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Camels and zebrafish, viruses and cancer: a microRNA update

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MicroRNAs (miRNAs) form an extensive class of RNA molecules that regulate gene expression at post-transcriptional level. In recent years, much progress has been made in dissection of biogenesis and functions of miRNAs. There are at least several hundred miRNA genes in the human genome, and the emerging evidence suggests that miRNAs are broadly implicated in gene regulation. Here, we review some recent advances, and particularly we discuss how comparative genomics helps to identify novel miRNA genes, how studies in zebrafish reveal roles of miRNAs in morphogenesis, how changes in miRNA expression patterns are connected with cancer and how host–virus coevolution exploits miRNA regulatory pathways.

INTRODUCTION

Non-coding RNAs (ncRNAs) are RNA molecules that do not encode proteins but have some other functions. The examples of well- and long-known ncRNAs include ribosomal, spliceosomal and transfer RNAs that perform essential housekeeping functions in a cell. Yet, recognition that other ncRNAs, such as introns and non-protein-coding transcripts, may also have crucial cellular functions, has emerged only during the last decade (1–3). Although only ~1.5% of the human genome encodes proteins, a substantially larger fraction of the genome is transcribed. The extent of genome transcription is best demonstrated in the recent work of Gingeras and colleagues, who used high-density tiling arrays to interrogate transcription of about one-third of the human genome at the 5 bp resolution level, and found that >15% of the examined regions were transcribed, which is an order of magnitude greater than expected from annotated exons and gene predictions (4). Interestingly, ~30% of the transcripts originate from unannotated genomic regions and thus are called transcripts of unknown function (TUFs). Two-thirds of these TUFs have a coding capacity of less than 100 amino acids and can very well be ncRNAs.

Information on the diversity of known ncRNAs is available from several databases (5–7). For example, RNAdb provides a listing of over 800 unique mammalian ncRNAs, including microRNAs (miRNAs), small nucleolar RNAs (snRNAs) and ncRNAs of unknown function but known to be developmentally regulated, disease-associated, imprinted, expressed pseudogenes or antisense transcripts (7). Among the variety of ncRNA classes, miRNAs have attracted most attention in recent years due to their abundance, unexpectedly broad involvement in gene regulation and intersection with RNA interference (RNAi) pathway. In this article, we review some recent advances in the miRNA field: from the many angles from which to view the field we have certainly chosen that of our own laboratory and we apologize for omissions or emphases. For information on snRNAs and other ncRNAs, we refer readers to other reviews (8–11).

miRNA MACHINERY

miRNAs form an extensive class of ncRNAs that regulate the expression of genes at post-transcriptional level. An unusual mechanism of gene regulation, in which a small 22 nt RNA molecule (*lin-4*) forms a duplex with the 3′UTR of a target mRNA (*lin-14*) and blocks its translation, was first discovered in *Caenorhabditis elegans* more than a decade ago (12,13). As *lin-4/lin-14* sequences were conserved only in nematodes, the generality of this regulatory mechanism was initially not recognized, until 7 years later another *C. elegans* RNA, *let-7*, was shown to regulate *lin-41* gene in the same manner (14,15). In contrast to *lin-4/lin-14* pair, the sequence and expression pattern of *let-7* RNA, as well as *lin-41* target sites, appeared to be conserved in a wide variety of animals (16,17), suggesting an ancient origin of this type of gene regulation and the existence of a larger class of regulatory RNAs of this kind. Indeed, efforts of different laboratories for cloning small RNAs soon resulted in identification of more than a hundred additional genes that, like *lin-4* and *let-7*, expressed

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~22 nt RNAs potentially processed from stem–loop precursors (18–20). The newly discovered RNAs were collectively named miRNAs.

Understanding of miRNA biogenesis was greatly facilitated by advances in the field of RNAi, because it appeared that miRNA and siRNA pathways partially overlap (21–26). miRNA genes are transcribed by RNA polymerase II as large primary miRNA (pri-miRNA) transcripts that have CAP structures and poly(A) tails (27,28). Some miRNAs are arranged in clusters and transcribed as polycistrons (29), whereas ~40% of miRNAs reside in introns of protein-coding genes (30) and are presumably co-transcribed with host genes. Pri-miRNA transcripts form characteristic fold-back structures that are recognized and processed into ~70 nt imperfect stem–loop miRNA precursors (pre-miRNA) by a so-called microprocessor complex (31,32). This complex includes RNAse III enzyme Drosha (33) and its cofactor, the DiGregory syndrome critical region gene 8 (DGCR8) protein, also known as Pasha in *D. melanogaser* and in *C. elegans* (31,32,34,35). It is believed that Drosha needs Pasha/DGCR8 cofactor for recognition of pri-miRNA tertiary structure, which seems to be a primary determinant for substrate specificity (33,36,37).

After processing by the microprocessor complex, pre-miRNAs are exported by Ran-GTP dependent transporter exportin-5 (38–40) from the nucleus into cytoplasm, where they are processed by another RNAse III enzyme, Dicer, into imperfect dsRNA duplexes that contain both mature and complementary miRNA strands (41–43). The thermodynamic energy of the 5′ ends of a miRNA–miRNA* duplex is usually different and the strand with lower 5′ end stability is preferentially loaded into RNA-induced silencing complex (RISC), thus becoming a mature miRNA, and the complementary miRNA* strand is rapidly degraded (44,45). RISCs are ribonucleoprotein complexes that exist in different subtypes that probably reflect different functions of RISCs. The core components of RISC are proteins from the extensive Argonaute family (46–48). For example, the human miRNA-containing RISC contains Argonaute protein eIF2C2 and helicases Gemin3 and Gemin4 (49). Additional identified proteins that associate with RISC include nuclease Tudor-SN (50) and RNA-binding VIG and fragile-X related protein (51,52). Despite the extensive characterization of RISC complexes, exact biochemical mechanisms of RISC function remain largely unknown. Good progress in this direction was achieved with recent identification of Argonaute2 as a catalytic center of target mRNA cleavage by siRNA-containing RISC (53). As an alternative route to degradation, RISC can impose translational block of a target mRNA, and it is thought that the mode of RISC action is largely determined by the extent of complementarity between a effector RNA (siRNA or miRNA) and a target mRNA, with a perfect identity leading to mRNA cleavage and imperfect matching to translational block (47,54–56). Whereas in plants most miRNAs target mRNA to degradation (57–60), in animals all known miRNA–mRNA interactions lead to translational block (61), and only one case of miRNA-mediated cleavage of target mRNA has been reported so far (62). Recently, Lim *et al*. (63) used microarrays to analyze the effects of transfection of human cells with particular miRNAs and found that levels of many direct mRNA targets decreased, thus suggesting that influence of miRNAs on the levels of target mRNAs can be widespread. A more detailed recent reviews on miRNA biogenesis are described earlier (26,64).

IDENTIFICATION OF miRNA GENES

Information about all known miRNAs is accumulated in the miRNA registry—a database of published miRNA sequences (65), the recent release of which (6.0) contains 227 human, 230 mouse and 191 rat miRNA genes. Essentially, there are two approaches for the identification of novel miRNA genes: cloning of size-fractionated (18–25 nt) RNAs and computational prediction based on different structural features of miRNAs. The cloning approach was particularly efficient at the initial stages of miRNA hunting and allowed identification of more than two hundred miRNAs from human, mouse, Drosophila and *C. elegans* (18–20,49,66–73). Although powerful, the cloning approach has limitations and depends on the abundance and spatio-temporal expression patterns of miRNAs for their detection. The large number of miRNAs identified by cloning, however, formed a solid data set essential for designing efficient prediction algorithms, and several groups have developed such computational algorithms for identification of miRNAs in different organisms (67,74–80). All these algorithms follow one common theme (81) and search for fold-back structures that have characteristics similar to those of known miRNAs and are evolutionarily conserved. On the basis of the correlations between the number of computationally predicted miRNA candidates, the fraction of known miRNAs included in the predictions, frequencies of cloning of known miRNAs and verification rates for novel candidates, Bartel and colleagues (76) estimated that the human genome contains at most 255 miRNA genes. Similarly, it was estimated that there are about 120 and 110 miRNAs in *C. elegans* and in *D. melanogaster* genomes, respectively (75,77). An alternative estimate of 140–300 miRNA genes in *C. elegans* genome is provided by Ruvkun and colleagues (74).

We have recently revisited the estimate of a possible number of miRNA genes in the human genome (80). Our original intention was to investigate conservation patterns of different miRNA genes and their flanking sequences, and to that end, we sequenced genomic regions encompassing 122 miRNA genes in 10 primate species. This approach, known as phylogenetic shadowing (82), provides fine-grain resolution of sequence conservation and allows identification of functional elements that would have been missed in comparisons of evolutionary distant species. The main goal of our analysis was to probe for the presence of conserved elements in sequences immediately flanking miRNA genes. For example, such conserved elements, if found, could play a role in miRNA biogenesis. The obtained data suggested, however, that there are no such conserved elements common to different miRNAs. Instead, we found that immediate flanks of most miRNAs analyzed are not conserved, which lead to the realization that flanks variability may be a good filter for improving existing algorithms of miRNA gene prediction. In its most prominent form, miRNA conservation profiles resembled camel's humps (Fig. 1), and therefore, the typical profile

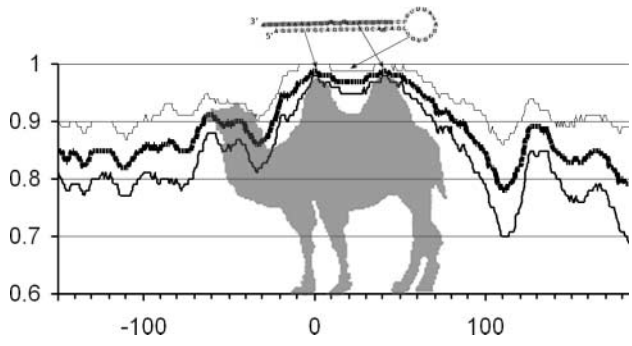

Figure 1. Camel-shaped conservation profile is characteristic for miRNA genes. An average conservation level (middle line) with 95% confidence intervals (upper and lower lines) was calculated for 64 miRNAs. Corresponding regions of the precursor miRNA (top) are shown by arrows. The figure is modified from Berezikov *et al*. (80).

received a code-name 'camel'. We next developed a computational pipeline for identification of regions in human/rodent whole-genome alignments (WGA) that have 'camel'-like conservation profiles and are able to form fold-back structures. Besides structural and conservation/variation criteria, our computational approach for identification of miRNA genes included an additional filter—the recently discovered property of miRNAs to have a lower folding free energy than random sequences of the same nucleotide composition (83). Analysis of human/mouse and human/rat WGAs identified a total of 978 candidate miRNA regions that satisfied the earlier mentioned criteria and included 158 known miRNAs (>80% of known miRNAs present in initial alignments). Searches in dog, cow, opossum, chicken and zebrafish genomes revealed that 678 of the predicted miRNAs could be also identified in at least one more species besides rodents. In addition, we confirmed a number of novel miRNA candidates by northern blot analysis. The conservative interpretation of our results suggested that there may be between 200 and 300 novel miRNAs in the candidate data set, bringing the total number of miRNA genes in the human genome to around 500. We also speculated that the actual number of miRNAs may be even higher, up to 1000, if highly restricted temporal and spatial expression patterns as well as taxon-specificity of some miRNAs are taken into account. Microarray and cloning experiments are currently underway to interrogate the complete set of predictions.

A number of candidates from our data set are already proved to be real miRNAs by independent works. Margalit and colleagues (84) performed analysis of conservation and clustering patterns of known human miRNAs and used conservation profiles similar to 'camel', to predict a number of novel miRNA genes in the vicinity of known miRNAs. They then experimentally confirmed the expression of 14 predicted miRNAs, of which nine were also on our candidates list.

A higher estimate of the total number of miRNA genes in the human genome is also supported by the recent original work of Xie *et al*. (85), who analyzed conservation patterns of promoter and 3′ UTR sequences in human, mouse, rat and dog genomes, and found a number of over-represented conserved regulatory motifs. Unexpectedly, motifs found in 3′ UTRs showed a length distribution with a prominent peak at an eight base length (72 of 106 conserved motifs), which led authors to speculate that these motifs might be miRNA target sites. In addition, indeed, about half of known miRNAs matched through Watson-Crick pairing to the highly conserved 8mer motifs. The discovered 8mers were next used to search for novel miRNA genes and 242 conserved hairpin sequences were identified that included 113 known miRNAs and 129 novel predictions. Experimental verification confirmed the expression of six out of 12 tested candidates, allowing the authors to conclude that many of their predictions are real miRNAs. It is worth noting that from 129 candidate miRNAs identified by Xie *et al*., 76 are overlapping with our predictions, further strengthening the notion that there is still a substantial number of miRNAs in mammalian genomes waiting for experimental confirmation.

miRNA FUNCTIONS

The functions of some miRNAs were elucidated by forward genetics screens (61). In *C. elegans lin-4* and *let-7* miRNAs were shown to be involved in developmental timing (12,14) and *lsy-6* and *miR-273* in neuronal cell fate (86,87); *bantam* and *miR-14* in *D. melanogaster* are responsible for cell death, proliferation and fat storage (88,89). Cloning of miRNAs from different tissues and/or establishment of miRNA expression patterns may also provide clues for the function of particular miRNAs. For example, *miR-181* was cloned from mouse bone marrow and thymus, where it is predominantly expressed and was consequently shown to be involved in B- and T-cell development (90). Poy *et al*. (91) found that *miR-375*, cloned from pancreatic cells, targets myotrophyn gene, involved in insulin secretion.

Functions of miRNAs can also be predicted computationally—the approach actively explored by different groups (58,92–98). However, the initial algorithms for miRNA target predictions were far from perfect, with little or no overlap among top-predicted targets (99), which were attributed to the lack of experimental data necessary to formulate clear rules for miRNA target recognition. To fill this gap, several research groups performed systematic analysis of pairing requirements between miRNA and target mRNA and revealed that first eight bases of miRNA starting from its 5′ end are most important for miRNA–mRNA interaction (55,100,101). Surprisingly, these eight bases, also known as miRNA seed, can alone, with little or no pairing in the 3′ end of the miRNA, be sufficient for a functional miRNA–mRNA duplex. The importance of the miRNA seed in target recognition was also recognized by computational analysis: it appeared that many of the conserved 8mer motifs in 3′ UTRs of genes correspond to miRNA seeds (85,93,102). However, perfect matching at the 5′ end of a miRNA is not an absolute requirement, because Brennecke *et al*. (101) found that sites with mismatches in the seed can also be functional if there is strong compensatory pairing at the 3′ end of a miRNA. Equipped with the better understanding of miRNA target recognition principles, Kerk *et al*. (98) and Lewis *et al*. (102) recently developed improved algorithms for miRNA target prediction and independently came to the similar estimates

that in vertebrates miRNAs can target on average 200 transcripts, resulting in regulation of as much as 30% of human genes. Thus, there is a growing evidence of a much broader involvement of miRNAs in gene regulation than previously anticipated.

Information on miRNA expression patterns will be essential for dissection of miRNA-containing gene regulatory networks. We have recently used a combination of microarray and *in situ* hybridization techniques to establish expression profiles of 115 conserved vertebrate miRNAs in zebrafish embryos (103). *In situ* hybridizations using LNA (locked-nucleic-acid)-modified probes revealed that most (68%) of the investigated miRNAs are expressed only in specific tissues, e.g. muscles, nerves, sensory organs or digestive system (Fig. 2). In addition, the expression of miRNAs was detected starting at segmentation stages of zebrafish embryo development but not at earlier stages. On the basis of these observations, we concluded that miRNAs are not crucial for early patterning but play a role in morphogenesis and maintenance of tissue identity. Similar conclusions came from the work of Giraldez *et al*. (104), who used germline replacement technique to generate Dicer-deficient zebrafish embryos completely void of mature miRNAs. This trick was necessary to circumvent the problem of maternally inherited miRNAs in Dicer mutants (105). It appeared that the maternal-zygotic Dicer null mutants display no abnormalities in early development and undergo normal axis formation and regionalization, i.e. form all major subregions and cell types. However, morphogenesis at later stages of development was severely affected. For example, defects in gastrulation, somitogenesis, cardiovascular morphogenesis and brain development were observed. Interestingly, injection of synthetic miRNAs of a single subfamily (*miR-430*) was able to rescue the brain morphogenesis defects (104), demonstrating a fundamental role of miRNAs in organ formation.

Differential gene expression is a principal driving force behind developmental processes such as morphogenesis. Lim *et al*. (63) has recently shown that a single miRNA can influence the entire transcriptome profile of a cell. The authors transfected human cells with *miR-124* and *miR-1* miRNAs, which are specifically expressed in brain and muscles, respectively, and demonstrated by microarray analysis that in case of *miR-124*, the expression profile was shifted towards that of brain, whereas *miR-1* transfection resulted in the expression profile characteristic for muscles. These experiments provide additional support for the notion that miRNAs help to establish and maintain the identity of different cell types.

miRNAS AND CANCER

A connection between miRNAs and cancer was suggested not long after the discovery of miRNAs. First, Calin *et al*. (106) found that miRNAs *mir-15* and *mir-16* are located in a locus 13q14, which is frequently deleted in B-cell chronic lymphocytic leukemias. Extension of that study led to the discovery that, in fact, about half of miRNA genes (98 of 186 studied) are situated in cancer-associated genomic regions (107) and expression profiling revealed that both up- and down-regulation of many miRNAs occurs in cancer tissues (108–110).

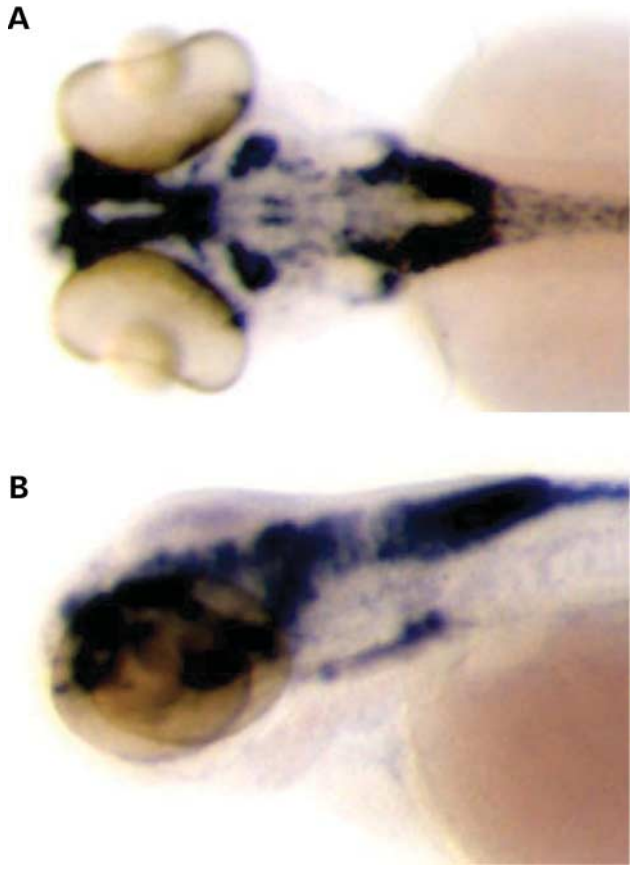

Figure 2. Example of a tissue-specific miRNA expression pattern. (**A**) Ventral and (**B**) lateral view of *miR-137* whole-mount *in situ* expression in brain structures and spinal cord of zebrafish embryos. The pictures are kindly provided by Erno Wienholds (103).

For example, miRNAs from the *let-7* family appeared to be poorly expressed in lung cancers (107,111) but not in other types of cancer. In addition, Johnson *et al*. (112) demonstrated that *let-7* family members regulate RAS oncogenes in human. He *et al*. (113) found that the *mir-17-19* cluster of miRNAs is overexpressed in certain human lymphomas and demonstrated that its increased expression, mediated by *c-MYC* oncogene, accelerates tumor development. O'Donnell *et al*. (114) also showed that c-Myc activates expression of *mir-17-19* miRNA cluster, whereas two miRNAs from this cluster, *miR-17-5p* and *miR-20a*, negatively regulate E2F1 transcription factor (TF) involved in cell cycle progression. Interestingly, E2F1 and c-Myc are also known to activate each other. Thus the role of c-Myc-regulated miRNAs can be in preventing a positive-feedback loop between c-Myc and E2F1, which otherwise could lead to overexpression of both genes and failure in cell cycle regulation (115).

Altered expression of particular miRNAs in different types of cancer can possibly be used for cancer diagnosis and prognosis. Brown *et al*. (116) profiled the expression of miRNAs in more than 60 patients with lung, colon, breast, bladder, pancreatic, prostate or thymus cancer and found that different types of tumor have different miRNA expression signatures. For example, in 70% of the patients with lung cancer, six miRNAs (*miR-30a, -126, -143, -146, -188*, and *-331*) were

expressed at significantly lower levels, whereas three (*miR-21, -189* and *-200b*) were significantly over-expressed. Similarly, colon cancers had a characteristic set of five over-expressed and four under-expressed miRNAs. Notably, there are many known oncogenes among putative targets of differentially expressed miRNAs. Lu *et al*. (117) demonstrated recently that miRNAs can indeed be developed into potent cancer markers. Expression profiling of 217 mammalian miRNA using bead-based flow cytometry revealed that most miRNAs are downregulated in tumor tissues and that different types of tumors can be distinguished by miRNA expression patterns. Moreover, Lu *et al*. showed that miRNA profiles can be used for highly reliable diagnosis of tumors of histologically uncertain cellular origin.

Taken together, there is growing evidence that miRNAs can act as both oncogenes and tumor suppressors. Further studies are certainly required to reveal the full breadth of miRNA involvement in cancerogenesis.

VIRUS CONNECTIONS

Besides animals and plants, miRNAs were also recently discovered in viruses (118–120). Pfeffer *et al*. (118) performed a small RNA profile of human cells infected by Epstein-Barr virus, a member of the human herpesvirus family, to study the role that RNA silencing can play during viral infection in animals. Besides host miRNAs they identified five miRNAs originating from the virus. They next developed a computational algorithm to identify miRNAs in virus genomes and surveyed a wide range of viruses (119). Experimental verification of predictions in herpes viruses resulted in identification of 28 novel miRNAs from Kaposi sarcoma-associated virus, mouse gamma-herpes virus and human cytomegalovirus. Unexpectedly, virus miRNAs appeared to have no homology between each other or with human miRNAs. Positions of miRNAs within different virus genomes were also not conserved. Lack of sequence and positional conservation suggests that miRNAs are likely to be recent acquisitions in viral genomes evolved to perform very specific functions. The first example of such a specific function of viral miRNA in a polyomavirus was recently published by Sullivan *et al*. (120), who discovered that simian virus 40 (SV40) encodes a miRNA that regulates one of the viral genes called T antigene. The authors found that SV40 miRNA accumulates in late stages of infection and targets a early viral T antigene mRNA for degradation, thus reducing expression of viral T antigens. Importantly, besides involvement in virus replication, T antigenes also trigger the cytotoxic response of T lymphocytes. Therefore, reduced levels of T antigene expression enhance chances of the virus to escape lysis by T-cells. Although the exact roles of many other viral miRNAs remain to be established, the available evidence suggests that DNA viruses evolved to exploit miRNA silencing pathway for their advantage.

Interestingly, miRNAs were found in different DNA viruses but not in small-genome retroviruses (119). In addition, no viral siRNAs were detected in human cells infected with different RNA viruses, whereas RNAi machinery of the cells remained unaffected, suggesting that RNA viruses do not interfere with RNA silencing pathways in mammalian cells (119). This is in contrast to plants and insects, where RNA silencing is used as an immune response to RNA viruses (121,122). It was discovered recently that mammalian cells actually do use RNA silencing against retroviruses but utilize miRNA pathway instead of RNAi. Lecellier *et al*. (123) reported that in cells expressing a suppressor of RNA silencing, accumulation of a retrovirus called primate foamy virus type 1 (PFV-1) was strongly enhanced, indicating involvement of siRNAs or miRNAs in the control of virus replication. However, similar to the results of other researchers, no viral siRNAs were detected in infected cells. Instead, computational analysis suggested that human miRNA miR-32 can target one of viral ORF sequences, and experimental verification proved that predicted *miR-32* target sequence is functional. In addition, depletion of *miR-32* from infected cells resulted in an increased accumulation of viral mRNA, proving antiviral properties of *miR-32*. Leicellier *et al*. (123) also demonstrated that one of PFV-1 proteins, Tas, is a general suppressor of RNA silencing that can offset the *miR-32* effect. Thus, a picture of host–virus interactions is emerging in which cellular miRNAs play an important role in limiting virus replication, and viruses counteract this cellular defense by hampering RNA silencing pathways. The authors suggested that miRNAs might be broadly implicated in viral infection of mammalian cells, and virtually every miRNA, disregarding its primary cellular function, can have a fortuitous antiviral potential (123). Indeed, as matching of eight bases in the seed of a miRNA to a target sequence is often sufficient for a functional interaction (101), host's miRNAs relations to viruses may be seen as a mammalian version of the bacterial restriction enzyme defense system against phages. To test this hypothesis and to establish the extent of host's miRNA involvement in virus regulation, a survey of a broad range of viruses will be required.

CONCLUSIONS

It is no exaggeration to say that we are evidencing a paradigm shift in our understanding of the complexity in gene regulatory networks, provoked by the discovery of miRNAs. The recent findings that miRNAs provide a new layer in gene regulation, additional to TFs and other regulatory systems, constitute the essence of this shift. The similarity in the logics of action of TFs and miRNAs was discussed by Hobert (124), who noticed that different combinations of miRNAs in different cell types, or 'miRNA codes', can have roles conceptually similar to 'TF codes' in establishing these cell types. Bartel and Chen (125) speculated that differential expression of miRNAs in different cell types provides a 'miRNA milieu' that influences expression of thousands of mRNAs. Recent experimental data confirm these concepts (63,103,104). Tissue-specific expression of many miRNAs, their necessity for morphogenesis but not for early development, and ability to shift expression profiles of cells to that of specific tissues, suggest that establishment and maintenance of cells in specific differentiated states is the main function of many miRNAs. Thus, by analogy to housekeeping genes that provide basic functions in all the organisms, miRNAs can be called

roomkeeping genes, because they are to a large extent responsible for keeping differentiated cells in order, by dumping expression of genes that should not be expressed in particular cell types. Connection between miRNAs and cancer immediately becomes obvious from this analogy: perturbations in miRNA expression patterns may lead to cell dedifferentiation and can be a cause as well as a consequence of tumorogenesis. Apparently, roomkeeping is not the only function of miRNAs. The recently discovered involvement of miRNAs in host–virus coevolution is spectacular: at least some miRNAs evolved to play defensive roles against viruses, while viruses also evolved to use miRNA machinery for their advantage.

Although a steady progress in understanding the miRNA world has been made in recent years, there is still a long way ahead before miRNAs reveal all their secrets. The issue on the number of miRNAs in the human genome is not settled and continuing cloning and other experimental efforts are required to identify all miRNA genes. Knowledge of the complete microRNome is essential for experimental and computational dissection of miRNA-containing gene regulatory networks. Knockout and knockdown experiments are required to reveal specific functions of particular miRNA genes, and technologies for this type of experiments are available (126). Although miRNA knockouts/knockdowns will provide loss of function phenotypes, computational approaches will be necessary to assist in identification of mRNA targets behind these phenotypes and further improvements in miRNA target prediction algorithms can be expected. As computational predictions require an experimental confirmation, a high-throughput technology for miRNA target verification, if developed, will greatly benefit the field. Finally, we foresee a substantial increase in volume of research on connection between miRNAs and human disease.

Conflict of Interest statement. None declared.

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