Cell. Mol. Life Sci. 65 (2008) 545 – 562
1420-682X/08/040545-18
DOI 10.1007/s00018-007-7355-9

Cellular and Molecular Life Sciences

Review

Functional aspects of animal microRNAs

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Received 3 August 2007; received after revision 3 October 2007; accepted 5 October 2007
Online First 29 October 2007

Abstract. MicroRNAs (miRNAs) are a recently discovered family of small regulatory molecules that function by modulating protein production. There are approximately 500 known mammalian miRNA genes, and each miRNA may regulate hundreds of different protein-coding genes. Mature miRNAs bind to target mRNAs in a protein complex known as the miRNA-induced silencing complex (miRISC), sometimes referred to as the miRNP (miRNA-containing ribonucleoprotein particles), where mRNA translation is inhibited or mRNA is degraded. These actions of miRNAs have been shown to regulate several developmental and physiological processes including stem cell differentiation, haematopoiesis, cardiac and skeletal muscle development, neurogenesis, insulin secretion, cholesterol metabolism and the immune response. Furthermore, aberrant expression has been implicated in a number of diseases including cancer and heart disease. The role of miRNAs in these developmental, physiological and pathological processes will be reviewed.

Keywords. MicroRNA, gene regulation, development, cancer.

Introduction

MicroRNAs (miRNAs) are a family of non-coding RNAs that post-transcriptionally regulate gene expression by inhibiting mRNA translation or by mRNA degradation. Many hundreds or even thousands of miRNA genes may exist throughout the human genome [1]. Each miRNA gene encodes a mature miRNA approximately 22 nucleotides in length. These miRNAs are thought to regulate the expression of hundreds of target mRNA molecules. However, the function of most miRNAs is unknown, even though many are phylogenetically conserved [2]. The first miRNA to be assigned a specific function was lin-4 in the nematode worm *Caenorhabditis elegans*, which targets and inhibits lin-14 during temporal pattern formation [3]. Another miRNA important to *C. elegans* development, let-7, was later identified in several animal species [4]. The expression of many miRNAs has now been associated with developmental processes in plants, *Drosophila*, zebrafish, mice and humans among other species. Furthermore, miRNAs function in the regulation of a range of physiological responses including stem cell differentiation, haematopoiesis, cardiac and skeletal muscle development, neurogenesis, insulin secretion, cholesterol metabolism and the immune response.

Biosynthesis of miRNAs

RNA interference (RNAi), mediated by double-stranded 19- to 23-nucleotide RNA duplexes, has been identified as a mechanism for the sequence-specific degradation of mRNA [5]. Initial studies revealed that virus-derived double-stranded RNA was cleaved by the RNase III-type enzyme Dicer to produce characteristic RNA duplexes [6–8]. These

were called short interfering RNA (siRNA) in order to differentiate them from longer double-stranded RNA (>30 nucleotide duplexes) that induce an interferon-mediated anti-viral response. The biological action of siRNAs is mediated *via* the RNA-induced silencing complex (RISC), which utilises the antisense strand (the complementary strand of the siRNA) as a template to cleave the sequence-specific target mRNA and attenuate subsequent protein production [9]. The experimental use of siRNA to inhibit functional gene expression is now commonplace [10].

Recent studies have also identified the sequence of events required for the production of mature miRNAs [11, 12] (Fig. 1). These genes are transcribed in the nucleus as large non-coding genes approximately 2 kb in length and are known as primary miRNA transcripts (pri-miRNAs). Within the nucleus the RNase III-type enzyme Drosha, which forms a micro-processor complex with the double-stranded RNA-binding protein DGCR8 [13], processes the pri-miRNAs into smaller, stem-loop miRNA precursors (pre-miRNAs) of ~70 nucleotides [14]. The pre-miRNAs are then exported across the nuclear membrane and into the cytoplasm by the Exportin-5/Ran complex [15–17]. Like siRNAs, these pre-miRNAs are then cleaved by the highly conserved RNase III-type enzyme Dicer to produce a 19- to 23-nucleotide RNA duplex. The miRNA is then incorporated into a ribonucleoprotein complex (RNP) called the RISC-like complex, referred to as the microRNA-induced silencing complex (miRISC) or miRNP [18, 19]. Only one strand of the miRNA, known as the mature miRNA, is incorporated into the miRISC, while the other strand, referred to a miRNA*, is degraded [20].

miRNAs predominately block mRNA translation following imperfect complimentary binding to miRNA-recognition elements (MRE) within the 3′-untranslated region (UTR) of target mRNA [21, 22]. Although translation is blocked, the target mRNA is thought to be degraded only by those miRNAs with high complementarity, and in human cells this process is likely to be mediated by Argonaute2 (Ago2). The Ago2 protein is the main component of the miRISC and provides a scaffold for the effector functions of miRNA [23, 24]. The Ago2 protein also mediates transfer of the mature miRNA from Dicer into the miRISC [25] and appears to be responsible for cleavage of the mRNA [26].

Although the biosynthesis of miRNAs *via* the Drosha pathway is likely to be the standard, recent findings have highlighted alternative processing pathways. Several intronic sequences have been identified in *Drosophila* and *C. elegans* that mimic pre-miRNAs and therefore bypass Drosha-mediated cleavage [27, 28]. miRNAs that enter this alternative pathway have been termed 'mirtrons' and function in much the same way as miRNAs. The mirtrons enter the miRNA processing pathway at the Exportin-5 stage (Fig. 1) and are cleaved in the cytoplasm by Dicer. It is thought that mature mirtrons enter the miRISC in association with Ago1 [28], which primarily results in translational repression rather than mRNA cleavage [26].

miRNA targeting of mRNAs

The algorithms used to predict the specific mRNA targets for mammalian miRNAs were originally based on the complementarity properties of known miRNAs such as lin-4 and let-7 from *C. elegans* [29, 30]. It is possible that as few as 6 of the 22 nucleotides that comprise a particular miRNA provide the necessary binding specificity for a target mRNA. As predicted from mutation analysis of several miRNAs, of particular importance may be the pairing of bases 2–7, often known as the seed sequence [31–33]. The pairing between miRNA and target RNA is assisted by Ago2 [34].

The major regulatory function of miRNAs is to prevent protein translation from the target mRNA, although a decrease in mRNA stability is a function of some miRNAs [35]. Although the precise mechanism of translational inhibition is still uncertain, one way in which miRNAs are thought to inhibit translation is through interference with proteins involved in initiation and elongation events during translation [36, 37]. Another way in which translational repression may take place is by sequestration of mRNAs into a cytosolic site called the P-body [34]. However, it is unclear whether P-body localisation is a prerequisite for translational repression [34, 38] or whether inhibition of translation occurs initially within the miRISC, and mRNA degradation takes place in the P-body following re-localisation. The P-body may also act as an intracellular store for mRNAs that can subsequently allow mRNAs to escape and initiate translation [39].

miRNAs may inhibit protein translation by more than one mechanism [34]. Using a luciferase reporter system in HeLa cells, a cognate miRNA co-transfected with its CXCR4 target was shown to inhibit translation initiation by inhibiting eukaryotic initiation factor 4E (eIF4E) [40]. The initiation of translation involves the cap structure and poly(A) tail of mRNA, which are also required for miRNA inhibition. It was suggested that the miRNA may interfere with eIF4E binding to the mRNA or block its function once bound [40]. Indeed, let-7 inhibited translation initiation, possibly by interfering with m^7GpppG cap

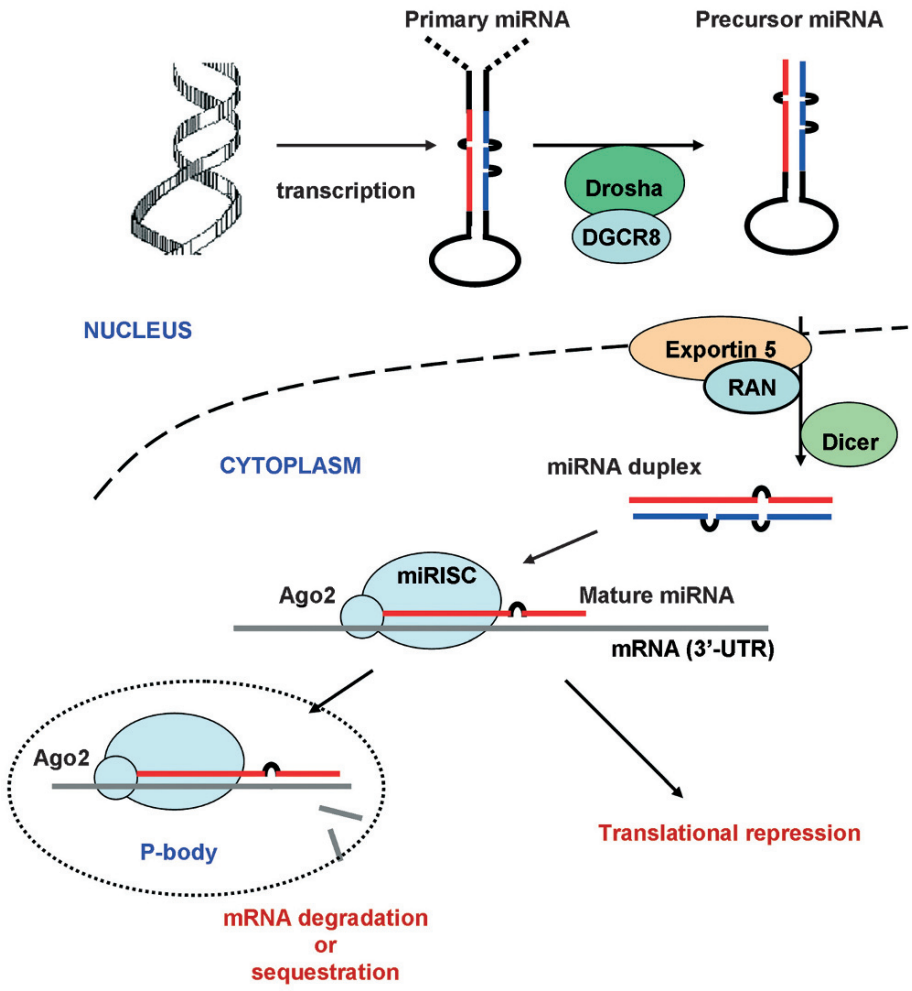

Figure 1. MicroRNA (miRNA) biosynthesis. miRNAs are transcribed from the nucleus as primary miRNAs ~2000 nucleotides in length. They are processed by the RNase III-type enzyme Drosha in association with the RNA-binding protein DGCR8 into smaller precursor miRNAs, which are then transported across the nuclear membrane by Exportin-5. In the cytoplasm the precursor miRNAs are cleaved by another RNase III-type enzyme, Dicer, into mature double-stranded miRNAs. Only the guide strand of the mature miRNA enters the miRNA-induced silencing complex (miRISC), where it binds to target mRNA at the 3′UTR in a partially complementary manner. This results in inhibition of mRNA translation and modulation of protein expression. In addition, miRNA bound to target mRNA can enter specialised protein complexes known as P-bodies, where mRNA sequestration or degradation may take place.

recognition by initiation factors [41]. Let-7 and target mRNAs were also associated with miRNPs and Ago proteins, and subsequent accumulation was observed in P-bodies [42], although mRNA degradation was not a prerequisite for translational inhibition [43]. It was also suggested that Ago2 prevents the recruitment of eIF4E by binding to a similar m^7G cap motif on the target mRNA, thus inhibiting initiation [44]. Another eIF member implicated in miRNA function is the anti-association factor eIF6, which prevents ribosomal assembly. Depletion of eIF6 abrogated miRNA function, resulting in elevated target protein and mRNA levels [45]. In addition, in a *Drosophila* cell-free system, translation initiation was inhibited by miR-2 through the formation of miRNPs and was dependent on an m^7GpppG cap structure [46].

Alternatively, let-7a blocked lin-41 translation by interfering with polypeptide accumulation on actively translating polyribosomes, an event that occurs after translation initiation [47]. This mechanism was further supported by demonstrating that miRNAs associate with mRNAs in polyribosomes even during active, steady-state translation [48]. However, this inhibition did not result in degradation of the mRNA. Moreover, subsequent re-localisation to P-bodies still remains a possibility [40] although not a necessity for translation inhibition [47]. It may be the case that miRNAs inhibit protein production at the initiation and elongation stages of translation, while P-bodies function to prevent mRNA from interacting with the translation machinery on ribosomes.

It has been suggested that miRNAs may act by inhibiting those genes that should not be expressed in a particular cell type and therefore function to reinforce or fine-tune gene expression (Fig. 2). Indeed, Bartel et al. proposed that as many as 30% of all mRNA may be under the control of miRNAs [49]. It is often the case that the expression of a particular miRNA tends to not co-localise with the high expression of a predicted mRNA target either spatially or temporally [50]. This situation may be the result of the miRNA preventing mRNA expression in a particular tissue. On the other hand, mRNAs that are highly expressed in a given tissue may have

evolved 3′UTR sites that do not match miRNA seed sequences [50]. Overall, the expression patterns of both mRNAs and miRNAs appear to have the general effect of fine-tuning tissue-specific gene expression.

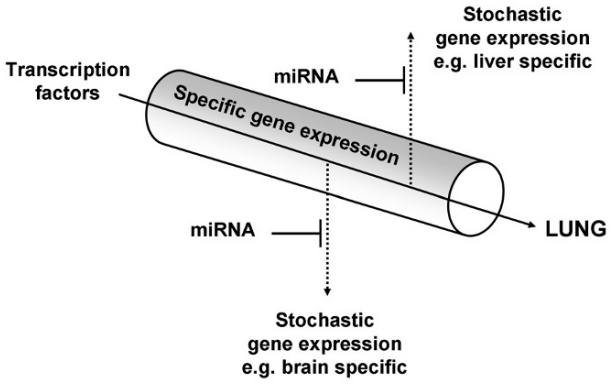

Figure 2. MicroRNAs (miRNAs) fine-tune gene expression in a tissue-specific manner. It is often observed that a particular miRNA in not expressed in the same cell type as its target mRNA. This is probably due to inhibition of target gene expression by that miRNA. Tissue-specific gene expression often results in stochastic gene expression, *i.e.* the expression of genes not necessarily involved in a particular cellular programme. miRNAs in a given tissue target these unwanted genes and in so doing canalize and reinforce tissue specificity.

miRNAs and development in *C. elegans*

Lin-4 and let-7 are considered to be the archetypal miRNAs and were originally known as small temporal RNAs (stRNAs) because of their sequential expression and role in temporal patterning [51]. The target of lin-4 is lin-14, which is critical in regulating stage-specific larval development. The timing of cell division in several larval stem cells, known as seam cells, is of vital importance in synchronising the L1–L2 moulting stage during *C. elegans* development. A mutant worm deficient in lin-4 fails to undergo L1–L2 development, as the seam cells repeat the cell division pattern of the L1 stage. Overexpression of lin-4 strengthens lin-14 repression, resulting in a complete lack of cell division in the seam cells. Both situations lead to a similar phenotype where the worm is stuck at the L1 stage. Further key stages of development are regulated by miRNAs, including the control of L2–L3 transition by miR-48, miR-84 and miR-241 [52], while let-7 controls L4-adult stage transition [53]. Vulval development in *C. elegans* also involves let-7 along with miR-48 and has revealed the importance of miRNAs during organogenesis [54]. The vulva is a ring-like structure formed from a sequence of cellular differentiations directed by let-60 expression (the nematode homologue of the *RAS* gene) from the gonadal anchor cell. Mutants defective in let-7 eventually die as a result of vulval cell hyperplasia, a phenotype that can be abrogated by simultaneously inhibiting let-60. Reduced expression of let-7 has recently been implicated in the development of certain human cancers associated with overexpression of *RAS* [55].

A genetic screen of *C. elegans* with mutations in asymmetric chemosensory neurons (ASE) revealed a novel gene with no protein end product. *C. elegans* possesses bilateral taste receptor neurons known as ASE left (ASEL) and ASE right (ASER). The gene lys-6 codes for a miRNA with partial complementarity to the 3′UTR of cog-1 mRNA, which in turn functions as a homeobox gene that controls the differential development of ASEL or ASER [56]. Worms deficient in lys-6 do not develop ASEL neurons but develop compensatory ASER neurons. Therefore, lys-6 was the first miRNA to be shown to actively determine neuronal patterning by inhibiting cog-1 activity in ASEL neurons but not ASER neurons [57]. Another miRNA, miR-273, has also been implicated in the ASEL/ASER asymmetric patterning in the worm head and is thought to target the transcription factor die-1 [58].

miRNAs and *Drosophila* development

The fruit fly *Drosophila melanogaster* has long been used as a tool to study developmental processes and has provided essential clues to the function of miRNAs [59, 60]. One of the first miRNAs to be assigned a specific target in *Drosophila* was that encoded by the *bantam* gene [61]. The expression of *bantam* is related to patterning during development, while *bantam* miRNA stimulates cell proliferation and inhibits apoptosis by targeting the pro-apoptotic gene *hid*. Several miRNAs have since been demonstrated to exhibit temporal and spatial expression patterns during development [62, 63]. Injection of anti-sense molecules specific to certain miRNAs into embryos resulted in a number of abnormal developmental phenotypes [64]. In particular, miR-2 family members were shown to target the pro-apoptotic factors *hid*, *grim*, *reaper* and *sickle* and resulted in aberrant apoptosis and embryonic lethality. Interestingly, each member of the miR-2 family exhibited distinct targeting effects, suggesting that target specificity involves differential 3′UTR pairing [64]. Other miRNAs were shown to affect segmentation (miR-31), dorsal closure (miR-310 family) and cellularisation (miR-9) [64].

Photoreceptor differentiation in the *Drosophila* eye was shown to be mediated by miR-7, which acted to reinforce a change in gene expression between

progenitor cells and differentiated photoreceptor cells [65]. Signalling through the epidermal growth factor (EGF) receptor causes phosphorylation of the transcription factor Yan, which is necessary for progenitor cell differentiation. In unstimulated cells Yan acts to suppress the expression of miR-7 and competes with another transcription factor, Pointed-P1, that controls downstream gene expression. Inhibition of Yan expression by EGF receptor activation allows Pointed-P1 to mediate the expression of miR-7, which in turn inhibits further translation of Yan protein. This negative feedback loop allows a more stable switch from one gene expression state to another by moderating unwanted gene expression and corresponds with differentiation into photoreceptor cells. miRNAs also play a role in sensory organ precursor (SOP) development. miR-9a was shown to control the formation of SOPs in the imaginal disks of adult flies by inhibiting senseless expression and preventing excessive neuronal cell development [66]. Other aspects of *Drosophila* development have been shown to be controlled by miRNAs [67], including suppression of cell death required for fat metabolism by miR-14 [68], regulation of metamorphosis and developmental timing by let-7, miR-100 and miR-125 [69, 70], regulation of the Notch pathway by miR-1 [71, 72], control of muscle cell growth by *Drosophila* miR-1 [73] and transformation of halteres to wings by miR-iab-4 (homologous to miR-196) through inhibition of Ubx activity [74].

miRNAs and vertebrate development

The demonstration that many miRNAs are organ-specific indicates that they are central to cell differentiation and tissue development. Mutant mice that possess a specific genetic knockout are useful models to study the function of individual genes. However, miRNA genes are often located in multi-gene loci or are part of large gene clusters, and due to the potential redundancy of miRNAs, specific deletion of miRNA genes may result in problematic analyses of phenotype. Instead, genetic removal of the enzyme Dicer has been achieved in zebrafish and mice. Dicer is essential for miRNA processing, and therefore a Dicer-deficient animal is unable to synthesise new miRNAs. The loss of Dicer resulted in embryonic lethality in both species, 10 days after fertilization in zebrafish [75] and on embryonic day 7.5 (E7.5) or E12.5 [76–78] in mice (depending on the transgenic), demonstrating that Dicer plays a vital modulatory role during animal development. Dicer was also found to be important in the maintenance of embryonic stem cells, as Dicer-deficient mice had reduced numbers of pluripotent stem cells within the blastocyst [79].

Using a conditional Dicer-knockout mouse in which Cre expression was driven by the *Sonic Hedgehog* (*Shh*) promoter, Dicer was shown to be vital for the normal development of the murine lung. Inactivation of Dicer in mouse embryos (E9 to E12.5) resulted in defective branching of lung epithelium and abnormal patterns of programmed cell death [80]. Overexpression of fibroblast growth factor 10 (FGF10) and subsequent high expression of *BMP4* and *Spry2* in Dicer-deficient cells was thought to be the reason for the abnormal morphogenesis. The regulation of FGF10 expression is vital for normal lung development, especially within the mesenchyme cells that govern epithelial morphogenesis. Micro-environmental control of growth factors during morphogenesis may well be under the control of miRNAs. Indeed, a large-scale expression screen of fetal, neonatal and adult lung revealed a number of differentially expressed miRNAs in developing tissue [81], including a cluster of maternally imprinted miRNAs on human chromosome 14q32.31.

Dicer is also required for angiogenesis in the mouse embryo [78]. Mice deficient in Dicer exhibit defective blood vessel and yolk sac formation and, in addition, excessive VEGF, flt1 and kdr expression. Developing blood vessels are extremely sensitive to VEGF, and overexpression tends to result in disorganised patterning, vessel leakage and the up-regulation of hypoxia-inducible gene expression.

A conditional knockout Dicer allele, using transgenes specific to the limb mesoderm and driven by the *Shh* promoter, showed the potential importance of miRNAs during patterning of the murine limb [82]. The absence of Dicer resulted in excess cell death, overexpression of *Spry* and smaller limb buds, although the tissue patterning and differentiation was not altered. Furthermore, it was recently reported that miR-196 acts upstream of Hoxb8 and Shh specifically in the hindlimb of mouse embryos [82]. In the forelimb, Hoxb8 expression results in the up-regulation of *Shh* in response to retinoic acid (RA), but in the hindlimb RA does not induce Hoxb8. However, a conditional Dicer-deficient mouse did express Hoxb8 in the hindlimb, revealing the importance of miRNAs in inhibiting Hoxb8 expression, and a microarray screen revealed that miR-196 was expressed 20-fold higher in the hindlimb than in the forelimb. By switching to the more accessible chick embryo, miR-196 was shown to decrease the accumulation of both Hoxb8 and Shh in the forelimb [83]. This suggests that the function of miR-196 is to prevent unwanted gene expression during limb development.

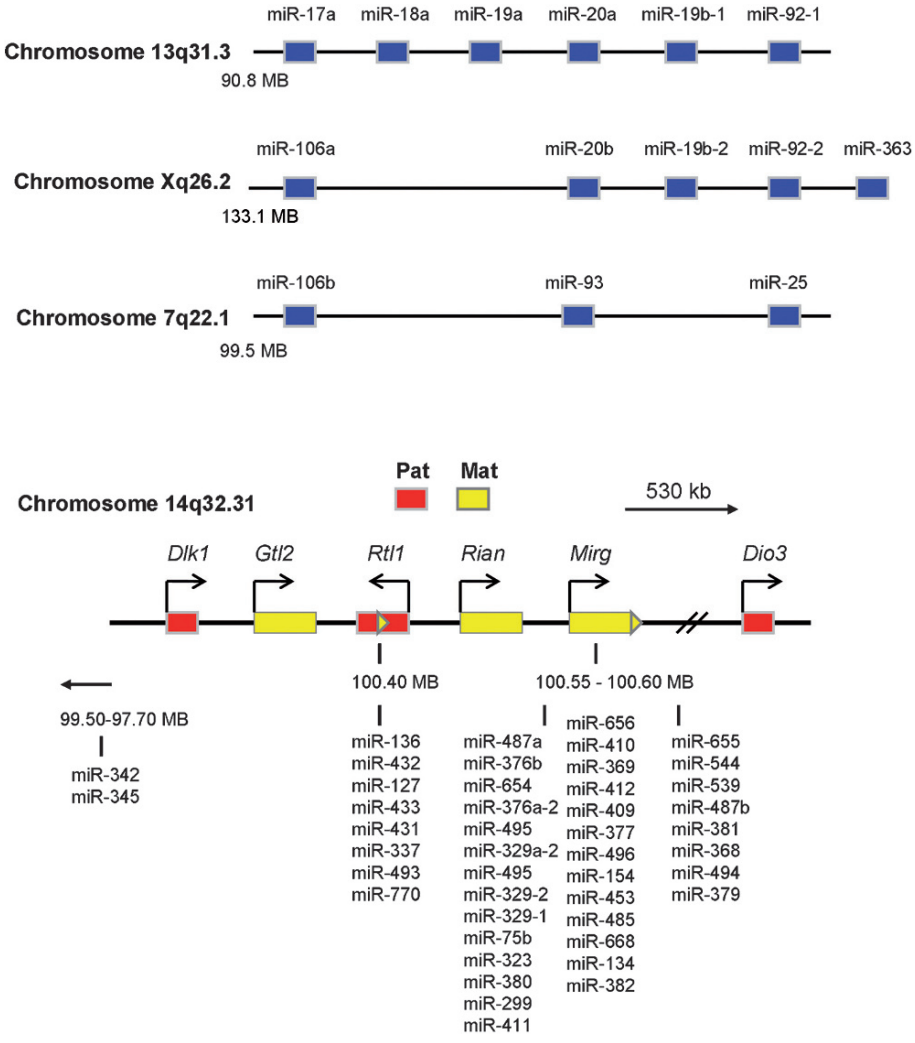

Figure 3. MicroRNA (miRNA) gene clusters. The miR-17-92 miRNA gene cluster is located at human chromosome 13q31.3. The miR-17-92 cluster has homologous clusters on the X chromosome and chromosome 7. The large cluster at human chromosome 14q32.31 is located within the microRNA-containing gene (*Mirg*) in the *Gtl2-Dio3* region. This cluster is highly imprinted and only expressed from the maternally derived chromosome (Pat, paternally-expressed; Mat, maternally-expressed).

miRNA gene clusters are associated with disease and development

miRNA genes are located throughout animal genomes in both intergenic and intronic sites. Many miRNAs are also located in gene clusters, the largest of which are located at human chromosome 13q31.3 and 14q32.31. The miRNA cluster miR-17–92 is located at 13q31.3 and has been associated with several types of cancer, including colorectal cancer [84] and lung cancer [85], and is thought to be a potential oncogene [86, 87] that enhances cell proliferation [88]. This cluster has homologous regions located at Xq26.2 and 7q22.1 (Fig. 3), which have also been implicated in cancer [89].

Probably the largest miRNA gene cluster is located at the imprinted Dlk1-Gtl2 domain (otherwise referred to as the Gtl2-Dnchc1 cluster) at chromosome 14q32.31 [90], with potentially 35 expressed miRNAs concentrated in the miRNA-containing gene (*Mirg*). This region is also highly conserved in the mouse on the syntenic region of chromosome 12 [90]. The cluster includes several separately encoded sequences, although a single oligonucleotide is also expressed and subsequently processed into separate miRNAs [91]. The region contains a number of imprinted genes, and the miRNAs seem to be expressed from only the maternal chromosome (Fig. 3). For example, miR-136 and miR-127 are located within the retrotransposon-like gene (*Rtl1*) and are transcribed in an antisense direction [92]. Interestingly, *Rtl1* is transcribed only from the paternal chromosome, while the miRNAs are transcribed from only the maternal chromosome. Moreover, miR-136 and miR-127 are thought to negatively regulate *Rtl1* expression *via* a direct mRNA degradation pathway [93]. This cluster of maternally imprinted miRNAs was also shown to be highly expressed in the developing lung of both mice and humans [81].

The imprinted genomic region (14q32.31) has also been associated with the Callipyge phenotype exhibited by sheep, which is characterised by muscular

hypertrophy [31]. Only heterozygous offspring that have inherited the mutation from the male exhibit the Callipyge phenotype, a non-Mendelian event known as polar overdominance [94]. It appears that heterozygous offspring with a paternally inherited Callipyge mutation overexpress the paternally imprinted genes (*e.g. DLK1/PEG11*), leading to the phenotype. The expression of these genes is normally repressed due to imprinting. Likewise, a maternally inherited Callipyge mutation causes the overexpression of maternally expressed genes (*e.g. GTL2*), which then decrease the expression of the paternally imprinted genes [95]. The regulation of gene expression at this imprinted region is thought to act *in trans*, possibly by interfering with *DLK1* or *PEG11* transcription or translation. The maternally imprinted miRNAs miR-127 and miR-136 have now been demonstrated to regulate *PEG11* expression by miRISC-mediated cleavage of the mRNA, demonstrating the involvement of miRNAs at this imprinted region [93].

A clustering of small RNAs in the imprinted region at chromosome 15q11.13 has been associated with Prader-Willi-Angelman syndrome [96], further demonstrating the influence of non-coding RNAs, including putative miRNAs, in gene regulation [97]. At least two families of miRNAs are also located in intergenic locations within *Hox* gene clusters, both of which follow similar patterns of evolution within vertebrate genomes [98]. The translation products of *Hox* genes are homeodomain transcription factors, which are involved in morphogenesis, and have been shown to be regulated by the miRNAs located in the same region [99]. For example, miR-10 is predicted to target the *Src/Hox5* gene in *Drosophila* [60], while the *Drosophila* miRNA iab-4 transforms halteres to wings, possibly through direct inhibition of Ubx activity [60]. *Drosophila* iab-4 is analogous to mammalian miR-196, itself located in a *hox* gene cluster, which has been shown to inhibit Hoxb8 and influence cell differentiation [83], suggesting miRNAs are able to modulate *Hox*-mediated gene expression.

miRNAs regulate stem cell differentiation

Several miRNAs are specifically expressed in embryonic stem (ES) cells and may be essential in maintaining a pluripotent state [100]. By comparing undifferentiated with differentiated mouse ES cells, a small miRNA cluster, from which miR-293 was highly expressed, was identified to be ES cell-specific. Many other miRNAs were up-regulated following RA-induced ES cell differentiation and may be involved in cell lineage determination during early development. The observation that differences exist in the miRNA expression profile between undifferentiated and differentiated ES cells in zebrafish and pufferfish may also suggest that developmental events are controlled by miRNAs [100]. Interestingly, three human miRNAs (miR-302b, miR-302c and miR-302d) cloned from human ES cells were identical to miRNAs derived from mouse ES cells, suggesting a certain amount of conservation across species [101]. Furthermore, many clusters of human miRNA clones from ES cells share homologues with those miRNAs cloned from mouse ES cells, including the miR-371 and miR-302 families. Those miRNAs expressed in ES cells may be involved in maintaining pluripotency, while those expressed in differentiated cells may be involved in lineage specificity, and those expressed in individual cell types may play a role in general cell homeostasis. The recent development of a method for the analysis of miRNAs from single stem cells using a sensitive PCR-based technique may aid in the elucidation of the developmental pathways controlled by miRNAs [102].

The significance of miRNAs during stem cell division was highlighted in Dicer-deficient mice, in which the number of stem cells within the blastocyst cell mass was markedly reduced [79]. Dicer-1 (Dcr-1) mutant *Drosophila* exhibited germline stem cells with a normal phenotype but a reduced capacity to pass through the G1/S phase transition in the cell cycle [103]. It was suggested that miRNAs inhibit expression of the cyclin-dependent kinase inhibitor Dacapo (Dap), which prevents cell cycle progression.

miRNAs are involved in cardiac and skeletal muscle development

In mouse embryos, miR-1-1 and miR-1-2 have been shown to be selectively expressed in cardiac tissue and have been implicated in cardiogenesis [104]. In addition to these observations, it was demonstrated that serum response factor (SRF) up-regulated miR-1-1 and miR-1-2, leading to inhibition of cardiomyocyte proliferation. A possible target for miR-1-1 and miR-1-2 is the transcription factor Hand2, which promotes cardiomyocyte differentiation. Yang et al. [105] demonstrated that miR-1 is overexpressed in patients with coronary artery disease and that overexpression of miR-1 in a rat model of cardiac infarction exacerbated arrhythmogenesis. The authors suggested that miR-1 targets the mRNA that encodes the potassium channel subunit KCNJ2 as well as GJA1, which encodes connexin 43. Cardiac hypertrophy may also be regulated by miR-1, which is significantly down-regulated in hypertrophic tissue [106]. Indeed, a targeted knockout of miR-1-2 in

mouse cardiac muscle revealed its essential role in heart biogenesis [107]. The majority of miR-1-2-deficient animals died *in utero*, while 15% of the surviving animals had severe heart defects and died prematurely. The remaining adult mice had physiological defects such as cardiomyocyte hyperplasia, reduced heart rate and abnormal electrical activity [107]. It is likely that miR-1-2 targets multiple genes involved in cardiogenesis and normal physiological function.

The *Drosophila* homologue of miR-1 is also regulated through an SRF-like binding site and acts to prevent cardiac differentiation by targeting and inhibiting the Notch ligand Delta [72]. Deletion of miR-1 in *Drosophila* led to embryonic or larval death, emphasizing the importance of this miRNA in cardiac and somatic muscle development [73]. Furthermore, miR-208 was recently shown to enhance stress-related cardiac muscle growth by up-regulating a contractile protein, βMHC, possibly by repressing translation of thyroid hormone receptor-associated protein 1 (THRAP1) [108].

Chen et al. [109] showed that miR-133 is transcribed together with miR-1 in mouse myoblasts and in a tissue-specific manner in mouse embryos and neonates. In *Xenopus* embryos miR-1 promoted myogenesis by inhibiting histone deacetylase 4 (HDAC4), which itself inhibits muscle gene expression, while miR-133 enhanced myoblast proliferation by inhibiting SRF [109]. miR-1 and miR-133 are derived from the same chromosomal cluster, and although both promote muscle development and are overexpressed in diseased hearts, they have distinct biological properties. miR-133 was shown to inhibit the expression of ERG (ether-a-go-go-related gene) and cause depression of the potassium channel I(Kr) [110]. This inhibition contributed to long QT syndrome and arrhythmia in a diabetic rat model. Yet another target during myoblast differentiation is Hox-A11, which was targeted by miR-181 in mouse embryos [111]. Hox-A11 negatively regulated the differentiation of myoblasts, and miR-181 seemed to inhibit its expression, thereby further supporting the participation of miRNAs in mammalian development.

Neurogenesis is regulated by miRNAs

The significance of miRNAs in the morphogenesis of the brain and neuronal system has received much attention. A maternal-zygotic Dicer-mutant zebrafish revealed the importance of miRNAs during the later stages of embryonic development, as gastrulation, somitogenesis, cardiogenesis and neurogenesis were abnormal [75]. Rescue of normal brain morphogenesis was achieved by injecting miR-430 duplexes, demonstrating the specific importance of miRNAs. Selective microarray analysis of mouse brain showed that a number of miRNAs are differentially regulated throughout brain development [112]; some of these may be conserved in mammals [113], while others are orthologous to miRNAs involved in *C. elegans* development [114]. Although large-scale profiling of brain tissue suggests that miRNAs are largely involved in later stages of development, Krichevsky et al. recently found that miR-124a and miR-9 affect the differentiation of ES cells into neural progenitor cells [115]. They also proposed a mechanism whereby overexpression of these miRNAs results in reduced phosphorylation of the signal transducer and activator of transcription 3 (STAT3). The small C-terminal domain phosphatase 1 (SCP1) possesses anti-neural properties and was shown to be down-regulated by miR-124, further establishing the role of miR-124 in neuronal development [116]. Makeyev et al. [117] recently demonstrated that miR-124 directly targets PTBP1 (polypyrimidine tract-binding protein 1) mRNA during nervous system (NS) differentiation. PTBP1 is a universal repressor of alternative mRNA splicing. Inhibition of PTBP1 by miR-124 allows the accumulation of a homologous splicing protein, PTBP2, which results in the transition from non-NS to NS-specific mRNA alternative splicing patterns. Furthermore, others have suggested that miR-124 acts to repress non-neuronal gene expression in order to reinforce neuronal differentiation [118].

In addition, the brain-specific miR-134 has been shown to inhibit the growth of dendritic spines from rat hippocampal neurons [119]. This effect was mediated by miR-134 inhibition of the protein kinase Limk1, high expression of which results in dendrite growth. Furthermore, transfection of human epithelial HeLa cells with miR-1 or miR-124 resulted in the down-regulation of approximately 100 mRNAs [120]; this resulted in a shift toward muscle-specific gene expression following miR-1 administration or brain-specific gene expression following miR-124 administration. These data may suggest that miRNAs involved in skeletal muscle or brain development are able to modify the normal expression profile of a cell and modulate its phenotype.

The function of miRNAs during haematopoiesis

The differentiation of multipotent stem cells into the varied and terminally differentiated cell types of the blood is known as haematopoiesis. Recently it was suggested that miRNAs are key regulators of this process. Using a well-characterised microarray, Mon-

ticelli et al. compared the expression of 181 miRNAs between cell line-derived PU.1$^{-/-}$ haematopoietic cells and neuronal tissue [121]. Of particular interest was the differential expression of miR-150, which was highly expressed during early B and T cell differentiation but down-regulated during Th1 and Th2 cell differentiation. miR-146 was also differentially expressed: it was up-regulated in Th1 cells but not Th2 cells, possibly functioning in the maintenance of lymphocyte lineage specificity. miR-181 was preferentially expressed in cells of the B cell lineage, and ectopic expression of miR-181 enhanced the population of CD19$^+$ B lineage cells *in vitro* [122]. T cell lineage differentiation was also shown to be modified by miR-181a, as an antagomir to miR-181 inhibited negative selection of double-positive thymocytes and significantly impaired positive selection of CD4$^+$ T cells [123].

A conditional allele knockout system was used to study the function of Dicer during T cell development [124]. Survival of αβ T cells was reduced following deletion of Dicer at an early stage of T cell development, but γδ T cells and later stage CD4$^+$ or CD8$^+$ T cells were unaffected. The authors suggest that Dicer, and therefore miRNA expression, may not be as critical during lineage-specific differentiation as it is during embryonic development. However, Ramkissoon et al. demonstrated differences in the level of miRNA expression between normal human haematopoietic cells and those derived from malignant haematopoietic cell lines, demonstrating the importance of miRNAs in the maintenance of healthy tissue [125]. In particular, miR-142, miR-155, miR-188 and miR-233 have discrete expression profiles in B cells, T cells, monocytes and granulocytes [122], the patterns of which change in malignant cells. Moreover, the expression profile in humans is different from that observed in mouse haematopoietic cells [125], suggesting important species variation. miRNAs are also differentially expressed in CD34$^+$ hematopoietic progenitor cells depending on the stage of haematopoiesis [126]. For example, miR-155 is thought to inhibit genes related to myeloid and erythroid differentiation, while the expression of miR-15b, miR-16, miR-22 and miR-185 correlates with erythropoiesis in umbilical cord-derived CD34$^+$ haematopoietic progenitor cells [127].

The regulation of human granulopoiesis also involves miRNAs. The transcription factors NFI-A and C/EBPα establish a mini-circuit with miR-223 [128]. NFI-A and C/EBPα both compete for access to the promoter region of miR-223, but while NFI-A maintains low-level expression of miR-223, C/EBPα upregulates miR-223 in response to retinoic acid (RA). In the absence of RA, NFI-A is the dominant transcription factor, and so miR-223 expression remains low. Addition of RA to the system increases C/EBPα, which replaces NFI-A at the miR-223 promoter. C/EBPα enhances miR-223 expression, and the mini-circuitry is completed by miR-223 inhibiting the translation of NFI-A, lowering the levels of protein and in effect promoting differentiation to the granulocyte lineage (Fig. 4). This pathway was confirmed by overexpression of miR-233, which enhanced differentiation, while miR-223 knockdown inhibited differentiation in response to RA. In addition, a number of other miRNAs (*e.g.* miR-10a, miR-10b and miR-126) were shown to be down-regulated during magakaryocytopoiesis, possibly so that inhibitory signals are removed for differentiation to take place [129]. This provides a fine example of how miRNA can fine-tune gene expression through the establishment of a regulatory mini-circuit.

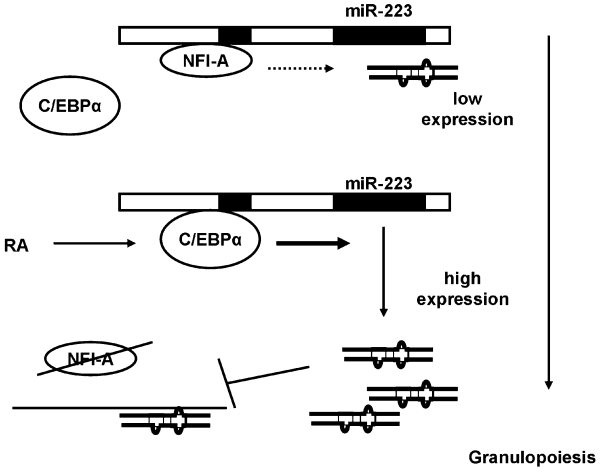

Figure 4. miR-223 mini-circuitry. The expression of miR-223 is controlled by two transcription factors, NFI-A and C/EBPα. While NFI-A leads to low expression of miR-223, C/EBPα leads to high expression of miR-223; this high expression is reinforced by inhibition of NFI-A by miR-223, thereby maintaining miR-223 levels and driving granulopoiesis in response to retinoic acid (RA).

miRNAs involved in immune function

Recently, miR-155 was shown to play important roles in normal immune function. Mice deficient in *bic/miR-155* are rendered immunodeficient and display increased lung airway remodelling [130]. Analysis of *bic/miR-155*-deficient CD4$^+$ T cells revealed that this miRNA is a key regulator of several genes, including cytokines (IL-4), chemokines (CCL5) and transcription factors (c-Maf). miR-155 is also involved in regulating T helper cell differentiation and mediating the T cell-dependent antibody response [110]. Mutant mice deficient in miR-155 were shown to be deficient in germinal centre (GC) B cells, whereas mice over-

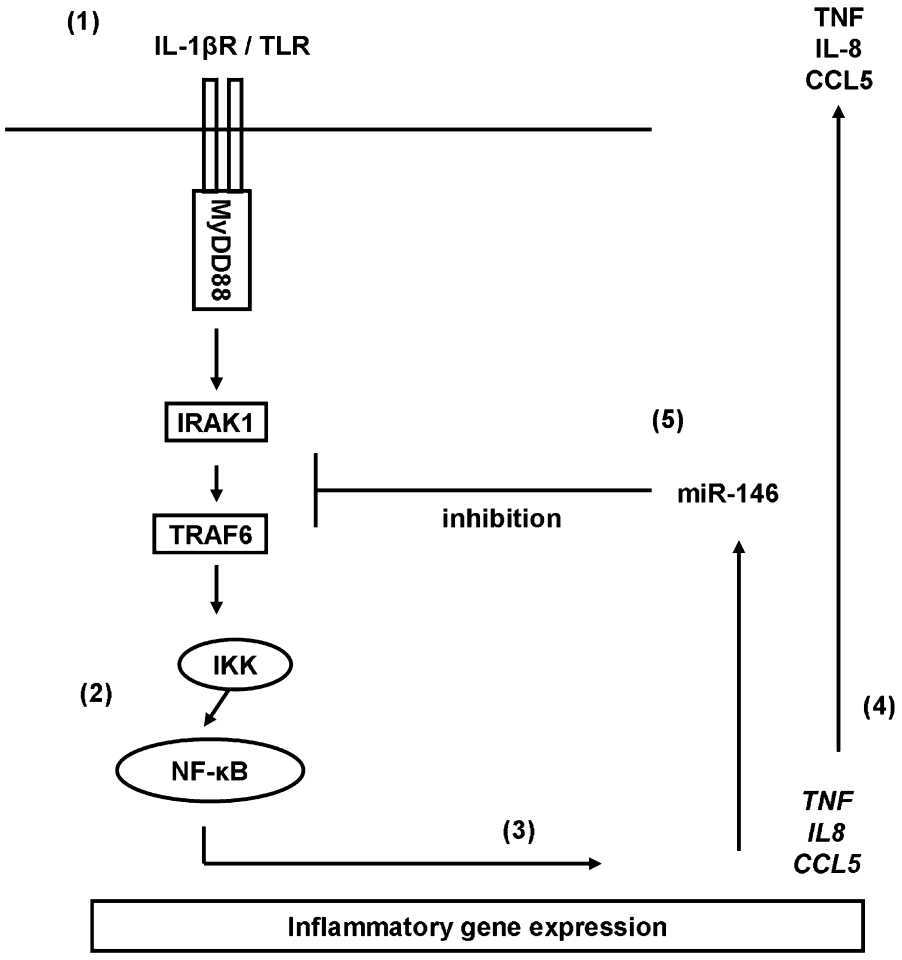

Figure 5. Inflammation is modulated by miR-146. (1) Induction of inflammation occurs following ligation of the IL-1β receptor or Toll-like receptors (TLRs). (2) This ultimately leads to the activation of the NF-κB pathway and (3) the expression of inflammatory genes. (4) For example, gene expression leads to the release of cytokines such as TNF and chemokines such as IL-8 and CCL5. (5) NF-κB activation also results in the increased expression of miR-146, which putatively inhibits members of the signalling pathway, IRAK1 and TRAF6. This feedback mechanism then reduces the magnitude of the inflammatory response.

expressing miR-155 had increased numbers of GC B cells in Peyer's patches and gut-associated lymph nodes [131]. Mice deficient in miR-155 also had diminished T cell-dependent antibody responses, most likely due to a reduction in TNF production by B cells and possibly through enhancement of the regulatory T cell compartment. In addition, macrophages up-regulate miR-155 in response to IFN-α/β or indirectly following activation by a range of Toll-like receptor ligands such as poly:IC or LPS; this up-regulation was dependent on MyDD-88 and the JNK pathway [132]. Another study demonstrated that the expression of a number of miRNAs, including miR-223, increased in the lung following LPS challenge [133], suggesting that miRNAs are able to modulate innate and adaptive immune responses.

Another miRNA, miR-146, was shown to regulate the innate immune response by potentially interfering with components of the NF-κB pathway, namely interleukin-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) [134]. It was suggested that, as miR-146 is up-regulated following stimulation, it may then act by down-regulating elements essential for inflammatory cytokine release *via* a negative feedback mechanism (Fig. 5). miR-146 is also expressed highly in the skin of psoriasis patients and in activated $CD4^{+}CD25^{high}$ T cells [135]. In the same study, miR-203 was up-regulated within inflamed skin, while one of its predicted targets, suppressor of cytokine signalling-3 (SOCS-3), was down-regulated.

Furthermore, the sensitivity of mature T cells was shown to be modified by miR-181a [123]. Increased miR-181a expression resulted in an enhanced sensitivity to a T cell agonist, probably through increased CD28 expression and decreased CTLA-4 expression. The basal activation level for T cell receptor signalling was also enhanced following miR-181a overexpression due to, for example, higher levels of ERK, although miR-181a is likely to exert its effects through multiple targets [123]. These data demonstrate that miRNAs can modulate important immunological responses through subtle regulation of gene expression.

miRNAs associated with cancer

Several techniques have been utilised to study the miRNA expression profile of human cancers, including microarray and flow cytometry-based assays [136, 137]. As miRNAs have been shown to control cell fate and cell proliferation, the expression of miRNAs might be closely associated with the development of human cancers. Indeed, miRNA expression levels differ between healthy tissue and cancerous tissue. Many miRNAs are expressed at much lower levels in tumours, suggesting that miRNAs play a role in controlling cell proliferation and preventing tumourigenesis [138]. Furthermore, inhibition of DGCR8, Drosha or Dicer using siRNA led to decreased miRNA expression and an enhancement of transformation and tumour growth [139]. However, a large-scale microarray screen of several solid tumours identified many miRNAs that are overexpressed, including miR-17-5, miR-21 and miR-155 (among others), suggesting that these particular miRNAs may inhibit tumour suppressor genes [140, 141]. Indeed, inhibition of miR-17-5 (and miR-20a) using antisense oligonucleotides enhanced apoptosis in lung cancer cells, further supporting an important role for the miRNA cluster miR-17-92 in tumour progression [85]. Intriguingly, the proto-oncogene *c-myc* encodes a transcription factor that controls cell proliferation through the activation of another transcription factor, E2F1. The c-myc protein also activates the miR-17-92 cluster, of which miR-17-5p and miR-20a were shown to negatively regulate translation of the transcription factor E2F1, resulting in tight control of the cell proliferative signal [142].

miR-155 has also been implicated in a number of cancers including Burkitt's lymphoma [143], Hodgkin lymphoma [144] and lung cancer [145]. The overexpression of miR-21 was recognized in malignant brain tumours, and knockdown of this miRNA in cultured glioblastoma cells resulted in caspase activation and apoptosis [146]. The tumour suppressor tropomyosin 1 (TPM1) was demonstrated to be a target for miR-21, explaining why inhibition of miR-21 results in reduced tumour growth [147]. Numerous miRNAs are also up-regulated in papillary thyroid carcinoma, including miR-221, miR-22 and miR-146 [148]. This up-regulation was associated with a down-regulation in the expression of the oncogene *KIT*, a tyrosine kinase important for the control of cell proliferation. Furthermore, miR-372 and miR-373 have been implicated in testicular germ cell tumours [149]. The miRNA expression profile can also distinguish the developmental history of certain tumours: for example, similar profiles were obtained from tumours derived from embryonic endoderm [136].

Chronic lymphocytic leukaemia (CLL) is characterised by overexpression of the anti-apoptotic B cell lymphoma protein Bcl-2 in B cells and represents the most common human leukaemia. In less than 5% of cases, overexpression of Bcl-2 is due to a translocation of the *BCL2* gene putting it under the control of the immunoglobulin heavy chain promoter [150]. For the majority of CLL cases, no explanation for the dysregulation of Bcl-2 has been reported; however, it has been demonstrated that a unique miRNA expression profile exists in CLL cases, with a mutation in the immunoglobulin heavy chain variable region [151]. In addition, mutations in genomic regions containing miRNAs were associated with disease progression in a number of CLL patients. Although miR-15a and miR-16-1 have been shown to be deleted or translocated in approximately 65% of CLL patients, Cimmino et al. were the first to show that these miRNAs directly inhibit Bcl-2 activity and activate apoptotic processes [152]. miR-15a and miR-16 are often deleted from chromosome 13q14.2 in patients with B cell CLL, suggesting that these miRNAs may be involved in tumour suppression [153]. Additionally, introduction of exogenous miR-16 into a malignant B-1 cell line resulted in increased apoptosis [154]. Post-transcriptional repression of Bcl-2 by miR-15a and miR-16-1 is an important event in preventing B cell leukaemia and may represent an alternative pathway for cancer therapy. In a similar manner, miR-34a induces apoptosis in neuroblastoma cells, possibly by targeting the transcription factor E2F3 [155].

Several independent studies have recently demonstrated that miR-34 is an important component of the p53 tumour suppressor network. Indeed, p53 is a predicted target for members of the miR-34 family (miR-34a-c), while the expression of miR-34a is often up-regulated in cells expressing p53 [156] or following p53 activation and DNA damage [157]. This p53-induced up-regulation of miR-34a results in an enhanced reduction in cell proliferation, strongly suggesting that miR-34a reinforces the tumour suppressor function of p53. The transactivation of miR-34a by p53 was also shown to promote apoptosis [158], and ectopic miR-34a arrested cells in the G1 phase of the cell cycle [157], while inhibition of miR-34a reduced p53-mediated apoptosis [159].

miRNAs may also act as oncogenes, as it has been suggested that a mutation in (or loss of expression of) a miRNA results in either a loss of function, so that a target mRNA is overexpressed, or a gain in function, so that the target mRNA is repressed [160]. Both these scenarios could result in aberrant cell proliferation. Alternatively, a mutation in the target sequence could result in either a loss or gain of miRNA specificity and

result in a similar disease outcome. Calin et al. demonstrated that approximately 50% of miRNA genes are located in fragile sites or genomic regions associated with cancers [151]. Overexpression of the miRNA gene cluster miR-17-92 was associated with human lung cancer and cell proliferation [88, 161]; this cluster includes miR-17, miR-18a, miR-19a and miR-20a (Fig. 3) located upstream of the gene for glypican-5, which itself may be up-regulated by a member of the oncogenic c-myc family. Conversely, miR-17-5p was down-regulated in breast cancer cells, and enhanced expression decreased tumour cell proliferation [162]. The disparity in function for miR-17, as an oncogene or conversely as a tumour suppressor gene, may be the result of cell type differences between lung and breast cancer cells. This may further highlight target gene preferences in certain cells, possibly due to contrasting co-localisation of mRNA molecules.

Many miRNAs are located at chromosomal break points or fragile sites associated with cancer. Indeed miR-29a and miR-29b are associated with the fragile site FRA7H. This fragile site is the only one of its kind not associated with a known tumour suppressor gene, further indicating a role for miRNAs in cancer [153]. In addition, miR-29b along with miR-181b may target the oncogene TCL1 in CLL patients [163], while miR-29b reduced Mcl-1 protein and rendered cholangiocarcinoma cells more susceptible to apoptosis [164]. The significance of miRNAs during cancer was further established by finding down-regulation of Dicer in a significant proportion of lung cancer patients [165]. Furthermore, a reduction in the expression of let-7 was associated with lung cancers and in particular with reduced survival [166, 167]. Mayr et al. demonstrated that a chromosomal translocation at 12q5 disrupts let-7 expression and consequently curtails inhibition of the oncogene *High Mobility Group A2* (*Hmga2*) [168, 169]. Let-7 has also been implicated in the development of colon cancers [170] and the progression of colorectal cancers [171]. Modulation of miRNA expression and/or activity may therefore provide a novel means of cancer treatment, either by utilizing their natural tumour suppressor properties or by inhibiting oncogenic function (reviewed further [172]).

Metabolism

miRNAs also appear to be involved in the regulation of insulin release, cholesterol metabolism and renal function. For instance, miR-375 was shown to directly regulate insulin secretion from pancreatic islet cells [173]. Overexpression of miR-375 led to an enhanced inhibition of insulin exocytosis, whereas anti-sense to miR-375 enhanced insulin secretion by blocking the effects of the miRNA. It was also suggested that myotrophin may be the target for miR-375 and that manipulation of this pathway could be useful in treating diabetes. Anti-sense targeting was also used to demonstrate that miR-122 is involved in the regulation of lipid metabolism in the liver. Inhibition of miR-122 resulted in decreased levels of cholesterol in the plasma and improved liver function in obese mice [174]. miRNAs have been implicated in other metabolic processes; most notably, miR-155 was shown to decrease the expression of angiotensin II type 1 receptor in human primary fibroblasts [175]. This suggests that miR-155 may regulate a diverse set of physiological functions including fluid homeostasis and renal function.

Summary

miRNAs are able to regulate a myriad of developmental and physiological processes in invertebrate and vertebrate animals in a remarkably evolutionarily conserved manner. These relatively recent findings undoubtedly add another layer of complexity to established gene regulatory networks. In addition, miRNAs have now been implicated in transmitting heritable epigenetic traits as a result of maternal or paternal expression and in influencing embryonic gene expression [176]. Gene clusters containing miRNAs have even been implicated in genetic diseases such as fragile X and Prader-Willi-Angelman syndromes [97, 177] and in several types of cancer, broadening the potential modalities that may be regulated by miRNAs. Immune defence against pathogens is also modulated by miRNAs [178], while certain viruses retort by expressing their own inhibitory miRNAs [179–181]. However, to date the most important function of miRNAs appears to be in the regulation and consolidation of gene expression patterns that govern developmental cell fate, proliferation and the maintenance of homeostasis [182].

It is becoming increasingly important to identify the mRNA targets of the known miRNAs and attribute a specific biological function. However, this analysis is made problematic by the lack of complementarity between miRNA and mRNA and the way in which most miRNAs modulate translation rather than mRNA levels. Subtle downstream effects may have to be relied upon in order to ascertain significant biological function. The difficultly in predicting target sequences is further compounded by the discovery of miRNA editing [183], which may increase the potential number of target sequences and complicate current target prediction algorithms. It is clear, how-

ever, that miRNAs reinforce gene expression patterns and regulate many signalling pathways in several developmental and biochemical programmes. They act by fine-tuning gene expression and reducing the unwanted effects of excessive genetic noise. The complete extent of their biological influence remains to be revealed.

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