

# miRNAs in human cancer

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## Abstract

Mature microRNAs (miRNAs) are single-stranded RNA molecules of 20–23 nucleotide (nt) length that control gene expression in many cellular processes. These molecules typically reduce the stability of mRNAs, including those of genes that mediate processes in tumorigenesis, such as inflammation, cell cycle regulation, stress response, differentiation, apoptosis and invasion. miRNA targeting is mostly achieved through specific base-pairing interactions between the 5' end ('seed' region) of the miRNA and sites within coding and untranslated regions (UTRs) of mRNAs; target sites in the 3' UTR lead to more effective mRNA destabilization. Since miRNAs frequently target hundreds of mRNAs, miRNA regulatory pathways are complex. To provide a critical overview of miRNA dysregulation in cancer, we first discuss the methods currently available for studying the role of miRNAs in cancer and then review miRNA genomic organization, biogenesis and mechanism of target recognition, examining how these processes are altered in tumorigenesis. Given the critical role miRNAs play in tumorigenesis processes and their disease-specific expression, they hold potential as therapeutic targets and novel biomarkers.

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## miRNA overview

miRNAs were originally shown to be important in the timing of larval development in *C. elegans*, identifying the miRNAs *lin-4* and *let-7* [1,2]. Our initial understanding of miRNA–mRNA target recognition came from observations of sequence complementarity of the *lin-4* RNA to multiple conserved sites within the *lin-14* 3' UTR [1,3]; molecular genetic analysis had shown that this complementarity was required for the repression of *lin-14* by *lin-4* [4]. Homologues of *let-7* or *lin-4/miR-125* were thereafter shown to have temporal expression in other organisms, including mammals, and to regulate mammalian development [5–8]. Given their integral role in development, it was no surprise that miRNAs were soon found to be important in tumorigenesis, and since their discovery close to 3000 publications associate miRNAs to cancer, including over 700 reviews (examples include [9–11]). miRNAs were initially linked to tumorigenesis due to their proximity to chromosomal breakpoints [12] and their dysregulated expression levels in many malignancies [13,14].

Given the wealth of rapidly accumulating information implicating miRNAs in cancer, to allow the reader to critically assess the reports exploring the function of miRNAs in malignancies, we first review the methods used to study the expression and role of miRNAs in tumours, and then review the evidence

that relates miRNA genomic organization, biogenesis, target recognition and function to tumorigenesis. An overview of miRNA cisgenic expression and sequence similarity allows a better understanding of the regulation of miRNA expression and the factors contributing to technical limitations in accuracy of miRNA detection. Understanding the regulatory potential of miRNAs based on sequence similarity families and miRNA abundance allows evaluation of which miRNAs are important regulators of tumorigenesis pathways.

## Methods for studying miRNA genetics and expression

### miRNA profiling

The main methods currently used for miRNA profiling are sequencing, microarray and real-time RT–PCR-based approaches (reviewed in [15–17]). The input material initially used for these studies comprised high-quality preserved fresh-frozen samples, but recently it has been possible to obtain reproducible and comparable profiles using formalin-fixed paraffin-embedded tissues (FFPE), making these archived tumour collections accessible for study [18–20].

Microarrays generally provide fold changes in miRNA expression between samples, with members of miRNA sequence families prone to cross-hybridization

[21–25]. More recently, calibration cocktails of synthetic miRNAs were used in array experiments to derive absolute abundance of miRNAs [23]. RT-PCR methods are lower throughput and require normalization [26]; if external miRNA standards are used for quantification, the most abundant miRNA, which may vary in length due to 3' end heterogeneity, should be used as a calibration standard. Sequencing methods, besides their obvious potential to identify new miRNAs, editing and mutation events, estimate miRNA abundance based on the frequency of sequence reads (eg [5,7,8,27–30]). Given the dramatic increase in sequencing power, bar-coding samples can allow multiple specimens to be processed at the same time, reducing the cost and effort of profiling and paving the way for large specimen studies [30,31]. Ligation biases exist in sequencing methods, which may affect comparable representation of certain miRNAs obscuring absolute expression levels, but are irrelevant when monitoring fold changes between samples. A study with a synthetic pool of 776 miRNA sequences showed that overall these biases do not prevent identification of miRNAs and allow estimation of these biases for the small subset of sequences affected (Hafner, unpublished data). Finally, choosing the appropriate statistical analysis to evaluate the data depends on the methodology used to obtain the profiles, ranging from established Significance Analysis of Microarrays (SAM) [32], to newly developed techniques for sequencing data [30,33,34]. Recent *in situ* hybridization (ISH) advances allowed sensitive detection of miRNAs in heterogeneous tissues, defining miRNA cellular localization [35–37]. The potential of miRNA localization to suggest function for a subpopulation of cells was demonstrated early on, as in the case of *lxy-6*, expressed in <10 neurons in *Caenorhabditis elegans*, controlling left/right asymmetry [38].

#### miRNA databases and validation

It is critical to know which miRNAs are validated and have the potential to regulate cellular functions, especially given the frequent revisions of the miRNA database, miRBase (www.mirbase.org) [39] and the dramatic increase in the number of novel and re-annotated miRNAs through the use of deep-sequencing technologies. It is extremely challenging to establish the validity of novel miRNAs, particularly when their definition is based on a handful of sequence reads. The latest release of miRBase (version 16) includes 121 novel human miRNA precursors, 13 miRNA mature and precursor name changes, four miRNA precursor sequence revisions and the removal of 13 miRNA precursors. A recent study of 60 million small RNA sequence reads, generated from a variety of adult and embryonic mouse tissues, confirmed 398 annotated miRNA genes and identified 108 novel miRNA genes but was unable to find sequencing evidence for 150 previously annotated mouse miRNAs. Ectopic expression of the confirmed and newly identified miRNA

hairpin sequences yielded small RNAs with the classical miRNA features but failed to support other previously annotated sequences (of the 17 tested miRNAs with no read evidence, only one yielded a single sequence read, while of 28 tested miRNAs with insufficient number of reads, only four were verified) [40]. Deep sequencing of large human tissue sample collections will allow us to assess the validity of the human miRBase entries in a similar fashion.

#### Mechanisms of alteration of miRNA levels in malignancy

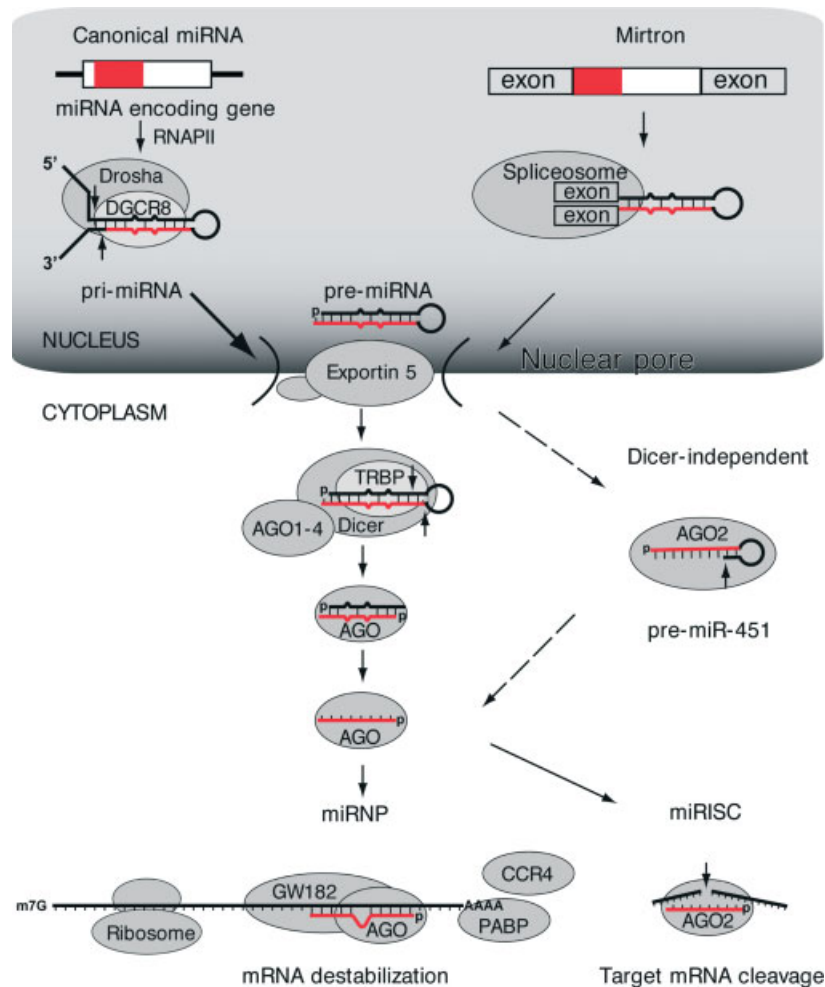
We review miRNA biogenesis (Figure 1) and illustrate which steps of the biogenesis pathway are linked to malignancy, starting from miRNA genomic localization, transcriptional regulation, processing steps and post-transcriptional modification. There is evidence supporting the association of the first three processes and/or the factors that control them with tumorigenesis, whereas evidence relating post-transcriptional miRNA modifications to cancer is not clear-cut.

#### General principles of miRNA genomic organization

miRNAs are frequently expressed as polycistronic transcripts. To date, 1048 human miRNA precursor sequences have been deposited in miRBase [39]. Approximately one-third (390) of these miRNAs are located in 113 clusters, each measuring  $\leq 51$  kb in the human genome (51 kb being the longest distance between miRNAs belonging to the same cluster; Figure 2). These miRNA clusters are co-expressed based on evidence from miRNA profiling data from a variety of tissues and cell lines [22,29,30,40]. The genomic organization of representative oncogenic (*mir-17-92* and *mir-21*) and tumour suppressor (*let-7/mir-98* and *mir-141/200*) families is illustrated in Figure 2. Presentation of miRNA profiles in the form of expression clusters provides a readily interpretable summary of expression data and stresses the importance of cistronic expression regulation; dysregulation of one member of the cluster should be accompanied by similar dysregulation of other cluster members. Since miRNA genes are frequently multi-copy, determining the relative contribution of each genomic location to mature miRNA expression is challenging.

#### Alterations in genomic miRNA copy numbers and location

Changes in miRNA expression between normal and tumour specimens are often attributed to the location of miRNAs in regions of chromosomal instability (amplification, translocation or deletion) or nearby chromosomal breakpoints, initially locating 52.5% of miRNA genes in cancer-associated regions or fragile sites [12]. The miRNA cluster *15a/16-1* is located in a frequently deleted genomic locus containing a putative



**Figure 1.** miRNA biogenesis pathway. miRNAs are transcribed by RNAPII to produce pri-miRNAs. Canonical miRNAs are processed by the endoribonuclease Drosha in partnership with its RBP partner DGCR8; mirtrons are instead processed by the spliceosome. The processed pre-miRNA is transported to the cytoplasm through an export complex consisting of Exportin 5. The pre-miRNA is subsequently processed in the cytoplasm by another endoribonuclease, Dicer, in partnership with its RBP partner TRBP, to form the final 21–23 nucleotide miRNA product. *miR-451* is not processed by Dicer but is rather cleaved by Ago2. Mature miRNAs (indicated in red) are then incorporated into Ago 1–4, forming miRNPs, also known as miRISC. miRNPs also incorporate other proteins, such as GW182. miRNPs are thought to direct miRNA-mediated destabilization (ie through interaction with CCR4) or miRNA-mediated translational repression (ie through interaction with ribosomes) of miRNAs without perfectly complementary mRNA targets. miRISC is thought to direct Ago2-catalysed target mRNA cleavage of miRNA fully or nearly fully complementary mRNA targets.

tumour suppressor-containing region in chronic B cell lymphocytic leukaemia (B-CLL) [41]. Other examples include deletion of *let-7g/mir-135-1* in a variety of human malignancies [12], amplification of the *mir-17-92* cluster in lymphoma [42], translocation of *mir-17-92* in T cell acute lymphoblastic leukaemia (T-ALL) [43] and amplification of *mir-26a* in glioblastoma [44].

#### Alterations in miRNA transcriptional regulation

Some autonomously expressed miRNA genes have promoter regions that allow miRNAs to be highly expressed in a cell type-specific manner, and can even drive high levels of oncogenes in cases of chromosomal translocation. The *mir-142* gene, a marker of haematopoietic cells, is located on chromosome 17 and was found at the breakpoint junction of a t(8;17) translocation, which causes an aggressive B cell leukaemia due to strong up-regulation of a translocated

*MYC* gene [45]. The translocated *MYC* gene, which was also truncated at the first exon, was located only four nucleotides from the 3' end of the *mir-142* precursor, placing the translocated *MYC* under the control of the upstream *mir-142* promoter. In an animal model for hepatocellular carcinoma (HCC), a similar event placed *MYC* downstream of the *mir-122a* promoter active only in hepatocytes [46].

Many transcription factors regulate miRNA expression in a tissue-specific and disease state-specific fashion and some miRNAs are regulated by well-established tumour suppressor or oncogene pathways, such as TP53, MYC and RAS (reviewed in [47]). The miRNA and its transcriptional regulators can participate in complex feedback regulation loops. Examples include the TP53-regulated *miR-34a* [48,49], the RAS-regulated *miR-21* [29,50,51] and the MYC-regulated *miR-17-92* cluster [52,53].



miRNA dysregulation has also been linked to changes in epigenetic regulation, such as the methylation status of miRNA genes, which results in alterations in their expression levels [54,55]. Examples of methylated miRNAs include *mir-127* in bladder cancer cells [56] and *mir-9-1* in breast cancer [57].

#### miRNA biogenesis pathway in tumorigenesis

miRNA biogenesis has been reviewed extensively [47,58–64] (Figure 1). miRNA pathway components could either be mis-expressed in tumours or mutated (reviewed in [65,66]). Post-transcriptional regulation of miRNAs themselves through RNA editing or terminal modifications was shown to alter miRNA targeting, processing and stability, but connection of these modifications to tumorigenesis has not yet been definitive (reviewed in [47,66,67]).

#### miRNA biogenesis

Briefly, the mature 20–23 nt miRNA molecules are excised in a multi-step process from primary transcripts (pri-miRNAs) that contain one or more 70 nt hairpin miRNA precursors (pre-miRNA) and have their own promoters or share promoters with coding genes. These hairpin structures are recognized in the nucleus by DGCR8, a double-stranded RNA-binding protein (dsRBP) and RNASEN, also known as RNase III Droscha, and excised to yield pre-miRNAs. These molecules are subsequently transported by XPO5 (exportin 5) to the cytoplasm, where they are further processed by DICER1 (Dicer) in complex with the dsRBPs TARBP2 (TRBP) and/or PRKRA to yield an RNA duplex processing intermediate, composed of mature miRNA and miRNA\* sequences. Some miRNAs bypass the general miRNA processing order and their maturation can be independent of DGCR8 and RNASEN, such as *miR-320* or *miR-484* [68], or are DICER1-independent, such as erythropoiesis-related *miR-451* [69,70]. DGCR8- and RNASEN-independent miRNAs include mirtrons and tailed mirtrons, which release their pre-miRNA by splicing and exonuclease trimming [71,72].

While the mature miRNA is loaded into the Argonaute/EIF2C (Ago) proteins that are at the core of the miRNA-containing ribonucleoprotein complex (miRNP), sometimes also referred to as miRNA-induced silencing complex (miRISC), the miRNA\* is released and degraded. miR-451 is generated from an unusual hairpin structure that is processed by Ago2 instead of DICER1 [69,70]. The miRNPs contain a member of the Ago family (1–4), which binds the miRNA and mediates target mRNA recognition. Several other RBPs have been implicated in miRNA biogenesis, including DHX9, DDX6, MOV10, DDX5, DDX17, LIN28A, HNRNPA1 and KSRP [47]. Following transcription, miRNAs can be modified by several enzymes, including deaminases, resulting in miRNA editing, and terminal uridylyl transferases, leading to pre-miRNA uridylation, potentially affecting the amount

and ratio of miRNA and miRNA\* (eg [73]) or their sequences (eg [74]).

#### Alterations in RNASEN/DGCR8 and DICER1/TARBP2

Inhibition of the miRNA biogenesis pathway leads to severe developmental defects and is lethal in many organisms (reviewed earlier in [75]; recent examples include [68,69]), and perturbations of this pathway predispose to tumorigenesis [76]. Initial miRNA expression profiling experiments suggested that miRNAs are less abundant in tumours compared to their normal tissue counterparts [14], leading to the proposal that miRNAs are predominantly tumour suppressors rather than oncogenes. Quantification of absolute miRNA levels, not only relative abundance, in miRNA profiling methods is necessary to clarify these observations. 27% of various tumours are found to have a hemizygous deletion of the gene that encodes DICER1 [77]. Global knockdown of mature miRNAs by targeting DICER1, RNASEN and its cofactor DGCR8 increases the oncogenic potential of already transformed cancer cell lines and accelerates tumour formation [76]. Reductions in the amount of DICER1 resulting in impaired miRNA processing have also been shown to increase the rate of tumour formation in two different cancer mouse models, a K-RAS-driven lung cancer [77] and an Rb-driven retinoblastoma [78]. DICER1 is a haploinsufficient tumour suppressor, requiring partial deletion for its associated tumorigenesis phenotype [78]. The phosphorylation of DICER1 cofactor TARBP2 by the mitogen-activated protein kinase Erk enhances pre-miRNA processing of oncogenic miRNAs, such as *miR-21*, and decreases production of tumour suppressor *let-7a* [79]. Moreover, TARBP2 is mutated in some colon and gastric cancers with microsatellite instability, and TARBP2 frameshift mutations correlate with DICER1 destabilization; in cell line and xenografts with TARBP2 mutations, reintroduction of wild-type TARBP2/DICER1 slowed tumour growth [80].

#### Alterations in other pathway-related RBPs

First, LIN28A blocks processing of tumour suppressor pri- and pre-let-7 [81–85], thus maintaining expression of genes that drive self-renewal and proliferation (reviewed in [86]); tumours that express LIN28A were indeed shown to be poorly differentiated and more aggressive than LIN28A-negative tumours. Second, the helicases DDX5 and DDX17 are thought to stimulate processing of one-third of all murine miRNAs, by acting as a scaffold and recruiting factors to the RNASEN complex and thereby promoting pri-miRNA processing [87]. Association of DDX17 and DDX5 RNA helicases through interaction mediated by the tumour suppressor TP53 with the RNASEN/DGCR8 complex facilitates the conversion of pri- to pre-miRNAs [88]. Specifically, the DDX5-mediated interaction of the RNASEN complex with the tumour suppressor TP53 was shown

to have a stimulatory effect on the tumour suppressor pri-miR-16-1, pri-miR-143 and pri-miR-145 processing in response to DNA damage in cancer cells [88]. Thus, TP53 mutations, often observed in malignancies, led to a decrease in pre-miRNA production. Third, oncogenic SMADs, downstream effectors of the TGF $\beta$  superfamily pathways, have been shown to control RNASEN-mediated miRNA maturation through interaction with DDX5, promoting production of oncogenic miR-21 [89]. In a final example, KSRP promotes the biogenesis of a subset of miRNAs, including *let-7a*, by serving as a component of both DICER1 and RNASEN complexes affecting proliferation, apoptosis and differentiation [90].

### miRNA–mRNA target recognition dysregulation

#### miRNA function/mechanism

As described above, miRNAs function through the Ago proteins, containing both RNA-binding domains and RNase H domains (reviewed in [91]). The four human *Ago* genes are co-expressed and bind to miRNAs irrespective of their sequence. Ago2, in contrast to the other members, retains an active RNase H domain, able to directly cleave target RNAs with extensive complementarity to the bound miRNAs. The assembly of the miRNP complex involves multiple Ago conformational transitions captured in a series of crystal structures (reviewed in [92]). The mRNA target is recognized by pairing of the miRNA seed region (positions 2–8) to complementary sequences located mainly in the target 3' UTR but also in the coding regions. Target mRNA recognition and regulation involves members of the GW182/TNRC6 family. Moreover, Ago and TNRC6 proteins co-localize in mRNA-processing bodies (P bodies) that are important for mRNA storage and RNA turnover. Proteomic approaches identified additional Ago-interacting proteins, some of which likely represent mRNA-interacting partners that co-purified with miRNA-targeted mRNPs; their function in RNA silencing processes and potentially tumorigenesis remains to be established.

In mammalian cells miRNAs destabilize targeted transcripts, without significantly perturbing translation of mRNAs, through various mechanisms, such as decapping and de-adenylation, in addition to P-body localization [93,94]. mRNA profiling and proteomic studies, following the introduction of miRNAs, inhibition of miRNAs using antagomirs or miRNA knockout in mouse models, have shown that miRNAs destabilize mRNAs through binding sites located mainly in their 3' UTRs, without a measurable effect on translation [95–103]. Ribosome profiling studies demonstrated that the ribosome density of miRNA targets was unaltered, while changes in miRNA levels were inversely correlated to mRNA and protein abundance, emphasizing the role of miRNAs in regulation of mRNA stability but not translation [104].

#### Organization of miRNAs into sequence families

Certain miRNAs share sequence similarity in regions that are critical for mRNA target recognition, specifically the seed region, and are best viewed as a family when considering mRNA target regulation and the functional consequences of altered miRNA expression. miRNAs can be grouped in sequence families, based not only on their seed sequence similarity but also on overall sequence similarity, given that the miRNA 3' end also contributes to miRNA targeting, although to a lesser extent (reviewed in [59]) (Figure 2). Changes in the overall abundance of miRNA sequence families relate directly to target regulation. In a MYC-driven B cell lymphoma mouse model, a conditional knockout of the oncogenic *mir-17-92* cluster induces apoptosis, which can be reduced by reintroduction of only one of the four sequence families produced from the cluster [105].

#### miRNA–mRNA stoichiometry

The majority of miRNA profiling studies do not provide an estimate of miRNA abundance, which is critical in our understanding of the role of miRNA–mRNA-mediated regulation in tumorigenesis. Only the most abundantly expressed miRNAs occupy a substantial fraction of their available mRNA target sites and affect target mRNA stability [100]. Abundant miRNAs that behave as 'switches', turning on or off during the tumorigenesis process, as shown in developmental processes, have the most significant regulatory potential, given that miRNAs usually lead to modest 1.5–4-fold regulation of their target expression [95,97,102]. However, miRNAs that have lower expression can, in combination, fine-tune target expression, since a given mRNA may be subject to regulation by multiple miRNAs of unrelated families [106–108]. To conclude, the interplay between miRNAs expressed in particular tissues, the levels of their respective expressed targets, as well as other post-transcriptional gene regulatory mechanisms (such as regulation by RBPs or other competing interactions—see below) is likely responsible for balancing miRNA conferred regulation.

#### Changes in the miRNA targets

The binding sites of miRNAs in mRNAs can be altered through a variety of mechanisms, such as point mutations, translocations, shortening of the 3' UTR, competition with other RBPs or decoy molecules for mRNA binding. Point mutations in miRNA targets can both create or destroy a miRNA binding site [109–111]. Chromosomal translocations can remove miRNA binding sites from their regulated oncogenes, such as in the case of *let-7* targeting of the 3' UTR of the *Hmga2* gene [112]. Shortening of the 3' UTR through alternative polyadenylation can relax miRNA-mediated regulation of known oncogenes, such as *IGF2BP1/IMP1*, and lead to oncogenic transformation [113], as does use of decoy pseudogenes, as in the case

of PTEN, by saturating miRNA binding sites [114]. Finally, cooperativity or competition of miRNAs for mRNA target site binding with other RBPs, such as ELAVL1 (HuR), DND1 and PUM1, can also de-repress target expression [115–118].

### Cancer tissues have distinct miRNA profiles

We will first briefly discuss the state of current miRNA profile databases and explore the issue of tissue heterogeneity in the tissue profiles before summarizing the role of miRNA dysregulation in malignancies.

#### miRNA cancer database

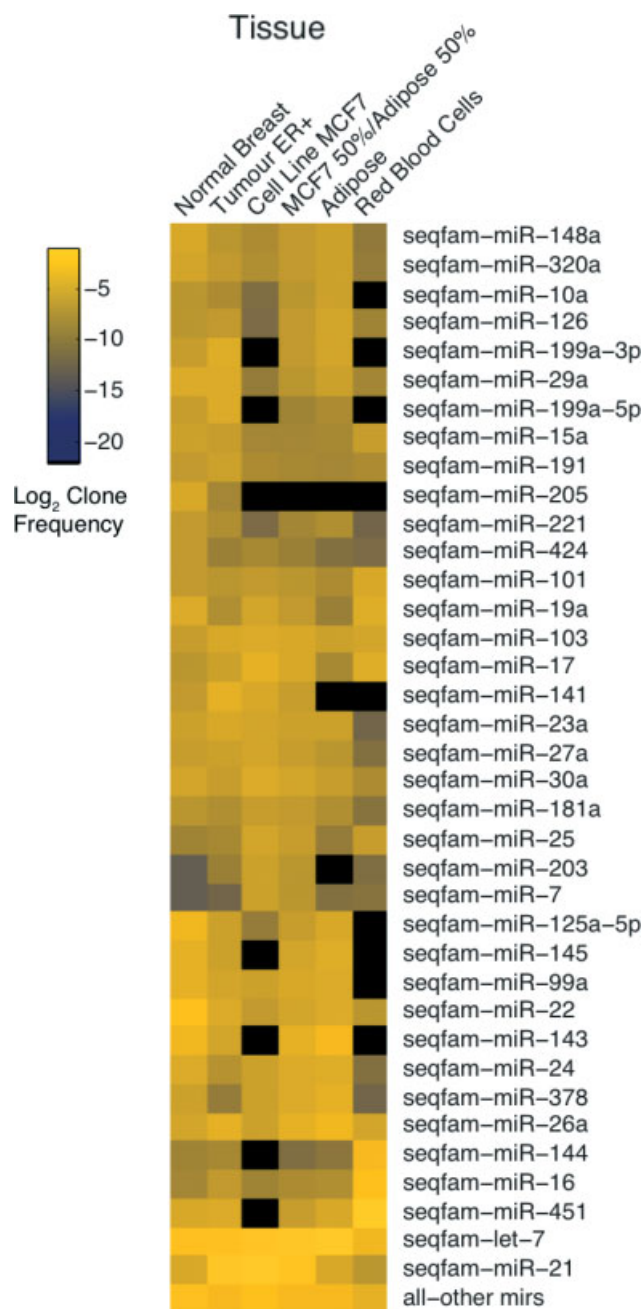
The development of miRNA microarrays, RT-PCR platforms and deep sequencing methodologies have resulted in an exponential acquisition of miRNA profiles. Some of the published miRNA profiles are available in the NCBI Gene Expression Omnibus, similarly to mRNA profiles (other resources include: [www.microna.org](http://www.microna.org); <http://www.mirz.unibas.ch>). However, there is no database or viewer that allows for cross-platform comparison of existing data.

#### Tissue heterogeneity

Tissues are generally composed of multiple cell types, each with its distinct gene expression programme. Disease not only alters the expression programme of the affected cell type but often also its cell type composition. To best separate these effects in the profiling of heterogeneous tumour samples, it may be useful to profile tumour cell lines and individual cell types that may be present in a tumour sample, or define miRNA cellular localization by performing ISH. Figure 3 compares miRNA abundance profiles of normal breast, an oestrogen receptor-positive invasive ductal breast carcinoma, the oestrogen receptor-positive ductal cell line MCF7, human fat and blood (Farazi and Horlings, unpublished data). Strikingly, we can model the profile of a human cancer by simply combining tumour cell line and human fat profiles at equal ratio. This exercise demonstrates that the MCF7 tumour cell line may be a good disease model for deciphering miRNA regulatory networks, expressing many of the miRNAs present in the predominant tumour-derived cell type and highlighting the need for individual cell type miRNA profiles.

#### miRNAs as tumour suppressors and oncogenes

miRNA dysregulation could be used as a diagnostic tool, even if the particular miRNAs do not serve any regulatory function. Alternatively, miRNA dysregulation could drive tumorigenesis, through the roles miRNAs can adopt as tumour suppressors or oncogenes. miRNAs that are up- or down-regulated in malignancies are respectively referred to as oncogenic or tumour-suppressor miRNAs, sometimes even if there



**Figure 3.** miRNA breast tumour and cell line profiles. Comparison of abundance profiles of the top expressed miRNA sequence families of normal breast, an oestrogen receptor-positive invasive ductal carcinoma breast tumour (ER<sup>+</sup>), the MCF7 ductal derived cell line, human subcutaneous adipose tissue and red blood cells.

is no evidence for their causative role in tumorigenesis. Some of the most commonly dysregulated miRNAs are summarized in Table 1 (reviewed in [11]).

Functional studies performed in cancer cell lines or mouse models of various malignancies through over-expression or knockdown of miRNAs have supported a role for some of these miRNAs in tumorigenesis. Over-expression of tumour suppressor miRNAs, such as *let-7g*, reduced tumour burden in a K-RAS murine lung cancer model [156]. Over-expression of the oncogenic *mir-17-92* cluster led to a lymphoproliferative disorder, and higher level expression of the

Table 1. Some of the most common cancer-associated miRNAs

miRNA	Tissue type specificity	Chromosomal location	Property	Malignancy
<i>let-7</i> family	Ubiquitous	Multiple members (chromosomes 3, 9, 11, 19, 21, 22)	TS	CLL [119], lymphoma [120], gastric [121], lung [122], prostate [9], breast [123], ovarian [121], colon [121], leiomyoma [121], melanoma [121]
<i>miR-15a/16-1</i> cluster	Ubiquitous	13q14.2	TS	CLL [124], lymphoma [9], multiple myeloma [9], pituitary adenoma [125], prostate [125], pancreatic [125]
<i>miR-17-92</i> family	Ubiquitous	Multiple members (chromosomes 7, 13, X)	OG	Lymphoma [126], multiple myeloma [9], lung [122], colon [126], medulloblastoma [127], breast [123], prostate [128]
<i>miR-21</i>	Ubiquitous	17q23.1	OG	Lymphoma, breast, lung, prostate, gastric, cervical, head and neck, colorectal, glioblastoma (for all: [129])
<i>miR-26a</i>	Ubiquitous	3p22.2 (-1) 12q14.1 (-2)	TS OG	Lymphoma [130], hepatocellular carcinoma [131], thyroid carcinoma [132] Glioblastoma [44,133]
<i>miR-34a/b/c</i>	Ubiquitous	1p36.22 (a) 11q23.1 (b) 11q23.1 (c)	TS	CLL [119], lymphoma [9] Pancreatic [9], colon [9], neuroblastoma [134] Glioblastoma [135]
<i>miR-155</i>	Haematopoietic system	21q21.3	OG	Lymphoma (ie Burkitt's, Hodgkin's, non-Hodgkin's) [9], CLL [9,18], breast [123], lung [9], colon [9], pancreatic [9]
<i>miR-200/141</i> family	Epithelial-specific	Multiple members (chromosomes 1, 12)	TS	Breast [123,136], renal clear cell carcinoma [137], gastric [138], bladder [139]
<i>miR-205</i>	Epithelial-specific	1q32.2	OG/TS TS	Ovarian [140-142] Prostate [143,144], bladder [145], breast [136,146,147], oesophageal [148]
<i>miR-206</i>	Skeletal muscle-specific	6p12.2	OG TS	Ovarian [149] Rhabdomyosarcoma [150], breast [151]
<i>miR-9</i>	Nervous system-specific	1q22 (-1) 5q14.3 (-2) 15q26.1 (-3)	TS OG/TS	Medulloblastoma [152], ovarian [153] Breast [57,154,155]

miRNAs that are up- or down-regulated in malignancies are respectively referred to as oncogenic (OG) or tumour-suppressor (TS), but their role in malignancy is not always experimentally validated. Given the number of manuscripts providing evidence for the role of each miRNA based on patient, cell culture or animal model studies, reviews are often cited instead of original reports to limit the number of references, and only a few selected reports are presented if no review is available.

cluster in MYC-driven B cell lymphomas dramatically increased tumorigenicity [53,157]. Over-expression of another oncogene, mir-21, frequently highly expressed in solid and haematological malignancies, resulted in a pre-B malignant lymphoid-like phenotype, whereas subsequent mir-21 inactivation in the same model led to apoptosis and tumour regression [158]. Transgenic mouse models with loss and gain of function of mir-21 combined with a model of lung cancer confirmed the role of mir-21 as an enhancer of tumorigenesis when over-expressed, or a partial protector when genetically deleted [50]. Ectopic expression of mir-155 in bone marrow induced polyclonal pre-B cell proliferation progressing to B cell leukaemia or myeloproliferation in mice [159,160].

Metastasis-related miRNAs have been identified in various malignancies, mainly from cell line and xenograft experiments (reviewed in [161]). Examples include breast cancer-related *miR-10b*, *miR-9*, *miR-31* and *miR-335*, among others. The interesting regulatory roles of these miRNAs cannot easily be validated in large clinical studies. Two clinical studies with long-term follow-up data instead identified miR-210 associated with tumour aggressiveness [162,163], pointing to difficulties reconciling cell line, xenograft model and

patient materials, due to the tissue heterogeneity discussed earlier, the heterogeneous nature of the malignancy and timing of clinical specimen acquisition. Tumour miRNA profiles cannot dissect contributions from sub-populations of cells that may be important for tumour characteristics, such as metastasis, while cell line miRNA profiles cannot capture the cellular interactions from supporting cell types in the tumour microenvironment. Patient samples are often collected at time of diagnosis, by which time a tumour is already well established and cannot unravel early changes that may be critical in tumour initiation or later changes important in metastasis.

#### miRNA-regulated pathways

The observed effects of miRNA mis-expression on tumour initiation, maintenance or metastasis can be explained by the mRNA targets and pathways they regulate, which include known tumour suppressors and oncogenes (reviewed in [11]). miRNAs regulate a large number of genes, some estimates reporting miRNA regulation of up to 60% of the human genome, making it challenging to attribute a phenotype after mis-expression of a particular miRNA through its action on only a subset of targets [94,164]. If a few of these targets control rate-limiting steps in the



studied tumorigenesis processes within the specified tissues and cell types, such as metastasis, then miRNA regulation of a handful of targets could potentially explain the phenotype resulting from miRNA mis-expression [165].

Examples of miRNA-regulated cancer pathways include differentiation, apoptosis, proliferation and stem cell maintenance, a process important for disease relapse and/or metastasis. The skeletal muscle-specific *miR-206* blocked human rhabdomyosarcoma growth in mouse xenograft models by inducing myogenic differentiation [150], while the *miR-200/141* family was critical in the epithelial to mesenchymal transition (EMT) in various malignancies (reviewed in [166]). Sustained expression of the endogenous *mir-17-92* cluster was required to suppress apoptosis in MYC-driven B cell lymphomas in a conditional knockout allele of the *mir-17-92* cluster [105]. TP53-regulated ectopically expressed *miR-34* induced cell cycle arrest in both primary and tumour-derived cell lines, down-regulating genes promoting cell cycle progression (reviewed in [49]). In a final example of miRNA-regulated cancer pathways, isolation of a subset of highly tumorigenic breast cancer cells that were thought to have stemness properties showed that these cells do not express *let-7* family members and that expression of *let-7* or its known target RAS leads to loss of self renewal [167].

#### Alterations of miRNA sequence

miRNA dysregulation could be a result of mutations in miRNA genes in well-conserved regions in their mature sequence affecting mRNA targeting, or the remainder of the miRNA precursor potentially affecting processing and stability of the mature miRNA (reviewed in [66]). For example, a mutation in the seed region of *mir-96* was shown to lead to hearing loss in a mouse model [168] and was identified in families with non-syndromic progressive sensorineural hearing loss [169], while a point mutation in the viral *mir-K5* precursor stem loop was shown to interfere with its processing and reduce mature miR-K5 accumulation [170]. If miRNAs are drivers of oncogenic and tumour suppressor pathways, we would expect to find miRNA mutations that can also be causative of the disease. So far the only mutation identified in a miRNA that could lead to malignancy is *miR-16*, where a germline mutation potentially affects *miR-16* biogenesis and abundance in a kindred with familial CLL [171] and New Zealand black mice that naturally develop CLL-like disease [172]. Single nucleotide polymorphisms (SNPs) located in both precursor and mature miRNA sequences have been examined in the context of disease risk for various malignancies but have not been validated as causative (reviewed in [66]).

#### miRNA target identification

The currently available target prediction databases (reviewed in [59]) do not easily allow the involvement

of reported targets in certain phenotypes to be prioritized, thus necessitating the selection of a few targets from a list of hundreds for further study and validation, based on *a priori* knowledge of potentially involved biological pathways. Since the prediction algorithms do not always produce identical target lists, use of multiple algorithms and comparison or intersection of their results narrows the list to higher confidence targets. Targets are only relevant to a specific phenotype if they are expressed in the studied tissue, an issue not addressed by most computational prediction algorithms. Biochemical identification methods in cell lines and tissues are being established and further refine our understanding of miRNA–mRNA target binding recognition. These methods involve two approaches: over-expression or down-regulation of studied miRNAs, followed by assessment of transcriptome-wide mRNA levels by mRNA microarray analysis or proteomic analysis (eg [100]) or deep sequencing technology after immunoprecipitation of miRNAs and mRNAs complexed with the main component of the miRNA effector complex, Ago, not only to identify mRNA targets but also to localize their precise binding sites [173,174].

#### miRNAs as diagnostics

miRNAs demonstrated their potential as diagnostic tumour markers early on, when their profiles were shown to correlate with the tumour embryonic origin, thus defining tumours of unknown origin indistinguishable by histology and assigned based on clinical information [14]. miRNA expression patterns have been linked to clinical outcomes, given that miRNAs modulate tumour behaviour such as tumour progression and metastasis. Expression of *let-7* is down-regulated in non-small cell lung cancer patients [175] and is associated with poor prognosis [111,176], whereas an miRNA signature was identified to be associated with prognosis in CLL [171]. Advances in miRNA detection, such as ISH or RT–PCR, may allow miRNAs to be used as diagnostic and prognostic markers in the clinic.

#### miRNAs as therapeutics

Because miRNAs affect the expression of multiple genes and thereby tune multiple points in disease pathways, miRNAs and their regulated genes, represent interesting drug targets. Antisense oligonucleotide targeting experiments in mice [99,177,178] and non-human primates [179] have demonstrated the feasibility of manipulating miRNA levels. *miR-143* was initially shown to promote adipocyte differentiation and could be a target for therapies in obesity and metabolic diseases [180]. Alternatively, ‘miRNA sponges’ have been exploited to reduce miRNA expression in mammalian cells and mouse models by using RNA transcripts expressed from strong promoters containing

miRNA-complementary binding sites (reviewed in [181]). Systemic administration of antisense oligonucleotide therapeutics to *miR-122*, a liver-enriched miRNA, in mice and primates was shown to alter lipid metabolism and hepatitis C viral load, resulting in reduced liver damage [99,177–179,182,183]. At the same time, systemic delivery of a miRNA mimic for *miR-26a* in a murine model of HCC reduced tumour size [131]. The new and exciting advances in delivery of miRNA inhibitors and mimics hold the promise of quickly translating our knowledge of miRNAs into treating disease.

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### Author contributions

TAF wrote the manuscript, JIS and PM were responsible for table and figure generation, and TT supervised the work.

### Teaching Materials

PowerPoint slides of the figures from this review are supplied as supporting information in the online version of this article.

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