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Gene expression regulators —MicroRNAs

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Abstract **A large class of non-coding RNAs found in small molecule RNAs are closely associated with the regulation of gene expression, which are called microRNA (miRNA). MiRNAs are coded in intergenic or intronic regions and can be formed into foldback hairpin RNAs. These transcripts are cleaved by Dicer, generating mature miRNAs that can silence their target genes in different modes of action. Now, research on small molecule RNAs has gotten breakthrough advance in biology. To discover miRNA genes and their target genes has become hot topics in RNA research. This review attempts to look back the history of miRNA discovery, to introduce the methods of screening miRNAs, to localize miRNA loci in genome, to seek miRNA target genes and the biological function, and to discuss the working mechanisms of miRNAs. Finally, we will discuss the potential important roles of miRNAs in modulating the genesis, development, growth, and differentiation of organisms. Thus, it can be predicted that a complete understanding of miRNA functions will bring us some new concepts, approaches and strategies for the study of living beings.**

Keywords: miRNA, gene regulation, RNAi, bioinformatics.

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MicroRNAs (miRNAs) are a large class of ~22 nt small RNAs (sRNA) discovered recently in varieties of species from plants to netamode to human beings[1]. The first discovered miRNA is *lin-4* and its target gene *lin-14*[2]. Lee et al.[2] cloned *lin-4* gene in *C. elegans* by genetic screening and position cloning. Clear-cut evidence suggests that the *lin-4* gene does not code for a protein but generates a small RNA about ~22 nt in length. By imperfect complementarity to the 3′ UTR of *lin-14*, *lin-4* blocks the production of protein but does not alter the mRNA levels of *lin-14*. This phenomenon is dubbed translation repression. It has been shown that the biological effects of *lin-4* are associated with the transformation of worms from L1 to L2 larva. How does a single small RNA containing ~22nt make such a great contribution to the development of nematodes? No body could give a reasonable explanation at that time. It was considered as only a very rare phenomenon in nature. However, it became hot topics in the biological field when the second miRNA, *let-7*[3], and the orthologues were discovered in human and flies, suggesting that miRNAs are a large and conservative family of noncoding RNAs that play important regulatory roles in lives. Now, the endogenous RNA interfering (RNAi) mediated by miRNAs[4,5] in different manners such as translation repression, mRNA cleavage or other modes is uncovered gradually and extensively. miRNAs and their targets seem to constitute high complex regulatory networks since a single miRNA derived from intergenic region or intronic segment can bind to and regulate many different mRNA targets, and meanwhile several different miRNAs can also bind to and cooperatively control a single mRNA target. Many research workers are interested in the biological functions of miRNAs in modulating expression of genes encoding proteins. Thus the biogenesis, sequence context, regulatory networks and target molecules of miRNAs are widely investigated. The discovery of miRNAs expands the knowledge of translational fine-tuning of protein synthesis, rapid and efficient regulation of mRNA, and interplay network among genes in the RNA levels[6]. The function identification of miRNAs enriches and enforces the roles of RNAs in the central dogma of genetics. All these promote people to ponder again upon the problems of genetic and developmental regulation of the cells in new ways.

1 miRNA biogenesis

1.1 miRNA transcription and maturation

miRNAs are coded in genomes and formed into hairpin RNAs (Fig. 1). Primary transcripts of the miRNA genes, dubbed pri-miRNAs, with a 5′,7-methyl guanosine cap and a 3′ polyadenylated tail are generated by polymerase II[7]. The best representative of human pri-miRNAs is miR-23a~27a~24-2, a 2.2 kb transcript containing three miRNAs. Pri-miR-23a~27a~24-2 is ~1.8 kb in size including a 5′ capped and polyadenylated sequence from downstream of the 3′ end of miR-24-2. A minimal (~600 bp) polymerase II-dependent promoter has been identified at this miRNA gene[7]. Although the promoter containing ~600 bp (minimal) is able to direct polymerase II-dependent transcription of miRNAs, it still does not share the same typical promoter elements required for transcription initiation as that of the other genes does. On the other hand, a subset of miRNA genes are located within introns of pre-mRNA, called host gene, which make people come to infer that they are transcribed at the same time as the host genes by polymerase II. Rodriguez et al.[8] explored the transcriptional mode of miRNA throughout the genome, and found that all the miRNAs located in introns were transcriptionally consistent with 90 host genes. The biogenesis of miRNAs from introns suggests that miRNAs seem to act as a vehicle between their host genes and target genes. However, other transcriptional modes of miRNAs located within the flanking non-coding sequence are still unknown. Do all the

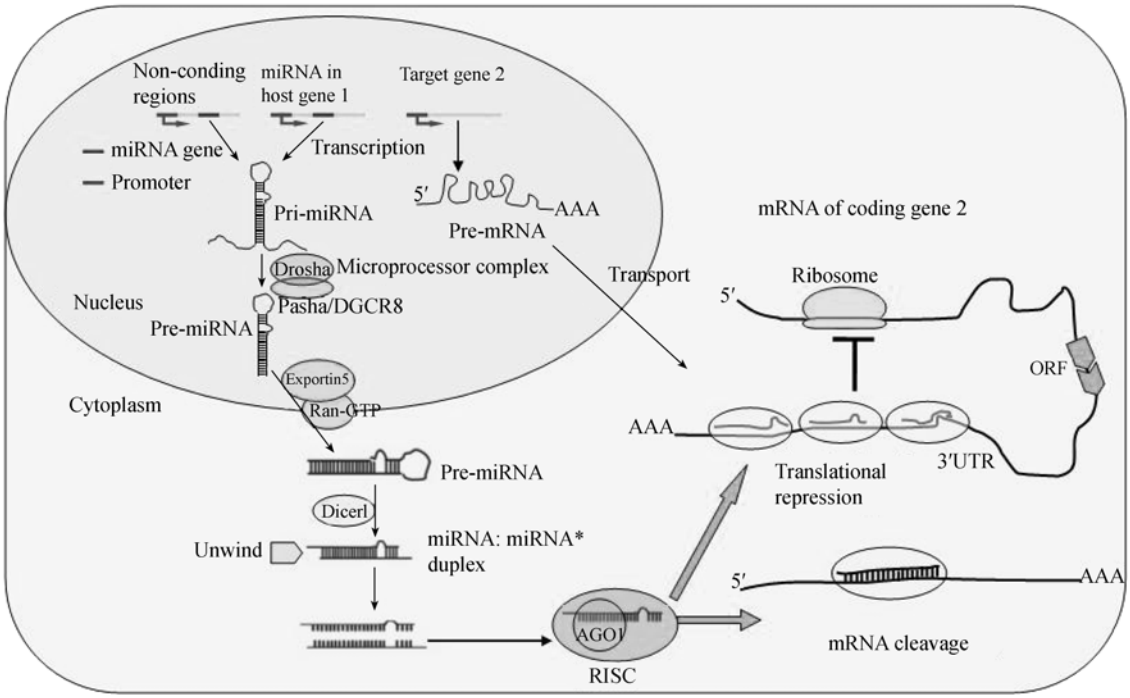

Fig. 1. Mechanisms of miRNA biogenesis and function.

miRNAs share the same polymerase? Do they possess the same promoter? Does the promoter contain general motifs? All these remain to be extensively and profoundly researched.

The processing of pri-miRNAs in the nucleus is shown to be mediated by Drosha, an RNase III endonuclease[9]. Drosha asymmetrically cleaves both strands of hairpin stem at sites near the base of the primary stem loop. Thus, miRNA is released as a 60- to 70-nucleotide pre-miRNA that has a 5′ phosphate and a 2-nucleotide 3′ overhang. Specific RNA cleavage by Drosha predetermines the mature miRNA sequence and provides the substrate for subsequent processing events. The pre-miRNAs are transported to the cytoplasm by Exportin-5 and meanwhile a GTP-dependent protein Ran is required[10−13]. The recognition of pre-miRNA by Expotin-5 is dependent on the correct cleavage by Drosha and the special hallmark of the "hairpin" structure of the pre-miRNA[10,11]. In the cytoplasm, another RNase III endonuclease, Dicer, comes to consummate the pre-miRNA. It is thought that the PAZ domain of Dicer recognizes the 2-nucleotide 3′ overhanging at the base of the stem loop of pre-miRNA. Dicer cleaves both strands of the duplex[14]. The resulting miRNA:miRNA* duplex containing 5′ phosphates and a 2-nucleotide 3′ overhangs are generated. Usually, the mature miRNAs asymmetrically come from one arm of the stem of pre-miRNA. The mature miRNA is subsequently incorporated into an effector complex termed RISC (RNA-induced silencing complex), which directly mediates mRNA cleavage or translational inhibition[15−17].

1.2 Primary miRNAs processed by the microprocessor complex

After transcription, the primary transcript of miRNA comes through several processing steps. For example, the polyadenylated transcript of *let-7* gene undergoes trans-splicing to the spliced leader 1 (SL1) RNA[18] followed by the well-known cleavage by Drosha. As discussed, Drosha plays an essential role in the genesis of miRNAs. Recently, four groups independently investigated the potential involvement of related proteins in pri-miRNA processing[19−22]. Gregory et al.[20] purified Drosha from human cells and fractionated two complexes: a larger one and a smaller one (~600 kD). In the latter, a new protein DGCR8 was found to be involved in the pri-miRNA processing. Another group[19] assayed the processing complex in *Drosophila* and found an orthologue of DGCR8, named Pasha (partner of Drosha). Both the DGCR8 and Pasha belong to the family of RNA-binding protein, and the processing complex containing Pasha /DGCR8 and Drosha was named Microprecessor complex[19−21]. Subsequently, the third group[21] also provided very strong support for the existence of Microprocessor, a Droshacontaining complex of around ~600 kD that is conserved from flies to human. The DGCR8 protein contains two double-stranded RNA binding domains. The role of DGCR8 in the Microprocessor may be to recognize pri-miRNA and orient the catalytic RNase III core of Drosha for correct cleavage site selection at the stem of the hairpin RNA structure. Interestingly, the

DGCR8 gene with monoallelic deletion accounts for >90% of patients with Digeorge syndrome[22]. Given the developmental functions of miRNA, the DGCR8 connects the miRNA biogenesis with DiGeorge syndrome, suggesting that the DiGeorage syndrome patients are due to the miRNA perturbation caused by the DGCR8 gene deletion.

1.3 Dicer and RISC

Dicer was initially identified in *Drosophila* and subsequently found in diverse eukaryotic organisms including worms, human and plants. In *Drosophila*, two Dicer paralogues were discovered, Dicer1 and Dicer2[23,24]. Dicer2 is responsible for siRNA production, whereas Dicer1 is involved in the maturation of miRNA. Dicer1 is the only homologue in human beings that is involved in both siRNAs and miRNAs production[25]. In plant, the homologues of Dicer are termed DCL (Dicer-like protein), which includes three paralogues, DCL-1, DCL-2 and DCL-3. DCL-1 is the enzyme that produces miRNAs and interacts with HYL1 and HEN1 to initiate the miRNA pathway.

RISC (RNA-induced silencing complex) is the effector complex involved in the working mechanism of miRNAs. An important feature of RISCs is that AGO proteins, a large gene family in diverse organisms, are associated with RISCs, and determine the mRNA cleavage or translation repression[24]. In *Drosophila*, different from AGO2, AGO1 interacts with Dicer1 and is involved in miRNA pathway. In contrast to *Drosophila*, human beings contain only one Dicer1 but contain several AGO proteins. Components of AGO1 and AGO3 form non-cleavage RISC and interact with Dicer1 in the miRNA pathway. But in the cases of some miRNAs playing the role of mRNA cleavage, such as miR-196 and HoxB8[26,27], the function of AGO protein recruited in RISC remains unclear. It is worth pointing out that the type of AGO protein rescuited in RISC may determine the modes of mRNA cleavage or translation repression.

2 Search for miRNA genes

2.1 Discovery of miRNAs by cloning

In 2001, three groups simultaneously published their works in *Science* reporting over 100 miRNA in human beings, *Droshophila*, and *C. elegans*[28−30], which served as the prelude to the search for small regular RNAs and miRNAs. Lagos-Quintana et al.[28] established a method for cDNA cloning of small RNA with about 22 nucleotides (shown in panel A of Fig. 2), and by it they cloned 37 novel miRNA genes in the lysate of *Droshophila* embryo and HeLa cell line. Using similar approach, Lau et al.[29] found 55 new miRNAs in *C. elegans*. With bioinformatics and cDNA cloning, Lee and Ambros[30] found 15 new miRNA genes in *C. elegans* (shown in panel B of

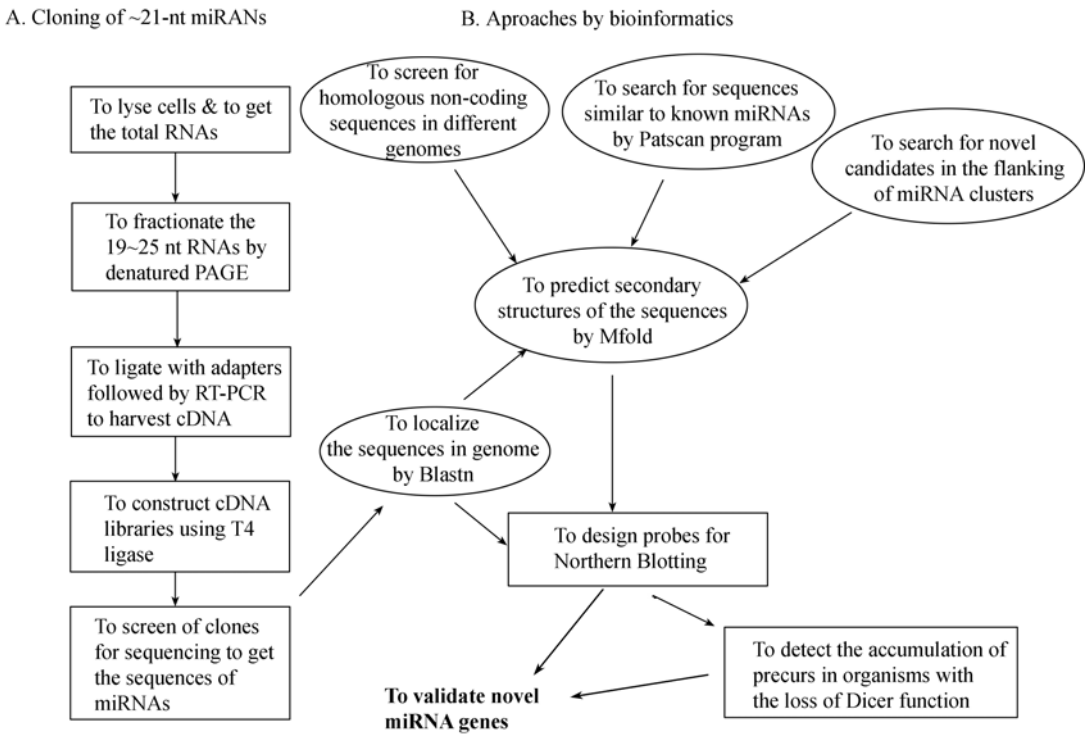

Fig. 2. Approaches to finding and identifying miRNA genes by combining cDNA clone technology with bioinformatics (Panel A, Roadmap for cDNA clone; Panel B, bioinformatics).

Fig. 2). After their initiatory work, Lagos- Quintana et al. have endeavored to search for the miRNA genes, and in 2002[31] and 2003[32] they respectively reported 34 and 31 novel miRNAs, which were identified by cloning in mouse tissues and the human Saos-2 cell line. They reported that no more miRNAs could be identified by cloning because of the recurring identification of already known miRNAs and the unavoidable background of ribosomal RNA breakdown products[32], which was consistent with the point of view that there remain ~40 unknown miRNAs in human beings, as predicted by Lim et al.[33,34] with the programme MiRscan. Recently, the cloning method has become more and more prevalent. Ambros and Lee[35] reviewed and summarized the methods of small RNA cloning.

However, a comprehensive collection of miRNAs is important for identification of novel miRNAs in special tissues and special phases of development[36−41]. Mourelatos et al.[37] identified 31 miRNAs in the HeLa cells by small RNA cloning, and Houbavity et al.[38] found 15 miRNAs in embryo stem cells (ES cells) of mouse. Dostie et al.[39] and Kim et al.[40] revealed 53 and 40 miRNAs in the neural cells in mammals, respectively. Ambros et al.[42] found another 21 new miRNAs in *C. elegans*. 63 miRNAs were identifed in different developmental time in *Drosophila*[34]. In China, Wang et al.[43] found 20 miRNAs in *O. sativa*. 5 miRNAs in EB virus genome made up the family of miRNA in virus[44]. After that, other 29 miRNAs were found in other three viruses such as the Kaposi sarcoma-associated virus (KSHV or HHV8), the mouse gammaherpesvirus 68 (MHV68) and human cytomegalovirus (HCMV or HHV5) by the same group[45]. Suh et al.[46] found 36 miRNAs in human EC cells, some of which are negatively expressed with the embryo development, indicating certain developmentally regulatory function of these miRNAs. By using a computer-assisted approach, Seitz et al.[47] identified 46 potential miRNA genes in the human imprinted 14q32 region, among which 40 are organized as a large cluster. In addition, they found that the miRNA genes were only expressed from the maternally inherited chromosome and that their imprinted expression was regulated by an intergenic germline-derived differentially methylated region (IG-DMR) approximately 200 kb upstream from the miRNA cluster. By cDNA cloning, Kasashima et al.[48] investigated miRNAs using TPA (12-O-tetradecanoylphorbol-13-acetate)-induced differentiation of human leukemia cells (HL-60) and found 3 novel miRNAs. Until this review publication, a total of 1650 miRNAs have been found in a variety of species.

2.2 Methods to detect the expression of miRNAs

Northern blotting is a potential and direct method for detecting the expression of miRNA in cells. All the miRNAs acquired by small RNA cloning or predicted by bioinformatics have been detected and identified by northern blotting[28,30]. In addition to the Northern blotting, more and more methods such as RT-PCR, liquid hybridization and microarrays have been established to identify miRNAs. In order to investigate the transcriptional mode of miRNAs, Lee et al.[7] knockdowned the enzyme processing pri-miRNA, Drosha, by introducing Drosha-siRNA to the cells. They discovered the accumulation of pri-miRNAs by RT-PCR. Using northern blot assay, Sempere et al.[49] characterized the expression of 119 known miRNAs in adult organs from mouse and human, and pointed out that some of the miRNAs specifically express or get greatly enriched in a particular organ. They suggested that miRNAs might have organ- or tissue-specific functions. A subset of brain-expressed miRNAs, whose expression behavior is conserved in both mouse and human differentiating neurons, has been identified, implying that these miRNAs may play certain roles in mammalian neuronal development or growth. By introducing fluorescence detection in microtiter plates into the research of miRNAs, Allawi et al.[50] developed a quantitative, sensitive, and rapid miRNA assay, called the Modified RNA Invader Assay. Using it, several miRNA were successfully detected using as little as 50—100 ng of total cellular RNA or as few as 1000 lysed cells.

Methodology based on the development of microchips is more rapid, comprehensive, and promising for the study of miRNA expression. Liu et al.[51] first reported that microarrays could be used to obtain highly reproducible results. These results revealed tissue-specific miRNA expression signatures, which had been confirmed by Northern blotting and real-time RT-PCR. Afterwards, 254 miRNAs in differentiating adipocytes were also examined on miRNA microarrays[52] and meanwhile the miR-143 and its target gene in the regulation of adipocyte differentiation were identified. Using microarrays, Calin et al.[53] identified significant differences in miRNA expression between CLL and normal CD5+ B cells, indicating a novel way to target micro-RNAs (miRNAs) for clinical therapy of cancer. Moreover, Miska et al.[54] detected 138 mammalian miRNAs with the same sequences of the miRNAs cloned from developing mouse brain, suggesting that miRNAs play important roles in the development of the mammalian brain.

In addition to the microarray analysis to assess the miRNA expression profiling rapidly, another method termed the RNA-primed, array-based Klenow enzyme (RAKE) assay, was developed for high-throughput miRNA detection by Nelson et.al.[55]. *In situ* hybridization technologies[56,57] have been used in plant to exhibit more direct and distinct miRNA expression profilings. Moreover, a GFP report protein gene was fused with the downstream of the promoter of *let-7* to show the transcriptional regulation of *let-7*[58]. An enhancer element, the temporal regulatory element (TRE), localized upstream of the start

of the mature *let-7* RNA, was found to bind to a TRE binding factor (TREB) for regulating the transcription of *let-7*. In addition, another group[26] developed an interesting technique that took *Laz* as the report gene to visualize detailed miRNA expression termed "miRNA sensor".

2.3 Bioinformatics to predict of miRNA genes

The discovery of *let-7* in *C. elegans* and its orthologues in other species including *Droshophila*, mouse and human makes computational approaches viable and promising for prediction of novel miRNAs. Using bioinformatics methods and cDNA cloning to find out novel miRNAs, Lee and Ambros[30] did precursory work in bioinformatics of miRNAs. The features of miRNA such as ~70 nt pre-miRNA with hairpin structure, conservation in different species, are considered as the rules for development of computational approaches to predicting miRNA genes.

MiRscan[33,34] used for identifying potential homologous stem-loop structures through scanning genomes of two species is a program based on the secondary structure prediction program (RNAfold). Over 35000 stem-loop sequences conserved between *C. elegans* and *C. briggsae* were predicted by this method. With 50 known miRNA genes used as a training set[34], 30 additional miRNAs were found. Additionally, MiRscan was used to estimate the upper and lower limit of miRNAs in *C. elegans*. Another work[33] was performed to screen miRNAs in human, mouse and pufferfish genomes. The upper limit of ~255 human miRNA genes and the lower one between 180 to 200 miRNA genes were estimated using MiRscan[33]. Recently, MiRscan was improved and used to align sequences upstream and downstream of orthologous nematode miRNA foldbacks[59]. These alignments showed a pronounced peak in sequence conservation about 200 bp upstream of the miRNA foldback, and revealed a highly significant sequence motif with consensus CTCCGCCC that is present upstream of almost all independently transcribed nematode miRNA genes. MiRscan also assessed Orthology of host genes for intronic miRNA candidates. 9 new miRNAs were found by MiRscan[59], and were validated by PCR detection.

MiRseeker[60] is another program examining the stem-loop structure of RNA sequences for miRNA screen based on the following three criteria: the 70—100 nt stem-loop secondary structure of pre-miRNA, the conservation of pre-miRNA in different species genomes, and the nucleotide divergence of miRNA candidates. The process consisted of three main steps: (i) to scan the conserved sequences in two *Droshophila* genomes by AVID programme; (ii) to fold the sequences by Mfold for assessing the stability of stem-loops; (iii) to estimate nucleotide divergence in different genomes. After all, 48 conserved novel miRNAs were found in *Drosophila*, and around 110 miRNA genes were estimated as the drosophila upper bound.

"ERPIN"[61] is an alignment programme similar to "Blast", constructed to search sequences similar to miRNA on animal genomes. Very recently, a particular phylogenetic approach, termed phylogenetic shadowing, has been used to identify potential novel human miRNAs[62]. The method is based on the sequence comparison of closely related species and is able to accurately identify conserved regions at the nucleotide level. The authors compared the sequences of 122 miRNA regions in 10 different primates and revealed a strong conservation in stems of miRNA hairpins, an increasing variation in loop sequences and a striking dropping conservation in flanking sequences. 976 potential human RNAs were identified. Northern blotting analyses combined with database searches have reached a conservative estimate of 200—300 novel human miRNAs[62].

The RNA folding programme maybe play greater and more comprehensive roles in miRNA discovery than other special soft in miRNA screen. Mfold is the most popular programme for predicting RNA secondary structure, which is integrated in the MiRseeker[60] for miRNA identification. Srnaloop is another RNA secondary structure prediction programme used in candidate miRNAs folding analysis. A platform[63] is provided on Vienna RNA secondary structure server (http://rna.tbi.univie.ac.at/) for RNA folding.

2.4 Annotation of miRNAs and miRNA database

As more and more miRNAs were identified in a variety of species and complex genotypes of miRNAs, a profound nomenclature is required to prvent confusion. The precursory researchers have given a set of rules to name the mina genes[26]:

(i) The miRNAs are abbreviated to miR-No., and the genes encoding miRNA are named *mir-No.* (such as miR-1 and *mir-1*).

(ii) Highly homologous miRNAs are named by the same No, but followed by a lower case letter (usual from a, just like miR-26a and miR-26b).

(iii) Multiple genomic copies of miRNAs are annotated by adding a dash and a number (such as miR-6-1 and miR-6-2).

Besides these, some additional rules are suggested for concrete details[64,65]. The orthologous miRNAs in different species are assigned the same name. In the case of some miRNA hairpin precursors producing one miRNA from each arm, an asterisk has been used to denote the less predominatnt form, that is, miR-56* and miR-56 although previous reports have also denoted these kinds of miRNAs by miR-142-s (5′ arm) and miR-142-as (3′ arm). It is desirable for current reports to name miRNAs as miR-142-5p and miR-142-3p for miRNAs cleaved from the 5′ and 3′ arms, respectively. In the database of Rfam, each miRNA is assigned the abbreviation of organism in front of the miRNA name, such as has-miR-138. However, the miRNAs in plants are named MIR156 and the miRNA genes are named *MIR156*.

A miRNA registry system has been constructed in the Rfam database (http://www.sanger.ac.uk/Software/Rfam/mirna/). Two aims of the registry are achieved: the first is to provide service for the assignment of miRNA gene names for publication; the second is to provide an interface for search and download by researchers. Until this review publication, the database has been updated to release 6.0 (April 2005), which contains 1650 entries (Table 1).

Table 1 Summary of miRNAs (Data from Rfam)[65]

Species	Number	Species	Number
D. melanogaster	78	*X. laevis*	7
D. pseudoobs	73	*D. rerio*	33
A. gambiae	38	*A. thaliana*	114
A. mellifera	25	*O. sativa*	173
C. elegans	116	*S. bicolor*	64
C. briggsae	79	*Z. mays*	40
G. gallus	122	*Epstein Barr* virus	5
H. sapiens	227	Kaposi sarcoma-associated herpesvirus	11
M. musculus	230	Human cytomegalovirus	9
R. norvegicus	191	Mouse gammaherpesvirus 68	9
C. familiaris	6		

3 Identification of miRNA target genes

Though numerous miRNAs are found in many species and are pointed out to play great roles in cell proliferation, differentiation, metabolism and cell death, so far, the mRNA targets of only a few specific miRNAs were discovered[1,66,67]. From the first miRNA and its target gene, *lin-4* and *lin-14*, found in *C. elegans* in 1993 to the publication of this review, miRNAs with experimentally validated functions and targets are no more than a dozen in animals (Table 2).

3.1 To search miRNA target genes by forward and reverse genetic analysis

Previously, the discovery of miRNA functions was dependent on the forward genetics[2,3], including production of mutants, screen of phenotype, positional cloning and identification of miRNA and its target genes, just like the discovery of *lin-4* and *let-7* and their target genes.

In *Drosophila*, the finding of miRNA *bantam*[68,69] and its target gene *hid* stood for the classical procedure for identifying an miRNA gene and its target gene by forward genetics (Fig. 3). The procedure contains two main steps. Firstly, one should get the evidence that gene *bantam* screened genetically encode an miRNA including following points: (i) The *bantam* locus is identified by EP element insertions that are transposable elements designed to allow inducible expression of sequences flanking the insertion site under control of the yeast transcription factor Gal4. And the expression of the EP elements inserted at the *bantam* locus causes an overgrowth. (ii) The mutants with the *bantam* deletion grow poorly and die as early pupae. (iii) Alignment of the sequence near the locus in two genomes (*Drosophila* and *Anopheles*) by ClustalW identifies a block of ~ 90 nt residues with considerable similarity and 30/31 residues. With Mfold, the sequences are predicted to fold into stable hairpin secondary structures. (iv) By Northern blotting analysis, a small RNA of ~21 nt and its larger precursor are detected in the total RNA from third instar larvae using an end-labeled probe complementary to the conserved 31 nt sequence. Secondly, one should find the target gene, *hid*, of *bantam*. The procedure is as follows: (i) by bioinformatics analysis, five target sites are located in the 3′ UTR of *hid*; (ii) target sites share high conservation either in these five or in inter-organisms (*D. pseudoobscura* and *D. melanogaster*); (iii) a report gene is constructed with a 3′ UTR of *hib* inserted into the tubulin-EGFP, and the result shows that GFP is downregulated by *bantam*; (iv) *hid* protein is reduced by coexpression with *bantam* endogenously but *hid* transcript levels are comparable, suggesting that *bantam* plays certain roles in translation repression just like other miRNAs. Taken together, *hid* is identified as a target of *bantam*, thus explaining the fact that overexpression of *bantam* can induce tissue overgrowth, and *bantam* may play the role of blocking *hid*-induced apoptosis.

Table 2 miRNAs and their target genes with known functions

miRNA	Species	Target genes	Function	Refs.
lin-4	*C. elegans*	*lin-14* & *lin-28*	developmental timing	[2]
let-7	*C. elegans*	*lin-41* & *hbl-1*	developmental timing	[3]
lsy-6	*C. elegans*	*Cog-1* transcriptional factor	neuronal patterning	[71]
miR-273	*C. elegans*	*die-1* transcriptional factor	neuronal patterning	[72]
bantam	*Droshophila*	*Hid*	cell death, proliferation	[68], [69]
miR-14	*Droshophila*	unknown	cell death, fat storage	[70]
miR-181	mammals	unknown	haematopoiesis	[73]
miR-196	mammals	*HOXB8*	development of vertebrate	[26], [27]
miR-143	mammals	*ERK5*	differentiation of Adipocyte	[51]
miR-375	mammals	*Mtpn*	regulation of insulin secretion	[74]
miR-430	zebrafish	unknown	regulation of brain Morphogenesis	[75]

Similar genetic screens have identified a locus affecting cell death and fat storage in the fly, which, when cloned, is found to correspond to the *mir-14* gene[70]. Though the targets for miR-14 in the control of apoptosis have not been identified, some genes involved in cell-death effectors may be predicted to be the prime targets. In worms, an exciting work was performed by Johnston and Hobert[71] and Chang et al.[72], in which an miRNA, *lsy-6* was cloned by screens for mutants with ASEL cells or ASER cells. The target gene of *lsy-6* is *cog-1*, an *Nkx*-type homeobox gene. Through repression of *cog-1*, *lsy-6* controls the neuronal left/right asymmetry of chemosen- sory receptor expression.

In the discovery of *lsy-6*, Northern blotting failed to detect some miRNAs because of *lsy-6* with very low expression level. This is a challenge. However, an alternative method was developed to predict miRNA target genes and test the function of special miRNAs. An miRNA, miR-273[72], was identified by methods of reverse genetics to be involved in some pathway regulated by *lsy-6*. For the rare expression in the worm, *mir-273* was only predicted computationally and validated by RT-PCT rather than Northern blotting. *die-1* , a zenc-finger transcription factor upstream *cog-1*, was predicted and validated as the target of miR-273, and was regulated by translation repression. Results showed that *die-1* might result in bias of expression of chemoreceptors in left/right asymmetric development of neuronal cells.

To test diverse miRNA candidates, miRNAs are often discovered by reverse genetics, including miRNA cloning or computational prediction. miR-181[73] is the classical miRNA identified by cDNA cloning. The role of miR-181 in haematopoiesis development is indicated by its substantial enrichment in mouse bone marrow and thymus. Though the target of miR-181 remains unknown, the function of miR-181 in directional differentiation of B cells is revealed by the evidence that miR-181 is preferentially expressed in the B-lymphoid cells of mouse bone marrow and its ectopic expression in hematopoietic stem/progenitor cells leads to an increasing fraction of B-lineage cells in tissue-culture differentiation assays. Very recently, another exciting advance comes from the work[74] that a pancreatic islet-specific miRNA, miR-375, is found to regulate the insulin secretion. The target gene of miR-375 is identified to be *Mtpn* that is validated from 64 putative target genes involved in insulin secretion.

Giraldez et al.[75] found an miRNA, miR-430, in zebrafish involved in regulation of brain morphogenesis using the methods of injuction of special miRNA into maternal-zygotic Dicer mutants. Functional research of Dicer is focused on RNAi field, but the loss of function of Dicer can be used as potential methods to investigate the roles of miRNAs. Obviously, target gene validation and special

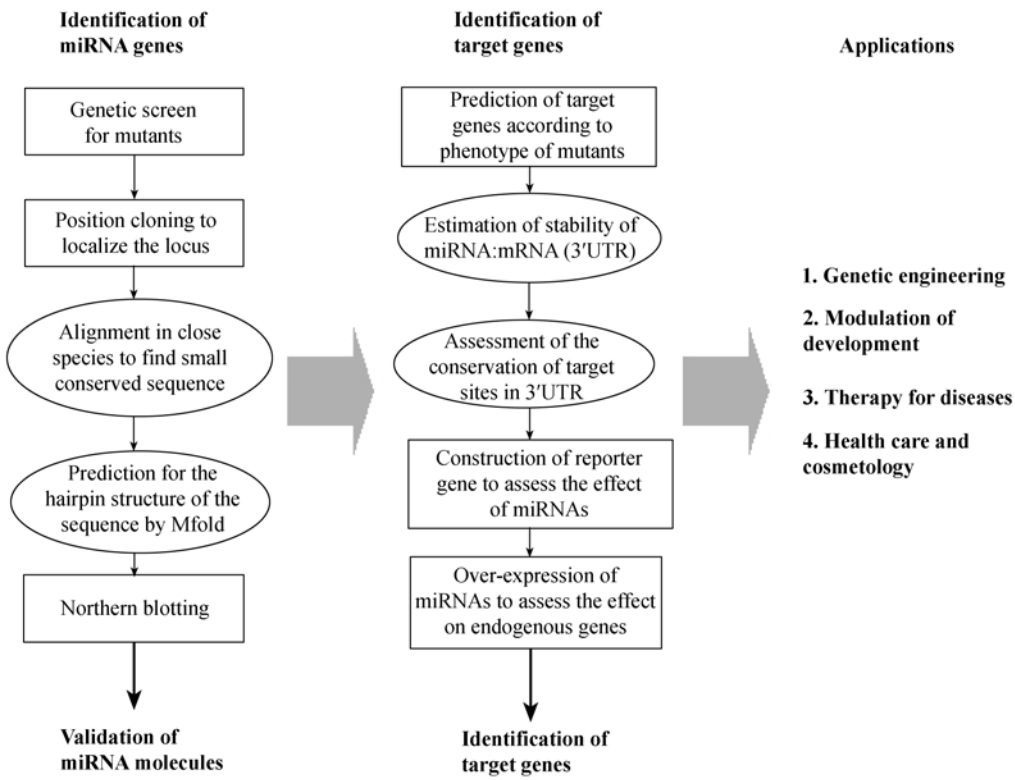

Fig. 3. Approaches to exploring the functions of miRNA (Square frames, molecular biological methods; elliptic frames, bioinformatical methods).

function investigation of miRNAs come to light and become direct aims (Fig. 3).

3.2 Soft to predict the target genes of miRNAs

Reverse genetic methods combined with bioinformatics, which is mainly composed of soft development to predict target genes of miRNAs providing guideline for molecular experiments, are greatly superior to forward genetic methods. So far, different computational approaches, which are mainly based on the nature of the pairing between miRNA and its target gene, have been developed and used to predict the target genes of miRNAs. The comparison of relation between known miRNAs and their target genes, such as *lin-4*, *let-7* and *bantam* etc., has given great knowledge for program development.

Stark et al.[76] first constructed an miRNA-Target predicting program to predict potential target genes of miRNAs in *Drosophila*. 3′ UTR databases are used and the conserved sequences in the 3′ UTR between two organism *D. melanogaster* and *D. pseadoobscura* can be screened. The alignment tool HMMer is used to search for sequences complementary to the first eight residues of the miRNAs, allowing for G:U mismatches. Mfold is used to estimate the free energy of folding (ΔG) suggesting energetically favorable RNA-RNA duplexes. Then a Z score can be calculated to evaluate the possibility of the 3′ UTR region as a target site. The approach has successfully identified five previously validated target genes and some new target genes of three miRNAs including *Notch* target genes for miR-7, proapoptotic genes for the miR-2 family, and enzymes from a metabolic pathway for miR-277 (Table 3). The soft and other results are to be found on http://www.rus- sell.embl.de/miRNAs.

MiRanda is an algorithm in the target gene prediction procedure constructed by Enright et al. to predict the target genes in *Drosophila*[77,78]. The upstream of MiRanda is to construct a 3′ UTR database by alignment using Blastn and AVID. The MiRanda algorithm is similar to the Smith-Waterman algorithm except for its scoring of complementarity of RNA-RNA duplexes (allowing G:U pairwise). Additionally, an RNA secondary structure folding program from the Vienna 1.3 RNA secondary structure programming library (RNAlib) is used. Three aspects should be considered: the local complement, the RNA-RNA duplex energy tendency and the conservation of target sites. The prediction results indicate that the potential target genes are rich in transcription factors. Recent analysis by MiRanda suggests that miRNA genes, which are about 1% of all human genes, work on regulating protein production for 10% or more of all human genes[78]. The details are shown on http://www.microrna. org.

TargetScan[79,80] is a widely used program for predicting miRNA targets in mammals. Two points should be considered in the program. The first is the energy stability of miRNA::mRNA duplexes. The second is the conservation of the target sites in different organisms. In its latest version, certain criteria[80] have been simplified, such as the omission of both thermodynamic stability of pairings and multiple target sites per UTR. In the original version, the concept of "seed matches" is presented that the 2—8 nucleotide of miRNA (from 5′ end) must be perfectly complementary to target sites, while in updated version of TargetScanS, the seed matches are relaxed to six-nucleotide pairs. ~451 regulatory target genes for the conserved vertebrate miRNAs by identifying mRNAs with conserved pairing to the 5′ region of the miRNA were predicted. Recently, a study on the overrepresentation

Table 3 miRNAs and possible target genes

miRNA	Species	Target gene	Function	Reference
miR-7	*Droshophila*	*HLHm3* and *hairy* basic HLH transcriptional repressor *m4* Brd family protein	Notch-mediated decisions in neuronal development	
miR-14	*Droshophila*	*grim*, *reaper*, *sickle*	promotes apoptosis	[76]
miR-1	human being	*BDNF*	neuronal development	[79]
		G6PD	Oxidative stress resistance	
miR-19a	human being	*PTEN*	tumor suppression	[79]
miR-23a	human being	*SDF-1*	growth and localization of	[79]
		BRN-3b POU-domain transcription factor	hematopoietic progenitor cells neuronal development	[79]
miR-26a	human being	*SMAD-1*	TGF-β pathway regulation	[79]
miR-34	human being	*Delta-1* *Notch1* transmembrane receptor for Delta	activation of Notch cell differentiation	[79]
miR-101	human being	*ENX-1* polycomb gene	proliferation of hemotpceitic cells	[79]
		N-MYC basic HLH transcription factor	regulation of oncogene cell differentiation and proliferation	[79]
miR-130	human being	*MCSF*	regulation of mononuclear phagocytic lineage	[79]

of conserved adenosines flanking the seed complementary sites indicated that the primary sequence determinants could supplement base pairing to specifiy miRNA target recognition. Thus, more than 5300 human genes have been predicted to be targets, amounting to 30% of human gene sets. The soft can be downloaded on http://genes.mit.edu/targetscan.

DIANA-microT is another program to predict human miRNA targets by Kiriakidou et al.[81]. The results obtained by DIANA-microT are shown on http://diana.pcbi.upenn.edu/DIANA-microT. RNA-hybrid developed by Rehmsmeier et al.[82] can predict the target genes of *Drosophila*, with details shown on http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/. Pic Tar is a prediction soft developed by Krek et al.[83]. This software has an excellent success rate in predicting targets for single miRNAs and for combinations of miRNAs.

In summary, the criteria for developing these computational pipelines are shown as follows: (i) the conservation of miRNA and its target sites, (ii) the complementarity of 3′ UTRs of the potential targets and miRNAs with emphasis on the critical pairing at the 5′ end of the miRNA; (iii) the kinetics and thermodynamics of the association between the miRNA and its target, as determined by RNA folding programs.

4 Mechanism and Function

4.1 Mechanism

It is well-known that the primary miRNA transcripts (pri-miRNA) are cleaved by the RNase III endonuclease Drosha to release ~70-nt stem-loop precursors (pre-miRNA). These pre-miRNA are actively transported from the nucleus to the cytoplasm with the help of Exportin-5, and subsequently processed into ~22-nt miRNAs duplexes by the cytoplasmic RNase III Dicer. The single strand miRNA enters into an miRNA-containing ribonucleoprotein complex (miRNP) that is identical to RISC in David's review article[1]. miRNAs direct miRNP complexes to target for cleavage or translational repression by pairing with the 3′ UTR of its target mRNA[15−17]. Cleavage or repression depends on the extent of sequence complementarity between miRNA and its target mRNA and the sequence context of miRNA. In plants, miRNAs with full or near complementarity to the 3′ UTR of target mRNAs direct the cleavage of target mRNAs at precise positions. However, in animals, miRNAs, partially complementary to the 3′ UTR of target mRNAs, act as repressors of translation.

In the *C. elegans*, *lin-4* downregulates the protein levels of *lin-14* and *lin-28*[2]. Similarly, *let-7* suppresses the expression of *lin-41* mRNA without cleavage by pairing with its 3′ UTR[3]. Now it is generally considered that miRNA directs miRNP to target mRNA for translation repression by partial complementarity with its 3′ UTR. This perfectly coincides with the idea that the main translational regulation motif of eukaryotes gene is located in 3′ UTR[84]. Recently, by blasting and analyzing genomes of human, mice, rat and dog，Xie et al. pointed out that numerous regulation motifs were included in 3′ UTR, and almost half of them had relationship with the miRNAs modulation[85]. Thereinto, residues 2—8 in the 5′ end of miRNAs play important roles in complementarity between miRNAs and 3′ UTR of their target mRNAs. First, residues 2—8 are perfectly complementary to single or several regions in 3′ UTR of target mRNA[86]. Second, the target sequences of residues 2—8 are usually conserved among diverse species[76]. Third, residues 2—8 are the most conserved among homologous miRNAs[77]. So people use them to predict the target genes of miRNAs[76]. The translational repression mechanism of miRNA acting on its target mRNA is an enigma so far[1]. David supposed two possibilities: miRNA might repress translation at a step after translation initiation in a manner that does not perceivably alter the density of the ribosomes on the message. An alternative possibility is that translation continues at the same rate but is nonproductive because the newly synthesized polypeptide is specifically degraded. The study mentioned above may be combined to the research of siRNA function. Recently, the determination of the crystal structure of Argonaute PAZ domain[87−89] unveils the interaction principles when RISC binds to RNA. Certainly, revealing the repression mechanism of miRNA requires deep-going research in miRNA field.

4.2 Functions

miRNAs are widespread molecules that are associated with micromanagement of gene expression. miRNA genes constitute ~1% of the predicted genes in humans. Diverse miRNAs are differentially expressed during different stages of development and differentiation, leading to the viewpoint that each type of cells might have a unique internal miRNA milieu, which controls the mRNA levels of thousands of coding genes and limits various protein expressions to a proper level[90]. Each miRNA complementary site within target mRNA 3′ UTR is analogous to an adjustable rheostat[66] that works together to dampen the productive translation of the mRNA for regulating optimal protein levels in cells. Like *lin-4* acting on *lin-14* and *lin-21*, *let-7* acting on *lin-41* and *hbl-1* can bring about apparent changes in phenotype. They are rarely cases in miRNA family where most of them act as a micromanager. Just as miR-7 and miR-14 that have several targets, miRNAs may mediate gene functions in an integrated way. To micromanage so many targets, many miRNA molecules need to be present in each cell. This is indeed the case: certain *C. elegans* miRNAs, such as miR-2, are present at an average of 50000 molecules per cell, an abundance that is several hundred, if not thousand, times greater than that of typical mRNAs. In addition, it has

been demonstrated that different miRNAs may regulate a single target mRNA.

As mentioned above, miRNAs execute the regulatory functions by repressing its target genes. miRNAs with upregulated function have not been found as yet. Tables 2 and 3 show that miRNAs play important roles in modulating the genesis, development, growth, differentiation and death of organisms. Lewis et al.[79] predicted that the target genes of miRNA might be enrolled in transcription, signal transduction, and tumor origin. Although the action of miRNA is specific, especially residues 2—8 in its 5′ end that are almost full complementary to the target mRNA, miRNA is distinct from siRNA because one miRNA may target multiple genes *in vivo*. *lin-4* acts on *lin-14* and *lin-28*[2], and *let-7* acts on *lin-41* and *Hbl-1*[3]. It is called the "multiple target" hypothesis. People also supposed that multiple miRNAs might target just one gene[3,91]. So the action manners of miRNA are likely a regulation network, in which it is to be stimulated by some signals, and meanwhile it also modulates life activities of organisms in an integrated way.

All of the miRNAs study remains at theoretical level and is far from complete. But as normally expressed genes *in vivo*, they are related to some human diseases and plant growth. People are now engaged to use them to target interesting genes and explore treatment approaches[92]. The technology of miRNA gene chip reported by Calin et al.[53] may serve as a useful tool for tumor diagnoses. Recently, Suh et al.[46] indicated that human embryonic stem cells could express a unique set of miRNAs. miRNA, as a modulator involved in directional differentiation of human organism, will promote the cultivation of human organism *in vitro*. A better understanding of working mechanisms and biological functions of miRNAs will be sure to bring us a broad perspective of their application.

5 Outlook

The discovery of ribozyme in tetrahymena has attracted biologists' attention to RNAs and their functions and the arguments on life genesis have lasted for several decades. While the debate had just been somewhat calmed down, Fire et al. reported the discovery of RNA interference in the *C. elegans*, which drew our eyes back to RNAs. RNAi is a powerful and special gene silencing system, a promising tool for the research of gene functions[93] and diseases therapy[94], as well as a breakthrough achievement in biological research field. The rapid progress in miRNA study is benefited from the discovery of RNAi too, but the difference is that miRNAs are endogenous small RNAs in organisms. Several investigations demonstrated that miRNAs are not only the metabolites but also important regulation molecules with some special functions. They are involved in modulating the genesis, development, growth, differentiation and death of organisms. miRNAs are sure to have influences on life genesis, species evolution, the complexity of gene expression and regulation, and the original and developmental principles of diseases. Meanwhile, the research of miRNA will offer new concepts, approaches and strategies for the application of RNAi technology. Now, some developed countries have applied the outcomes of miRNA deeply and comprehensively to the directional development of tissue and organism, the temporal and spatial regulation of cell growth and differentiation, switches of signal transduction pathways, supervision and modulation of cell cycle, study of memory, reverse differentiation of tumor cells, obesity, decrepitude and death, precaution and treatment of diseases, and regulation of gene expression.

To search for miRNA genes and their target genes and to better understand the underlying mechanisms is of great ponderability. From current reports comparing to great number of miRNAs discovered in diverse species, the target genes and functions of just a few miRNAs have been elucidated with definite evidence. It shows that there is a gap between the discovery of miRNA genes and the identification of their functions, and that there are some problems about the total number of miRNAs reported by current approaches. The current endeavors are to find out all miRNAs and their target genes in diverse species, and to identify their functions, which is also one inevitable question in the era of post-genome. Synthesis of small RNAs and bioinformatics application may open up a new path in miRNA field, and unveil their target genes and functions. Considering that miRNAs play a crucial role in growth, development and differentiation of organisms with particular temporal expression, people can design the miRNA-chip to outline miRNAs expression profiles of distinct organisms, developmental phases, and different tissues and cells. Revelation of the functions of all the miRNA genes may initiate a new revolution in our view of life phenomena.

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