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Review

The Emerging World of MicroRNAs

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Abstract. *MicroRNAs (miRNAs) are a family of naturally occurring, evolutionary conserved, small (approximately 19-23 nucleotides), non-protein-coding RNA molecules that generally negatively regulate post-transcriptional gene expression. miRNAs are estimated to account for >3% of all human genes and to control expression of thousands of target mRNAs, with multiple miRNAs targeting each mRNA. A role for miRNAs has been identified in both normal physiological and pathological conditions, including metabolism, proliferation, cell death, differentiation and development, insulin secretion from pancreatic β cells, viral infection and cancer. Antisense technologies have been successfully used to control miRNA expression in vitro and in vivo. Further analysis of this interesting class of small RNAs, in normal and pathological conditions, will enable us determine their potential to be exploited as therapeutic targets in disease.*

Small RNAs

Almost 30 years ago – with the identification of ribozymes – it was established that RNAs are much more complex and contribute much more actively to the dynamics of a cell than merely acting as a carrier of information between DNA and proteins, as had previously been considered. At the beginning of this century, the involvement of "small RNAs" regulating gene expression was described (1-3). Small RNAs are a family of regulatory non-coding RNAs of 14-28 nucleotides in length, derived from double-stranded RNA (dsRNA). This family includes small interfering RNAs (siRNAs), repeat associated small interfering RNAs (rasiRNAs) and microRNAs (miRNAs), which have been identified as key components of an evolutionary-conserved system of RNA-based regulators of gene expression in eukaryotes (4). These small RNAs are distinguished by their origins, rather than their function (5). [A comprehensive review of all small RNAs is beyond the space limitations of this manuscript which is focussed on miRNAs – for reviews of other small RNAs see: (6-8)].

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MicroRNAs

The first endogenous small RNAs to be discovered were miRNAs (7). miRNAs are approximately 22 nucleotide long, non-coding RNAs (ncRNAs) that bind to partially complementary sequences within the 3'-untranslated region (UTR) of target mRNAs and generally negatively regulate gene expression post-transcriptionally, although at least one human miRNA has recently been found to act positively on gene expression, *i.e.,* miR-122 enhances replication of hepatitis C virus (5). Mammalian miRNAs are described using the prefix *mir*, followed by a number, while miRNAs originally identified in *Caenorhabditis elegans* (*C. elegans*) are denoted by the prefix *lin* or *let* (9). Each miRNA apparently regulates multiple genes and hundreds of miRNA genes are predicted to be present in mammals (10). miRNAs are, thus, proposed to be involved in regulating at least 1/3 of all genes within the human genome (5), although, of the hundreds of miRNAs identified to date, the biological function(s) of very few has been elucidated so far (11).

In mammalian cells, miRNAs are transcribed, by RNA Polymerase II, as primary long pre-miRNA transcripts – ranging from 100s to 1,000s of nucleotides in length (12-14). These are capped and polyadenylated (classical characteristics of protein-coding mRNAs), spliced, and contain stem-loop or hairpin structures of approximately 70 nucleotides in length, from which mature miRNAs are produced by sequential processing in the nucleus and the cytoplasm (15). Most of the sequences encoding miRNAs occur in areas of the genome that are not associated with known genes, many of which were found in fragile sites in

human chromosomes (16). It is estimated that approximately 60% of miRNAs are independently transcribed (10, 17-19), with approximately 15% transcribed in clusters – suggesting that they are transcribed as polycistronic transcripts. Approximately 25% of miRNAs are encoded in introns of primary mRNA transcripts. Such intronic miRNAs are generally encoded in the same orientation as the pre-mRNA in which they exist, suggesting that expression of these miRNA genes is driven by mRNA promoters (10, 20-22).

In the nucleus, precusor miRNAs are cleaved by the dsRNA-specific RNase III-type endonuclease, *Drosha*, acting with its dsRNA-binding partner (*i.e.,* DGCR8), releasing pre-miRNAs of approx. 60-70 nucleotides in length. After their subsequent transport to the cytoplasm in a Ran-GTP-dependent manner [by exportin 5 (23)], pre-miRNAs are processed by the dsRNA-specific RNase III-type endonuclease, *Dicer*, acting with its dsRNA-binding partner [*i.e.,* the tar-binding protein (TRBP)]. This results in the generation of approximately 22 nucleotide duplexes, one strand of which is the mature miRNA – resembling siRNA (24). The mature miRNA is subsequently incorporated – together with the highly conserved argonaute (Ago) protein (25) – into the RNA-induced silencing complex (RISC), named miRNA-containing ribonucleoprotein particles (miRNPs) (26-28).

The Discovery of miRNAs

miRNAs were first described in 1993, by the identification of *lin-4* and its target, *lin-14*, in controlling timing events during larval development in *C. elegans* (29-30). The subsequent identification of a second miRNA, *let-7*, not only in *C. elegans*, but also in mammalian cells (31-32), indicated the evolutionary conservation of these RNAs and led to the further identification of many miRNAs in plants (19, 33-34), worms (18, 35), flies and mammals (36), by using prediction and cloning methodologies. However, it was as recently as 2001 that a direct connection was made between miRNA and siRNA, when *Dicer* – the enzyme previously shown to convert long dsRNA into siRNAs – was shown to have a role in the maturation of miRNAs.

Over the past 13 years, many miRNAs have been identified in plant and mammalian cells (a database of known and predicted endogenous miRNAs is available at http://www.sanger.ac.uk/Software/Rfam/mirna). Although the exact number of miRNA genes in the human genome has yet to be determined, current estimates range from 500 to 1,000 (37). As miRNA are short nucleotide sequences, it is thought that many new miRNA genes may evolve through duplication and mutation (5). Indeed, Blow *et al.* (38) have recently reported RNA editing (*i.e.,* site-specific modification of an RNA sequence to yield a product differing from that encoded by the DNA template) in at least 6% of human miRNAs, which may further increase the diversity of miRNAs and their targets.

At least fifty-three miRNAs have been identified as unique to primates (39), although the sequences of many of the miRNAs are homologous among organisms, suggesting that miRNAs represent a relatively old and important regulatory pathway (40). In fact, exact sequence matches for more than 90% of the sequence-verified human miRNAs can be found in the genomes of mouse and rat. Some miRNAs are widely expressed (*e.g., miR-15a* and *miR-16-1*), with others expressed in a tissue-specific and developmental-specific manner (28). The fact that many mRNAs have predicted target sites for numerous different miRNAs suggests that gene expression in tissues may be dependent on the miRNA population in those specific cells.

miRNAs are described as being present at very high steady-state levels – generally more than 1,000 molecules in the cells in which they are expressed, with some exceeding 50,000 molecules per cell. Such miRNAs have been implicated in providing specificity to a remarkable range of biological pathways and processes, including cell proliferation and cell death during development, stress resistance, fat metabolism, insulin secretion from pancreatic beta cells, neurological disease and cancer (41).

Mechanism(s) of Action of miRNAs

miRNAs post-transcriptional regulation of gene expression apparently occurs at a number of levels and has been described as "fine-tuning" of expression (42). miRNAs have also been found to co-operate in silencing; in fact, mRNAs containing multiple miRNA binding sites are more responsive to miRNA-induced translational repression than those containing a single miRNA binding site (43, 44). While early studies suggested that miRNAs regulate the accumulation of proteins from bound mRNAs without affecting the levels of the mRNA population (45), more recent studies have reported that miRNAs promote rapid mRNA degradation by accelerating decapping and deadenylation (46-48). Levels of control that have now been described include miRNAs acting on stability, compartmentalisation, and translation of mRNAs, with several miRNAs acting in reciprocal negative-feedback loops with protein factors to control cellular events triggered by signal transduction (48).

As described above, mature miRNA is assembled in effector complexes, termed miRNPs. Once the miRNP is assembled, by Watson-Crick base-pairing, the miRNA directs miRNP to partially complementary sites within target mRNA(s). Although plant miRNAs generally are 100% complementary to their mRNA target site, perfect base-pairing is rare between mammalian miRNAs and target genes studied to date. Strong recognition between the

5' end of the miRNA and its target appears to be sufficient for regulation of gene expression.

The "seed" nucleotides, *i.e.,* nucleotides 2-7 at the 5' end of the miRNA appear to be responsible, for the most part, for the specificity and functionality of the miRNA-mRNA target interactions, with Argonaute (Ago) proteins acting as key components of this complex (49-51). Ago are a diverse family of proteins, approximately 100 kDa in size. All members contain a PAZ domain, which is involved in miRNA binding, and a PIWI domain, which is related to R*Nase* H endonuclease activity and is involved in "slicer" activity, *i.e.,* cleavage of the target mRNA (52-53). It has been established that base-pairing is required at the site of cleavage, between bases 10 and 11, for efficient endonuclease activity (54-55). In humans, only one specific Ago protein – *i.e.,* Ago2 – has been shown to exhibit cleavage (56-58). Recent studies suggest that other sequence-specific RNA-binding proteins may also be involved in this process, adding a further degree of specific to such miRNA:mRNA interactions (59-61). The 3' region of the miRNA also contributes to efficient repression, possibly by working as a modulator of suppression (49, 62-63).

In addition to slicer-dependent mechanisms, apparently miRNAs are also involved in induction of mRNAs decay by a slicer-independent mechanism. The possibility that miRNAs may target decapping is supported by observations that Ago proteins are co-localised (realised by microscopy and by co-precipitation) with decapping enzymes and several activators of decapping in cytoplasmic foci (termed cytoplasmic processing bodies; P-bodies) (64-66). A proposed mechanism of action is that miRNAs target mRNA to P-bodies, increasing their association with the decapping machinery and, thus, reducing their expression levels by decapping and 5'-to-3' degradation (53).

miRNAs have also been found to regulate gene expression by directly affecting translation (29, 39). In fact, based on the observation that *lin-4* miRNA reduced the amounts of lin-14 protein without apparently reducing the levels of *lin-14* mRNA (29), it was initially thought that all actions of miRNA were likely to be directly on translation control. Although the mechanism(s) by which translation is repressed has yet to be defined, suggested models include reduced rates of translation or protein release by ribosome drop-off during elongation or by miRNA recruitment of protease(s) that degrades the protein being generated (45, 67). Other possibilities include accumulation, by miRNA, of mRNA targets in P-bodies, reducing their availability to the cell's translation machinery or direct or indirect functional inhibition of translational initiation factor(s) (53). Interestingly, it has been established that while a single complementary site is generally sufficient to result in down-regulation of gene expression by cleavage, with few exceptions, multiple sites of recognition and binding are necessary for efficient repression of translation (53).

Methods for miRNA Analysis

miRNAs have been identified and analysed using a broad range of techniques, including both computational prediction methods and experimental analysis. Computational-driven analysis (*e.g.,* applying MirScan software) involves candidate miRNA prediction, based on known structural features, followed by experimental analysis to validate the existence of the predicted sequence (68). Conversely, experimental-driven analysis involves the identification of a small RNA sequence, followed by bioinformatics analysis to determine if this sequence fulfils the defined structural characteristics of a miRNA (69-70).

miRNA target analysis has been greatly contributed to by *in silico* computational approaches. Based on the observation that the "seed" nucleotides within the 5' region of miRNAs are of significant functional relevance, bioinformatics has also been developed and applied to predict direct targets of specific miRNAs – generally by searching for seed complementarity in mRNAs 3' UTRs (39, 62, 71-75). Due to the short seed sequence, however, many potential mRNA targets are generally predicted for a given miRNA. Binding studies and functional analysis are essential to determine true mRNA targets.

De novo identification of miRNAs generally involves sequencing of size-fractioned cDNA libraries. To achieve this, small RNAs (approx. 20-28 nucleotides) are isolated from denaturing gels and, following 5' and 3' adapters attachment to the RNAs, RT-PCR is performed. The resulting cDNAs are cloned to form a cDNA library. To establish the genomic origin of the small RNAs, individual clones are sequenced.

In addition to identifying new miRNAs, large-scale cDNA cloning may also be used to evaluate the relative expression levels of miRNAs in a range of specimens. However, the most frequently, and least laborious methods, for global profiling of miRNAs involves the use of microarrays (76-88) or RNA-primed array-based Klenow enzyme (RAKE) assay (89). Bead-based flow cytometry assays have also been developed for miRNA analysis, whereby beads are coupled to probes ($\leq$100 probes) representing individual miRNAs. Following incubation with the specimen of interest, the beads are analysed by flow cytometry for expressed miRNA identification and quantification (90). Methods used for validation of results from global analysis – or for analysis of small numbers of miRNAs – include qPCR, Northern blotting, dot blotting, RNase protection assay, and a modified invader assay (91-92). Functional relevance of miRNAs may be investigated using precursor miRNAs (Pre-miR™ miRNA precusors) or miRNA inhibitors (Anti-miR™ miRNA inhibitors) (see: www.ambion.com). Similarly, antisense technologies have also been used to successfully regulate miRNA *in vitro* and *in vivo* (93-95).

miRNA in Normal and Disease Conditions

Correlations have been identified between miRNA expression and both normal physiological and pathological conditions. These include metabolism, proliferation, cell death, differentiation and development, insulin secretion from pancreatic β cells, spinal muscular atrophy, DiGeorge syndrome, viral infection and cancer. Some examples of these findings are described below.

miRNAs in β cell function. miRNAs have been shown to play a role in regulating insulin secretion from pancreatic beta cells. Following cloning of small RNAs from the glucose-responsive murine pancreatic β cell line, MIN-6, and the murine pancreatic α cell line, TC1, Poy *et al.* (96) identified 67 expressed miRNAs; the most abundant of which was *miR-375*. Antisense targeting *miR-375* was found to induce insulin secretion from MIN-6, while *miR-375* over-expression suppressed glucose-stimulated insulin secretion. Based on sequence information, the target for *miR-375* was predicted – and subsequently confirmed – to be myotrophin. The mechanism of action of *miR-375* is apparently directly on insulin exocytosis and is independent of glucose metabolism and calcium signalling. Using PicTar, a computational method for identifying common targets of miRNA, Krek *et al.* (97) suggest that two other miRNAs, which are highly expressed in MIN-6 cells, *i.e., miR-124* and *let-7b*, may act together with *miR-375* to repress myotrophin expression. *miR-143* has been shown to play a role in adipocyte differentiation (98), while *miR-14* is involved in regulating adipocyte droplet size and triacylglycerol levels in *Drosophila* (99). Results from these studies suggest that miRNAs may be involved, to some extent, in the manifestation of both types 1 and 2 diabetes. Further research is required to determine this possible involvement and examine the potential to therapeutically manipulate miRNAs in diabetes.

miRNAs in viral infection. The identification of miRNAs encoded by herpes DNA viruses, including Epstein-Barr virus and human cytomegalovirus, suggest a possible role for miRNAs in viral infection. Homologues in the host genome have yet to be identified and their function(s) is not yet defined (100-103). Again, further research in this area is necessary in order to define the involvement of miRNAs in infection.

miRNAs in cancer. Studies of miRNAs in fresh/frozen and formalin-fixed, paraffin-embedded primary tissues have indicated that miRNAs are differentially expressed in many cancer types. This is supported by the fact that studies of the genomic locations of genes encoding miRNAs indicate approximately 50% to be in cancer-associated genomic regions or in fragile sites (42). A high proportion of genomic loci containing miRNA genes has been found to display DNA copy number alternations in cancers, including melanoma (86%), breast cancer (73%) and ovarian cancer (37%) (104).

Some examples indicating the association of miRNA expression with cancer include a study of 143 lung cancers, reported by Takamizawa *et al.* (105), in which reduced expression of the miRNA *let-7* was found to be associated with reduced post-operative survival. In a microarray analysis of 104 paired primary lung tumours and matched non-cancerous lung tissues, Yanaihara *et al.* (106) identified a unique profile, confirmed by qPCR, that could distinguish lung cancer from non-cancerous tissue. Expression of *mi-15α* and *miR-16* have been shown to be reduced in chronic lymphocytic leukaemia (CLL) (in fact, the region of 13q14 to which *mi-15α* and *miR-16* are mapped is commonly deleted in CLL) (107). In a study of 28 miRNAs identified in colonic adenocarcinoma and normal mucosa, Michael *et al.* (108) reported human homologues of two murine miRNAs (namely, *miR-143* and *miR-145*) to consistently display reduced levels at the adenomatous and cancer stages of colorectal neoplasia, while Bandres *et al.* (109) have identified a group of 13 miRNAs whose expression is significantly altered in colorectal cancer (compared to non-tumour tissue). miRNA microarray analysis of 76 breast tumour biopsies has also indicated differential expression, particularly of *miR-21*, *miR-125b*, *miR-145* and *miR-155*, between tumour and normal breast tissue, with expression of specific miRNAs associated with oestrogen and progesterone receptor expression, tumour stage, vascular invasion and proliferative index (110). Similarly, profiling of miRNA expression in 363 solid tumours (including lung, breast, prostate, stomach, colon and pancreas cancers) and 177 normal specimens resulted in the identification of an expression signature for solid tumours, again supporting the proposal of miRNAs being oncogenic, *i.e.,* "oncomirs" (111-115). Microarray analysis has also identified a significantly increased expression of *miR-181b*, *miR-221* and *miR-222* in thyroid papillary carcinomas compared to normal thyroid tissue (116). Recent studies, using breast and prostate biopsies, aimed at minimising the quantity of miRNA required for high-throughout expression analysis, have identified miRNAs associated with normal, non-malignant precursor lesions and advanced metastasis (117). Furthermore, in a study of 67 non-small cell lung cancer (NSCLC), Karube *et al.* (118) found reduced levels of the endonuclease enzyme *Dicer* to be associated with poor prognosis in lung cancer.

It is likely that dys-regulation of miRNA expression and function will be found to be associated with many other pathological conditions in the future.

Potential miRNA Therapeutics

As miRNAs do not encode proteins, they have not – until recently – been considered as classical therapeutic targets. Initial studies, aimed at exploiting miRNAs as a form of therapy, have resulted in promising results. Following intravenous injection of modified antisense (termed antagomirs) into mice, Krutzfeldt *et al.* (93) have recently demonstrated *in vivo* inhibition of four miRNAs – namely: *miR-16*, *miR-122*, *miR-192* and *miR-194*. This approach resulted, not only in blockage of target miRNAs, but also in their degradation in almost all organs studied including liver, kidney, heart, lung, intestine, bone marrow, muscle, skin, fat, ovaries and adrenals, while no effect was observed in brain, possibly due to restricted diffusion of charged nucleic acids across the blood brain barrier. Future studies targeting miRNAs in pathological conditions, such as diabetes and cancer, may not only increase our understanding of such complex diseases but will, hopefully, aid in the identification of novel therapeutic targets.

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