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Review Article

Intronic MicroRNA (miRNA)

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Nearly 97% of the human genome is composed of noncoding DNA, which varies from one species to another. Changes in these sequences often manifest themselves in clinical and circumstantial malfunction. Numerous genes in these non-protein-coding regions encode microRNAs, which are responsible for RNA-mediated gene silencing through RNA interference (RNAi)-like pathways. MicroRNAs (miRNAs), small single-stranded regulatory RNAs capable of interfering with intracellular messenger RNAs (mRNAs) with complete or partial complementarity, are useful for the design of new therapies against cancer polymorphisms and viral mutations. Currently, many varieties of miRNA are widely reported in plants, animals, and even microbes. Intron-derived microRNA (Id-miRNA) is a new class of miRNA derived from the processing of gene introns. The intronic miRNA requires type-II RNA polymerases (Pol-II) and spliceosomal components for their biogenesis. Several kinds of Id-miRNA have been identified in *C elegans*, mouse, and human cells; however, neither function nor application has been reported. Here, we show for the first time that intron-derived miRNAs are able to induce RNA interference in not only human and mouse cells, but in also zebrafish, chicken embryos, and adult mice, demonstrating the evolutionary preservation of intron-mediated gene silencing via functional miRNA in cell and in vivo. These findings suggest an intracellular miRNA-mediated gene regulatory system, fine-tuning the degradation of protein-coding messenger RNAs.

INTRODUCTION

The first microRNA (miRNA) molecules, *lin-4* and *let-7*, were identified in 1993 [1]. Since then there have been rapid advances in small RNA research, with progress in identifying more miRNAs and understanding their biogenesis, functionality and target gene regulation. These early miRNAs were located in the noncoding regions between genes and transcribed by unidentified promoters; these are intergenic miRNAs. Most miRNAs studied at this stage were recognized as intergenic miRNA until 2003, when Ambros et al [1] discovered some tiny noncoding RNAs derived from the intron regions of gene transcripts.

In the meantime, Lin et al [1] demonstrated the biogenetic and gene silencing mechanisms of these intron-derived miRNAs, providing the first functional evidence for a new miRNA category: intronic miRNA. As shown in Table 1, several intronic miRNA molecules have been identified in *C elegans*, mouse, and human genomes [1–3] and some of their functions have been related to RNA interference (RNAi).

Introns occupy the largest proportion of noncoding sequences in the protein-coding DNA of a genome. The transcription of the genomic protein-coding DNA generates precursor messenger RNA (pre-mRNA), which contains four major parts including the 5′-untranslated region (UTR), the protein-coding exon, the noncoding intron, and the 3′-UTR. Broadly speaking, both the 5′- and 3′-UTR can be seen as a kind of intron extension; however, their processing during mRNA translation is different from the intron located between two protein-coding exons, termed the in-frame intron. The in-frame intron can range up to thirty or so kilobases and was initially thought to be a huge genetic waste in gene transcripts. Recently, this misconception was corrected by the observation of intronic miRNA. miRNA is usually about 18–25 oligonucleotides in length and is capable of either directly degrading its intracellular messenger RNA (mRNA) target or suppressing the protein translation of its targeted mRNA, depending on the complementarity between the miRNA and its target. In this way, the intronic miRNA is similar structurally and functionally to the previously described intergenic miRNAs, but differs from them in its unique requirement for Pol-II and RNA splicing components for biogenesis [2, 4, 5]. Approximately 10 ∼ 30% of a spliced intron is exported into the cytoplasm with a moderate half-life [6].

Table 1

miRNA	Species	Host gene (intron) (#)	Target gene(s)
miR-2a, -b2	Worm	Spi	
miR-7b	Mammal	Pituitary gland specific factor 1A (2) [NM174947]	Paired mesoderm homeobox protein 2b; HLHm5
miR-10b	Mammal	Homeobox protein HOX-4 (4)	
miR-11	*Drosophila*	E2F	
miR-13b2	*Drosophila*	CG7033	
miR-15b, -16-2	Mammal	Chromosome-associated polypeptide C	
miR-25, -93, -106b	Mammal	CDC47 homolog (13)	
miR-26a1, -26a2, -26b	Vertebrate	Nuclear LIM interactor-interacting factor 1, 2, 3	
miR-28	Human	LIM domain-containing preferred translocation parterner in lipoma [NM005578]	
miR-30c1, -30e	Mammal	Nuclear transcription factor Y subunit γ (5)	Transcription factor HES-1; PAI-1 mRNA-binding protein
miR-33	Vertebrate	Sterol regulatory element binding protein-2 (15)	RNA-dependent helicase p68; NAG14 protein
miR-101b	Human	RNA 3′-terminal phospate cyclase-like protein (8)	
miR-103, -107	Human	Pantothenate kinase 1, 2, 3	
miR-105-1, -105-2, -224	Mammal	γ-aminobutyric-acid receptor α-3 subunit precursor, epsilon subunit precursor	
miR-126, -126*	Mammal	EGF-like, Notch4-like, NEU1 protein (6) [NM178444]	
miR-128b	Mammal	cAMP-regulated phospho-protein 21 (11)	
miR-139	Mammal	cGMP-dependent 3′,5′-cyclic phosphodiesterase (2)	
miR-140	Human	NEDD4-like ubiquitin-protein ligase WWP2 (15)	
miR-148b	Mammal	Coatomer ζ-1 subunit	
miR-151	Mammal		
miR-152	Human	Coatomer ζ-2 subunit	N-myc proto-oncogene protein; noggin precursor
miR-153-1, -153-2	Human	Protein-tyrosine phosphatase N precursors	
miR-208	Mammal	Myosin heavy chain, cardiac muscle α isoform (28)	
miR-218-1, -218-2	Human	Slit homolog proteins [NM003062]	

RNA interference (RNAi) is a posttranscriptional gene silencing mechanism in eukaryotes, which can be triggered by small RNA molecules such as microRNA (miRNA) and small interfering RNA (siRNA). These small RNA molecules usually function as gene silencers, interfering with intracellular expression of genes either completely or partially complementary to the small RNAs. In principle, siRNAs are double-stranded RNAs capable of degrading target gene transcripts

with almost perfect complementarity [7, 8]. Unlike the stringent complementarity of siRNAs to their RNA targets, miRNAs are single-stranded and able to pair with target RNAs that have partial complementarity to the miRNAs [9, 10]. Numerous natural miRNAs are derived from hairpin-like RNA precursors in almost all eukaryotes, including yeast (*Schizosaccharomyces pombe*), plant (*Arabidopsis spp*), nematode (*Caenorhabditis elegans*), fly (*Drosophila melanogaster*), mouse, and human, functioning as a defense against viral infections and allowing regulation of gene expression during development [11–21]. In contrast, natural siRNAs are abundant in plants and relatively simple animals (worms and flies), but are rarely seen in mammals [10]. Because of the widespread presence of miRNAs in eukaryotes, these small RNAs have recently been used to design novel therapeutics for cancers and viral infections [4, 22]. In fact, gene-silencing mechanisms involving miRNA may be an intracellular defense system for eliminating undesired transgenes and foreign RNAs, such as viral infections and retrotransposon activities [22, 23].

Definition of miRNA: Biogenesis

The definition of intronic miRNAs is based on two factors; first, they must share the same promoter with their encoded target genes, and second, they are spliced out of the transcript of such encoded genes and further processed into mature miRNAs. Although some of the currently known miRNAs are encoded in the genomic intron region of a given gene but in the opposite orientation to the gene transcript (palindrome), such miRNAs are not intronic miRNAs because they neither share the same promoter with the gene nor need to be released from the gene transcript by RNA splicing. For the transcription of such palindromic miRNAs, the promoters are located in the antisense direction to the gene, likely using the gene transcript as a potential target for the antisense miRNA. A good example is *let-7c*, which is an intergenic miRNA located in the antisense region of a gene intron. Current computer programs for miRNA prediction cannot distinguish the intronic miRNA from the intergenic miRNA. Because intronic miRNAs are encoded in the gene transcript precursors (pre-mRNA) and share the same promoter with the encoded gene transcripts, the miRNA prediction programs tend to classify the intronic miRNAs along with the intergenic miRNAs located in the exonic regions. However, in view of their different biogenic mechanisms, these two types of miRNA may have different gene-regulatory functions in the adjustment of cellular physiology. Thus, a miRNA-prediction program utilizing a database of noncoding sequences located in the protein-coding pre-mRNA regions is urgently needed for thoroughly screening and understanding the distribution and variety of hairpin-like intronic miRNAs in the genomes.

The process of miRNA biogenesis in vertebrates involves five steps (Figure 1). First, a long primary precursor miRNA (pri-miRNA) is excised, probably by RNA polymerase type-II (Pol-II) [2, 24]. Second, the long pri-miRNA is further excised by Drosha-like RNase III endonucleases or spliceosomal components, depending on the origin of the pri-miRNA either in an exon or an intron, respectively [2, 25], to form a mature precursor miRNA (pre-miRNA), and third, the pre-miRNA is exported out of the nucleus by Ran-GTP and the receptor Exportin-5 [26, 27]. In the cytoplasm, Dicer-like nucleases cleave the pre-miRNA to form mature miRNA. Lastly, the mature miRNA is incorporated into a ribonuclear particle (RNP), which becomes the RNA-induced gene silencing complex (RISC), capable of executing RNAi-related gene silencing [28, 29]. Although an in vitro model of siRNA-associated RISC assembly has been generated, the link between the final miRNA maturation and RISC assembly remains to be determined. The characteristics of Dicer and RISC are distinctly different in the siRNA and miRNA mechanisms [30]. In zebrafish, we have recently observed that the stem-loop structure of pre-miRNA is involved in strand selection for mature miRNA during RISC assembly. These findings suggest that the duplex structure of siRNA may not be essential for the assembly of miRNA-associated RISC in vivo. The biogeneses of miRNA and siRNA seem to be very similar; however, the miRNA mechanisms previously proposed were based on the model of siRNA. In contrast, it will be necessary to distinguish the individual properties and differences in these two types of RNAs in order to understand the evolutionary and functional relationship of these gene-silencing pathways. In addition, the differences may provide a clue for understanding the prevalence of native siRNAs in invertebrates compared to that in mammals.

The proposed research will generate data from several transgenic zebrafish lines. It is our explicit intention that these data will be submitted in a readily accessible public database in the ZFIN website. All efforts will be made to rapidly release data through publication of results as quickly as possible to analyze the experiments. Data used in publications will be released in a timely manner. ZFIN data will be made accessible through a public site that allows querying as has been set up for a similar project.

Intronic miRNA and disease

The majority of human gene transcripts contain introns, phylogenetically conserved to a greater or lesser degree. Changes in these non-protein-coding sequences are frequently observed in clinical malfunction such as myotonic dystrophy and fragile X syndrome.

Numerous introns encode miRNAs which are involved in RNAi-related chromatin silencing mechanisms. Over 90 intronic miRNAs have been identified using the bioinformatic approaches to date, but the function of the vast majority of these molecules remains to be determined [3]. According to the strictly expressive correlation of intronic miRNAs with their encoded genes, one may speculate that the levels of condition-specific, time-specific, and individual-specific gene expression are determined by the influences of distinctive miRNAs on single or multiple gene modulation. This interpretation accounts for the heterogeneity of

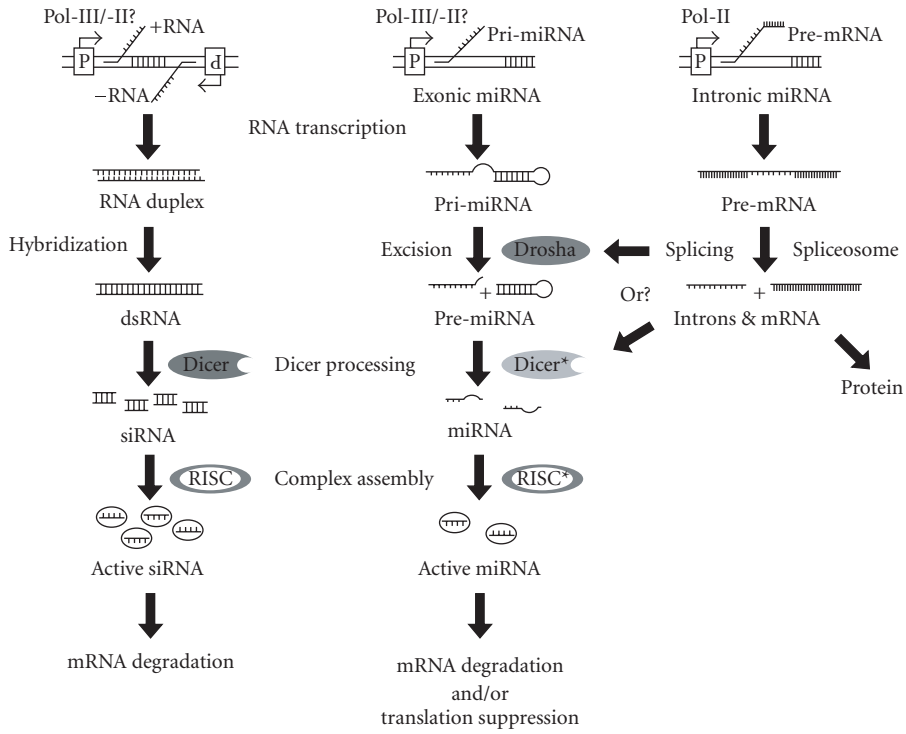

Figure 1: Comparison of biogenesis and RNAi mechanisms among siRNA, intergenic (exonic) miRNA, and intronic miRNA. siRNA is likely formed by two perfectly complementary RNAs transcribed from two different promoters (remaining to be determined) and further processing into 19–22 bp duplexes by the RNase III-familial endonuclease, Dicer. The biogenesis of intergenic miRNAs, for example, *lin-4* and *let-7*, involves a long transcript precursor (pri-miRNA), which is probably generated by Pol-II or Pol-III RNA promoters, while intronic miRNAs are transcribed by the Pol-II promoters of its encoded genes and coexpressed in the intron regions of the gene transcripts (pre-mRNA). After RNA splicing and further processing, the spliced intron may function as a pri-miRNA for intronic miRNA generation. In the nucleus, the pri-miRNA is excised by Drosha RNase to form a hairpin-like pre-miRNA template and then exported to the cytoplasm for further processing by Dicer* to form mature miRNA. The Dicers for siRNA and miRNA pathways are different. All three small regulatory RNAs are finally incorporated into an RNA-induced silencing complex (RISC), which contains either the strand of siRNA or the single-strand of miRNA. The action of miRNA is considered to be more specific and less adverse than that of siRNA because only one strand is involved. siRNA primarily triggers mRNA degradation, whereas miRNA can induce either mRNA degradation or suppression of protein synthesis depending on the sequence complementarity to the target gene transcripts.

genetic expression of various traits; dysregulation will result in genetic disease. For instance, monozygotic twins frequently demonstrate slight, but definitely distinguishing, differences in disease susceptibility and behavior. For example, a long CCTG expansion in intron 1 of the zinc finger protein ZNF9 gene has been correlated with type 2 myotonic dystrophy in whichever twin exhibits the higher susceptibility [31]. Since the expansion motif bound with high affinity to certain RNA-binding proteins, an interfering role of intron-derived expansion fragments is suggested. Another more-established example involving pathogenic intronic expansion fragments is fragile X syndrome, which accounts for about 30% of human inherited mental retardation. Intronic CGG repeat (rCGG) expansion in the 5′-UTR of the *FMR1* gene is the causative mutation in 99% of individuals with fragile X syndrome [32]. *FMR1* encodes an RNA-binding protein, FMRP, which is associated with polyribosome assembly in an RNP-dependent manner and is capable of suppressing translation through an RNAi-like pathway. FMRP also contains a nuclear localization signal (NLS) and a nuclear export signal (NES) for shuttling certain mRNAs between the nucleus and cytoplasm [33]. Jin et al proposed that RNAi-mediated methylation occurs in the CpG region of the *FMR1* rCGG expansion, which is targeted by a hairpin RNA derived from the 3′-UTR of the *FMR1* expanded allele transcript [32]. The Dicer-processed hairpin RNA triggers the formation of an RNA-induced initiator of transcriptional gene silencing (RITS) on the homologous rCGG sequences and leads to heterochromatin repression of the *FMR1* locus. These examples suggest that natural evolution gives rise to more intronic complexity and variety in higher animals and plants, allowing the coordination of their vast gene expression libraries and interactions. Any dysregulation of miRNA derivation from introns may then lead to genetic disease involving intronic expansion or deletion, such as myotonic dystrophy and fragile X mental retardation.

Man-made intronic miRNA

To understand the disease caused by the dysregulation of intronic miRNA, an artificial expression system is needed to recreate the function and mechanism of miRNA in vitro and in vivo. The same approach may be used to design and develop therapies. Several vector-based RNAi expression systems have been developed, using type-III RNA polymerase (Pol-III)-directed transcription activities, to generate more stable RNAi efficacy and lower interferon-related toxicity in several cell lines in vitro [34–37]. For gene therapy in vivo, a functional gene is preferably delivered into an animal or human being by expression-competent vector vehicles, such as retroviral vector, lentiviral vector, adenoviral vector, and adenoassociated viral (AAV) vector. The main purpose of these vector-based approaches is to maintain long-term and consistent gene modulation. Although some studies [38, 39] attempting to use the Pol-III-directed RNAi system have succeeded in maintaining constant gene silencing efficacy in vivo, their delivery strategies failed to target a specific cell population due to the ubiquitous existence of Pol-III activity in all cell types. Moreover, the requirement of using Pol-III RNA promoters, for example, U6 and H1, for small RNA expression is another problem. Because the read-through side-effect of Pol-III occurs on a short transcription template in the absence of proper termination, large RNA products longer than the desired 18–25 base pairs (bp) can be synthesized and cause unexpected interferon cytotoxicity [40, 41]. Such a problem can also result from competition between the Pol-III promoter and another vector promoter (ie, LTR and CMV promoters). We and others [42] have found that a high dosage of siRNA (eg, > 250 nM in human T cells) caused strong cytotoxicity similar to that of long double-stranded dsRNA [42, 43]. This toxicity is due to the double-stranded structure of siRNA and dsRNA, which activates interferon-mediated nonspecific RNA degradation and programmed cell death through signaling via the PKR and 2–5A systems. It is well known that the interferon-induced protein kinase PKR can trigger cell apoptosis, while activation of the interferon-induced 2′, 5′-oligoadenylate synthetase (2–5A) system leads to extensive cleavage of single-stranded RNAs (ie, mRNAs) [44]. Both the PKR and 2–5A systems contain dsRNA-binding motifs which are highly conserved, but these motifs do not bind either single-strand RNAs or RNA-DNA hybrids. These disadvantages limit the use of Pol-III-based RNAi vector systems for therapeutic purposes.

The intron-derived miRNA system is activated in a specific cell type under the control of type-II RNA polymerases (Pol-II)-directed transcriptional machinery. To overcome Pol-III-mediated siRNA side effects, we have successfully developed a novel Pol-II-based miRNA biogenesis strategy, employing intronic miRNA molecules [2] to knock down more than 85% of selected oncogene function or viral genome replication [45, 46]. Because of the flexibility in binding to partially complementary mRNA targets, miRNA can serve as an anticancer drug or vaccine, a major breakthrough in the treatment of cancer polymorphisms and viral mutations. We are the first research group to discover the biogenesis of miRNA-like precursors from the 5′-proximal intron regions of gene transcripts (pre-mRNA) produced by the mammalian Pol-II. Depending on the promoter of the miRNA-encoded gene transcript, intronic miRNA is coexpressed with its encoding gene in a specific cell population, which activates the promoter and expresses the gene. It has been noted that a spliced intron was not completely digested into monoribonucleotides for transcriptional recycling since approximately 10–30% of the intron was found in the cytoplasm with a moderate half life [6, 47]. This type of miRNA generation relies on the coupled interaction of nascent Pol-II-mediated pre-mRNA transcription and intron excision, occurring within certain nuclear regions proximal to genomic perichromatin fibrils [46, 48, 49]. After Pol-II RNA processing and splicing excision, some of the intron-derived miRNA fragments can form mature miRNAs and effectively silence the target genes through the RNAi mechanism, while the exons of pre-mRNA are ligated together to form a mature mRNA for protein synthesis (Figure 2(a)) [2]. Because miRNAs are single-stranded molecules insensitive to PKR- and 2–5A-induced interferon systems, the Pol-II-mediated miRNA generation can avoid the cytotoxic effects of dsRNA and siRNA in vitro and in vivo. These findings indicate new functions for mammalian introns in intracellular miRNA generation and gene silencing, which can be used both as tools for the analysis of gene functions and the development of gene-specific therapeutics against cancers and viral infections.

Using artificial introns carrying hairpin-like miRNA precursors (pre-miRNA), we have successfully generated mature miRNA molecules with full capacity for triggering RNAi-like gene silencing in human prostate cancer LNCaP, human cervical cancer HeLa, and rat neuronal stem HCN-A94-2 cells [2, 45]. As shown in Figure 2(b), the artificial intron (SpRNAi) was cotranscribed within a precursor messenger RNA (pre-mRNA) by Pol-II and cleaved out of the pre-mRNA by RNA splicing. Then the spliced intron containing the pre-miRNA was further processed into mature miRNA capable of triggering RNAi-related gene-silencing effects. Utilizing this artificial miRNA model, we have tested various pre-miRNA constructs, and observed that the production of intron-derived miRNA fragments originated from the 5′-proximity of the intron sequence between the 5′-splice site and the branching point. These miRNAs were able to trigger strong suppression of genes possessing more than 70% complementarity to the miRNA sequences, whereas nonhomologous miRNA intron, that is, empty intron without the pre-miRNA insert, with an off-target miRNA insert (negative control) and splicing-defective intron, showed no silencing effects on the targeted gene. The same results can also be reproduced in the zebrafish by directing the miRNA against target EGFP expression (Figure 2(c)), indicating the consistent preservation of the intronic miRNA biogenesis system in vertebrates. Furthermore, no effect was detected on off-target genes, such as RGFP and β-actin, suggesting the high specificity of miRNA-directed RNA interference (RNAi). We have confirmed the identity of the intron-derived miRNA, which comprised about 18–25 nucleotides

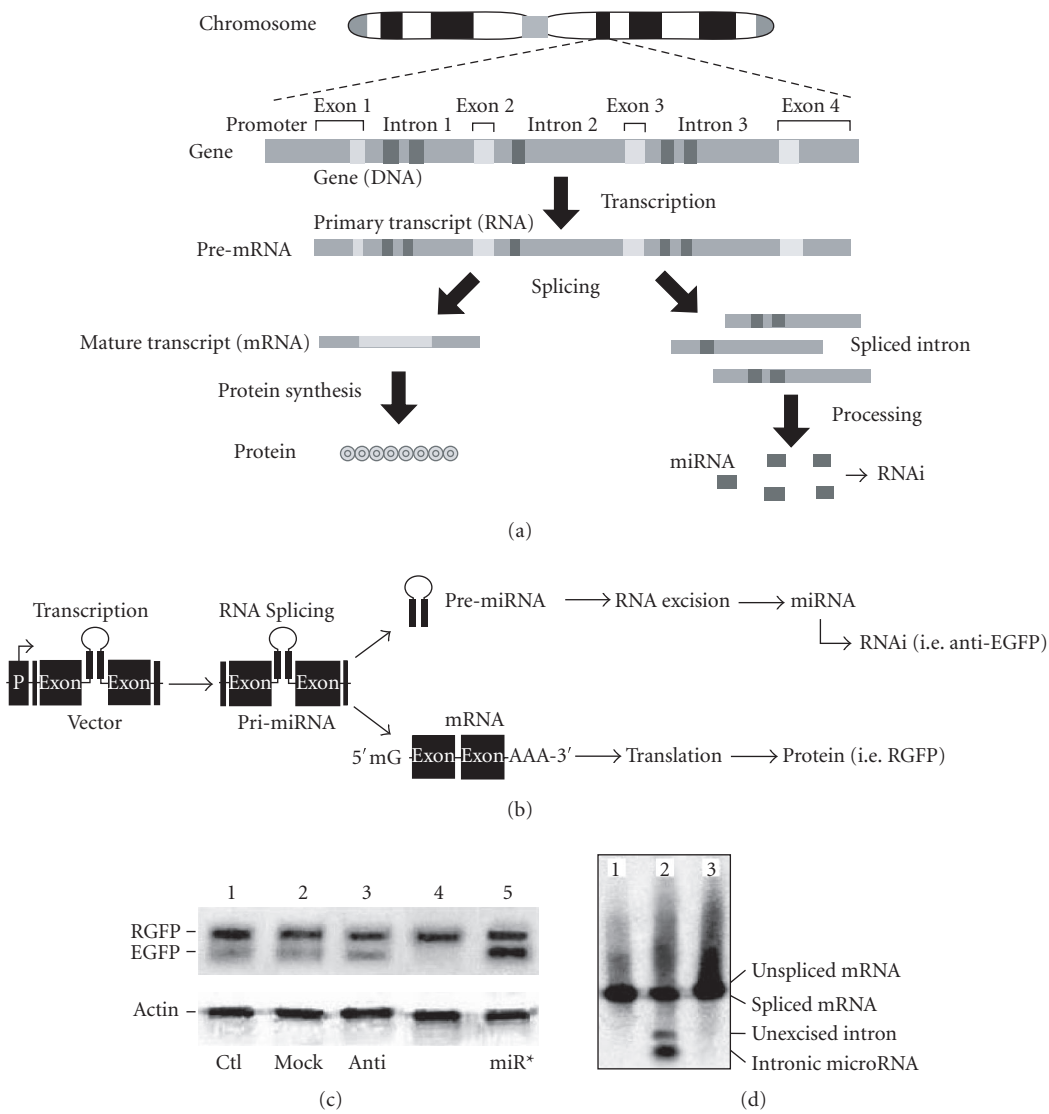

Figure 2: Biogenesis and function of intronic miRNA. (a) The native intronic miRNA is cotranscribed with a precursor messenger RNA (pre-mRNA) by Pol-II and cleaved out of the pre-mRNA by an RNA splicing machinery, the spliceosome. The spliced intron with hairpin-like secondary structure is further processed into mature miRNA capable of triggering RNAi effects, while the ligated exons become a mature messenger RNA (mRNA) for protein synthesis. (b) We designed an artificial intron containing pre-miRNA, namely SpRNAi, mimicking the biogenesis of the native intronic miRNA. (c) When a designed miR-EGFP(280–302)-stemloop RNA construct was tested in the EGFP-expressing Tg(UAS:gfp) zebrafish, we detected a strong RNAi effect only on the target EGFP (lane 4). No detectable gene-silencing effect was observed in other lanes; from left to right: 1, blank vector control (Ctl); 2, miRNA-stemloop targeting HIV-p24 (mock); 3, miRNA without stemloop (anti); and 5, stemloop-miRNA* complementary to the miR-EGFP(280–302) sequence (miR*). The off-target genes, such as vector RGFP and fish actin, were not affected, indicating the high target specificity of miRNA-mediated gene silencing. (c) Three different miR-EGFP(280–302) expression systems were tested for miRNA biogenesis; from left to right: 1, vector expressing intron-free RGFP, no pre-miRNA insert; 2, vector expressing RGFP with an intronic 5′-miRNA-stemloop-miRNA*-3′ insert; and 3, vector similar to the 2 construct but with a defected 5′-splice site in the intron. In Northern blot analysis probing the miR-EGFP(280–302) sequence, the mature miRNA was released only from the spliced intron resulting from the vector 2 construct in the cell cytoplasm.

(nt), approximately the length of the newly identified intronic miRNAs in *C elegans*. Moreover, the intronic small RNAs isolated by guanidinium-chloride ultracentrifugation can elicit strong, but short-lived, gene-silencing effects on the homologous genes in transfected cells, indicating a reversible RNAi effect. Thus, the long-term (> 1 month) gene-silencing effect that we observed in vivo, using the Pol-II-mediated intronic miRNA system, is likely maintained by constitutive miRNA production from the vector rather than the stability of the miRNA.

The components of the Pol-II-mediated SpRNAi system include several consensus nucleotide elements consisting of

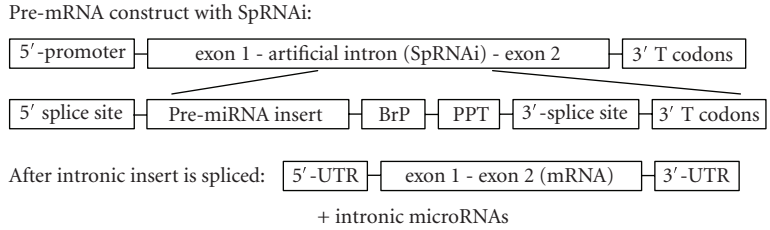

Figure 3: Schematic construct of the artificial SpRNAi intron in a recombinant gene SpRNAi-RGFP for intracellular expression and processing. The components of the Pol-II-mediated SpRNAi system include several consensus nucleotide elements consisting of a 5′-splice site, a branch-point domain (BrP), a poly-pyrimidine tract (PPT), a 3′-splice site, and a pre-miRNA insert located between the 5′-splice site and the BrP domain. The expression of the recombinant gene is under the regulation of either a mammalian Pol-II RNA promoter or a compatible viral promoter for cell-type-specific effectiveness. Mature miRNA molecules are released from the intron by RNA splicing and further Dicer processing.

a 5′-splice site, a branch-point domain, a poly-pyrimidine tract, and a 3′-splice site (Figure 3). Additionally, a pre-miRNA insert sequence is placed within the artificial intron between the 5′-splice site and the branch-point domain. This portion of the intron would normally form a lariat structure during RNA splicing and processing. We now know that spliceosomal U2 and U6 snRNPs, both helicases, may be involved in the unwinding and excision of the lariat RNA fragment into pre-miRNA; however, the detailed processing remains to be elucidated. Further, the SpRNAi contains a translation stop codon domain (T codon) in its 3′-proximal region to facilitate the accuracy of RNA splicing which, if present in a cytoplasmic mRNA, would signal the diversion of a splicing-defective pre-mRNA to the nonsense-mediated decay (NMD) pathway and thus cause the elimination of any unspliced pre-mRNA in the cell. For intracellular expression of the SpRNAi, we needed to insert the SpRNAi construct into the *Dra*II cleavage site of a red fluorescent membrane protein (RGFP) gene from mutated chromoproteins of coral reef *Heteractis crispa*. The cleavage of RGFP at its 208th nucleotide site by the restriction enzyme *Dra*II generates an AGGN nucleotide break with three recessing nucleotides at each end, which forms 5′ and 3′ splice sites, respectively, after the SpRNAi insertion. Because this intronic insertion disrupts the expression of functional RGFP, it becomes possible to determine the occurrence of intron splicing and RGFP-mRNA maturation through the appearance of red fluorescent emission around the membrane surface of the transfected cells. The RGFP also provides multiple exonic splicing enhancers (ESEs) to increase RNA splicing efficiency.

Intron-mediated gene silencing in zebrafish

The foregoing discussion establishes the fact that intronic miRNAs are an effective strategy for silencing specific target genes in vivo. We first tried to determine the structural design of pre-miRNA inserts for the best gene-silencing effect. We found that a strong structural bias exists for the selection of a mature miRNA strand during the assembly of the RNAi effector, the RNA-induced gene silencing complex (RISC). RISC is a protein: RNA complex that directs either target gene transcript degradation or translational repression through the RNAi mechanism. Formation of siRNA duplexes plays a key role in the assembly of the siRNA-associated RISC. The two strands of the siRNA duplex are functionally asymmetric, but the assembly into the RISC complex is preferential for only one strand. Such preference is determined by the thermodynamic stability of each 5′-end base-pairing in the strand. Based on this siRNA model, the formation of miRNA and its complementary miRNA (miRNA*) duplex was thought to be an essential step for the assembly of miRNA-associated RISC. If this were true, no functional bias would be observed in the stemloop of a pre-miRNA. Nevertheless, we observed that the stemloop of the intronic pre-miRNA was involved in the strand selection of a mature miRNA for RISC assembly in zebrafish. In these experiments, we constructed miRNA-expressing SpRNAi-RGFP vectors as previously described [2] and two symmetric pre-miRNAs, miRNA-stemloop-miRNA* (1) and miRNA*-stemloop-miRNA (2), were synthesized and inserted into the vectors, respectively. Both pre-miRNAs contained the same double-stranded stem arm region, which was directed against the EGFP nt 280–302 sequence. Because the intronic insert region of the SpRNAi-RGFP recombined gene is flanked with a *PvuI* and an *MluI* restriction site at the 5′- and 3′-ends, respectively, the primary insert can be easily removed and replaced by various gene-specific inserts (eg, anti-EGFP) possessing cohesive ends. By allowing a change in the pre-miRNA inserts directed against different gene transcripts, this intronic miRNA generation system provides a valuable tool for genetic and miRNA-associated research in vivo.

To determine the structural preference of the designed pre-miRNA, we have isolated the zebrafish small RNAs by mirVana miRNA isolation columns (Ambion, Austin, TX) and then precipitated all potential miRNAs complementary to the target EGFP region by latex beads containing the target RNA sequence. One full-length miRNA, miR-EGFP(280–302), was active in the transfections of the 5′-miRNA-stemloop-miRNA*-3′ construct, as shown in Figure 4(a) (gray-shading sequences). Since the mature miRNA was detected only in the zebrafish transfected by the 5′-miRNA-stemloop-miRNA*-3′ construct, the miRNA-associated RISC tends to preferably interact

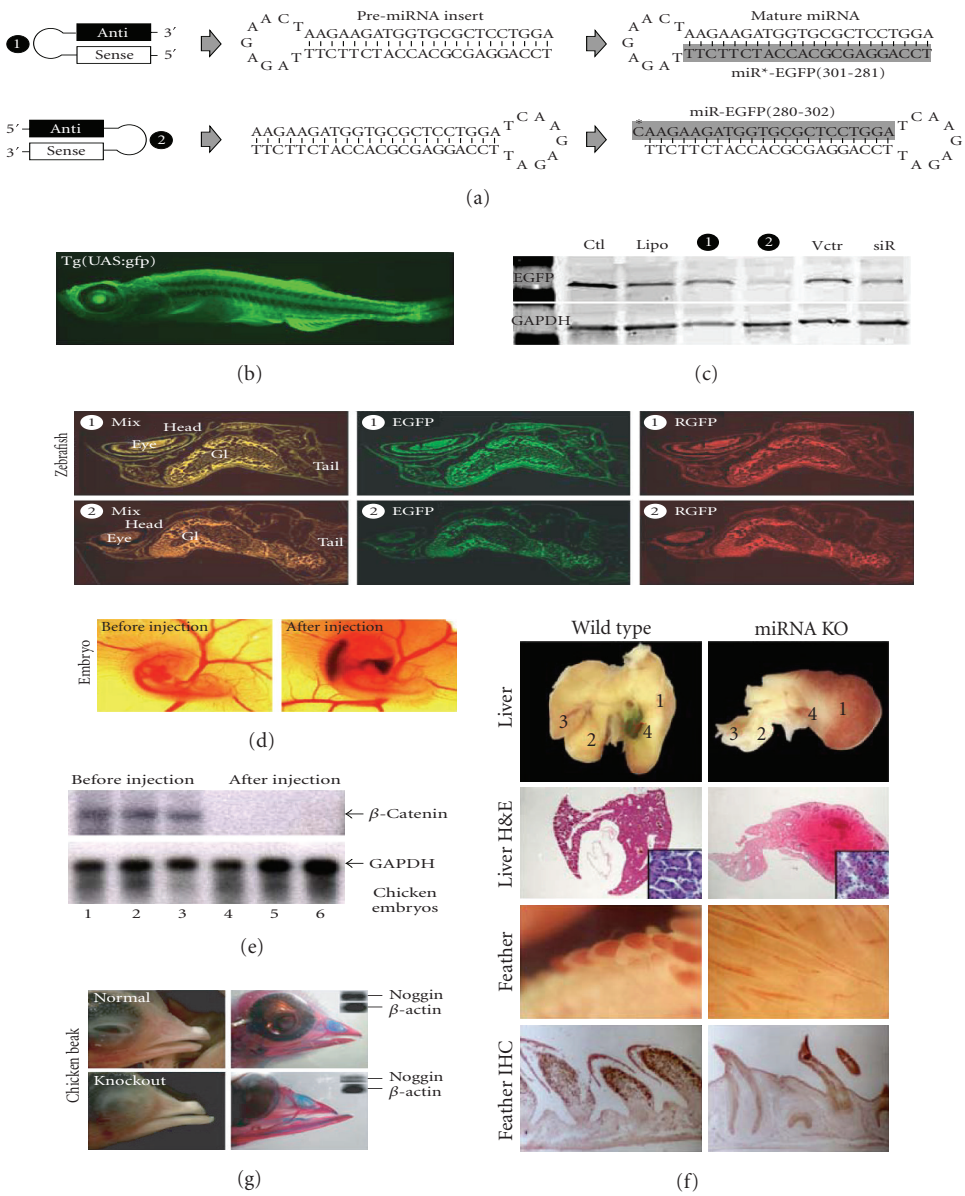

Figure 4: Intronic miRNA-mediated gene silencing effects in vivo. (a)–(c) Different preferences for RISC assembly were observed following the transfection of 5′-miRNA*-stemloop-miRNA-3′ (1) and 5′-miRNA-stemloop-miRNA*-3′ (2) pre-miRNA structures in zebrafish, respectively. (a) One mature miRNA, namely miR-EGFP(280/302), was detected in the (2)-transfected zebrafish, whereas the (1)-transfection produced another kind of miRNA, miR*-EGFP(301–281), which was partially complementary to the miR-EGFP(280/302). (b) The RNAi effect was only observed with the transfection of the (2) pre-miRNA, showing less EGFP (green) in (2) than in (1), while the miRNA indicator RGFP (red) was equally present in all vector transfections. (c) Western blot analysis of the EGFP protein levels confirmed the specific silencing result of (b). No detectable gene silencing was observed in fish without (Ctl) and with liposome only (Lipo) treatments. The transfection of either a U6-driven siRNA vector (siR) or an empty vector (Vctr) without the designed pre-miRNA insert resulted in no significant gene silencing. (d)–(g) Silencing of endogenous β-catenin and noggin genes in chicken embryos. (d) The pre-miRNA construct and fast green dye mixtures were injected into the ventral side of chicken embryos near the liver primordia below the heart. (e) Northern blot analysis of extracted RNAs from chicken embryonic livers with anti-β-catenin miRNA transfections (lanes 4–6) in comparison with wild types (lanes 1–3) showed a more than 98% silencing effect on β-catenin mRNA expression, while the house-keeping gene, GAPDH, was not affected. (f) Liver formation of the β-catenin knockouts was significantly hindered (upper right 2 panels). Microscopic examination revealed a loose structure of hepatocytes, indicating the loss of cell-cell adhesion due to breaks in adherents junctions formed between β-catenin and cell membrane E-cadherin in early liver development. In severely affected regions, feather growth in the skin close to the injection area was also inhibited (lower right 2 panels). Immunohistochemical staining for β-catenin protein (brown) showed a significant decrease in the feather follicle sheaths. (g) Lower beak development was increased by the mandibular injection of the antinoggin pre-miRNA construct (lowerpanel) in comparison to wild type (upper panel). Right panels showed bone (alizarin red) and cartilage (alcian blue) staining to demonstrate the outgrowth of bone tissues in the lower beak of the noggin knockout. Northern blot analysis (small windows) confirmed a $\sim$ 60% decrease of noggin mRNA expression in the lower beak area.

with the construct (2) rather than the (1) pre-miRNA. The green fluorescent protein EGFP expression was constitutively driven by the β-actin promoter located in almost all cell types of the zebrafish, while Figure 4(b) shows that transfection of the SpRNAi-RGFP vector into the Tg(UAS:gfp) zebrafish coexpressed the red fluorescent protein RGFP, serving as a positive indicator for miRNA generation in the transfected cells. This approach has been successfully used in several mouse and human cell lines to show RNAi effects [2, 45]. We applied the liposome-encapsulated vector (total 60 μg) to the fish and found that the vector easily penetrated almost all tissues of the two-week-old zebrafish larvae within 24 hours, providing fully systemic delivery of the miRNA effect. The indicator RGFP was detected in both of the fish transfected by either 5′-miRNA*-stemloop-miRNA-3′ or 5′-miRNA-stemloop-miRNA*-3′ pre-miRNA, whereas the silencing of target EGFP expression (green) was observed only in the fish transfected by the 5′-miRNA-stemloop-miRNA*-3′ pre-miRNA (Figures 4(b)–4(c)). The suppression level in the gastrointestinal (GI) tract was somewhat lower, probably due to the high RNase activity in this region. Because thermostability in the 5′ end of the siRNA duplexes resulting from both of the designed pre-miRNA molecules is the same, we suggest that the stemloop of pre-miRNA is involved in strand selection of mature miRNA during RISC assembly. Given that the cleavage site of Dicer in the stem arm determines the strand selection of mature miRNA [25], the stemloop may function as a determinant for the recognition of a special cleavage site. Therefore, the heterogeneity of stemloop structures among various species may help to explain the prevalence of native miRNA in vertebrates over invertebrates.

Intron-mediated gene silencing in chicken embryos

The in vivo model of chicken embryos has been widely utilized in developmental biology, signal transduction, and flu vaccine development. We have successfully demonstrated the feasibility of localized gene silencing in vivo by the intronic miRNA approach and also discovered that the interaction between pre-mRNA and genomic DNA may be essential for miRNA biogenesis. As an example, the β-catenin gene was selected because its products play a critical role in development [50]. β-catenin is involved in the growth control of skin and liver tissues in chicken embryos. The loss-of-function of β-catenin is lethal in transgenic animals. As shown in Figures 4(d)–4(g), experimental results demonstrated that the miRNAs derived from a long mRNA-DNA hybrid construct ($\geq$ 150 bp) were capable of inhibiting β-catenin gene expression in the liver and skin of developing chicken embryos. Homologous recombination between the intronic miRNA and genomic DNA may account for a part of the specific gene-silencing effect [46]. We have demonstrated that the [P^{32}]-labeled DNA component of an mRNA-DNA duplex construct in cell nuclear lysates was intact during the effective period of miRNA-induced RNA interference (RNAi) phenomena, while the labeled RNA component was replaced by cold homologues and excised into small RNA fragments within a 3-day incubation period. Since intronic miRNA generation relies on a coupled interaction of nascent Pol-II-directed pre-mRNA transcription and intron excision occurring proximal to genomic perichromatin fibrils, the above observation indicates that pre-mRNA-genomic DNA recombination may facilitate new miRNA generation by Pol-II RNA transcription and excision for relatively long-term gene silencing. Alternatively, Pol-II may function as an RNA-dependent RNA polymerase (RdRp) for producing small interfering RNAs, since mammalian Pol-II possesses RdRp activities [51, 52]. Thus, it appears that Pol-II-mediated RNA generation and excision is involved in both mRNA-DNA-derived and intron-derived miRNA biogenesis, resulting in single-stranded small RNAs of about 20 nt, comparable to the usual sizes of Dicer-processed miRNAs as observed in the regulation of numerous developmental events.

In an effort to test the pre-mRNA and genome interaction theory, we performed an intracellular transfection of the mRNA-DNA hybrid construct containing a hairpin anti-β-catenin pre-miRNA, which was directed against the central region of the β-catenin coding sequence (aa 306–644) with perfect complementarity. A perfectly complementary miRNA theoretically directs target mRNA degradation more efficiently than translational repression. Using embryonic day 3 chicken embryos, a dose of 25 nM of the pre-miRNA construct was injected into the ventral body cavity, which is close to where the liver primordia would form (Figure 4(d)). For efficient delivery into target tissues, the pre-miRNA construct was mixed with the DOTAP liposomal transfection reagent (Roche Biomedicals, Indianapolis, IN) at a ratio of 3:2. A 10% (v/v) fast green solution was concurrently added during the injection as a dye indicator. The mixtures were injected into the ventral side near the liver primordia below the heart using heat pulled capillary needles. After injection, the embryonic eggs were sealed with sterilized scotch tape and incubated in a humidified incubator at 39–40° C until day 12 when the embryos were examined and photographed under a dissection microscope. Several malformations were observed, although the embryos survived without visible overt toxicity or overall perturbation of embryo development. The liver was the closest organ to the injection site and its phenotype was most dramatically affected. Other regions, particularly the skin close to the injection site, were also affected by the diffused miRNA. As shown in Figure 4(e), Northern blot analysis for the targeted β-catenin mRNA expression in the dissected livers showed that β-catenin expression in the wild-type livers remained normal (lanes 1–3), whereas expression in the miRNA-treated samples was decreased dramatically (lanes 4–6). miRNA silencing degraded more than 98% of β-catenin mRNA expression in the embryonic chicken, but had no effect on the house-keeping gene GAPDH expression, indicating high target specificity and very limited interferon-related cytotoxicity in vivo for the miRNA construct.

After ten days of primordial injection with the anti-β-catenin pre-miRNA template, the embryonic chicken livers showed enlarged and engorged first lobes, but the sizes of the second and third lobes of the livers were dramatically

decreased (Figure 4(f)). Histological sections of normal livers showed hepatic cords and sinusoidal space with few blood cells. In the anti-β-catenin miRNA-treated embryos, the general architecture of the hepatic cells in lobes 2 and 3 remained unchanged; however, there were islands of abnormality in lobe 1. Endothelial development appeared to be defective and blood leaked from the blood vessels. Abnormal hematopoietic cells were also observed between hepatocytes, particularly dominated by a population of small cells with round nuclei and scanty cytoplasm. In severely affected regions, hepatocytes were disrupted (Figure 4(f), small windows) and the diffused miRNA also inhibited feather growth in the skin area close to the injection site. The results showed that the anti-β-catenin miRNA was very effective in knocking out targeted gene expression at a very low dose of 25 nM over a long period of time ($\geq$ 10 days). Further, the miRNA gene-silencing effect appeared to be very specific as off-target organs appeared normal, indicating that the small single-stranded miRNA herein produced no generalized toxicity. In an attempt to silence noggin expression in the mandible beak area using the same approach (Figure 4(g)), an enlarged lower beak morphology was produced similar to what is seen in BMP4-overexpressing chicken embryos [53, 54]. Skeleton staining showed outgrowth of bone and cartilage tissues in the injected mandible area (Figure 4(g), right panels) and Northern blot analysis further confirmed that about 60% of noggin mRNA expression was knocked out in this region (small windows). Since bone morphogenetic protein 4 (BMP4), a member of the transforming growth factor-β (TGF-β) superfamily, is known to promote bone development and since noggin is an antagonist of BMP2/4/7 genes, it is not surprising to find that our miRNA-mediated noggin knockouts exhibited a morphological change resembling the effects of BMP4-overexpression as reported in chicken and other avian models. Thus, gene silencing in the chicken by miRNA transfection has a great potential for localized transgene-like manipulation in developmental biology.

Development of miRNA therapy

The following experiments demonstrate silencing exogenous retrovirus replication in an ex vivo cell model of patient-extracted $CD4^+$ T lymphocytes. Specific anti-HIV SpRNAi-RGFP vectors were designed to target the *gag-pol* region from approximately nt +2113 to +2450 of the HIV-1 genome. This region is relatively conserved and can serve as a good target for anti-HIV treatment [55]. The viral genes located in this target region include 3′-proximal $Pr55^{gag}$ polyprotein (ie, matrix p17 + capsid p24 + nucleocapsid p7) and 5′-proximal $p66/p51^{pol}$ polyprotein (ie, protease p10 + reverse transcriptase); all these components have critical roles in viral replication and infectivity. During the early infection phase, the viral reverse transcriptase transcribes the HIV RNA genome into a double-stranded cDNA sequence, which forms a preintegration complex with the matrix, integrase, and viral protein R (Vpr). This complex is then transferred to the cell nucleus and integrated into the host chromosome, consequently establishing the HIV provirus. We hypothesized that, although HIV carries few reverse transcriptase and matrix proteins during its first entry into host cells, the co-suppression of $Pr55^{gag}$ and $p66/p51^{pol}$ gene expression by miRNAs would eliminate the production of infectious viral particles in the late infection phase. Silencing $Pr55^{gag}$ may prevent the assembly of intact viral particles due to the lack of matrix and capsid proteins, while suppression of protease in $p66/p51^{pol}$ can inhibit the maturation of several viral proteins. HIV expresses about nine viral gene transcripts which encode at least 15 various proteins; thus, the separation of a polyprotein into individual functional proteins requires the viral protease activity. As shown in Figure 5, this therapeutic approach is feasible [22, 43].

The anti-HIV SpRNAi-RGFP vectors were tested in $CD4^+$ T lymphocyte cells from HAART-treated, HIV-seropositive patients. Because only partial complementarity between miRNA and its target RNA is needed to trigger the gene silencing effect, this approach may be superior to current small molecule drugs since the high rate of HIV mutations often produce resistance to such agents. Northern blot analysis in Figure 5(a) demonstrated the ex vivo gene silencing effect of anti-HIV miRNA transfection ($n = 3$ for each set) on HIV-1 replication in $CD4^+$ T lymphocytes from both acute and chronic phase AIDS patients. In the acute phase ($\leq$ one month), the 50 nM miRNA vector transfection degraded an average of 99.8% of the viral RNA genome (lane 4), whereas the same treatment knocked down only an average of $71.4 \pm 12.8\%$ of viral genome replication in the chronic phase (about a 2-year infection). Immunocytochemical staining for HIV p24 marker protein confirmed the results of Northern blot analysis (Figure 5(b)). Sequencing analysis has revealed at least two HIV-1b mutations in the acute phase and seven HIV-1b mutations in the chronic phase within the targeted HIV genome domain. It is likely that the higher genome complexity produced by HIV mutations in chronic infections reduces miRNA-mediated silencing efficacy. Transfection of 50 nM miRNA* vector homologous to the HIV-1 genome failed to induce any RNAi effect on the viral genome, indicating the specificity of the miRNA effect (lane 5). Expression of the cellular house-keeping gene, β-actin, was normal and showed no interferon-induced nonspecific RNA degradation. These results suggest that the designed anti-HIV SpRNAi-RGFP vector is highly specific and efficient in suppressing HIV-1 replication in early infections. In conjunction with an intermittent interleukin-2 therapy [55], the growth of noninfected $CD4^+$ T lymphocytes may be stimulated to eliminate the HIV-infected cells.

CONCLUSION

The consistent evidence of miRNA-induced gene silencing effects in zebrafish, chicken embryos, mouse stem cells, and human disease demonstrates the preservation of an ancient intron-mediated gene regulation system in eukaryotes. In these animal models, the intron-derived miRNA produces an RNAi-like gene silencing effect. We herein provide the first

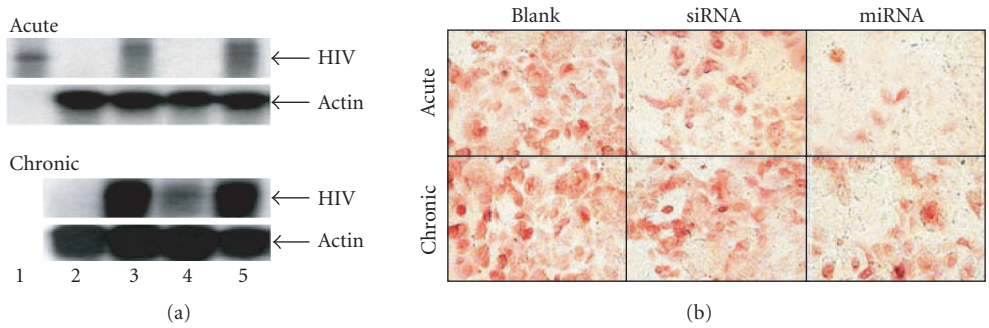

FIGURE 5: Silencing of HIV-1 genome replication using anti-*gag/pro/pol* miRNA transfection into $CD4^+$ T lymphocytes isolated from the acute and chronic phases of AIDS infections. (a) Northern blot analysis showed about 98% and 70% decreases of HIV genome in the acute and chronic infections after miRNA treatments (lane 4), respectively. No effect was detected in the T cells transfected with miRNA* targeting the same *gag/pro/pol* region of the viral genome (lane 5). The size of pure HIV-1 provirus was about 9,700 nucleotide bases (lane 1). RNA extracts from normal noninfected $CD4^+$ T lymphocytes were used as a negative control (lane 2), whereas those from HIV-infected T cells were used as a positive control (lane 3). (b) Immunostaining for HIV p24 marker confirmed the results in (a). Since the ex vivo HIV-silenced T lymphocytes were resistant to any further infection by the same strains of HIV, they may be transfused back to the donor patient for eliminating HIV-infected cells.

evidence for the biogenesis and function of intronic miRNA in vivo. Given that evolution has given rise to more complexity and more variety of introns in higher animal and plant species for the task of coordinating their vast gene expression libraries and interactions, dysregulation of these miRNAs due to intronic expansion or deletion will likely cause genetic diseases, such as myotonic dystrophy and fragile X mental retardation. Thus, gene expression produces not only gene transcripts for its own protein synthesis but also intronic miRNA, capable of interfering with the expression of other genes. Thus, the expression of a gene results in gain-of-function of the gene and also loss-of-function of other genes, with complementarity to the mature intronic miRNA. An array of genes can swiftly and accurately coordinate their expression patterns through the mediation of their intronic miRNAs, bypassing the time-consuming translation process in quickly changing environments. Conceivably, intron-mediated gene regulation may be as important as the mechanisms by which transcription factors regulate gene expression. It is likely that intronic miRNA is able to trigger cell transitions quickly in response to external stimuli without such tedious protein synthesis. Undesired gene products are reduced by both transcriptional inhibition and/or translational suppression via miRNA regulation. This could enable a rapid switch to a new gene expression pattern without the need to produce various transcription factors. This regulatory property of miRNAs may have modulated ancient gene even before the emergence of proteins in the post-RNA world. Considering the variety of microRNAs and the complexity of genomic introns, a thorough investigation of miRNA variants in the human genome will markedly improve the understanding of genetic diseases and also the design of miRNA-based drugs. Learning how to exploit such a novel gene regulation system for future therapeutic applications will be a great challenge.

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