1. ABSTRACT

MicroRNAs (miRNAs) are small single-stranded regulatory RNAs capable of interfering with messenger RNAs (mRNAs) through complete or partial complementarities. Partial complementarity gives miRNAs a flexibility which is useful for construction of new therapies against cancer polymorphisms and viral mutations. Varieties of miRNAs have been reported in diverse species; and they are believed to induce RNA interference (RNAi), a post-transcriptional gene silencing mechanism. Recently, many intronic sequences have been shown to encode miRNAs. Intrinsic miRNA, a new class of miRNAs, is derived from introns by RNA splicing and Dicer processing, and it differs uniquely from previously described intergenic miRNA in that intronic miRNAs require type II RNA polymerases (Pol-II) and spliceosomal components for their biogenesis. Several kinds of intronic miRNAs have been identified; however, their functions and applications have not been reported. Here, we show for the first time that intron-derived miRNAs are able to induce RNA interference in many cells demonstrating the evolutionary preservation of this post-transcriptional regulatory system in vivo.

2. INTRODUCTION

The first microRNA (miRNA) molecules, lin-4 and let-7, were identified in 1993 (1). Since then, a large variety of miRNAs have been identified, and understanding of their biogenesis, functionality, and gene regulation mechanisms has been remarkably increased. All miRNAs studied during the early stage of miRNA research are now recognized as intergenic miRNA. By definition, intergenic miRNAs are located in the non-coding regions between genes and they are transcribed by thus far un-identified promoters. In 2003, Ambros et al. (1) discovered another kind of tiny non-coding RNAs derived from the intron regions of gene transcripts. In the meantime, Lin et al. (2) demonstrated the biogenesis and related gene silencing mechanism of these intronic miRNAs, a new class of small single-stranded regulatory RNAs. Several intrinsic 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 (Table 1).

Non-coding DNA accounts for nearly 97% of the human genome, and introns constitute the largest extent of non-coding sequences in the gene-coding area of the genomic DNA. The transcription of the genomic gene-coding
**Intron-mediated RNA interference and microRNA**

Table 1. Currently studied intronic microRNA (Id-miRNA).

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Species</th>
<th>Host Gene (Intron)</th>
<th>Target Gene(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-2a, -b2</td>
<td>worm</td>
<td>Spi</td>
<td>paired mesoderm homeobox protein 2b; HHdm5</td>
</tr>
<tr>
<td>miR-7b</td>
<td>mammal</td>
<td>pituitary gland specific factor 1A (2) [NM174947]</td>
<td></td>
</tr>
<tr>
<td>miR-10b</td>
<td>mammal</td>
<td>homeobox protein HOX-4 (4)</td>
<td></td>
</tr>
<tr>
<td>miR-11</td>
<td>Drosophila</td>
<td>E2F</td>
<td></td>
</tr>
<tr>
<td>miR-12b</td>
<td>Drosophila</td>
<td>CG7033</td>
<td></td>
</tr>
<tr>
<td>miR-15b, -16-2</td>
<td>mammal</td>
<td>chromosome-associated polypeptide C</td>
<td></td>
</tr>
<tr>
<td>miR-17-92</td>
<td>human</td>
<td>CI3orf25</td>
<td></td>
</tr>
<tr>
<td>miR-25, -93, -106b</td>
<td>mammal</td>
<td>CDC47 homolog (13)</td>
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</tr>
<tr>
<td>miR-26a1, -26a2, -26b</td>
<td>vertebrate</td>
<td>nuclear LIM interactor-interacting factor 1, 2, 3</td>
<td></td>
</tr>
<tr>
<td>miR-28</td>
<td>human</td>
<td>LIM domain-containing preferred translocation partner in lipoma [NM005578]</td>
<td></td>
</tr>
<tr>
<td>miR-30c1, -30c</td>
<td>mammal</td>
<td>nuclear transcription factor Y subunit γ (5)</td>
<td>transcription factor HE8-1; PAI-1 mRNA-binding protein</td>
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<tr>
<td>miR-33</td>
<td>vertebrate</td>
<td>sterol regulatory element binding protein-2 (15)</td>
<td>RNA-dependent helicase p68; NAG14 protein</td>
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<tr>
<td>miR-101b</td>
<td>human</td>
<td>RNA 3’-terminal phosphatase cyclase-like protein (8)</td>
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<td>miR-103, -107</td>
<td>human</td>
<td>pantothenate kinase 1, 2, 3</td>
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<td>miR-105-1, -105-2, -224</td>
<td>mammal</td>
<td>γ-aminobutyric-acid receptor α-3 subunit precursor, epsilon subunit precursor</td>
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<tr>
<td>miR-126, -126*</td>
<td>mammal</td>
<td>TdT-like, Notch4-like, NEU1 protein (6)</td>
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<tr>
<td>miR-128b</td>
<td>mammal</td>
<td>cAMP-regulated phospho-protein 21 (11)</td>
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<tr>
<td>miR-139</td>
<td>mammal</td>
<td>cGMP-dependent 3’-5’ cyclic phosphodiesterase (2)</td>
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<tr>
<td>miR-140</td>
<td>human</td>
<td>NEDD4-like ubiquitin-protein ligase WWP2 (15)</td>
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<tr>
<td>miR-148b</td>
<td>mammal</td>
<td>coatomer γ-1 subunit</td>
<td></td>
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<tr>
<td>miR-151</td>
<td>mammal</td>
<td></td>
<td></td>
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<tr>
<td>miR-152</td>
<td>human</td>
<td>coatomer γ-2 subunit</td>
<td>N-myocyte proto-oncogene protein; noggin precursor</td>
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<tr>
<td>miR-153-1, -153-2</td>
<td>human</td>
<td>protein-tyrosine phosphatase N precursors</td>
<td></td>
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<tr>
<td>miR-208</td>
<td>mammal</td>
<td>myosin heavy chain, cardiac muscle α isoform (28)</td>
<td></td>
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<tr>
<td>miR-218-1, -218-2</td>
<td>human</td>
<td>Slit homolog protein [NM003062]</td>
<td></td>
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DNA generates precursor messenger RNA (pre-mRNA). Precursor messenger RNA contains four major parts: 5`-untranslated region (UTR), protein-coding exon, non-coding intron, and 3`-UTR. Broadly speaking, both 5`- and 3`-UTR can be considered intronic; however, UTR regions are processed differently from the in-frame introns during mRNA maturation. The in-frame introns can be as long as several 10 k-bases and were initially thought to be a huge genetic waste in gene transcripts. Recently, the discovery of intronic miRNA corrected this misunderstanding. MiRNA is usually 18–27 oligonucleotides long and 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 sense, the intronic miRNA is similar to previously described intergenic miRNAs structurally and functionally. However, intronic miRNAs differ from intergenic miRNAs in its requirement of Pol-II and RNA splicing components for biogenesis (2,4,5). Approximately 10–30% of a spliced intron are exported into the cytoplasm with a moderate half-life (6).

RNA interference (RNAi) is a posttranscriptional gene silencing mechanism in eukaryotes. This mechanism can be triggered by small RNA molecules, such as microRNA (miRNA) and small interfering RNA (siRNA). These two types of small RNA molecules usually interfere with intracellular expression of genes either by completely or partially complementing target gene sequences. Basically, siRNAs are double-stranded RNAs that degrade target RNAs based on nearly perfect complementarity (7,8). MiRNAs, not displaying the stringent complementarity of siRNAs to their RNA targets, are single-stranded and can partially pair with target RNAs (9,10). In almost all eukaryotes, including yeast (Schizosaccharomyces pombe), plant (Arabidopsis spp.), nematode (Caenorhabditis elegans), fly (Drosophila melanogaster), mouse and human, many natural miRNAs are driven from hairpin-like RNA precursors. These native miRNAs are involved in defense mechanisms against viral infections and post-transcriptional regulation during development (11–21). Natural siRNAs, however, were mainly found in plants and primitive animals (worms and flies), but rarely in mammals (10). Because miRNAs are widely detected in eukaryotes, they have recently been extensively utilized in designing novel therapeutics against cancer and viral infections (22,23). Indeed, gene silencing mechanisms involving miRNAs are probably an intracellular defense system for eliminating undesired transgenes and foreign RNAs, such as retrotransposons and viruses. (22,24).

2.1. Biogenesis and definition

By definition, intronic miRNAs are transcribed along with their encoded genes by the same promoter. Subsequently they are spliced out of the precursor messenger RNA and further processed into mature miRNAs. Therefore, intronic miRNAs share the same promoter as well as the same orientation as the original gene transcript. Based on this definition, some intron-derived miRNAs, although they are encoded in the genomic intron region of a gene, are not intronic miRNAs since they do not share the identical orientation as the gene transcript. This occurs 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 those miRNAs, the promoters are located in the antisense direction relative to the gene, likely using the gene transcript as a potential target for the antisense miRNAs. A good example is let-7c, which is an intergenic miRNA located in the antisense direction from a gene intron. Current computer programs for miRNA prediction cannot distinguish the intronic miRNAs from the intergenic miRNAs. Because
intronic miRNAs are encoded in the gene transcript precursors (pre-miRNAs) and share the same promoter with the encoded gene transcripts, the miRNA prediction programs tend to classify the intronic miRNAs the same as the intergenic miRNAs located in the exonic regions of genes. Reflecting the different biogenesis of intronic and exonic miRNAs, these two types of miRNAs may function as different gene regulators in the adjustment of cellular physiology. Thus, a miRNA-prediction program including databases of non-coding sequences located in the gene-coding precursor-mRNA regions is urgently needed for thorough screening and understanding of the distribution and variety of hairpin-like intronic miRNAs in the genome.

Intronic miRNA is a new class of small regulatory RNAs derived from the non-coding DNA regions of a gene, such as introns, and the 5'- and 3'-untranslated regions (UTR). Many introns and UTRs contain tri- or tetra-nucleotide repeat expansions, capable of being transcribed and processed into repeat-associated miRNAs (25,26). The biogenesis of intronic miRNA in eukaryotes involves five steps (Figure 1). First, miRNA is generated, as a part of a long primary precursor miRNA (pri-miRNA) located in the intron or UTR of a gene transcript, by type II RNA polymerases (Pol-II) (2). Second, after intron splicing, the long pri-miRNA is excised by spliceosomal components or partially processed by Drosha-like RNaseIII endonucleases/micro-processors to form precursor miRNA (pre-miRNA) (2,27). Third, the pre-miRNA is exported out of the nucleus, probably by Ran-GTP and a receptor for exportins (28,29) and a receptor for exportins (28,29). Fourth, once in the cytoplasm, Dicer-like nucleases cleave the pre-miRNA to form mature miRNA. Lastly, the mature miRNA is assembled into a ribonuclear particle (RNP) to form a RNA-induced silencing complex (RISC) or RNA-induced transcriptional silencing (RTS) complex for executing RNA interference (RNAi)-related gene silencing mechanisms (27,30). Although an in vitro model of siRNA-associated RISC assembly has been reported, the link between final miRNA maturation and RISC assembly remains to be determined. The characteristics of Dicer and RISC are different in the siRNA and miRNA pathways (31,32). In zebrafish, we have observed that the stem-loop structure of intronic pre-miRNAs is involved in strand selection for mature miRNA during RISC assembly (27). Further, excessive accumulation and potential cytotoxicity of intronic miRNAs can be prevented by the intracellular nonsense-mediated decay (NMD) mechanism, a specific RNA degradation system for spliceosomal introns. These findings suggest that the duplex structure of siRNA may be not essential for the assembly of miRNA-associated RISC in vivo. The biogenesis of miRNA and siRNA seems to share only a certain similarity. However, the miRNA mechanisms previously proposed were based on a model of siRNA. A preferable alternative is to contrast the individual properties and differences in these two types of RNAs in order to understand the evolutionary and functional relationships among different gene silencing pathways.

2.2. Intronic microRNA and disease
The majority of human gene transcripts contain introns, varying in number from species to species, and changes in these non-protein-coding sequences are frequently observed in clinical and circumstantial malfunction. Numerous introns encode miRNAs, which are involved in RNAi-related chromatin silencing mechanisms. Over 90 intronic miRNAs have been identified to date using bioinformatic approaches, but the function of the vast majority of these molecules remains to be determined (3). According to the strictly expressed correlation between intronic miRNAs and their encoded genes, one may speculate that the levels of condition-specific, time-specific, and tissue-specific miRNA expression are determined by the intracellular modulation of the encoded gene. This could allow more accurate genetic expression of various traits and any dysregulation of this miRNA–encoded gene correlation may result in genetic disease. For instance, many introns contain tri- or tetra-nucleotide repeat expansions capable of being transcribed and processed into repeat-associated miRNAs (25,26). They play an important role in the modulation of epigenetic alteration of genes which are involved in several genetic diseases caused by triplet repeat expansions in specific single genes, collectively termed triplet repeat expansion diseases (TREDS). TREDS include, but not limited to, fragile X syndrome (FXS), Huntington’s disease (HD), myotonic dystrophy (DM), and a number of spinocerebellar ataxias (SCAs).

Variation in intron-related TREDS is seen in monozygotic twins who frequently demonstrate slightly, but definitely distinguishing, disease susceptibility, physiology and behavior. A longer CCTG expansion in intron 1 of a zinc finger protein ZNF9 gene has been correlated with type 2 myotonic dystrophy (DM2) susceptibility in one twin compared to the other of a pair (33). Since the repeat expansion motifs often bind with high affinity to certain RNA-binding proteins, the interfering role of intron-derivated repeat expansion fragments in DM and HD can trigger RNA-like gene silencing (26); however, the mechanism involved is still unclear. Another more established example is fragile X syndrome (FXS), which represents about 30% of cases of human inherited mental retardation, affecting approximately one in 2000 males and one in 4000 females. This disease is caused by a dynamic mutation [expansion of microsatellite-like trinucleotide –(cytosine-guanine-guanine)– repeats or r(CGG)] at an inherited fragile site on the long-arm of the X chromosome, locus of the FMR1 gene (34–36). Because this mutation is dynamic, it can grow in length with a corresponding increase in syndrome severity from generation to generation, from person to person, and even within a given person. Patients with the FXS usually have an increased number of r(CGG), often greater than 230 copies in the 5'-UTR of the FMR1 gene, and this CpG-rich r(CGG) expansion region is often heavily methylated (37). Such r(CGG) expansion and methylation leads to physical, neurocognitive and emotional deficits linked to the inactivation of the FMR1 gene and the deficiency of its protein product (30). Two theories have been proposed to explain this FMR1 methylation mechanism in FXS. First, Handa et al. (25) suggested that non-coding RNA transcripts transcribed from the FMR1 5'-UTR r(CGG) expansion region can
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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 (remains to be determined) and further processing into 19-22 bp duplexes by RNase III-familial endonuclease, Dicer. The biogenesis of intergenic miRNAs, e.g. 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 their encoded genes and co-expressed 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 spliceosomal machineries and/or Drosha-like RNases to form a hairpin-like pre-miRNA template and then exported to cytoplasm for further processing by Dicer* to form mature miRNAs. For intronic miRNA, the Drosha-like RNases can be replaced by other RNasesIII, which remain to be determined. The Dicers for siRNA and miRNA pathways are different. All three small regulatory RNAs are finally incorporated into a RNA-induced silencing complex (RISC), which contains either strand of siRNA or the single-strand of miRNA. The effect of miRNA is considered to be more specific and less adverse than that of siRNA because only one strand is involved. On the other hand, siRNAs primarily trigger mRNA degradation, whereas miRNAs can induce either mRNA degradation or suppression of protein synthesis depending on the sequence complementarity to the target gene transcripts.

fold into RNA hairpins and are further processed by Dicer RNases to form microRNA (miRNA)-like molecules directed against FMR1 expression. Second, Jin et al. (38) proposed that miRNA-mediated gene methylation occurs in the CpG regions of the FMR1 r(CGG) expansion, which are targeted by hairpin RNAs derived from the 3’-UTR of the FMR1 expanded allele transcript. The Dicer-processed hairpin RNA may trigger the formation of an RNA-induced initiator of transcriptional silencing (RITS) on the homologous r(CGG) sequences, leading to methylated heterochromatin repression of the FMR1 locus in 99% of FXS patients.

Using a fish-compatible retrovector, pGABAR2-rT, to deliver an intronic transgene, SpRNAi-RGFP, containing the fmr1 5’-UTR r(CGG) expansion in zebrafish, Lin et al. (30, unpublished) have shown that a special type of intronic miRNA, the repeat-associated miRNA (ramRNA), is highly produced in the diseased neurons with fragile X syndrome. One of the most abundant ramRNA species is located in the nt 25–45 region of the fish fmr1 5’-UTR r(CGG) expansion (accession number NM152963), capable of causing r(CGG)-methylation and inactivation of the FMR1 gene. This ramRNA is specially characterized by its unique pre-miRNA structure consisting of (a) multiple loops and short stems in a relatively long hairpin region, (b) a nuclear import signal (NIS) motif to allow the re-entry of the mature ramRNA into the cell nucleus, and (c) a gene silencing motif to recruit the DNA methylation machinery. Recent FXS studies using this ramRNA-induced disease model have found that formation of synaptic connections was markedly reduced among the dendrites of the FMR1-deficient neurons, similar to diseased hippocampal neurons in human FXS. FMR1 deficiency often caused synapse deformity in the neurons essential for cognition and memory, damaging activity-dependent synaptic neuron plasticity. Furthermore, metabotropic glutamate receptor (mGluR)-activated long-term depression (LTD) was augmented after FMR1 inactivation, suggesting that exaggerated LTD may be responsible for abnormal behavioral responses in FXS, such as autism. These findings support a novel disease model in which mature ramRNAs originating...
from the triplet repeat expansion of a gene can reversibly bind back to the corresponding triplet repeat regions of the gene. More triplet repeats in the gene generate more mature ramiRNAs. With accumulated ramiRNAs binding to their targeted gene, DNA methylation then takes place in the triplet repeat regions, consequently inactivating the targeted gene. An animal model like this would be most suitable for studying TREDs involving epigenetic alterations.

2.3. Man-made intronic microRNA

To understand disease caused by dysregulation of miRNAs, an artificial expression system is needed to recapitulate the function and mechanism of miRNA in vitro and in vivo. The same strategy may be used to design and develop therapies for the disease. Several vector-based RNAi expression systems have been developed, using type-III RNA polymerase (Pol-III)-directed transcription, to generate higher RNAi efficacy in vitro as well as in vivo (39–43). Although these approaches have succeeded in maintaining constant gene silencing in vivo, the 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, e.g. U6 and H1, for small RNA expression creates another drawback. Because the read-through side-effect of Pol-III occurs on a short transcription template without proper termination, large RNA products longer than the desired 18-25 base-pairs (bp) can be synthesized and cause unexpected interferon cytotoxicity (44,45). Such a problem can also result from the competitive conflict between Pol-III promoter and another vector promoter (i.e.LTR and CMV promoters). Sledz et al. and our laboratory have found that a high dosage of siRNAs (e.g., > 250 nM in human T cells) caused strong cytotoxicity similar to that of long double-stranded dsRNAs (46,47). This toxicity is due to the double-stranded structure of siRNAs and dsRNAs, which activates interferon-mediated non-specific RNA degradation and programmed cell death through the signaling of PKR and 2-5A systems. It is known that the interferon-induced protein kinase PKR can trigger cell apoptosis, while activation of the interferon-induced 2'-5' oligoadenylate synthetase (2-5A) leads to extensive cleavage of single-stranded RNAs (i.e. mRNAs) (48). High siRNA/shRNA concentrations generated by the Pol-III-directed RNAi systems can also over-saturate the cellular microRNA pathway and thus cause global miRNA inhibition and cell death (49). In contrast, a Pol-II-directed intronic miRNA expression system does not show these problems due to the precise regulation mediated by cellular RNA splicing and nonsense-mediated decay (NMD) (50–54), which degrade excessive RNA to prevent cytotoxicity. For therapeutic purposes in vivo, the Pol-II-directed intronic miRNA expression system is likely a better solution than the Pol-III-based siRNA/shRNA expression systems.

The intron-derived miRNA system is activated in a specific cell type under the control of a type-II RNA polymerase (Pol-II)-directed transcriptional machinery. To overcome the 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 (22,23,47). Because of its flexibility in binding with partially complementary miRNA targets, miRNA can serve as an anti-cancer drug or vaccine, a potential 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-miRNAs) produced by mammalian Pol-II. Depending on the promoter of the miRNA-encoded gene transcript, intronic miRNA is co-expressed with its encoding gene, which activates the promoter and expresses the gene in the specific cell population. It has been noted that a spliced intron was not immediately completely digested into monoribonucleotides for transcriptional recycling since approximately 10–30% of the intron was found in the cytoplasm, suggesting a moderate half-life (6,55). This type of miRNA generation relies on the coupled interaction of nascent Pol-II-mediated pre-miRNA transcription and intron excision, occurring within certain nuclear regions proximal to genomic perichromatin fibrils (22,56–58). 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 2A). Because miRNAs are single-stranded molecules insensitive to PKR- and 2-5A-induced interferon systems, the utilization of this Pol-II-mediated miRNA generation system avoids the cytotoxic effects of dsRNAs and siRNAs. These findings indicate new functions for mammalian introns in intracellular miRNA generation and gene silencing, a potential tool for analysis of gene function and the development of gene-specific therapeutics against cancer and viral infection.

Using artificial introns carrying hairpin-like miRNA precursors (pre-miRNA), we have successfully generated mature miRNA molecules with full capacity in triggering RNAi-like gene silencing in human prostate cancer LNCaP, human cervical cancer HeLa and rat neuronal stem HCN-A94-2 cells (2,59,60) as well as in zebrafish, chicken and mouse in vivo (30,61). As shown in Figure 2B, the artificial intron (SpRNPai) was co-transcribed 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 a pre-miRNA structure was further processed into mature miRNAs capable of triggering RNAi-related gene silencing. With this artificial miRNA model, we have tested various pre-miRNA constructs, and observed that the production of intron-derived miRNA fragments was originated from the 5'-region of the intron sequence between the 5'-splice site and the branching point. These miRNAs were able to trigger strong suppression of genes possessing over 70% complementarity to the miRNA sequences, whereas non-homologous miRNAs, i.e. an empty intron without the pre-miRNA insert, an intron with an off-target miRNA insert (negative control), and a splicing-defective intron showed no silencing of the targeted gene. The same protocol can be directed against target EGFP expression in zebrafish (Figure 2C), 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 a high specificity of miRNA-directed RNA
Figure 2. Biogenesis and function of intronic miRNAs. (A) The native intronic miRNA is co-transcribed with a precursor messenger RNA (pre-mRNA) by Pol-II and cleaved out of the pre-mRNA by a RNA splicing machinery, spliceosome. The spliced intron with hairpin-like secondary structures is further processed into mature miRNAs 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 processes of the native intronic miRNAs. (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. (D) 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 bolt analysis probing the miR-EGFP(280-302) sequence, the mature miRNA was released only from the spliced intron resulted from the vector 2 construct in the cell cytoplasm.
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Pre-mRNA construct with SpRNAi:

\[
\text{5'-promoter} \rightarrow \text{exon 1} \rightarrow \text{artificial intron (SpRNAi)} \rightarrow \text{exon 2} \rightarrow \text{3' T codons}
\]

\[
\text{5' splice site} \rightarrow \text{pre-miRNA insert} \rightarrow \text{BrP} \rightarrow \text{PPT} \rightarrow \text{3' splice site} \rightarrow \text{T codon}
\]

After intronic insert is spliced:

\[
\text{5'-UTR} \rightarrow \text{exon 1} \rightarrow \text{exon 2 (mRNA)} \rightarrow \text{3'-UTR}
\]

+ Intrinsic microRNAs

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 expression system include several consensus nucleotide elements consisting of a 5'-splice site, a branch-point motif (BrP), a poly-pyrimidine tract (PPT), a 3'-splice site and a pre-miRNA insert located between the 5'-splice site and the BrP motif. 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 miRNAs are released from the intron by RNA splicing and further Dicer processing.

interference (RNAi). We have confirmed the identity of the intron-derived miRNAs, which were sized at about 18–27 base nucleotides (nt), approximately the same as the newly identified Piwi-interacting RNAs. Moreover, the intronic RNA isoforms isolated by guanidinium-chloride ultracentrifugation can elicit strong, but short-term, gene silencing effects on homologous genes in transfected cells. However, the long-term (> 1 month) gene silencing effect that we observed in vivo occurs only in nuclear transfection of the Pol-II-mediated intronic miRNA system by retroviral vectors.

The components of the Pol-II-mediated SpRNAi system include several consensus nucleotide elements consisting of a 5'-splice site, a branch-point motif, 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 motif. This portion of the intron would normally form a lariat structure during RNA splicing and processing. We suggest 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 region (T codon) in its 3'-proximal end to increase the accuracy of intronic RNA splicing. If present in a cytoplasmic miRNA, this codon 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 accumulation in the cell. For intracellular expression of the SpRNAi, we insert the SpRNAi construct into the DraII cleavage site of a red fluorescent membrane protein (RGFP) gene from mutated chromoproteins of the coral reef Heteractis crispa. The cleavage of RGFP at its 208th nucleotide site by the restriction enzyme DraII generates an AG-GN nucleotide break with three recessing nucleotides in each end, which will form 5' and 3' splice sites respectively after the SpRNAi insertion. Because this intronic insertion disrupts the expression of functional RGFP, it is possible to determine the occurrence of intron splicing and RGFP-mRNA maturation through the appearance of red fluorescent emission around the cell surface of the transfected cells. RGFP also provides multiple exonic splicing enhancers (ESEs) to increase RNA splicing efficiency.

2.4. Strand-specific gene silencing in zebrafish

The foregoing shows that intronic miRNAs can silence specific target genes in vivo (27). We first tried to determine the structural design of pre-miRNA inserts for the best gene silencing effect and found that a strong structural bias exists in the selection of a mature miRNA strand during 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 assembly of the siRNA-associated RISC. The two strands of the siRNA duplex are functionally asymmetric, but 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*) duplexes was thought to be an essential step in 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 nts 280–302 sequence. Because the intronic insert region of the SpRNAi-RGFP recombinant 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 (e.g. anti-EGFP) possessing cohesive ends. By changing 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-miRNAs, 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 identity, mir-EGFP(280–302), was found to be active in the transfections of the 5'-miRNA-stemloop-miRNA*-3' construct, as shown in Figure 4A (gray-shading sequences). Since the mature miRNA was detected only in the zebrafish transfected by the 5'-miRNA-stemloop-miRNA*-3'
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Figure 4. Intronic miRNA-mediated gene silencing effects in vivo. (A)-(C) Different preferences of RISC assembly were observed by 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 in the transfection of the [2] pre-miRNA, showing less EGFP (green) expression in the transfectant [2] than [1], while the miRNA indicator RGFP (red) was evenly 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 fishes 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 gene silencing significance. (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 the EGFP protein levels confirmed the specific silencing result of (B). No detectable gene silencing was observed in fishes 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 gene silencing significance. (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 adherins 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). Immunohistochemistry staining of β-catenin protein expression (brown) showed a significant decrease in the feather follicle sheaths. (G) The lower beak development was increased by the mandible injection of the anti-noggin pre-miRNA construct (down panel) in comparison to the wild type (up panel). Right panels showed bone (alizarin red) and cartilage (alcian blue) staining to demonstrate the out growth of bone tissues in the lower beak of the noggin knockdowns. Northern blot analysis (small windows) confirmed a ~60% decrease of noggin mRNA expression in the lower beak area.
construct, the miRNA-associated RISC must preferably interact 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 4B shows that transfection of the SpRNAi-RGFP vector into the Tg(UAS:gfp) zebrafish co-expressed 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 (59,60). We applied the liposome-capsulated 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, showing full systemic delivery of the miRNA. The indicator RGFP was detected in both of the fish transfected with either 5’-miRNA*-stemloop-miRNA-3’ or 5’-miRNA-stemloop-miRNA*-3’ pre-miRNA, whereas silencing of target EGFP expression (green) was observed only in the fish transfected by the 5’-miRNA*-stemloop-miRNA*-3’ pre-miRNA (Figures 4B, 4C). The suppression level in the gastrointestinal (GI) tract was lower, probably due to the high RNase activity in this region. Because thermostability in the 5’ end of the siRNA duplexes in both of the designed pre-miRNAs 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 stemarm determines strandness and strand-specificity, as well as the clustered arrangement of their origins (58). The piRNA class of small RNAs is likely transcribed by an intracellular RNA polymerase, similar to RdRp, from the pre-miRNA–perichromatin DNA duplex region of a replicating cell genome during mitosis or meiosis. Mammalian type-II RNA polymerases (Pol-II) possess RdRp-like activity (4,64,65). Nuclear transfection of long DNA–RNA duplex templates triggers piRNA-like gene silencing effects against viral infection and retrotransposon activity (22). In Drosophila and zebrafish, Piwi proteins are implicated in piRNA biogenesis to maintain transposon silencing in the germline genome (24,66). This function may be conserved in mice as loss of Miwi2, a mouse Piwi homolog, leads to germline stem cell and meiotic defects correlated with increased transposon activity (67). Because the RNAi effector of piRNA-mediated gene silencing requires Piwi proteins rather than siRNA/shRNA-associated Dicer RNases, this suggests that the piRNA-mediated RNAi mechanism is slightly different from the siRNA/shRNA-mediated RNAi pathway.

Recent studies have shown that the pre-miRNA–perichromatin DNA interaction results in the generation of Piwi-interacting RNAs (piRNA), which are similar to intronic miRNAs but distinct from other small double-stranded siRNAs and shRNAs in terms of their relatively larger size (approximately 26–31 nucleotides), single-strandness and strand-specificity, as well as the clustered arrangement of their origins (58).

In an effort to examine the pre-miRNA–perichromatin DNA interaction theory, we performed intracelular transfection of a long RNA–DNA hybrid construct containing a hairpin anti-β-catenin intronic pre-miRNA, which was directed against the central region of the β-catenin coding sequence (encoding aa 306–644) with perfect complementarity. A perfectly complementary miRNA theoretically directs target mRNA degradation more efficiently than translational repression. A dose of 25 nM of the pre-miRNA construct was injected into the ventral body cavity of embryonic day 3 chicken embryos, close to where the liver primordia would form (Figure 4D). For efficient delivery into target tissues, the pre-miRNA construct was mixed with a liposomal transfection reagent (Roche Biomedicals, Indianapolis, IN). 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 pipettes. 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 and there was no visible overt toxicity or overall perturbation of embryonic...
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development. The liver was the closest organ to the injection site and thus 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 4E, Northern blot analysis for the target β-catenin mRNA expression in the dissected livers showed that β-catenin expression in the wild-type livers remained normal (lanes 1-3), whereas that of the miRNA-treated samples was decreased dramatically (lanes 4-6). The miRNA silencing effect degraded more then 98% of β-catenin mRNA expression in the embryonic chicken, but had no influence on the house-keeping gene GAPDH, indicating high target specificity and very limited interferon-related cytotoxicity in vivo.

After ten days of primordial injection with the anti-β-catenin pre-miRNA template, the embryonic chicken livers showed an enlarged and engorged first lobe, but the sizes of the second and third lobes of the livers were dramatically decreased (Figure 4F). 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 abnormal regions in lobe 1. Endothelium development appeared defective and blood leaked outside 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 4F, small windows) and a diffused miRNA effect further inhibited feather growth in the skin area close to the injection site. The results discussed above showed that the anti-β-catenin miRNA was very effective in knocking out the targeted gene at a very low dose of 25 nM and was effective over a long period of time (≥10 days). Furthermore, the miRNA gene silencing effect appeared to be very specific as off-targeted organs appeared normal, indicating that the small single-strand composition of miRNA possessed no overt toxicity. In an attempt to silence noggin expression in the mandible beak area using the same approach (Figure 4G), it was observed that an enlarged lower beak morphology was reminiscent of that of BMP4-overexpressing chicken embryos (68,69). Skeleton staining showed the outgrowth of bone and cartilage tissue in the injected mandible area (Figure 4G, 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-beta) superfamily, is known to promote bone development and since noggin is an antagonist of BMP2/4/7, it is not surprising to find that our miRNA-mediated noggin knockouts created a morphological change similar to the BMP4-overexpressing embryo as in previous reports in chicken and other avian models. Thus, localized gene silencing in chicken by pre-miRNA transfection has a great potential for creating animal models for developmental biology research.

2.6. Localized RNA interference effects on mouse skin

To evaluate the effectiveness and safety of intronic miRNA in animals, we have tested vector-based intronic miRNA transfection in mice (61). As shown in Figure 5, patched albino (white) skins of mice (W-9 black) were created by a succession of intra-cutaneous (i.c.) injections of an anti-tyrosinase (Tyr) pre-miRNA construct (50 µg) for 4 days (total 200 µg). Tyr, a type-I membrane protein and copper-containing enzyme, catalyzes the rate-limiting step of tyrosine hydroxylation in the biosynthesis of melanin (black pigment) in skin and hair. After a 13-day incubation, the expression of melanin was blocked by the miRNA transfections. In contrast, blank controls and mice receiving Pol-III (U6)-directed siRNA presented a normal black skin color at the same dosage. Northern blot analysis using RNA–PCR-amplified mRNAs from hair follicles showed a 76.1±5.3% reduction of Tyr expression two days after the miRNA transfection, consistent with the immunohistochemical staining results from the same skin area, whereas mild, non-specific degradation of common gene transcripts was detected in the siRNA-transfected mouse skins (seen from smearing patterns of both house-keeping control GAPDH and targeted Tyr mRNAs). Given that Grimm et al. (49) have recently reported that high siRNA/shRNA concentrations generated by Pol-III-directed RNAi could over-saturate the cellular microRNA pathway and cause global miRNA dysregulation, the siRNA pathway may be incompatible with the native miRNA pathway in some tissues of mammals. Therefore, these findings show that the utilization of intronic miRNA expression systems provides a powerful new approach for transgenic animal generation and in-vivo gene therapy. It was noted that non-targeted skin hair appears normal after miRNA transfection. This underscores the fact that intronic miRNA is safe and effective in vivo. The results also indicated that miRNA-mediated gene silencing is stable and efficient in knocking down targeted gene expression over a relatively long period of time, since hair re-growth takes at least ten days. Thus, the intronic miRNA-mediated transgene approach may offer relatively safe, effective and long-term gene manipulation in animals, avoiding the non-specific lethal effects of the conventional transgenic methods.

More recent advances in the utilization of intronic miRNA expression systems have been reported in mice. Chung et al. (53) have succeeded in expressing a cluster of polycistronic miRNAs using the Pol-II-mediated intronic miRNA expression system. A polycistronic miRNA cluster can be processed into multiple miRNAs via the cellular microRNA pathway. This new RNAi approach has a few advantages over the conventional Pol-III-mediated shRNA expression systems. First, Pol-II expression is tissue-specific, whereas Pol-III expression is not. Second, Pol-II-mediated intronic miRNA expression is compatible with the native microRNA pathway, while Grimm et al. (49) have reported some incompatibility between the Pol-III-mediated shRNA and Pol-II-mediated native miRNA pathways. Third, excessive RNA accumulation and toxicity can be prevented by the NMD mechanism in a cellular Pol-II-mediated intronic miRNA mechanism in mice, but not in a Pol-III shRNA expression system.
2.7. Development of microRNA/piRNA-based gene therapy

The following experiments have been successful in 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 about 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 (71). The viral genes located in this target region include 3'-proximal Pr55Gag polyprotein (i.e., matrix p17 + capsid p24 + nucleocapsid p7) and 5'-proximal p66/p51pol polyprotein (i.e., 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 pre-integration 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 Pr55Gag and p66/p51pol gene expressions by miRNAs should eliminate the production of infectious viral particles in the late infection phase. Silencing Pr55Gag may prevent the assembly of intact viral particles due to the lack of matrix and capsid proteins, while suppression of protease in p66/p51pol should 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 polypeptide into individual functional proteins requires viral protease activity. As shown in Figure 6, this therapeutic approach is feasible (22,47).

The anti-HIV SpRNAi-rGFP vectors were tested in the 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 may help to overcome the daunting challenge of high HIV mutation, which frequently generates new drug resistance to current small molecule drugs. Northern blot analysis in Figure 6A demonstrated the ex-vivo gene silencing effect of anti-HIV miRNA transfections (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 (< 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±12.8% of viral genome replication in the chronic phase (about 2-years post-infection). Immunocytochemical staining for the HIV p24 marker protein confirmed the results of Northern blot analysis (Figure 6B). Sequencing analysis has revealed that at least two HIV-1b mutants in the acute phase and seven HIV-1b mutations in the chronic phase were found within the targeted HIV genome domain. It is likely that the higher genome complexity of HIV mutations in chronic infections counteracts to some extent 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 (lanes 5). Expression of the cellular house-keeping gene, β-actin, was at a normal level and showed no interferon-induced non-specific 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 (47), we may be able to stimulate the growth of non-infected CD4+ T lymphocytes to eliminate the HIV-infected cells.

3. PERSPECTIVE

Evidence of miRNA-induced gene silencing in zebrafish, chicken embryos, mouse stem cells and human disease demonstrates that this ancient intron-mediated gene regulation is highly efficient in vertebrates. The activation of RNAi-like gene silencing pathways is dominantly determined by intronic miRNAs in these animal models. We herein provide the first evidence for the biogenesis and function of intronic miRNAs in vivo. Given that natural evolution gives rise to more complexity and more variety of introns in higher animal and plant species for coordinating their vast gene expression libraries and interactions, dysregulation of these miRNAs due to intronic
Figure 6. Silencing of HIV-1 genome replication using anti-gag/pro/pol miRNA transfections 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 (lanes 4), respectively. No effect was detected in the T cells transfected by miRNA* targeting the same gag/pro/pol region of the viral genome (lane 5). The size of pure HIV-1 provirus was measured about 9,700 nucleotide bases (lanes 1). RNA extracts from normal non-infected CD4⁺ Th lymphocytes were used as a negative control (lanes 2), whereas those from HIV-infected T cells were used as a positive control (lanes 3). (B) Immunostaining of HIV p24 marker confirmed the results of (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.
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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 miRNAs, which are capable of interfering with other genes. Thus, the expression of a gene results in gain-of-function of the gene and also loss-of-function of some other genes, which are complementary to the mature intronic miRNAs. An array of genes can swiftly and accurately coordinate their expression patterns with each other through the mediation of their intronic miRNAs, bypassing the time-consuming translation processes under 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 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 serve as one of the most ancient gene modulation systems before the emergence of proteins. 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 disease and the design of miRNA-based drugs. Learning how to exploit such a novel gene regulation system in future therapeutic applications will be a challenge.

4. ACKNOWLEDGMENT

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5. REFERENCES

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**Key Words:** RNA interference (RNAi), intronic microRNA (miRNA), Piwi-interacting RNA (piRNA), Repeat-associated microRNA, RNA splicing, nonsense-mediated RNA decay (NMD), RNA-induced gene silencing complex (RISC), RNA-induced initiator of transcriptional silencing (RITS), triplet repeat expansion disease (TRED).

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