to promoter methylation, allelic loss caused by genomic instability, and mutation of p53 (reviewed in REFS 103,105). Because miR-34 levels are frequently decreased in cancers, various laboratories have examined the therapeutic benefit of miR-34 replacement therapy. A miR-34 mimetic, delivered either intratumorally or systemically through tail vein injection, impaired tumorigenesis of NSCLC xenografts^{90,106}, and systemic delivery reduced the burden of preformed *Kras*^{LSL-G12D/+} lung tumours⁹⁰ by reducing proliferation and inducing apoptosis.

Although these pioneering studies examined the use of miR-34 in lung cancer, miR-34 replacement therapy should also be considered for other cancers that have p53 mutations or attenuated miR-34 expression, including prostate and pancreatic cancers^{96,107}. miR-34 levels were decreased in a subset of prostate cancer stem cells that were purified from xenografts and primary tumours¹⁰⁷. Enforced expression of miR-34 in the cancer stem cell population repressed clonogenic expansion, tumour regeneration and metastasis. Most notably, prostate cancer metastasis was impaired and mouse survival was extended through systemic delivery of lipid-based miR-34 mimetics. In another model, systemic delivery of a lipid-based nanovector expressing miR-34 inhibited the growth of subcutaneous and orthotopic MiaPaCa2 pancreatic tumour xenografts by suppressing proliferation and inducing apoptosis and tissue necrosis⁹⁶. Reduced expression of target genes *SIRT1* and *CD44* confirmed that sufficient miR-34 had reached the tumour tissue following systemic delivery; because suboptimal blood flow is a characteristic of pancreatic lesions, the delivery of therapeutics to these tumours is a frequent concern¹⁰⁸.

miR-122. Replacement of miR-122, a miRNA that is often correlated with high plasma cholesterol levels, is postulated to reduce HCC metastasis^{109–111}. Reduced miR-122 levels are correlated with intrahepatic metastasis and the loss of crucial liver functions in HCC, supporting the therapeutic potential of re-introducing miR-122. Although re-expressing miR-122 in cells reduced tumorigenesis, angiogenesis and metastatic potential in an orthotopic liver cancer model, systemic delivery of miR-122 has not yet been reported in cancer¹¹¹. However, antagomirs of miR-122 have been delivered systemically in hepatitis C virus (HCV)-infected non-human primates to suppress HCV viraemia¹¹², and in mice to decrease plasma cholesterol levels¹¹³. The pleiotropic role of miR-122 in multiple pathophysiologicals suggests that caution should be exercised before using miR-122 therapeutically.

miR-26a. Levels of miR-26a are also decreased in HCC^{114,115}. Systemic administration of miR-26a in a HCC model using an adeno-associated virus inhibited cancer cell proliferation, induced apoptosis and protected animals from disease progression¹¹⁴. Likewise, levels of miR-26a are also reduced in nasopharyngeal carcinoma and lymphoma^{116,117}. In both tumour types, miR-26 restoration reduced proliferation and colony formation through G1 arrest and through direct repression of the histone-lysine *N*-methyltransferase, EZH2, a global regulator of gene expression. Contrary to these findings, miR-26a was one of five miRNAs that independently promoted T-ALL through the inhibition of PTEN³⁴. In the background of activating mutations in *Notch1*, miR-26a overexpression decreased the latency of T-ALL more than the other miRNAs in the signature, including the bona fide oncomirs miR-20, miR-19 and miR-92. Indeed, the contextual nature of miR-26 function needs to be considered when advancing miR-26 therapies *in vivo*.

Targeting oncogenic miRNAs

miR-10b. Although miRNAs are frequently downregulated in cancer, some upregulated oncomirs have been therapeutically targeted *in vivo* with antagomirs. Proof-of-concept was established in 2005 when intravenous administration of antagomirs in mice impaired the functions of several miRNAs in multiple organs¹¹⁸. Also, miRNA silencing in HCV-infected non-human primates was successful with no toxicity¹¹². *In vivo* evidence supporting the use of antagomirs in cancer has also recently been established. Systemic intravenous delivery of antagomir-miR-10b — the effects of which are constrained to metastases — to tumour-bearing mice reduced miR-10b levels and suppressed the metastasis of 4T1 breast cancer cells to the lung⁸⁴. Antagomir-miR-10b treatment had no effect on the primary tumour but reduced pulmonary metastases by >80%. The crucial stages of metastasis targeted by antagomir-miR-10b were proposed to be the steps before extravasation (FIG. 4) because systemic antagomir-miR-10b did not prevent metastasis following the tail vein injection of 4T1 cells. Therefore, antagomir-miR-10b may prevent metastasis of highly invasive cancers containing elevated miR-10b levels, but may not be beneficial against already established metastatic lesions.

Sensitizing tumours to chemotherapy

Owing to the ability of miRNAs to target signalling pathways that are often perturbed in cancer, the potential of miRNAs or antagomirs to sensitize resistant cells to commonly used cancer therapies is being evaluated. Because this is an emerging area, limited *in vivo* analysis is available; however, based on promising *in vitro* experiments, more miRNA-based sensitization studies in preclinical animal models will probably be reported soon.

Multi-drug resistance. miRNAs that target genes that encode proteins involved in multi-drug resistance in tumours could sensitize otherwise impervious tumour cells. Multi-drug resistance usually involves the increased excretion of a drug through ATP-binding cassette (ABC) transporters. Two of these, *ABCC3* and *ABCC6*, are induced directly by *SOX2* (REF. 119). As such, when *SOX2* expression is elevated, cells indirectly dispense drugs back into the extracellular environment. Kim and colleagues¹¹⁹ recently identified miR-9 as a negative regulator of *SOX2*. Forced expression of miR-9* (miR-9 star strand) in a chemotherapy-resistant glioma stem cell line suppressed *SOX2* expression, which led to reduced ABC transporter expression and hence drug retention. *SOX2* is also one of a few transcription factors that is capable of inducing pluripotent stem cells. Therefore, miR-9* replacement therapy has the potential to decrease stemness as well as to reduce drug efflux.

Tamoxifen. miRNAs have also been shown to sensitize cancer cells to tamoxifen, the most widely used selective oestrogen receptor modulator (SERM). Tamoxifen treatment, although effective for many individuals, can result in recurrent breast cancer. Truncations of *ER α* or loss of its expression contribute to resistance. This is especially

problematic in individuals whose tumours overexpress the receptor tyrosine kinase *ERBB2*, which promotes growth and differentiation. One mechanism of *ERBB2*-induced survival involves the direct repression of the tumour-suppressive miRNAs miR-15 and miR-16 to restore anti-apoptotic *BCL-2* expression¹²⁰. Accordingly, reintroduction of miR-15 and miR-16 decreases *BCL-2* levels, thus sensitizing cells to tamoxifen. *ERBB2* also induces the oncomir miR-21, suggesting that antagonizing miR-21 may further sensitize cells to tamoxifen. Similar findings were identified for miR-342, which was downregulated in tamoxifen-resistant cells; overexpression sensitized cells to tamoxifen-induced apoptosis^{121,122}. Lastly, overexpression of the miR-221~222 cluster was associated with tamoxifen resistance through downregulation of the cell cycle inhibitor, p27^{KIP1}; ectopic p27^{KIP1} expression restored tamoxifen-induced cell death¹²². If these data are confirmed *in vivo*, miR-15, miR-16, miR-342 or anti-miR-221~222 could be used to enhance tamoxifen sensitivity.

Gefitinib. Levels of miRNAs that target cell survival signalling pathways are often decreased in chemotherapy-resistant cells; their re-expression affords the chemotherapy a better chance at treating the tumour. For example, forced overexpression of miR-126 sensitized cells to the small molecule epidermal growth factor receptor (EGFR) inhibitor gefitinib¹²³. Gefitinib is often a first-line treatment for EGFR-positive cancers, especially those of the lung and breast, to suppress downstream signalling by RAS and PI3K and thus to prevent uncontrollable growth and proliferation. However, point mutations within EGFR, or changes that activate the RAS and PI3K signalling network, can reduce gefitinib efficacy (reviewed in REF. 124). miRNAs targeting these pathways are often altered in cancers, especially in those that are resistant to therapeutics such as gefitinib. For example, levels of miR-126, which targets the PI3K pathway, are decreased in cancer; however, its overexpression was recently reported to resensitize two gefitinib-resistant NSCLC cell lines¹²³. Curiously, levels of miR-126 were also decreased in docetaxel-resistant breast cancer cells¹²⁵, so miR-126 replacement could also sensitize cells to conventional chemotherapeutics. There is also a rationale for using *let-7* to sensitize tumours to gefitinib, especially those with constitutively active *KRAS* alleles, as *let-7* can repress this pathway¹²⁶ and is known to radiosensitize lung cancer cells *in vitro*¹²⁷.

Taxanes. Taxanes are used to treat multiple cancers, and although individuals initially respond favourably, resistance can develop. Treatment with taxanes alters the expression of various genes that confer drug resistance. Many of these genes have since been found to be regulated by miRNAs, such as the oncogenic targets, mitogen- and stress-activated protein kinase 1 (MSK1; which is regulated by miR-148a), CASP8 and FADD-like apoptosis regulator (CFLAR, also known as c-FLIP; which is inhibited by miR-512-3p), *BCL-2* antagonist killer 1 (BAK1; which is repressed by miR-125b), SIRT1 and *BCL-2* (which are inhibited by miR-34) and inositol monophosphatase 1 (IMPA1; which is inhibited

star strand (miRNA*). The passenger strand of the mature microRNA (miRNA), originally thought not to be involved in miRNA-induced silencing; however, deep sequencing followed by functional studies have determined that the some of the miRNA* products are abundant and functional.

Selective oestrogen receptor modulator (SERM). An agent that targets the oestrogen receptors, *ER α* and *ER β* , which are often upregulated in breast cancer.

Docetaxel
A cytotoxic antimicrotubule agent that is used to treat breast, ovarian, prostate and non-small-cell lung cancer.

by *let-7* family members)^{128–132}. In all of these cases, forced expression of the individual tumour-suppressive miRNAs sensitized cells to taxanes. Conversely, levels of the oncomir miR-135a were significantly elevated in ovarian, NSCLC and uterine cells that were resistant to the taxane *paclitaxel*. Anti-miR-135a treatment in paclitaxel-resistant NSCLC xenografts restored sensitivity to paclitaxel, in part through the direct inhibition of adenomatous polyposis coli (APC) expression¹³³.

5-Fluorouracil. Based on studies showing that *5-fluorouracil* (5-FU) can alter protein-coding gene abundance, a comprehensive study was carried out to determine whether miRNA expression was altered in colorectal cancer cells that were treated with 5-FU. Among others, miR-21 levels were predictive of treatment response; high miR-21 levels correlated with poor therapeutic outcome¹³⁴. Some of the therapeutic resistance was attributed to miR-21-directed downregulation of core mismatch repair mutator genes¹³⁵. Similar findings were reported in HCC¹³⁶ and pancreatic cancer¹³⁷. In concordance, antagomirs directed against miR-21 were able to sensitize cultured cells to 5-FU treatment¹³⁷, suggesting that antagomir-miR-21 may sensitize tumours that are otherwise refractory to 5-FU.

Other chemotherapies. Many other studies have reported the ability of miRNAs and antagomirs to sensitize cells to cancer chemotherapeutics. These include antagonizing miR-21 and miR-221, or overexpressing miR-200 and *let-7*, in cells that are resistant to gemcitabine^{138,139}. In the case of cisplatin, overexpressing miR-34, miR-200c, miR-181 or miR-630 sensitized cells, as did antagonizing PTEN translation using miR-214 (REFS 140–142). The advancement of this knowledge in *in vivo* systems will help to guide the clinical development of miRNA-based therapies for sensitization to chemotherapy.

The forefront of miRNA-based therapeutics

It is likely, in light of recent advances in the field, that we will see the transition of therapeutics that are based on miRNAs into the clinic in the not-so-distant future. These master gene regulators are no longer the novel molecules once described as ‘oddities’¹⁴³. They are similar to protein-coding genes in that they regulate many survival-signalling pathways, are themselves subject to mutagenesis and often have conflicting roles in various disease states. They differ however in their therapeutic potential. In essence, miRNA replacement therapies have the capacity to do what protein-coding gene replacement therapies have tried to but with fewer obstacles to overcome. miRNAs are much smaller and less antigenic than their protein-coding counterparts and, as such, cellular delivery is possible without the use of potentially harmful viral-based delivery mechanisms that are needed for the cellular uptake of larger protein-coding genes.

Likewise, effective tools for systemically silencing miRNAs have been developed that specifically and safely target miRNAs. These antagomirs act as small sponges that soak-up miRNAs, resulting in subsequent

miRNA degradation and thus the upregulation of predicted targets with an *in vivo* effect that can be sustained for over 3 weeks¹¹⁸. This is in contrast to standard anti-sense miRNA targeting, which has a limited ability to suppress miRNA function and often leads to toxicity. Thus far, antagomirs have proven to efficiently and selectively silence miRNA function with limited side effects. Prime examples include systemically delivered antagomir-miR-122 in both primate and mouse models^{112,113,118} and antagomir-miR-10b against metastatic cancer in mice⁸⁴.

Indeed, multiple groups have explored effective mechanisms to deliver miRNAs in both a targeted and ubiquitous fashion with a good deal of success. Because many of these delivery strategies have recently been reviewed for small interfering RNAs (siRNAs)¹⁴⁴, we cover them minimally. Although the initial proof-of-principle studies using miRNAs as therapeutics took advantage of adenoviral-based¹⁴⁵ and lentiviral-based^{62,146} delivery methods, translation into clinical practice requires the development of safer delivery vehicles. These include packaging mature miRNAs into lipid-based nanoparticles (neutral or charged) that can be delivered locally to the tumour tissue or systemically, in which case they have been found to accumulate and to therapeutically regulate their targets in the lung^{90,106}, pancreas⁹⁶ and prostate¹⁰⁷. Expanding on this, physical and chemical moieties of the particles that facilitate the targeted distribution and the controlled and sustained release of miRNAs — including liposomes, polymers and dendrimer conjugates — are under clinical investigation¹⁴⁷. Furthermore, external moieties, such as aptamers and ligands that enhance cellular uptake by cancer cells, are being developed to direct the particles to a particular tissue^{148,149}. The challenge is to further refine the chemistry to allow the crossing of tissue barriers, especially in the case of treating glioblastoma with miRNAs, which has shown promise *in vitro* and in xenograft studies but is hindered owing to the inability of miRNAs to cross the blood–brain barrier⁹¹.

Certainly we must be cautious of the possible side effects of these molecules in human trials, independently of the side effects that could be associated with the delivery agents. Even in situations in which the miRNA is delivered directly to the tumour, miRNAs can escape from the tumour cells and become systemic, either through microvesicle exocytosis or secretion of miRNA–Argonaute 2 complexes that can be delivered to (and induce silencing of mRNA in) other cells^{150–153}. Furthermore, although there is value in overexpressing tumour-suppressive miRNAs, it is also important that this is monitored. It is plausible that miRNA overexpression could lead to the saturation of the miRNA machinery and non-specific effects. Previous reports suggest that the functions of two key miRNA silencing components, exportin 5 and Argonaute 2, are diminished when miRNA or siRNA levels are elevated^{154,155}. Furthermore, because DICER functions as a haploinsufficient tumour suppressor⁶⁴, overexpressing miRNAs may dampen DICER function and induce a tumour-prone

Gemcitabine

A nucleoside analogue that is used to treat multiple forms of cancer.

Cisplatin

A platinum-containing cytotoxic cancer drug.

environment. However, decreased DICER function due to the overexpression of a single miRNA has yet to be reported. Furthermore, increasing the cellular concentration of miRNAs may suppress lower-affinity targets that might not be targeted at endogenous miRNA levels. Similarly to current chemotherapies, concentration and dosing schedules need to be evaluated for efficacy

and toxicity, and the long-term effects following treatment must be assessed. The literature reviewed here suggests limited toxicity of some of these miRNAs in various model systems, whereas other miRNAs and antagomirs still need to be evaluated. Finally, clinical trials will have to evaluate whether this preclinical promise will be recapitulated in human patients.

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Competing interests statement

F.J.S. declares [competing financial interests](#). See Web version for details.

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