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Point of View

The multifaceted small RNAs

Long-Cheng Li

Department of Urology; University of California San Francisco; San Francisco, California USA

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Small RNA molecules are now being recognized as master regulators of gene activity. Although known initially for their gene-silencing functions, recent discoveries have revealed that small RNAs can also activate gene expression. These two opposing activities of gene silencing and activation together constitute an integrated gene regulatory mechanism.

Low molecular weight noncoding RNA (ncRNA) molecules such as small interfering RNA (siRNA), microRNAs (miRNAs), repeat-associated siRNAs (rasiRNAs) and Piwi-interacting RNAs (piRNAs) have been known for some time to negatively affect gene expression. However, two studies recently demonstrated that gene transcription can be stimulated by transfecting cells with small double-stranded RNA (dsRNA) constructs designed to target gene promoter sequences.[1,2] Thus, these small activating RNAs (saRNAs) result in RNA-induced gene activation (RNAa). These studies established that the transcription of a number of genes is enhanced by saRNAs, including those encoding E-cadherin, $p21^{WAF1/CIP1}$ (p21), vascular endothelial growth factor (VEGF), progesterone receptor (PR), and major vault protein (MVP).[1,2] These observations indicate that small RNAs have more diverse roles in gene regulation than previously recognized.

dsRNA-Induced Transcriptional Activation

Gene transcription is a major control point for regulation of gene expression. It has long been speculated that ncRNAs could function to activate gene transcription.[3,4] Indeed, RNA has many of the features found in protein transcriptional activators, such as a lack of positive charges and the ability to undergo hydrophobic interactions. These features provide the chemical repertoire required for these molecules to interact with the transcriptional machinery.[5] Examples that naturally occurring ncRNAs can positively regulate gene transcription include the roX RNA-mediated dosage compensation mechanism in Drosophila[6,7] and the transcriptional induction caused by steroid receptor RNA activators.[8]

Although the observation that targeting gene promoters with 21-nucleotide (nt) synthetic RNA results in transcriptional activation comes as a surprise, these studies establish that RNAa is quite potent in inducing gene expression. In cells with low basal levels of target gene expression, five to ten-fold amplification of gene expression can be achieved at both the mRNA and protein levels via RNAa. Even cells that already express high levels of target gene products exhibit increased transcriptional activity in response to RNAa.[1,2] One effect of RNAa is the reinstatement of endogenous gene functions, leading to phenotypic changes in transfected cells similar to those that occur in response to ectopic gene expression originating from an expression vector. For example, RNAa of the p21 and E-cadherin genes causes cell growth inhibition. Activation of PR by saRNAs results in expression changes of PR-regulated genes.[2] These observations point towards promising possibilities for therapeutic uses of saRNAs.

RNAa has very different kinetics from that of RNAi. The latter can be induced within hours in cells transfected with siRNA and the effect usually lasts for a period of 5–7 days.[9] In contrast, the effects of RNAa following transfecting cells with saRNA do not appear for about 48 hours. A possible explanation for this delay is that cytoplasmic small RNA gains access to genomic DNA targets only during cell division when the nuclear membrane is no longer a barrier. Another interesting difference is the long-lasting effects of RNAa-induced gene expression, which lasts for 10–15 days following a single transfection of saRNA.[1,2] This prolonged effect has been attributed to saRNA-induced epigenetic changes such as demethylation of histone 3 at lysine 9 (H3K9) 1 and increased methylation of histone 4 at lysine 4 (H4K4), alterations which are considered heritable across cell divisions.[10] This feature of RNAa is highly attractive from a therapeutic standpoint.

Critical questions regarding the mechanism of RNAa include how saRNA targeting of the promoter activates gene expression and what the nature of the effector complex is (if, indeed, there is one). By chromatin immunoprecipitation (ChIP) assays, chromatin modifications such as acetylation and methylation have been shown to be associated with saRNA's target regions in gene promoters. For example, loss of di- and tri-methylation at H3K9 is associated with RNAa of E-cadherin in PC-3 prostate cancer cells whereas no significant changes in methylation of H3K4[1] or in global acetylation at H3 and H4 (Li, unpublished observations) have been found. Reduced acetylation at H3K9 and H3K4, and increased di- and tri-methylation at H4K4 are associated with RNAa of the PR gene in MCF-7 breast cancer cells.[2] The diversity in saRNA-induced chromatin modifications may reflect gene-specific epigenetic changes associated with RNAa.[2] Even so, the question remains as to whether these changes are the cause of gene activation or simply events secondary to transcriptional induction.

Correspondence to: Long-Cheng Li; Box 0738; Department of Urology; University of California San Francisco; 533 Parnassus Ave; San Francisco, California 94143 USA; Tel.: 415.476.3802; Fax: 415.476.3803; Email: lilc@urology.ucsf.edu

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siRNA-mediated RNAi requires a perfect complementary match between the inhibitory molecule and the target mRNA sequence, whereas miRNA-mediated inhibition of protein translation or mRNA destabilization does not require such exactness if the so-called 'seed' sequence of the miRNA matches its target well. It appears that saRNA is similar to miRNA in this regard. For example, mutating the 5'-end of saRNA duplexes targeting the E-cadherin and p21 promoters completely disrupted their activity, whereas mutating the 3'-end had minimal effect on RNAa. Furthermore, mutations in the middle region of the p21 saRNA duplex produced constructs that retain partial activity in transcription induction.[1] These observations appear to rule out the possibility that transcriptional activation induced by dsRNA results from eliminating repressive promoter transcripts via RNAi.

The location of a dsRNA target sequence can have a great impact on the efficacy of the saRNA. Shifting a targeting sequence by just a few bases may change a potent saRNA into a less effective one or even into an siRNA. The basis for this spatial sensitivity is unclear, but it is known that dsRNAs targeting CpG islands (GC-rich areas) tend to function as siRNAs[11,12] and that these regions should be avoided as saRNA targets.[1] One plausible explanation for this observation is that dsRNAs targeting CpG island regions may downregulate gene expression by inducing CpG methylation, although it is still controversial whether dsRNAs induce DNA methylation in mammalian cells. An alternative explanation is that different target locations result in complementary dsRNAs with varying thermodynamic properties that determine which strand is used by Argonaute (Ago) proteins as the guide strand[13] and how efficiently it may affect gene transcription.

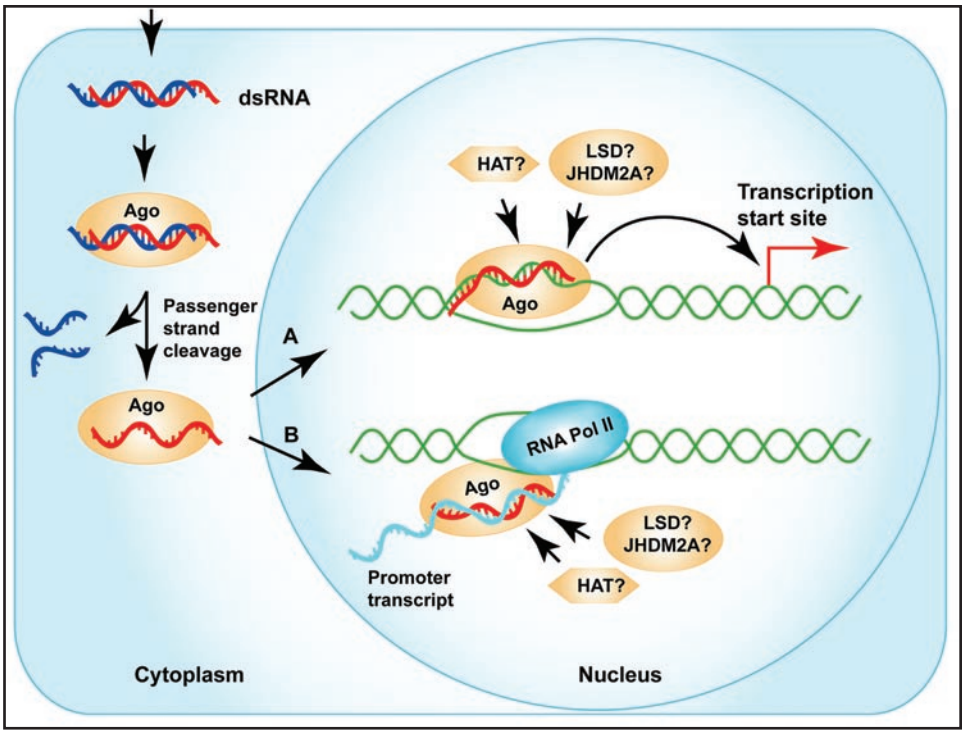

Figure 1. A proposed model for RNAa. An exogenously introduced or naturally occurring dsRNA is loaded onto an Ago protein where the passenger strand is cleaved and discarded. The remaining RNA strand serves as the guide strand and forms an active RNA-Ago complex. This RNA-programmed complex then gains access to chromosomal DNA through active transport into the nucleus or during mitosis (when the nuclear envelope disappears), and binds to its DNA target, whereupon the complex acts as a reaction platform for recruiting histone-modifying activities such as histone demethylase [lysine-specific demethylase (LSD), JHDM2A] and HATs, resulting in transcriptionally active chromatin. Alternatively, the RNA-Ago complex is tethered through base-pairing to a nascent transcript transcribed from the target promoter and further recruits active chromatin-modifying activities, leading to enhanced transcription.

A Model for dsRNA-Mediated RNAa

Based on the observations on RNAa described above, and considering other related mechanisms, we propose a model for RNAa in mammalian cells as described below (Fig. 1). In this model, the small RNA trigger is either exogenously introduced dsRNAs or naturally occurring molecules. Ago proteins (particularly Ago2) complexed with the RNA are required for initial strand processing and target recognition,[1] in a way similar to RNAi. The RNA-Ago complex then gains access to the nucleus, either during cell division when the nuclear envelope disappears or via active transport. The Ago-bound RNA may then bind to its DNA target or to cryptic promoter transcripts derived from the target promoter where the Ago protein serves as a chromatin remodeling platform and recruits histone-modifying activities such as histone acetyltransferase (HAT) or histone demethylase, leading to increased transcriptional activity.

miRNAs and positive gene regulation

miRNAs are a large class of small regulatory RNAs that mediate posttranscriptional gene silencing through one of two mechanisms: inhibition of mRNA translation at the initiation and elongation steps, or reduction of mRNA levels via de-adenylation, decapping and decaying of target mRNAs within the P-bodies.[14] miRNAs are involved in diverse biological processes such as organism development, morphogenesis, and stem cell division, and the etiology of diseases such as diabetes and cancer.[15,16] However, only a few miRNA targets have been experimentally verified as miRNA-silenced genes. Given the functional complexity of miRNA-mediated gene regulation, it is unlikely that the effects of these molecules are limited to gene silencing. Like exogenously introduced dsRNAs, it is possible that miRNAs also positively regulate gene expression. The initial evidence that miRNAs activate genes containing their target sequences arose from a study by Jopling et al. who established that a miRNA (miR-122) expressed in human liver enhances hepatitis C viral gene transcription by targeting its 5' noncoding region.[17] Very recently, a miRNA (miR-369-3) was shown to activate mRNA translation by targeting the AU-rich element (ARE) of 3' untranslated regions in cells experiencing stress conditions. This activation relies on Ago2 and FXR1, proteins already known to be involved in miRNA-mediated regulation of translation.[18,19] piRNAs also positively regulate gene expression by affecting mRNA stability, translation in mammalian germ cells,[20,21]

and transcription in Drosophila,[22] although they have primarily been found to silence gene activity.[23,24]

Gene expression profiling is often used to screen for miRNA target genes and has established that many genes, apart from those thought to be downregulated by miRNAs, show unexpected divergent changes in expression following manipulation of miRNAs or the miRNA machinery. These effects are frequently regarded as secondary to those on the primary target and largely ignored.[25-29] However, genes exhibiting divergent changes in expression often outnumber those downregulated by miRNAs[27,29] and are significantly overrepresented in distinct gene ontology categories.[29] These observations strongly suggest that the divergence in miRNA-regulated gene expression cannot simply be explained as secondary effects and instead may be the consequence of gene activation via miRNA targeting.

Hypothetical Mechanisms for miRNA-Mediated Positive Regulation of Gene Expression

miRNAs may stimulate gene expression both at the transcriptional level by targeting regulatory DNA sequences, and at the posttranscriptional level by enhancing mRNA stability and translation. For miRNAs to regulate DNA targets, and possibly also for the regulation of mRNA stability, miRNAs must gain access to the nucleoplasm. Several lines of evidence indicate that small RNAs, including exogenously introduced dsRNAs and endogenous miRNAs, indeed function in the nucleus.[1,2,11,12,30-32] In the case of RNAa induced by exogenous dsRNAs, the 'seed' sequence of the trigger RNA is critical to activity while mismatches of non-seed sequences with their targets are tolerated,[1] an aspect similar to the sequence specificity requirement of miRNA targeting. Therefore, it is tempting to hypothesize that miRNAs likewise bind to partially homologous target sites of gene promoters and activate gene transcription in the same way as exogenous dsRNAs.

It is also possible that positive gene expression regulation at the posttranscriptional level is effected by miRNA via stabilization of steady-state mRNA. It is estimated that the expression of 50% of eukaryotic genes is regulated by altered mRNA turnover[33] achieved by a variety of signals acting on specific sequences within the RNA. For example, the ARE located in the 3′ untranslated region is a well-known *cis*-acting element. The function of AREs is regulated by ARE-binding proteins (ARE-BPs). These proteins either promote RNA degradation[34] or increase stability.[35,36] The *BCL2* gene, for example, contains an ARE in the 3' UTR that regulates the rate of RNA degradation. Antisense oligoribonucleotides targeting the *BCL2* ARE can slow the rate of RNA decay, perhaps because their binding to the ARE sequences blocks interactions with destabilizing ARE-BPs. It is reasonable to speculate that endogenously generated antisense RNAs such as miRNAs that have imperfect complementarity with destabilizing AREs may also upregulate the levels of the target mRNA.

Taken together, evidence accumulated so far suggests that evolution has adapted small RNA molecules to regulate gene expression via a surprisingly diverse repertoire of mechanisms. Their effects include activation as well as inhibition, and may occur at the levels of transcription, translation, and RNA stability. The current thrust of research in this area is focused on identifying additional genes that are subject to such regulation, and determining the underlying molecular mechanisms in more detail.

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