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## The microcosmos of cancer

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### Abstract

The discovery of microRNAs (miRNAs) almost two decades ago established a new paradigm of gene regulation. During the past ten years these tiny non-coding RNAs have been linked to virtually all known physiological and pathological processes, including cancer. In the same way as certain key protein-coding genes, miRNAs can be deregulated in cancer, in which they can function as a group to mark differentiation states or individually as bona fide oncogenes or tumour suppressors. Importantly, miRNA biology can be harnessed experimentally to investigate cancer phenotypes or used therapeutically as a target for drugs or as the drug itself.

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MicroRNAs (miRNAs) are small, evolutionarily conserved, non-coding RNAs of 18–25 nucleotides in length that have an important function in gene regulation. Mature miRNA products are generated from a longer primary miRNA (pri-miRNA) transcript through sequential processing by the ribonucleases Drosha and Dicer1 (ref. 1). The first description of miRNAs was made in 1993 in *Caenorhabditis elegans* as regulators of developmental timing<sup>2,3</sup>. Later, miRNAs were shown to inhibit their target genes through sequences that are complementary to the target messenger RNA, leading to decreased expression of the target protein<sup>1</sup> (Box 1). This discovery resulted in a pattern shift in our understanding of gene regulation because miRNAs are now known to repress thousands of target genes and coordinate normal processes, including cellular proliferation, differentiation and apoptosis. The aberrant expression or alteration of miRNAs also contributes to a range of human pathologies, including cancer.

The control of gene expression by miRNAs is a process seen in virtually all cancer cells. These cells show alterations in their miRNA expression profiles, and emerging data indicate that these patterns could be useful in improving the classification of cancers and predicting their behaviour. In addition, miRNAs have now been shown to behave as cancer ‘drivers’ in the same way as protein-coding genes whose alterations actively and profoundly contribute to malignant transformation and cancer progression. Owing to the capacity of miRNAs to modulate tens to hundreds of target genes, they are emerging as important factors in the control of the ‘hallmarks’ of cancer<sup>4</sup>. In this Review, we summarize the findings that provide evidence for the central role of miRNAs in controlling cellular transformation and tumour progression. We also highlight the potential uses of miRNAs and miRNA-based drugs in cancer therapy and discuss the obstacles that will need to be overcome.

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## miRNAs are cancer genes

In 2002, Croce and colleagues first demonstrated that an miRNA cluster was frequently deleted or downregulated in chronic lymphocytic leukaemia<sup>5</sup>. This discovery suggested that non-coding genes were contributing to the development of cancer, and paved the way for the closer investigation of miRNA loss or amplification in tumours. Subsequently, miRNAs were shown to be differentially expressed in cancer cells, in which they formed distinct and unique miRNA expression patterns<sup>6</sup>, and whole classes of miRNAs could be controlled directly by key oncogenic transcription factors<sup>7</sup>. In parallel, studies with mouse models established that miRNAs were actively involved in tumorigenesis<sup>8</sup>. Collectively, these findings provided the first key insights into the relevance of miRNA biology in human cancer.

Despite these results, the sheer extent of involvement of miRNAs in cancer was not anticipated. miRNA genes are usually located in small chromosomal alterations in tumours (in amplifications, deletions or linked to regions of loss of heterozygosity) or in common chromosomal-breakpoints that are associated with the development of cancer<sup>9</sup>. In addition to structural genetic alterations, miRNAs can also be silenced by promoter DNA methylation and loss of histone acetylation<sup>10</sup>. Interestingly, somatic translocations in miRNA target sites can also occur, representing a drastic means of altering miRNA function<sup>11,12</sup>. The frequent deregulation of individual or clusters of miRNAs at multiple levels mirrors the deregulation for protein-coding oncogenes or tumour suppressors (Table 1).

In principle, somatic mutations that change an miRNA seed sequence could lead to the aberrant repression of tumour-suppressive mRNAs, but these seem to be infrequent<sup>13</sup>. Further sequencing could change this view, but this observation suggests that the intensity of miRNA signalling (altered by miRNA overexpression or underexpression) is more crucial than the specificity of the response. However, recent data indicate that miRNAs with an altered sequence can be produced through variable cleavage sites for Drosha and Dicer1, and that the presence of these variants can be perturbed in cancer<sup>14</sup>. Although the function of the variant 'isomiRs' remains unclear, in principle they could alter the quality of miRNA effects. State-of-the-art sequencing techniques will help to unmask mutations or modifications that otherwise would remain undetected. Whatever the mechanism, the widespread alteration in the expression of miRNAs is a ubiquitous feature of cancer.

## miRNAs as cancer classifiers

Aberrant miRNA levels reflect the physiological state of cancer cells and can be detected by miRNA expression profiling and harnessed for the purpose of diagnosis and prognosis<sup>15,16</sup>. In fact, miRNA profiling can be more accurate at classifying tumours than mRNA profiling because miRNA expression correlates closely with tumour origin and stage, and can be used to classify poorly differentiated tumours that are difficult to identify using a standard histological approach<sup>6,17</sup>. Whether or not this increased classification power relates to the biology of miRNAs or the reduced complexity of the miRNA genome still needs to be determined.

The special features of miRNAs make them potentially useful for detection in clinical specimens. For example, miRNAs are relatively resistant to ribonuclease degradation, and they can be easily extracted from small biopsies, frozen samples and even formalin-fixed, paraffin-embedded tissues<sup>18</sup>. Furthermore, relatively simple and reproducible assays have been developed to detect the abundance of individual miRNAs, and methods that combine small RNA isolation, PCR and next-generation sequencing, allow accurate and quantitative assessment of all the miRNAs that are expressed in a patient specimen, including material that has been isolated by laser capture microdissection. The detection of global miRNA

expression patterns for the diagnosis of cancers has not yet been proved; however, some individual or small groups of miRNAs have shown promise. For example, in non-small cell lung cancer, the combination of high miR-155 and low let-7 expression correlates with a poor prognosis, and in chronic lymphocytic leukaemia a 13 miRNA signature is associated with disease progression<sup>15,16</sup>. Further advances in the technology of miRNA profiling could help to revolutionize molecular pathology.

Perhaps the most appealing application of miRNAs as a cancer diagnostic tool comes from the discovery of circulating miRNAs in serum. For example, miR-141 expression levels in serum were significantly higher in patients with prostate cancer than in healthy control individuals<sup>19</sup>. Although the analysis of circulating miRNAs is only just beginning, the successful advancement of this technology could provide a relatively non-invasive diagnostic tool for single-point or longitudinal studies. With such diagnostic tools in place, miRNA profiling could be used to guide cancer classification, facilitate treatment decisions, monitor treatment efficacy and predict clinical outcome.

### When miRNA biogenesis goes awry

Although the expression of some miRNAs is increased in malignant cells, the widespread underexpression of miRNAs is a more common phenomenon. Whether this tendency is a reflection of a pattern associated with specific cells of origin, is a consequence of the malignant state or actively contributes to cancer development is still unclear. Because miRNA expression generally increases as cells differentiate, the apparent underexpression of miRNAs in cancer cells may, in part, be a result of miRNAs being 'locked' in a less-differentiated state. Alternatively, changes in oncogenic transcription factors that repress miRNAs or variability in the expression or activity of the miRNA processing machinery could also be important.

Two main mechanisms have been proposed as the underlying cause of the global downregulation of miRNAs in cancer cells. One involves transcriptional repression by oncogenic transcription factors. For example, the MYC oncoprotein, which is overexpressed in many cancers, transcriptionally represses certain miRNAs, although the extent to which this mediates its oncogenic activity or reflects a peripheral effect is still unknown<sup>20</sup>. The other mechanism proposed involves changes in miRNA biogenesis and is based on the observation that cancer cells often display reduced levels, or altered activity, of factors in the miRNA biogenesis pathway<sup>21</sup> (Box 1, Fig. 1).

*In vivo* studies have provided the most direct evidence of an active role for miRNA downregulation in at least some types of cancer. For example, analysis of mouse models in which the core enzymes of miRNA biogenesis have been constitutively or conditionally disrupted by different mechanisms suggests that these molecules function as haploinsufficient tumour suppressors. Thus, the repression of miRNA processing by the partial depletion of Dicer1 and Drosha accelerates cellular transformation and tumorigenesis *in vivo*<sup>22</sup>. Furthermore, deletion of a single *Dicer1* allele in lung epithelia promotes *Kras*-driven lung adenocarcinomas, whereas complete ablation of *Dicer1* causes lethality because of the need for miRNAs in essential processes<sup>23</sup>. Consistent with the potential relevance of these mechanisms, reduced Dicer1 and Drosha levels have been associated with poor prognosis in the clinic<sup>24</sup>. In addition to the core machinery, modulators of miRNA processing can also function as haploinsufficient tumour suppressors. Hence, point mutations that affect *TARBP2* or *XPO5* are correlated with sporadic and hereditary carcinomas that have microsatellite instability<sup>25,26</sup>. Other miRNA modulators that influence the processing of only a subset of miRNAs could also be important. For example, LIN28A and LIN28B can bind and repress members of the *let-7* family (which are established

tumour-suppressor miRNAs; Table 1), but this binding can be counteracted by KHSRP (KH-type splicing regulatory protein), also a factor involved in miRNA biogenesis; together this binding and counteracting dictate the level of mature let-7. The processing of miRNAs can be regulated by other genes including *DDX5* (helicase p68) or the SMAD 1 and SMAD 5 proteins, which may contribute to cancer development through the deregulation of miRNAs<sup>27</sup>. Collectively, the global changes in miRNA expression that are seen in cancer cells probably arise through multiple mechanisms; the combined small changes in the expression of many miRNAs seem to have a large impact on the malignant state.

## miRNAs as cancer drivers

Functional studies show that miRNAs that are affected by somatic alterations in tumours can affect cancer phenotypes directly, therefore confirming their driver function in malignancy. As drivers of malignancy, mechanistic studies show that these miRNAs interact with known cancer networks; hence, tumour-suppressor miRNAs can negatively regulate protein-coding oncogenes, whereas oncogenic miRNAs often repress known tumour suppressors (Fig. 2a). Perhaps the best example of this is the oncogenic miR-17-92 cluster, in which individual miRNAs suppress negative regulators of phosphatidylinositol-3-OH kinase signalling or pro-apoptotic members of the BCL-2 family, which disrupts the processes that are known to influence cancer development<sup>28</sup> (Table 1).

Cancer-associated miRNAs can also alter the epigenetic landscape of cancer cells. The cancer 'epigenome' is characterized by global and gene-specific changes in DNA methylation, histone modification patterns and chromatin-modifying enzyme expression profiles, which impact gene expression in a heritable way<sup>29</sup>. In one way, miRNA expression can be altered by DNA methylation or histone modifications in cancer cells<sup>10,30</sup>, but miRNAs can also regulate components of the epigenetic machinery, therefore indirectly contributing to the reprogramming of cancer cells. For example, miR-29 inhibits *DNMT3A* and *DNMT3B* expression in lung cancer<sup>31</sup>, whereas miR-101 regulates the histone methyltransferase *EZH2* in prostate cancer<sup>32</sup>. The presence of mature miRNAs in the nucleus<sup>33</sup> is another indication of the potentially direct role that miRNAs have in controlling epigenetic modifications, such as DNA methylation and histone modifications — a hypothesis that has been established in plants<sup>34</sup> but still needs to be demonstrated with certainty in mammals.

In the same way as protein-coding genes, miRNAs can be oncogenes or tumour suppressors depending on the cellular context in which they are expressed, which means that defining their precise contribution to cancer can be a challenge (Fig. 2b). The fact that miRNAs show tissue-specific expression and their output, shown in the cell's physiology, is dependent on the expression pattern of the specific mRNAs that harbour target sites could explain this apparent paradox. For example, the miR-29 family has a tumour-suppressive effect in lung tumours but appears oncogenic in breast cancer because of its ability to target the DNA methyltransferases *DNMT3A* and *DNMT3B*, and *ZFP36*, respectively<sup>31,35</sup> (Table 1).

To further complicate the process, some miRNAs repress several positive components of a pathway, whereas others target both positive and negative regulators, possibly to buffer against minor physiological variations that could trigger much larger changes in the cell physiology<sup>36</sup>. In cancer cells, this buffering role can mean that some miRNAs could simultaneously target oncogenes and tumour-suppressor genes. In addition, combinations of miRNAs can cooperate to regulate one or several pathways, which increases the flexibility of regulation but confounds experimentalists<sup>37</sup> (Fig. 2c). Consequently, the way in which miRNAs contribute to cancer development is conceptually similar to cancer-associated transcription factors such as MYC and p53, which are mediated through many targets that

depend on contextual factors that are influenced by cell type and micro-environment. From a practical perspective it is crucial that miRNA targets are studied in a context that is appropriate to the environment that is being studied to determine what impact they will have on tumour cell behaviour (Fig. 2b).

## Oncogenic pathways

Beyond the impact of somatic genetic and epigenetic lesions, the altered expression of miRNAs in cancer can arise through the aberrant activity of transcription factors that control their expression. Interestingly, the same transcription factors are often targets of miRNA-mediated repression, which gives rise to complex regulatory circuits and feedback mechanisms. Thus, a single transcription factor can activate or repress several miRNAs and protein-coding genes; in turn, the alteration in miRNA expression can affect more protein-coding genes that then amplifies the effects of a single gene.

As already mentioned, MYC directly contributes to the global transcriptional silencing of miRNAs<sup>20</sup>. This repression involves the downregulation of miRNAs with antiproliferative, antitumorigenic and pro-apoptotic activity such as, let-7, miR-15a/16-1, miR-26a or miR-34 family members<sup>38</sup> (Fig. 2d; Table 1). Initial studies indicate that Myc uses both transcriptional and post-transcriptional mechanisms to modulate miRNA expression. This phenomenon could be due to LIN28A and LIN28B being the direct target of MYC, and that they are required for MYC-mediated repression of let-7 (ref. 38). Furthermore, MYC directly activates the transcription of miR-17-92 polycistronic cluster and, given its oncogenic role, it may contribute to MYC-induced tumorigenesis<sup>39</sup>.

MYC-driven reprogramming of miRNA expression could also be a factor in hepatocellular carcinoma, because of the contribution the reprogramming has to the aggressive phenotype of tumours originating from hepatic progenitor cells<sup>40</sup>. Some miRNAs, such as let-7, also regulate MYC, closing the regulatory circuit<sup>37</sup>.

miRNAs are embedded in many other oncogenic networks, including *KRAS* activation, which leads to the repression of several miRNAs. For example, in pancreatic cancer with mutant *KRAS*, RAS-responsive element-binding protein 1 (*RREB1*) represses miR-143 and miR-145 promoter, and at the same time both *KRAS* and *RREB1* are targets of miR-143 and miR-145, revealing a feedforward mechanism that increases the effect of RAS signalling<sup>41</sup>. Similarly, *KRAS* is a target for several miRNAs, of which the let-7 family is the most representative example<sup>42</sup>. The integration of miRNAs into key oncogenic pathways, and the generation of feedforward and feedback loops that have a balancing effect, creates intricate ways to incorporate intracellular and extracellular signals in the decisions of cell proliferation or survival, and further implicates miRNAs in the pathogenesis of cancer.

## *TP53* is a master regulator of miRNAs

The *TP53* tumour suppressor is perhaps the most important and well-studied cancer gene, and it is not surprising that several studies have suggested that miRNA biology can have a role in its regulation and activity (Fig. 2e). The p53 protein acts as a sequence-specific DNA-binding factor that can activate and repress transcription. Although there is no doubt that most of the actions of p53 can be explained by its ability to control canonical protein-coding targets such as *CDKN1A* and *PUMA*, it can also transactivate several miRNAs. One of the best-studied classes is the miR-34 family (Table 1), which represses genes that can promote proliferation and apoptosis — plausible targets in a p53-mediated tumour-suppressor response<sup>43</sup>. In principle, the action of p53 to induce the expression of miR-34 and other miRNAs can explain some of its transcriptional repressive functions.

The discovery of additional p53-regulated miRNAs, and the targeting of p53 or its pathway by other miRNAs, has provided general insights into the miRNA-mediated control of gene expression and the potential therapeutic opportunities for targeting the p53 network (Fig. 2e). Several p53-activated miRNAs, such as miR-192, miR-194, miR-215 and miR-605, can target *MDM2*, which is a negative regulator of p53 and a therapeutic target. These potentially relevant miRNAs can be epigenetically silenced in some types of cancer; however, their reactivation or reintroduction (see the section miRNAs as drugs and drug targets) offers an intriguing therapeutic opportunity for inhibiting *MDM2* in tumours that harbour wild-type p53 (refs 44, 45). Similarly, p53 can also activate miR-107, miR-200 or miR-192, which are miRNAs that inhibit angiogenesis and epithelial-to-mesenchymal transition<sup>46–48</sup>. Conversely, p53 can be repressed by certain oncogenic miRNAs including miR-380-5p, which is upregulated in neuroblastomas with *MYCN* amplification, or miR-504, which decreases p53-mediated apoptosis and cell-cycle arrest and can promote tumorigenesis<sup>49,50</sup>. However, the extent to which these miRNAs control life and death decisions in the p53 network still needs to be shown decisively to determine whether these miRNAs are valid therapeutic targets.

The studies mentioned have extended our understanding of the roles and regulation of p53 into the world of small non-coding RNAs, but the action on miRNA biology may be even more complex. For example, one study<sup>51</sup> suggests that p53 can affect miRNA biogenesis by promoting pri-miRNA processing through association with the large Drosha complex (Fig. 2e), but the precise mechanism remains unclear<sup>51</sup>. In a more conventional way, the p53 family member p63 transcriptionally controls *Dicer1* expression. Mutant *TP53* can interfere with this regulation, which leads to a reduction in *Dicer1* levels and reduces the levels of certain cancer-relevant miRNAs<sup>52</sup>. Thus, with the p53 network as a typical example, it is clear that miRNAs can interact with cancer-relevant pathways at multiple and unexpected levels and that a better understanding of miRNA biology will help to decipher the role and function of other important cancer genes.

## Micromanagement of metastasis and beyond

In addition to promoting cancer initiation, miRNAs can modulate processes that support cancer progression, including metastasis<sup>53–56</sup>. As indicated earlier, changes in miRNA levels can occur through effects on their transcription or by global changes in the RNA interference (RNAi) machinery, and both mechanisms seem to be important for this process. For example, in breast cancer, miR-10b and miR-9 can induce metastasis, whereas miR-126, miR-335 and miR-31 act as suppressors. The miR-200 family inhibits epithelial-to-mesenchymal transition, which influences one aspect of the metastatic process<sup>57</sup>. However, miR-200 could also promote the colonization of metastatic cells in breast cancer, which provides yet another example of the opposing activities of some miRNAs<sup>58</sup>. Conversely, in head and neck squamous-cell carcinomas, lung adenocarcinomas and breast cancers, the reduced levels of certain miRNAs that arise from *Dicer1* downregulation also promote cell motility and are associated with enhanced metastasis in experimental models<sup>52,59</sup>.

The pleiotropic effects of miRNA biology on cancer extend to virtually all acquired cancer traits, including cancer-associated changes in intracellular metabolism and the tissue microenvironment. For example, most cancer cells display alterations in glucose metabolism termed the Warburg effect<sup>60</sup>. miRNAs may contribute to this metabolic switch because, in glioma cells, miR-451 controls cell proliferation, migration and responsiveness to glucose deprivation, thereby allowing the cells to survive metabolic stress<sup>61</sup>. The enhanced glutaminolysis observed in cancer cells can be partially explained by MYC-mediated repression of miR-23a and miR-23b (ref. 62) (Fig. 2d). In some cases, the control of these cancer-related processes by miRNAs creates an opportunity for new therapeutic approaches.

Hence, miR-132, which is present in the endothelium of tumours but not in normal human endothelium, induces neovascularization by inhibition of p120RasGAP, a negative regulator of KRAS<sup>63</sup>. The delivery of a miR-132 inhibitor with nanoparticles that target the tumour vasculature suppresses angiogenesis in mice; this indicates there is a potential for the development of new antiangiogenic drugs. Further studies are likely to implicate miRNAs in the modulation of every tumour-associated pathway or trait.

## Big lessons from mice

Much of what we have learnt concerning the functional contribution of miRNA biology to cancer development comes from studies in genetically engineered mice. These systems provide powerful tools for the genetic and biological study of miRNAs in an *in vivo* context, which is particularly important given the contextual activity of most miRNAs. In addition, owing to the ability of these models to recapitulate the behaviour of some human malignancies, they are useful in preclinical studies to evaluate new therapeutics.

Perhaps the most widespread use of mice for characterizing miRNA biology in cancer is the validation of miRNAs that are altered in cancer cells, as bona fide oncogenes and tumour suppressor genes. As already mentioned, the first direct evidence that miRNAs have a function in cancer came from mouse models, in which it was shown that expression of the miR-17-92 cluster — which is amplified in some human B cell lymphomas — cooperates with Myc to promote B-cell lymphoma in mice<sup>8</sup>. Subsequent studies that have used genetically engineered or transplantation-based systems identified the relevant miRNA components, showing that the miR-19 family (including miR-19a and miR-19b) represents the most potent oncogenes in this cluster<sup>28,64,65</sup>.

Another example is miR-155 overexpression in the lymphoid compartment, which triggers B-cell leukaemia or a myeloproliferative disorder depending on the system used to drive expression of the transgene; this was the first example of an miRNA that initiates cancer in a transgenic setting<sup>66,67</sup> (Table 1).

Gene targeting has been used extensively to delete miRNAs for the purpose of characterizing their physiological roles or action as candidate tumour suppressors. Gene targeting has suggested that miRNAs from similar families have redundant or compensatory functions, which has been shown for *C. elegans*<sup>68</sup>. Ablation of the miR-15a and miR-16-1 cluster, which is often deleted in human chronic lymphocytic leukaemia, predisposes mice to B-cell lymphoproliferative disease<sup>69</sup> (Table 1). Importantly, the ability to produce mouse strains with different gene dosage through heterozygous or homozygous gene deletions has revealed that *Dicer1*, which if lost completely has a deleterious effect, can promote malignant phenotypes as a haploinsufficient tumour suppressor<sup>23</sup>. Such a conclusion could not be formed from studies that examined only genomic data.

Conditional gene expression systems in mice have allowed researchers to determine cancer gene dependencies, as well as whether genes that initiate cancer also participate in tumour maintenance. In many cases, withdrawal of the initiating oncogenic transgene (or restoration of the deleted or lost tumour suppressor) leads to the collapse of the tumour; this validates the transgene or pathway that is controlled by these genes, as a therapeutic target. Similar studies have also been applied to miR-NAs; for example, conditional expression of miR-21, which is broadly deregulated in cancer, can promote lymphomagenesis in mice<sup>70</sup> (Table 1). Silencing of miR-21 leads to disease regression, in part, by promoting apoptosis<sup>70</sup> (Fig. 3a). Likewise, the use of miRNA inhibitors (for example, antagomirs) directed against miR-21 can inhibit the proliferation of human cancer cells that overexpress miR-21 (ref. 71). Together, these studies suggest that miR-21 antagonists have the potential to be effective therapies for at least some cancers.

The development of new technology has meant that mouse models are increasingly used to study gene function on a large if not genome-wide scale, and miRNAs are at the forefront of this revolution. Recently, a vast collection of mouse embryonic stem-cell clones that harbour deletions that target 392 miRNA genes was generated<sup>72</sup>. This unique and valuable toolbox, termed 'mirKO', will allow the creation of mice that lack specific miRNAs, express mutant miRNAs or the study of their expression. In a converse strategy, a collection of embryonic stem cells engineered to inducibly express the vast majority of known miRNAs is in production (S.W.L., Y. Park and G. Hannon, manuscript in preparation) and will allow the *in vivo* validation of miRNAs as oncogenes or as anticancer therapies. With a different strategy, miRNA sponges (Fig. 3b), which are oligonucleotide constructs with multiple complementary miRNA binding sites in tandem, have already been used to deplete individual miRNAs in transgenic fruitflies, in transplanted breast cancer cells in mice and in a transgenic mouse model<sup>56,73,74</sup>. Although these sponges provide a scalable strategy for miRNA loss-of-function studies, more work is needed to rule out off-target effects and assess their potency before conclusions can be made. However, the availability of such resources will help with the functional study of miRNAs in normal development and disease, and will be useful to the wider scientific community.

Finally, genetically engineered mouse models of human cancers are a testing ground for preclinical studies. For example, in *Myc*-induced liver tumours, miR-26 delivery by adeno-associated viruses suppresses tumorigenesis by inducing apoptosis<sup>75</sup>. The increasing use of state-of-the-art mouse models is likely to uncover new *in vivo* functions, such as metastasis and angiogenesis, that otherwise would have remained hidden *in vitro*. They will also provide key preclinical systems for testing miRNA-based therapeutics.

## Constructing and deconstructing cancer

The use of RNAi technology — a tool that exploits miRNA pathways — has revolutionized the study of gene function in mammalian systems and has provided a powerful means to investigate the function of any protein-coding gene. Experimental triggers of RNAi exploit different aspects of the pathway and result in the downregulation of gene expression through incorporation into the miRNA biogenesis machinery at different points<sup>76</sup>. Small-interfering RNAs (siRNAs), which function at the level of Dicer1, can transiently and potently lead to gene suppression; these RNAi triggers, or their variants, are probably the structural 'scaffold' for miRNA therapeutics (see the section miRNAs as drugs or drug targets).

Stable RNAi can be activated by the expression of miRNA mimetics, that are either the so-called stem loop short-hairpin RNAs (shRNAs) or shRNAs that incorporate a larger miRNA fold. One example of the latter is based on miR-30 (known as miR-30-based shRNAs or 'shRNAmirs'). These shRNAs, as occurs naturally for many miRNAs, can be embedded in non-coding sequences of protein-coding transcripts or linked in tandem, which allows, for example, the linkage of the shRNA with a fluorescent reporter or the simultaneous knockdown of two different genes<sup>77,78</sup>. Advances in the shRNAmir methodology have allowed the development of versatile vectors for the study of proliferation and survival genes, strategies for optimizing the potency of shRNAs, and rapid and effective systems for conditional shRNA expression in mice<sup>79–81</sup>. The last of these, together with systems based on short stem-loop shRNAs<sup>82</sup>, could eventually allow the spatial, temporal and reversible control of any gene *in vivo*.

Regardless of the platform, RNAi technology provides an effective tool to investigate cancer phenotypes and identify therapeutic targets. For example, RNAi has been used to identify and characterize tumour-suppressor genes, which if inhibited promote cancer development. Early studies, using the same system that validated miR-17-92 as an oncogene,



demonstrated that inhibition of *TP53* could produce phenotypes that were consistent with *TP53* loss<sup>83</sup>. Later studies showed that tumour suppressors could be identified prospectively using *in vitro* and *in vivo* shRNA screens, (for examples see refs 84 and 85). By conditionally expressing shRNAs that target tumour suppressors in mice, tumour-suppressor function in advanced tumours can be re-established by silencing the shRNA<sup>86</sup>. Tumour-suppressor reactivation leads to a marked (if not complete) tumour regression, which validates these pathways as therapeutic targets.

RNAi technology can be exploited more directly to identify genotype-specific cancer drug targets. Although there may be differences in the outcome of RNAi and small-molecule-mediated protein inhibition, siRNAs and shRNAs have been widely used to determine whether a candidate target is required for the proliferation of cancer cells. Moreover, the availability of RNAi libraries that target portions of, or all, the human genome allows genetic screens to identify 'synthetic lethal' genes, for which, if combined, the attenuation triggers the death of the cell. In principle, the identification of an RNAi target, the inhibition of which is selectively lethal to cells harbouring a particular oncogenic alteration, should identify cancer-specific targets. Such approaches have identified potential targets for *KRAS*-expressing tumours<sup>87-89</sup> and leukaemias with deregulated *MYC* (ref. 90). Application of these approaches could potentially be complementary to the traditional drug-target discovery approach, and possibly a systematic way to identify the combination of therapies that will ultimately be needed to combat cancer.

## miRNAs as drugs and drug targets

Despite advances in techniques to inhibit protein-coding genes using small molecules or biologicals, many cancers are unresponsive to the agents currently in use or become resistant to them; new and more creative approaches are therefore required for the treatment of cancer. Perhaps one of the most exciting opportunities that has arisen from our understanding of miRNA biology is the potential use of miRNA mimics or antagonists as therapeutics. Owing to the ability of miRNAs to simultaneously target multiple genes and pathways that are involved in cellular proliferation and survival<sup>38</sup>, the targeting of a single miRNA can be a form of 'combination' therapy that could obstruct feedback and compensatory mechanisms that would otherwise limit the effectiveness of many therapies in current use. In addition, because miRNA expression is often altered in cancer cells, agents that modulate miRNA activity could potentially produce cancer-specific effects<sup>10,91,92</sup>. Based on this, anticancer therapies that inhibit or enhance miRNA activity are being developed (Fig. 4). Evidence for this is shown by the inhibition of oncogenic miRNAs or the expression of tumour suppressor miRNAs in mice that harbour tumours, which have a significant effect on the outcome of cancer. Oncogenic miRNAs can be blocked by using antisense oligonucleotides, antagomirs, sponges or locked nucleic acid (LNA) constructs<sup>93</sup>. The use of LNAs has achieved unexpected success *in vivo*, not only in mice but also for the treatment of hepatitis C in non-human primates<sup>94</sup>. The downregulation of miR-122 can lead to a significant inhibition of replication of the hepatitis C virus. This inhibition is thought to decrease the risk of chronic hepatitis and hepatocellular carcinoma in patients who are hepatitis C-positive. Early clinical studies using SPC3649, an miR-122 antagonist, in healthy individuals to assess toxicity will provide valuable information about pharmacokinetics and safety of the treatment. LNAs have been optimized to target miRNAs by reducing their molecular size and this, along with developing strategies for more efficient delivery, has increased their therapeutic potential<sup>95</sup>. By contrast, another strategy involves the restoration of tumour-suppressor miRNA expression by synthetic miRNA mimics or viral delivery<sup>93</sup>. Both of these approaches have yielded positive results in mouse models of cancer<sup>75,96</sup>. Adeno-associated virus delivery of miRNAs or miRNA antagonists has the advantage of being efficient and, because the virus does not integrate into the genome, non-

mutagenic. However, the delivery and safety of treatment needs to be improved before this approach can achieve widespread clinical use.

In principle, the use of miRNA mimetics as therapeutics would allow ‘drugging the undruggable’ or the therapeutic inhibition of virtually any human gene. If this were possible it would undoubtedly impact many diseases including cancer by allowing the targeting of oncogenic transcription factors that are difficult to inhibit through traditional medicinal chemistry<sup>97</sup>. Furthermore, owing to the similar chemistry that is used to create drugs that target diverse molecules, the implementation of miRNA-based therapies could allow a more uniform drug development pipeline than is possible for more conventional treatments. Although experimental studies have validated the underlying biological impact of achieving miRNA modulation, there are still practical challenges that prevent the use of miRNA mimetics and antagonists clinically, including uncharacterized off-target effects, toxicities and poor agent-delivery. Concerning the last of these, most miRNA mimetics and antagonists rely on the delivery of molecules that mimic or inhibit the ‘seed’ sequence of an miRNA (typically molecules that consist of 6 nucleotides or related structures) across the plasma membrane — a particular challenge in the treatment of cancer, in which missing even a few cancer cells could lead to tumour relapse and progression. Extensive research is now focused on the viral and non-viral strategies required to meet this challenge, and results in the preclinical setting are promising<sup>75,94–96</sup>. Despite the considerable hurdles that have to be overcome, it seems likely that miRNAs will find a place alongside more conventional approaches for the treatment of cancer.

## Perspectives

Since the discovery of miRNAs in model organisms, miRNAs have emerged as key regulators of normal development and a diversity of normal cellular processes. Given what we know now, it is not surprising that perturbations in miRNA biogenesis or expression can contribute to disease. In cancer, the effects of miRNA alteration can be widespread and profound, and they touch on virtually all aspects of the malignant phenotype. Yet, precisely how miRNAs regulate the expression of protein-coding genes is not completely understood, and the underlying mechanism remains an important basic-science question that will have a significant impact on our understanding of gene regulation and its alteration in disease. In addition, we still lack effective approaches to understand and predict miRNA targets. New strategies to identify and characterize the targets of individual miRNAs, and to determine how they function in combination to regulate specific targets, will be required to understand their action on cell physiology. Because miRNAs can also regulate other non-coding RNAs (for example, long non-coding RNAs), which have a role in cancer development and vice versa<sup>98</sup>, these interactions will increase the complexity of gene regulation and are likely to produce regulatory processes that are currently hidden. Pioneering knowledge, gained through the study of miRNA function and regulation, will undoubtedly provide methodological and theoretical insights that will help in our understanding of the more recently identified non-coding RNA species.

Understanding miRNA biology and how it contributes to cancer development is not only an academic exercise, but also provides an opportunity for the generation of new ideas for diagnosis and treatment. RNAi-based technology has allowed sophisticated loss-of-function experiments that were previously impossible and has revealed therapeutic targets that, when inhibited, can lead to cancer cell elimination. In addition, miRNAs themselves are being used directly in the diagnosis of cancer and, in the future, will probably be exploited in therapy to identify drug targets or as the drug treatment. However, cost-effective miRNA profiling strategies and larger studies are needed to determine whether miRNA profiling provides an advantage for cancer classification compared with a more traditional approach.

Although drugs that function as miRNA mimetics, antagonists or synthetic siRNAs form the core of what is fundamentally a new class of drugs that are capable of targeting molecules outside the range of traditional medicinal chemistry, their clinical implementation will require improvements in drug composition and delivery; these challenges lie outside the scope of molecular biology and instead involve the fields of chemistry and nanotechnology. Nevertheless, the successful development of these technologies could ultimately translate our understanding of miRNA biology in cancer into strategies for the control of cancer.

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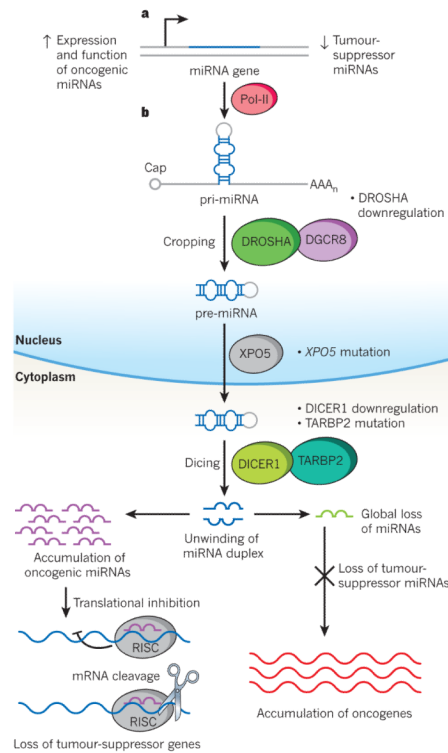
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**BOX 1****Biogenesis and function of miRNAs**

miRNAs are subjected to a unique biogenesis that is closely related to their regulatory functions. As the pathway in Fig. 1 shows, in general miRNAs are transcribed by RNA polymerase II into primary transcripts called pri-miRNAs<sup>76</sup>. The primary transcripts contain a 5' cap structure a poly(A)<sup>+</sup> tail and may include introns, similar to the transcripts of protein-coding genes<sup>76</sup>. They also contain a region in which the sequences are not perfectly complementary, known as the stem-loop structure, which is recognized in the nucleus by the ribonuclease Drosha and its partner DGCR8, giving rise to the precursor miRNA (pre-miRNA) by cropping<sup>76</sup>. However, some intronic miRNAs (called mirtrons) bypass the Drosha processing step and, instead, use splicing machinery to generate the pre-miRNA<sup>99</sup>. The pre-miRNA is exported from the nucleus to the cytoplasm by XPO5 and is further cleaved by the ribonuclease Dicer1 (along with TARBP2) into a double-stranded miRNA (process known as dicing)<sup>76</sup>. Again, this cleavage can be substituted by Argonaute-2-mediated processing<sup>100</sup>.

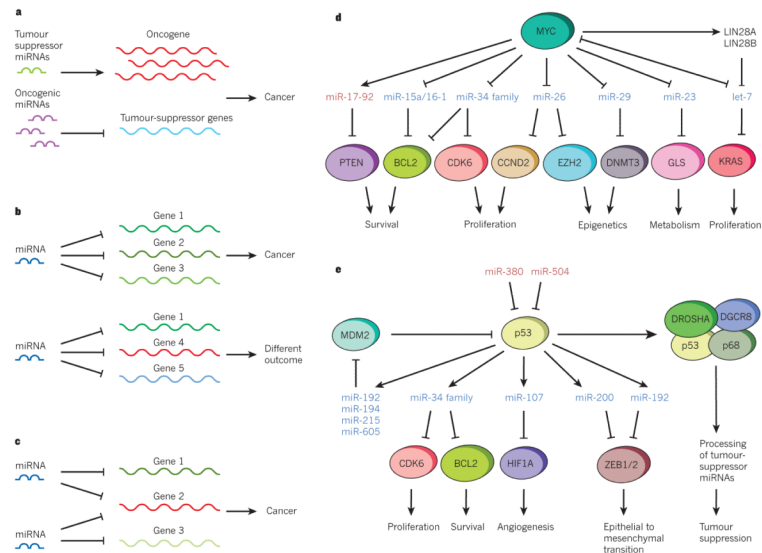
After strand separation, the guide strand or mature miRNA forms, in combination with Argonaute proteins, the RNA-induced silencing complex (RISC), whereas the passenger strand is usually degraded. The mature strand is important for specific-target mRNA recognition and its consequent incorporation into the RISC<sup>1</sup>. The specificity of miRNA targeting is defined by how complementary the 'seed' sequence (positions 2 to 8 from the 5' end of the miRNA) and the 'seed-match' sequence (generally in the 3' untranslated region of the target mRNA) are. The expression of the target mRNAs is silenced by miRNAs, either by mRNA cleavage ('slicing') or by translational repression<sup>1</sup>. In addition, miRNAs have a number of unexpected functions, including the targeting of DNA, ribonucleoproteins or increasing the expression of a target mRNA<sup>93</sup>. Overall, data indicate the complexity of miRNA-mediated gene regulation and highlight the importance of a better understanding of miRNA biology.





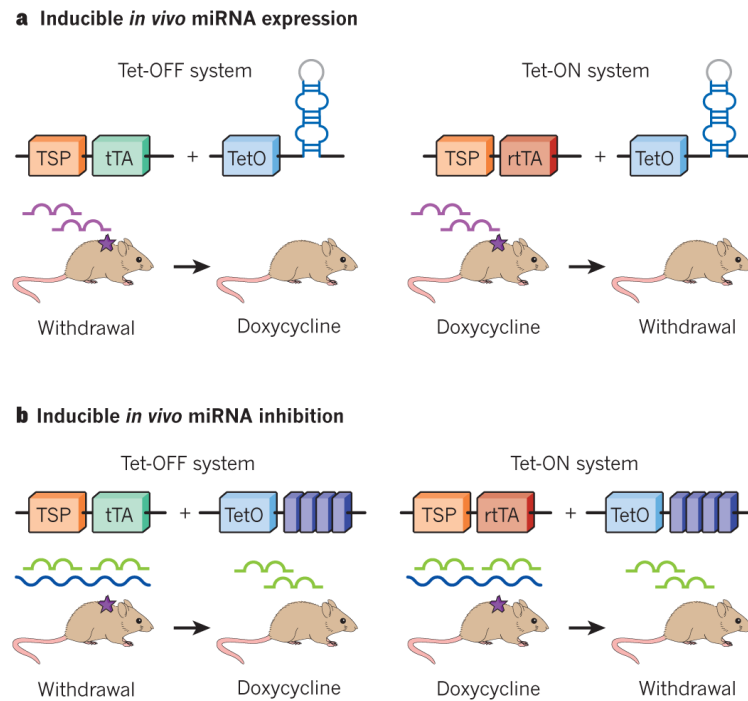
### Figure 1. Mechanisms of miRNA perturbation in cancer

Cancer cells present global downregulation of miRNAs, loss of tumour-suppressor miRNAs and specific accumulation of oncogenic miRNAs. The alteration in miRNA expression patterns leads to the accumulation of oncogenes and downregulation of tumour-suppressor genes, which leads to the promotion of cancer development. **a**, The expression and function of oncogenic miRNAs is increased by genomic amplification, activating mutations, loss of epigenetic silencing and transcriptional activation. By contrast, tumour-suppressor miRNAs are lost by genomic deletion, inactivating mutations, epigenetic silencing or transcriptional repression. **b**, After transcription, global levels of miRNAs can be reduced by impaired miRNA biogenesis. Inactivating mutations and reduced expression have been described for almost all the members of the miRNA processing machinery. If there is a downregulation of DROSHA this can lead to a decrease in the cropping of primary miRNA (pri-miRNA) to precursor miRNA (pre-miRNA). In the case of *XPO5* mutation, pre-miRNAs are prevented from being exported to the cytoplasm. Mutation of *TARBP2* or downregulation of DICER1 results in a decrease in mature miRNA levels. Pol II, RNA polymerase II; RISC, RNA-induced silencing complex.



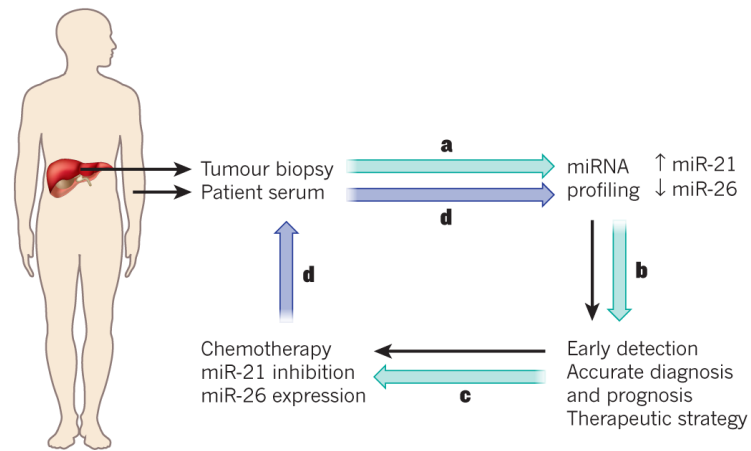
### Figure 2. Contribution of miRNAs to cancer pathways

**a**, Tumour-suppressor miRNAs, which repress oncogenes in healthy cells, are lost in cancer cells, leading to oncogene upregulation, whereas oncogenic miRNAs inhibit tumour-suppressor genes, giving rise to cancer. **b**, The presence of different target genes in different cell lines can modify the function of a miRNA, both in healthy cells and cancer cells, which can lead to the development of cancer or a different outcome. **c**, Two miRNAs can function together to regulate one or several pathways, which reinforces those pathways and can result in the development of cancer. **d**, The oncogene MYC can either repress tumour-suppressor miRNAs (in blue) or activate oncogenic miRNAs (in red) and can therefore orchestrate several different pathways. MYC can repress let-7, directly, or indirectly, through LIN28 activation. Conversely, let-7 can also repress MYC, which closes the regulatory circle. **e**, Tumour suppressor p53 can regulate several tumour suppressor miRNAs (blue), activating different antitumoral pathways. The regulation of *MDM2* by some of these miRNAs leads to interesting feedforward loops. At the same time, p53 can be negatively regulated by oncogenic miRNAs (in red). In addition, p53 is involved in the biogenesis of several tumour suppressor miRNAs.



**Figure 3. *In vivo* miRNA expression or inhibition ‘à la carte’**

**a**, Tetracycline (Tet)-mediated miRNA inactivation or activation by doxycycline administration using Tet-OFF, in which a tissue-specific promoter (TSP) is combined with a transactivator (tTA) to turn on expression of oncogenic miRNA (purple) and induce tumorigenesis (purple star) and subsequent tumour regression, revealing dependence on the oncogenic miRNA, or Tet-ON systems in which a reverse transactivator (rtTA) switches on oncogenic miRNA when the drug is applied. Drug withdrawal leads to tumour regression. **b**, Tet-mediated miRNA activation or inactivation by doxycycline administration using Tet-OFF or Tet-ON systems. miRNAs (green) can be inhibited by miRNA sponges (dark blue), with the same effects as miRNA expression, leading to tumorigenesis and subsequent tumour regression, which indicates a dependence on tumour-suppressor loss.



**Figure 4. Proposed scheme for the treatment of liver cancer with combined chemotherapy and miRNA-based therapy**

**a**, miRNA expression profiles of potential patients could be assessed by measuring circulating miRNAs in patient serum or tumoral miRNAs from a biopsy. For example, miR-21 expression and miR-26 loss could be detected in serum and tumour samples. **b**, This profile could be used for early detection of cancer, accurate diagnosis and prognosis, and choosing the best therapeutic strategy. The best available chemotherapeutic option could be combined with miRNA-based therapy. **c**, The oncomiRs detected in miRNA profiling and those present in the tumour, such as miR-21, could be inhibited by using different strategies, such as locked nucleic acid constructs. By contrast, the expression of tumour-suppressor miRNAs downregulated in the tumour could be restored and miR-26 levels could be increased with miRNA mimics. **d**, After treatment, the patient could be checked for relapse by periodically studying circulating miRNAs from serum in a non-invasive manner. The presence of miR-21 could indicate a potential relapse, and treatment would resume (black arrows).

Table 1

## Key microRNAs involved in cancer

MicroRNA	Function	Genomic location	Mechanism	Targets	Cancer type	Mouse models	Clinical application
miR-17-92 cluster	Oncogene	13q22	Amplification and transcriptional activation	BIM, PTEN, CDKN1A and PRKAA1	Lymphoma, lung, breast, stomach, colon and pancreatic cancer	Cooperates with MYC to produce lymphoma. Overexpression induces lymphoproliferative disease	Inhibition and detection
miR-155	Oncogene	21q21	Transcriptional activation	SHIP1 and CEBPB	Chronic lymphocytic leukaemia, lymphoma, lung, breast and colon cancer	Overexpression induces pre-B-cell lymphoma and leukaemia	Inhibition and detection
miR-21	Oncogene	17q23	Transcriptional activation	PTEN, PDCD4 and TPM1	Chronic lymphocytic leukaemia, acute myeloid leukaemia, glioblastoma, pancreatic, breast, lung, prostate, colon and stomach cancer	Overexpression induces lymphoma	Inhibition and detection
miR-15a/16-1	Tumour suppressor	13q31	Deletion, mutation and transcriptional repression	BCL2 and MCL1	Chronic lymphocytic leukaemia, prostate cancer and pituitary adenomas	Deletion causes chronic lymphocytic leukaemia	Expression with mimics and viral vectors
let-7 family	Tumour suppressor	11 copies (multiple locations)	Transcriptional repression	KRAS, MYC and HMGA2	Lung, colon, stomach, ovarian and breast cancer	Overexpression suppresses lung cancer	Expression with mimics and viral vectors
miR-34 family	Tumour suppressor	1p36 and 11q23	Epigenetic silencing, transcriptional repression and deletion	CDK4, CDK6, MYC and MET	Colon, lung, breast, kidney, bladder cancer, neuroblastoma and melanoma	No published studies	Expression with mimics and viral vectors
miR-29 family	Oncogene	7q32 and 1q30	Transcriptional activation	ZFP36	Breast cancer and indolent chronic lymphocytic leukaemia	Overexpression induces chronic lymphocytic leukaemia	No published studies
	Tumour suppressor		Deletion and transcriptional repression	DNMTs	Acute myeloid leukaemia, aggressive chronic lymphocytic leukaemia and lung cancer		No published studies

BCL2, B-cell lymphoma protein-2; BIM, BCL-2-interacting mediator of cell death; CDKN1A, cyclin-dependent kinase inhibitor 1A; CEBPB, CCAAT/enhancer binding protein  $\beta$ ; HMGA2, high mobility group AT-hook 2; CDK4, cyclin-dependent kinase 4; CDK6, cyclin-dependent kinase 6; DNMT, DNA methyltransferase; MCL1, myeloid cell leukaemia sequence 1; PTEN, phosphatase and tensin homologue; PRKAA1, protein kinase, AMP-activated, alpha 1 catalytic subunit; PDCD4, programmed cell death 4; SHIP1, Src homology 2 domain-containing inositol 5-phosphatase 1; TPM1, tropomyosin 1; ZFP36, zinc finger protein 36.