

## ROLE OF micro-RNAs IN THE REGULATION OF GENE EXPRESSION

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**Summary:** Recent discovery of a new class of non-coding interfering RNAs (ncRNAs) enhanced dramatically our knowledge regarding detailed mechanisms for regulation of gene expression. Several subtypes are already described, including microRNAs (miRNAs), small interfering RNAs (siRNAs) and piwi-interacting RNAs (piRNAs). These RNAs are very short in length (20-22 bp) and play significant role in highly specialized non-protein regulatory system for gene activity. Recent years witnessed intensive accumulation of new knowledge regarding their function and understanding the mechanism of action. miRNAs are critical post-transcriptional modulators and bind to complementary sequences on target messenger RNA transcripts (mRNAs), leading to repression of translation or degradation of target RNAs. Until now, more than several hundred miRNAs have been identified in the human genome and it is assumed that 30% of mammalian gene expression is directly regulated by these small RNAs. Dysfunction of miRNA is associated with abnormally expressed and disease related genes.

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First miRNAs were described in a flatworm species *C. elegans*, which led to an understanding of their important regulatory role in cell differentiation (Lee et al., 1993; Wightman et al., 1993). Another critical step was the discovery of the second miRNA named *let-7*, which is a product of expression of a heterochronic switch gene in *C. elegans* (Reinhart et al., 2000). Loss of *let-7* gene activity causes reiteration of larval cell fates during the adult stage, whereas increased *let-7* gene dosage causes precocious expression of adult fates during larval stages. *Let-7* gene encodes a temporally regulated

21-nucleotide RNA that is complementary to elements in the 3' untranslated regions of the heterochronic genes such as *lin-14*, *lin-28*, *lin-41*, *lin-42* and *daf-12*, indicating that expression of these genes may be directly controlled by *let-7*. It was proposed that the sequential stage-specific expression of the *lin-4* and *let-7* regulatory RNAs triggers transitions in the complement of heterochronic regulatory proteins to coordinate developmental timing.

These short RNAs represent entirely new class of non-coding small RNAs, which can regulate the expression of vast

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number of protein-coding genes at the transcriptional and posttranscriptional level (Carthew et al., 2006). Each individual miRNA can control hundreds of different gene targets by targeting their respective mRNA cleaving them, and also can induce mRNA degradation initiated by miRNA-guided deadenylation (Guarnieri et al., 2008). Usually they bind to 3' untranslated regions (UTR), different coding sequences or 5' UTR of targeted mRNAs ultimately leading to repression of translation or fast mRNA degradation (Filipowicz et al., 2008; Ambros 2004). Interfering RNAs are divided in three major subclasses depending on their size, mode of action, specificity and abundance. Fig. 1 shows all distinctive characteristics for these major subclasses.

### Biogenesis of miRNAs

miRNAs usually are encoded in the genome as long primary transcripts

(named pri-miRNAs) that contain an additional cap structure at the 5' end and are poly-adenylated at the 3' end. In the nucleus, the pri-miRNA is "cropped" into a ~70 nucleotide (nt) hairpin-structured precursor (pre-miRNA) by a multiprotein complex called the Microprocessor (Siomi et al., 2010). The two core components of the Microprocessor are Drosha, an RNase III enzyme, and DGCR8/Pasha, a double-stranded RNA-binding domain (dsRBD) protein. Drosha is the core nuclease that executes the initiation step of miRNA processing in the nucleus by cleaving pri-miRNA to release pre-miRNA *in vitro* (Lee et al., 2003). Furthermore, RNA interference of Drosha resulted in the strong accumulation of pri-miRNA and the reduction of pre-miRNA and mature miRNA *in vivo*. Thus, the two RNase III proteins, Drosha and Dicer, may collaborate in the stepwise processing of miRNAs, and have key roles in miRNA-

Properties	siRNA	miRNA	piRNA
Length	~21 nt	~22 nt	~24–30 nt
Strand	Double-stranded RNA	Single-stranded RNA	Single-stranded RNA
Number	NA	28654 mature miRNA (Sanger – miRBase 21)	~50 000 – 200 000
Origin	Exogenous RNA	Endogenous RNA	Endogenous RNA
Expression	Not expressed in tissues or cells	Various tissues and cells	Spermatocytes, spermatids
Binding targets	mRNA	Ago subclass	Piwi subclass
Dicer	Dicer required	Dicer required	Dicer independent
Function	mRNA degradation posttranscriptional silencing	mRNA instability translation inhibition	Repression of retrotransposon & post-transcriptional regulation

**Figure 1.** Comparison of the properties of small interfering RNA (siRNA), microRNA (miRNA), and piwi-interacting RNA (piRNA). The number of siRNA corresponds to the number of genes, known or unknown in rodents and humans and each gene can have many siRNAs targeting its various regions. Binding targets refer to the targets that small RNAs bind to, as an example, siRNAs can bind to gene transcripts (mRNA). NA, not applicable (Tolia & Joshua-Tor, 2007).

mediated gene regulation in processes such as development and differentiation. Resulting duplex is then unwound by an helicase into a mature miRNA, which is then incorporated in a multicomponent complex constituted by Argonaute proteins, known as RNA-induced silencing complex (RISC).

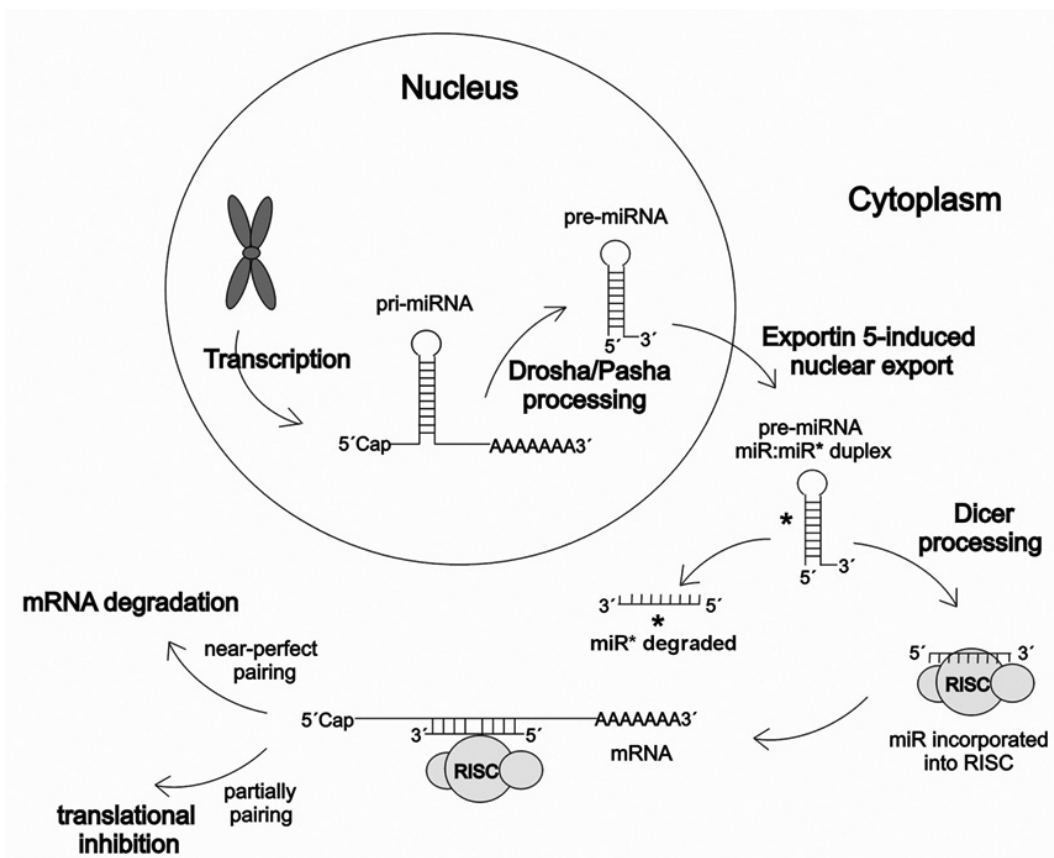
Several articles were critical for understanding the details of miRNA biogenesis, proposing involvement of RNAi pathway components in miRNAs maturation (Grishok et al., 2001; Hutvagner et al., 2001). Homolog of *Drosophila* Dicer (*dcr-1*), and two homologs of *rde-1* (*alg-1* and *alg-2*) were essential for *lin-4* and *let-7* activity. Inactivation of these genes caused phenotypes similar to *lin-4* and *let-7* mutations (Grishok et al., 2001). In the same time it was shown that *let-7* pre-miRNA is cleaved by Dicer. When cells were transfected with siRNA duplex corresponding to human Dicer enzyme, pre-*let-7* accumulated into the cells (Hutvagner et al., 2001). These data support the idea for eventual crosstalk between RNAi and miRNA pathways and lead to better understanding the formation of mature miRNAs. Two important enzymes involved in the miRNAs biogenesis pathway, Drosha and Dicer are spatially separated, being localized respectively in the cell nucleus and in the cytoplasm. Fig. 2 shows the possible pathways for miRNA biogenesis, as well as the enzymes involved in the process.

Nuclear export of the short miRNA precursors into the cytoplasm is mediated by Exportin-5 (Lund et al., 2004). However a number of miRNAs such as miR-29b are predominantly located in the nucleus (Hwang et al., 2007). These particular miRNAs contain additional

sequences elements that control their specific subcellular localization. miR-29b contains a hexanucleotide terminal motif that directs its import into the nucleus. Despite their small size, miRNAs can contain additional cis-acting regulatory motifs that might influence their posttranscriptional behavior and miRNAs with common 5' ends, predicted to regulate the same targets, might have distinct functions.

miRNAs are also found in many plant species and the current miRBase release (version 10.1) lists 39 miRNA families present in two or more phylogenetically distant plant species; many of these annotations are supported by robust experimental data in multiple species, whereas some have required more experimental data to support their annotated conservation patterns (reviewed by Axtell & Bowman 2008). It is evident that miRNA genes are rapidly evolving. Each plant species has miRNAs that are not present in other, even closely related species. Among the 100 miRNA families from *Arabidopsis*, only 21 are present in the monocot rice, which suggests that many miRNAs evolved after the dicot-monocot split (Chen 2009). DNA sequencing techniques have enabled more detailed explorations of the set of miRNA genes of individual plant species, and have revealed a large number of 'non-conserved' miRNAs (at least 48 in *Arabidopsis*), outnumbering the 'conserved' miRNAs (Lu et al. 2005; Hinas et al. 2007). "Non-conserved" miRNAs are defined as those with a limited phylogenetic distribution and are characterized by primarily being single-copy genes (Axtell & Bowman 2008).

Remarkable adaptation in plant is



**Figure 2.** miRNA biogenesis. MiRNAs are transcribed by RNA polymerase II from the genomic DNA as long (hundred or thousand nucleotides) primary miRNA transcripts (pri-miRNAs). A local stem-loop structure of pri-miRNAs is then cleaved in the nucleus by the dsRNA-specific ribonuclease Drosha/Pasha to produce a 70 nucleotides long precursor miRNA (pre-miRNA). Pre-miRNAs in form of hairpins are then actively transported from the nucleus to the cytoplasm. In the cytoplasm, pre-miRNAs are subsequently cleaved by RNase III Dicer into ~22-nt miRNA duplexes, consisting of the “guide” (miR) strand and the “passenger” (miR\*) strand. The “passenger” strand is degraded, the “guide” strand is incorporated into the RNA-induced silencing complex (RISC) and serves as a functional, mature miRNA, acting by two different mechanisms according to the complementarity with the target mRNA (adapted from Tomankova *et al.*, 2010).

the availability of two additional RNA polymerases, Pol IV and Pol V, which are specific for siRNA biogenesis (Herr *et al.* 2005, Pontier *et al.* 2005). Plant specific Pol IV is solely responsible for the posttranslational splicing of over 90% of all endogenous siRNAs in plant species (Mosher *et al.* 2008). Pol IV-dependent siRNAs are mostly localized

in pericentromeric regions, and originate from different repeats and transposable elements (Kanno *et al.* 2005, Zhang *et al.* 2007).

### Function of miRNAs

Small interfering RNAs inhibit the expression of homologous target genes in three different ways:

- Induce destruction of mRNA encoded by the target gene;
- Inhibit the translation of mRNA;
- Induce modifications of the chromatin within the target gene, and thereby block its transcription.

It is noteworthy that, irrespective of the route used to degrade the targeted mRNA, most of the components of the inhibitory complex are practically the same. The critical complex involved directly in modulation of mRNA activity is RISC and it contains in addition to the small interfering RNA various other components, including members of the Argonaute family of proteins. RISC complex is directed to a target mRNA comprising a nucleotide sequence complementary to that of the guiding RNA strand. Consequently these target mRNAs are degraded or their translation is suppressed. Typically, the selection depends in part on how similar are the interfering RNAs and the target mRNAs: if the sequences are highly complementary (as is usually the case with siRNAs), the target mRNA is degraded; if the similarity is not so close (if there are several different bases pairing, as often happens with miRNAs), then the translation is often inhibited. In those cases in which the target RNA is degraded, argonaute protein is the catalytic subunit that performs initial cleaving mRNA; for this reason often Argonaute protein is called Slicer and degradation of mRNA is also called slicing.

miRNAs main biological function is to inhibit the expression of protein-coding genes, either by interfering with translation or mRNA degradation. Until recently, the relative importance of each mechanism for repression was not well known particularly

for endogenous targets expressed at low-to-moderate levels. Exploring the approach of ribosome profiling to measure the overall effects on protein production and compare these to simultaneously measured effects on mRNA levels Guo et al., find that mRNA destabilization was the predominant miRNAs mechanism of action to decrease their targets levels. For both ectopic and endogenous miRNA regulatory interactions, lowered mRNA levels account for most of the decreased protein production. These results show that changes in mRNA levels closely reflect the impact of miRNAs on gene expression and indicate that degradation of targeted mRNAs is the predominant reason for reduced protein output (Guo et al., 2010)

In addition to mRNAs repression, miRNAs have been also reported to activate translation of targeted mRNAs (Fabian et al., 2010). Upon cell cycle arrest, the AU-rich elements (ARE) in tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) mRNA is transformed into a translation activation signal, recruiting Argonaute proteins and fragile X mental retardation-related protein 1 (FXR1), factors associated with micro-ribonucleoproteins (microRNPs). In human cells microRNA miR369-3 directs association of these proteins with the AREs to activate translation. Furthermore, the two well-known microRNAs-Let-7 and the synthetic microRNA miRcxcr4-likewise induce translational up-regulation of target mRNAs on cell cycle arrest and repress translation in proliferating cells. Activation of translation is certainly a common function of microRNPs on cell cycle arrest. It was proposed that translation regulation by microRNPs oscillates between repression

and activation during the cell cycle (Vasudevan et al., 2007). New evidences were accumulated demonstrating that miRNA may also function to induce gene expression. By scanning gene promoters in silico for sequences complementary to known miRNAs, a putative miR-373 target site was identified in the promoter of E-cadherin. Transfection of miR-373 and its precursor hairpin RNA (pre-miR-373) into PC-3 cells significantly elevated E-cadherin gene expression. Knockdown experiments confirmed that induction of E-cadherin by pre-miR-373 required the miRNA maturation protein Dicer. Furthermore, enrichment of RNA polymerase II was detected at both E-cadherin and CSDC2 promoters after miR-373 transfection. Transfection of promoter-specific dsRNAs revealed that the concurrent induction of E-cadherin and CSDC2 by miR-373 required the miRNA target sequences in both promoters. These findings reveal a new mechanism by which miRNAs may regulate gene expression by targeting specific promoter sites (Place et al., 2008).

Convincing data suggest that association with any position on a target mRNA is mechanistically sufficient for miRNA to inhibit the translation at some step downstream of initiation (Lytle et al., 2007). For instance, in animals, miRNAs bind to the 3' UTRs of their target mRNAs and interfere with translation despite that functional miRNA-binding sites in the coding regions or 5' UTRs of endogenous mRNAs have not been identified. Studies on the effect of introduced miRNA target sites into the 5' UTR of luciferase reporter mRNAs containing internal ribosome entry sites (IRESs) in human HeLa cells, which express endogenous let-7a miRNA,

demonstrated that the translational efficiency of these IRES-containing reporters was repressed.

Studies on mouse Nanog, Oct4 and Sox2 genes demonstrate the existence of many naturally occurring miRNA targets in their amino acid coding sequence (CDS). miR-134, miR-296 and miR-470, upregulated on retinoic-acid-induced differentiation of mouse embryonic stem cells, target the CDS of each transcription factor in various combinations, leading to transcriptional and morphological changes characteristic of differentiating mouse embryonic stem cells. These data demonstrate the abundance of CDS-located miRNA targets, some of which can be species-specific, and support a new model whereby miRNAs regulate mRNAs through targets that can reside beyond the 3' untranslated region (Tay et al., 2008).

Recently, Eiring et al., published a detailed analysis bringing a new understanding how miRNAs can interfere with the targeted mRNAs. They report that loss of miR-328 occurs in blast crisis chronic myelogenous leukemia (CML-BC) in a BCR/ABL dose- and kinase-dependent manner through the MAPK-hnRNP E2 pathway. Restoration of miR-328 expression rescues differentiation and impairs survival of leukemic blasts by simultaneously interacting with the translational regulator poly(rC)-binding protein hnRNP E2 and with the mRNA encoding the survival factor PIM1, respectively. These data reveal the dual ability of a microRNA to control cell fate both through base pairing with mRNA targets and through a decoy activity that interferes with the function of regulatory proteins (Eiring et al., 2010).

Small interfering RNAs are localized

in different tissues and their activity can be regulated specifically only in these areas. For instance, miRNAs are abundantly expressed in the brain and detailed characterization of the brain-enriched miRNAs revealed that a specific miR-124 is the most abundant and well conserved brain-specific miRNA. It was exclusively present presynaptically in a sensory-motor synapse where it constrains serotonin-induced synaptic facilitation through regulation of the transcriptional factor CREB. (Rajasekharan et al. 2009). These data are direct evidence that a modulatory neurotransmitter important for learning can regulate the levels of small RNAs and present a role for miR-124 in long-term plasticity of synapses in the mature nervous system.

Expression of miRNA decoys as single or heteromeric non-cleavable miRNAs sites embedded in either non protein-coding or within the 3' untranslated region of protein-coding transcripts can regulate the expression of one or more miRNA targets (Ivashuta et al. 2011). By altering the sequence of the miRNA decoy sites, it was possible to attenuate miRNA inactivation, which allowed for fine regulation of native miRNA targets and the production of a desirable range of plant phenotypes. These data demonstrate that miRNA decoys are a flexible and robust tool, not only for studying miRNA function, but also for targeted engineering of gene expression in plants. Computational analysis of the Arabidopsis transcriptome revealed a number of potential miRNA decoys, suggesting that endogenous decoys may have an important role in natural modulation of expression in plants.

Both plants and animals express

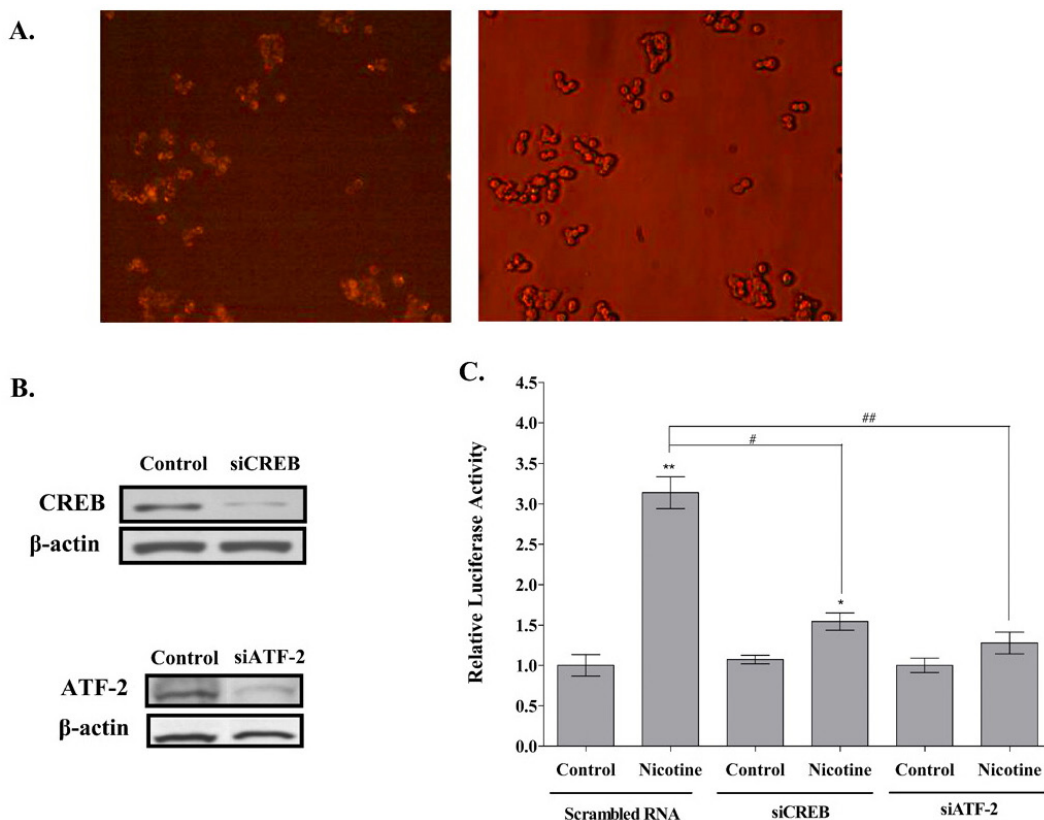
miRNAs and it is accepted that this class of noncoding RNAs has been regulating gene expression since at least the last common ancestor of these lineages (Reinhart et al., 2002). The major plant and animal miRNA pathways differ with respect to their biochemical mechanisms, the extent of their preferred target pairing, and numbers of functional targets (Axtell et al. 2011). These differences have resulted in distinct characteristics of the biological properties of plant and animal miRNAs. Co-evolution of target:miRNA pairs is common in plants, which is starkly different with the animal miRNAs tending to evolve and acquire target genes.

siRNA and direct regulation of gene expression. Small interfering RNAs (siRNAs) can control gene expression in eukaryotes. This RNA-mediated regulation generally results in sequence-specific inhibition of gene expression; this can occur at levels as different as chromatin modification and silencing, translational repression and mRNA degradation (Filipowicz et al. 2005). Many details of the biogenesis and function of siRNAs, and their complexes with different proteins with which they associate have been published. Biogenesis of siRNA is a process taking several critical steps. siRNAs are synthesized as a double stranded pre-siRNA, which undergo processing leading to formation of active double stranded siRNA.

To study the requirement for CREB and ATF-2 transcription factors in regulation of TH gene expression siRNAs were used to selectively knock down these genes (Gueorguiev et al., 2006). First, fluorescent dye-labeled siRNA was used to determine the efficiency of siRNA uptake by transfection. PC12 cells were transfected

with CyTM3-labeled glyceraldehyde-3-phosphate dehydrogenase siRNA. One day after transfection, 90% of the cells were labeled, demonstrating successful uptake of the siRNA (Fig. 3A). Additional preliminary experiments were performed to evaluate the ability of CREB or ATF-2 siRNAs to inhibit the levels of targeted proteins and to select the appropriate siRNA concentration and incubation time. PC12 cells were transfected with CREB

or ATF-2 siRNAs or scrambled RNA, and levels of CREB or ATF-2 proteins were determined by Western blots. After 2 days of transfection, both CREB and ATF-2 protein levels were greatly reduced compared with the controls (Fig. 3B). Therefore, cells were co-transfected with either CREB or ATF-2 siRNAs and TH-Luc(-272/-27). After 2 days, nicotine was added, and cells were further incubated for 16 h. Control cells were transfected with



**Figure 3.** Transfection with CREB or ATF-2 siRNAs inhibited the rise in TH promoter activity induced by nicotine. *A*, PC12 cells were transfected with CyTM3-labeled RNA as a positive control for evaluation of transfection efficiency and fluorescent image is shown (*left panel*). The same field of cells is also shown in phase contrast (*right panel*). *B*, Western blots were used to estimate the levels of CREB or ATF-2 proteins after transfection with siCREB or siATF-2 RNAs for 48 h. *C*, PC12 cells were co transfected with p5\_TH-Luc (-272/-27) and siCREB or siATF-2 RNAs or scrambled RNA as a control. After treatment with nicotine (24 h), luciferase activity was measured and standardized to the respective protein levels. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ , compared with the respective controls. #,  $p < 0.05$ ; ##,  $p < 0.01$  between different groups of nicotine-treated cells.



equal concentrations of scrambled RNA instead of the siRNA. Relative luciferase activity was significantly reduced, indicating that both CREB and ATF-2 transcription factors are required for the nicotine-triggered rise in TH promoter activity (Fig. 3C).

piRNA in the regulation of mammal spermatogenesis. The germ cells represent specialized cell lineage that store and transmits all genomic information from parents to the next generation. The genome stability and integrity of germ cells is of fundamental importance for maintaining phenotypic identity of individuals and species. One of the critical biological factors that induce genome mutations is mobile genetic elements, such as transposons and retrotransposons. A number of miRNAs are expressed abundantly in male germ cells throughout spermatogenesis, while piRNAs are only present in pachytene spermatocytes and round spermatids (He et al. 2009). Recently, a new class of 26-32 nt long small RNA termed as PIWI-interacting RNA (piRNA) was discovered in the germ cells of animals, revealing a distinct small RNA pathway as an innate immune system in germ cells that controls the activity of mobile genetic elements (Aravin et al. 2006; Girard et al. 2006). It is speculated that piRNA pathway may silence transposon and retrotransposon via novel mechanisms of epigenetic and posttranscriptional regulations (Aravin et al. 2007; Carmell et al. 2007; Lin 2007). Piwi proteins, the germ cell-specific Argonaute family proteins, are required for the biogenesis and function of piRNAs and act as the center in piRNA pathways (Aravin et al. 2006). Murine PIWI homologs, Miwi, Mili and Miwi2, are highly expressed in testis and are essential

for spermatogenesis. piRNAs are capable of regulation of gene expression during spermatogenesis. RNAi technology based on application of siRNAs can inhibit the expression of targeted specific genes during germ cell differentiation. It was demonstrated that glial cell line-derived neurotrophic factor receptor (GFRA-1) silencing by Gfra1 siRNAs leads to a switch from renewal to differentiation of mouse SSCs into A1–A4 spermatogonia, an initial step of mouse spermatogenesis (He et al. 2007).

In somatic Sertoli cells, knocking down either Partitioning defective 6 homolog (Par6) or Partitioning defective 3 homolog (Par3) genes by the respective RNAi induced a significant loss of the corresponding protein by approximately 60% in cells vs. controls was detected, alongside with a decline in aPKC after Par6 gene, but not Par3, knockdown (Wong et al. 2008).

## Conclusion

Intensive research in the recent years brought tremendous advancement in our understanding how small RNAs regulate gene expression on translational level. This also gave the modern biology another dimension of epigenetic regulation of the entire genome function. Two critical aspects of miRNAs properties are clearly outlined; first their application as a specific research tool for inhibiting gene expression and second their endogenous existence and regulatory functions in living cells. The use of siRNA technology for silencing specific genes is a powerful new approach to regulate individual gene expression and expression profiles for sets of genes. Functions of siRNAs, miRNAs and piRNAs have provided researchers

with more insights into the regulation of gene expression by different non-coding RNAs.

However, there are still a lot of questions regarding the exact mechanism of how miRNAs choose to silence their respective targets. Various signaling pathways activated in different cell types could lead to modulation of miRNAs specificity regarding some particular target genes. Most of the details regarding transcription and splicing of all different types of small interfering RNAs are largely unknown. Regulation, relative abundance and specificity of miRNAs in different tissues are still elusive and remain to be elucidated. Studies on miRNAs reveal their importance and their critical role in epigenetic regulation of genomic activity.

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