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RNAa: A Few Key Points

S Abdolhamid Angaji[1, *] and Somayeh Darvishani[1]

[1] *Department of Biology, Kharazmi University, Tehran, Iran*

ABSTRACT

RNAa is an emerging biological discovery involving double-stranded RNAs that target promoter regions in chromosomal DNA resulting in transcriptional activation of genes. The transcriptional activation or up-regulation of genes results in an increase in mRNA and protein production. Accordingly, this technology may have applications in a range of human disorders such as certain genetic diseases and cancer where the aberrant low expression of certain proteins is known to occur. In this article, The History of RNAa discovery, main mechanism of RNA activation and its applications were reviewed.

Key Words: dsRNAs; agRNA; RNAi; Gene activation

INTRODUCTION

Small dsRNAs were initially discovered as the trigger of RNA interference (RNAi), a mechanism by which homologous mRNA is degraded to result in posttranscriptional gene silencing [1, 2]. dsRNA is also involved in transcriptional gene silencing by directing DNA methylation in plants [3, 4] and heterochromatin formation in fission yeast [5] and *Drosophila* [6]. Only recently has transcriptional gene silencing been discovered to occur in mammals [7, 8]. Surprisingly, two recent studies [9, 10] have found that dsRNA can also activate gene expression at a transcriptional level, a mechanism that has been termed "small RNA-induced gene activation" or RNAa. Both studies demonstrated RNAa in human cells using synthetic dsRNAs termed small activating RNAs (saRNAs) [9, 10]. Endogenous miRNA that cause RNAa has also been found in humans [11] and it has been shown that RNAa exist in several mammalian species other than human including non-human primates, mouse and rat, suggesting that RNAa is a general gene regulation mechanism conserved at least in mammals [12]. Although the exact mechanism of RNAa is unknown at present, scientists is also investigating the endogenous RNAa mechanism, which may play important roles in cancer initiation and development via miRNA mediated oncogene activation and applying RNAa as therapeutics for the treatment of cancers [13].

Four decades ago, Britten and Davidson, proposed a theory in which so-called ‘‘activator’’ RNAs, transcribed from redundant genomic regions, activated a battery of protein coding genes [14]. After consecutive failed several attempts to convince theory of RNAa since 2004, young scientists from UCSF laboratory, Li, Dahiya and Place have showed small dsRNA induced transcriptional activation in human cells [9, 15, 16] with supporting study from separate team at UT Southwestern in Dec-2006 [10, 15, 16]. Firstly they transfected synthetic siRNAs to the PC-3 cells and that RNA targeted promoters of E-cadherin, vascular endothelial growth factor (VEGF) and p21 that observed to increase mRNA and protein levels of these genes [9, 17]. Two years later Place and co-workers reported that also the miRNA could target the promoter of E-cadherin and activate transcription. By scanning gene promoters

in silico for sequences complementary to known miRNAs, they identified a putative miR-373 target site in the promoter of E-cadherin [11]. These small RNAs (siRNAs and miRNAs) then called RNAa (RNA activation).

In most recently study by Li and his colleagues, they have replicated and have identified new examples of RNAa in non-human primate (e.g. Chimpanzee, Orangutan, and Rhesus monkey), mouse and rat cells. They showed that small activating RNAs derived from human sequences have RNAa activity in non-human primate cells [12].They confirmed same results about activation of E-cadherin, VEGF and p21 in these organisms and showed targeting and activation of new genes (e.g. p53, PAR4, WT1 and NKX3-1 genes), suggesting the mechanism of gene activation is conserved in other mammals [12].

RNAa could potentially be complementing the RNAi according to the Yin and Yang Philosophy (Figure 1). The dual concepts of yin and yang which describe two primal opposing but complementary principles or cosmic forces said to be found in all non-static objects and processes in the universe. This paradoxical concept can potentially be applied to elucidate the complex phenomenon of RNAa/RNAi molecular pathways in the RNAome [18].

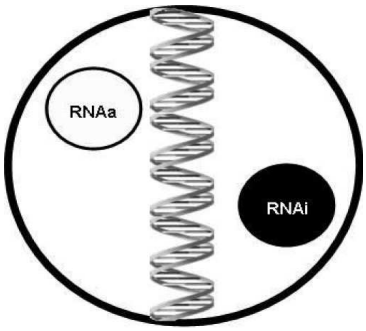

Figure 1. RNAa/RNAi constitutes the Yin and Yang of the RNAome.

Molecular mechanism of RNAa

The discovery that small double-stranded RNAs structured like small interfering RNAs (siRNAs) can also activate gene expression raised some exciting and challenging new questions about the mechanistic differences between silencing and activation [9, 10, 19]. A primary question was whether both mechanisms rely on the RNAi machinery [19].

RNAi is a precise and powerful gene knock down mechanism conserved in evolution from *Caenorhabditis elegans* to higher mammals [20, 21]. RNAi works through a number of RNA species including 1) siRNA–small, double-stranded RNA (long dsRNA) that degrade mRNA; 2) miRNA (micro RNA)-small, double-stranded RNA (hairpin dsRNA) that interfere with translation by imperfect base pairing with mRNA. Both siRNA and miRNA share common intermediates including Dicer, an RNase III endonuclease, RNA-induced silencing complex (RISC) [5, 22, 23] and a large family of homologous proteins including Argonaute [24, 25, 26, 27]. Small amounts of dsRNAs have been shown to silence a vast excess of target mRNA [21, 28].

Because Li and his team indicated that RNAa and RNAi have similar requirements for trigger dsRNAs, such as the importance of the “seed” sequence (the 5′ end of the antisense strand) and a preferred size of 21 nt, they decided to determine whether components of the RNAi pathway are also required for RNAa [9]. Accordingly, step by step they approved same features in silencing and activating such as necessity to Ago protein, details of methylation and elaboration of target sequence. One of the requirements was about Ago proteins. After transfections of Ago1- 4 in human prostate cancer cell (PC-3), they concluded that although other Ago family members may have played supportive roles in RNAa, Ago2 was indispensable for RNAa [9, 29]. Other item that has been approved was about DNA methylation. DNA methylation and chromatin modification operate along a common pathway to repress transcription [30, 31] and presumably inactivates the promoter by blocking its proper interactions with transcription factors or by attracting chromatin-remodeling proteins, which could lead to the heterochromatinization of the promoter sequence [32, 33, 34]. They reached to these results that although it currently have remained unclear how RNAa is linked to histone demethylation, it has been indicated that a decrease in H3-K9 methylation, [31, 35] that is associated with dsRNA-induced E-cadherin expression [9, 35]. In next step, they attempted for knowing about elaboration of target sequence for RNAa. A commonality in current reports of transcriptional activation is the

presence of retrotransposon or CpG repeat sequences in the promoter regions of such genes [37, 38]. In mammals, DNA methylation occurs predominantly at the dinucleotide CpG, and approximately 60% to 90% of the dinucleotides are modified [30, 31]. In normal cells, methylation involves mainly CpG-poor regions, while CpG-rich areas (CpG islands) located in regulatory regions of class II genes; seem to be protected from the modification [30, 39, 40]. Accordingly, Li and his colleagues selected regions in promoters of each target gene that avoided from CpG islands and best adhered to the rules of functional siRNA design (i.e., low GC content, lack of repeated or inverted sequences, bias toward low internal stability at the sense strand 3′ terminus, etc.)[9].

Most interesting event about RNAa is time of activation. Researches indicated that activation decreased over time [10] and RNAi typically silences genes for 5 to 7 days, but RNAa boosts gene activity for up to 13 days [9, 41]. It is not well characterized that how small dsRNAs could turn genes ON, especially for a very long period [9, 18].However, it is important to note that not all genes may be susceptible to RNAa. For instance, genes silenced by DNA methylation may confer resistance to RNAa [9]. For this reason, basal expression was evaluated to confirm that the targeted genes were not completely silenced. Regardless, RNAa offers a new approach to enhance endogenous gene expression, which may be manipulated to target a variety of genes [12].

In other research by Janowski et al [10], they work with synthetic antigene RNA (agRNA). Antigene RNAs (agRNAs) are small duplex RNAs that target gene promoters. Like siRNAs, they are generally 19 bp with 2-nt overhangs at the 3′-ends [42]. Depending on target sequence, cell type and basal level of expression, agRNAs can either silence [7, 43, 44] or activate [10, 45] gene expression at the transcriptional level and can act as endogenous [46] or exogenous [10]. Like gene silencing by siRNAs, modulation of gene expression by agRNAs involves argonaute (AGO) proteins [24, 47]. However, instead of targeting mRNA, agRNAs target non-coding RNA transcripts (ncRNAs) overlapping gene promoters [48, 49]. While agRNAs can target either sense or antisense non-coding transcripts, if it targets an antisense transcript overlapping the promoter, the gene is activated. Thus the guide strand of these agRNA duplexes is the sense strand, in contrast to siRNAs, for which the guide strand is by definition antisense (i.e. complementary to the mRNA) [42]. Janowski *et.al.* indicated that activation is accompanied by reduced acetylation at histones H3K9 and H3K14 and by increased di- and trimethylation at histone H3K4 [10, 35, 49] and concluded that remodeling of the histones, activates transcription from the agRNA-targeted promoter [18].

Both Li and Janowski with separate evidence indicated that activation was highly sequence specific. In the studies by Li *et al*. the most effective RNAa targets were far upstream of the transcriptional initiation sites of the E-cadherin, p21 and vascular endothelial growth factor promoters [9, 18] and Janowski *et.al.* found that single-base differences in the positioning of the agRNA within the progesterone receptor promoter can lead to either activation or repression of transcription and they were not able to find a general consensus of a silencing versus activation site [10, 18].

Applications of RNAa

Gene expression plays a critical role in the normal function of human cells. While changes in gene expression are normal for certain cell processes such as differentiation or activation, unintended changes in gene expression can lead to human disease [17]. It may be possible to simultaneously silence one gene and activate another for therapeutic purposes [18]. RNAa could potentially be complementing the RNAi. The use of RNAi is currently being proposed as a gene-specific approach for molecular medicine [34, 50, 51]. RNAa offers similar benefits as RNAi, while facilitating the exact opposite response – gene activation. This approach addresses a missing void in RNA-based gene therapies and offers a novel solution to provide greater efficacy in disease control. RNAa has already been shown to activate genes capable of suppressing cancer cell growth (e.g. p21, E-cadherin, p53, NKX3.1, etc.), triggering angiogenesis (e.g. VEGF), or influencing stem cell maintenance (e.g. CXCR4) [5,16, 18, 29]. As such, the ability to selectively up-regulate genes acting against a disease state can have far-reaching impacts in almost every therapeutic realm [52]. Since RNAa exists in mammalian species other than human, also can suggested that nonhuman primate disease models may have clinical applicability for validating RNAa-based drugs [12].

Application of RNAa is not limited to only cancer therapeutics. RNAa also has potential to function as a surrogate tool for vector-based gene overexpression systems. RNAa offers a new approach to enhance endogenous gene expression that may be manipulated to target a variety of genes. As a momentum within the biological sciences increases, RNAa may become an important technique to augment gene expression for therapeutics and functional gene studies [52]. Furthermore, RNAa also can play role in Apoptosis. In some studies on RNAa revealed that

dsP21 transfection to human bladder cancer cells [53] and human renal cell carcinoma [54] also enhanced apoptotic cell death and caused an accumulation in the G1 phase in both cell lines. Accordingly RNAa with increase in apoptosis can halt Cancers and even it can be say that RNAa have some effect in cellular pathway. In the study that resulted in discovering of agRNA as RNA activation, Up-regulation of PR protein reduced expression of the downstream gene encoding cyclooygenase 2 but did not change concentrations of estrogen receptor, which demonstrated that activating RNAs can predictably manipulate physiologically relevant cellular pathways [10].

Some applications of RNAa were summarized in table 1.

Table 1. Some of important applications of RNAa

Application			
Treatment of cancers	Disease	Target gene	References
	Prostate cancer	p21	9
		E-cadherin	9, 11, 55
		KLF4	56
		NKX3-1	12
		Ccnb1	12, 57
	Bladder Cancer	p21	47, 58
		E-cadherin	59
	Renal cancer	p21	53
	Breast cancer	PGR	10
Reprogramming of Stem Cells	Target gene	Definitions	References
	CXCR	applying RNAa as a tool for the derivation of virus-free induced pluripotent stem (iPS) cells through reprogramming [13] As such, RNAa may be useful in promoting stem cell phenotype by targeting genes involved in reprogramming	12
Triggering angiogenesis	Target gene	References	
	VEGF	60	
Apply in over expression systems	Target gene	Definitions	References
	CXCR	serve as a substitute for vector-based over expression in a variety of systems that traditionally have been done	12

Enhancement of RNAa-based drugs

The optimal window of RNAa activity was delayed by ~24-48 hours in comparison to RNAi. Perhaps, the delay in RNAa activity reflects a more complicated mechanism with additional rate-limiting steps. [28] and because RNAa is a nuclear process acting on gene transcription, acquiring access to the nucleus may serve as an additional rate-limiting step for RNAa. Changes in chromatin structure are also associated with RNAa [9,10, 60], which may further contribute to the delayed kinetics. Regardless, identifying the delay and defining the optimal window of RNAa activity allows for proper assessment or gene induction and functional analysis of saRNAs (small activating RNA). Assessing the rate of RNAa activity in cell culture also gives insight into the anticipated *in vivo* pharmacological properties of RNAa. For instance, RNAa-based drugs may require several days before target gene induction or beneficial changes in phenotype are evident. Moreover, the longer lasting effect of RNAa may result in less frequent administration of saRNA; a potential benefit as duplex RNA in excess can have toxic consequences [61]. Identifying features and key factors involved in the RNAa pathway can influence saRNA design. As such, defining Ago2 as an important mechanistic component implicated that chemically-modified saRNAs may function to manipulate RNAa activity in a manner similar to RNAi [62,63]. Utilizing dsP21-322 and dsEcad-215 as functional examples of saRNA molecules revealed that blocking the 5'-end or incorporating intentional mismatches can determine strand function. Studies have revealed an abundance of sense and antisense transcription within the promoters and flanking regions of active genes [64, 65]. Furthermore, overlapping noncoding RNAs and upstream cryptic transcripts have been shown to play substantial roles in regulating gene expression [66, 67]. As such, models for RNAa have included saRNAs targeting antisense transcripts and/or promoter-derived sequences to facilitate gene activation [9, 48]. RNAs transcribed in sense and antisense orientations have already been shown to serve as docking sites for transcriptional gene silencing (TGS) mediated by small duplex RNAs [68, 69]. Likewise, nascent sense and antisense transcripts may both serve as the targets for RNAa, as well. Utilizing modified saRNAs can not only improve mechanistic studies by defining strand activity, but also assist in determining orientation of such putative target transcripts. Identifying functional modifications is also necessary for therapeutic development in

order to improve the medicinal properties of saRNAs. In the case dsP21-322 and/or dsEcad- 215 (i) blocking the 5'-end of the sense strand completely inhibited its potential off-target effects; (ii) incorporating an intentional mismatch opposite the 5' most nucleotide in the antisense strand enhanced target gene induction, as well as reduced the off-target activity generated by the sense strand; (iii) 2'Ome modification to the sense strand inhibited RNAa activity, while the same modification to the antisense strand did not interfere with gene induction; (iv) modifying the 3'- end of either the sense or antisense strand had minimal effects on RNAa activity. Although the preferred guide strand may vary between the sense or antisense strand in different saRNAs, each modification may still be applied to manipulate saRNA activity or define strand function. As such, extrapolating these modifications to fit other saRNAs based on strand activity will also improve their medicinal properties. Development of saRNAs for therapeutic application may also require multiple modifications to optimize medicinal benefits. For instance, it is possible enhancing dsEcad-215 activity by blocking both the 5'-end of the sense strand and incorporating a mismatch opposite the 5' most nucleotide of the antisense strand. The combination of both modifications alleviated any potential off-target effects that would arise from improper use of the sense strand and enhanced gene induction; features needed to manipulate in order to develop RNAa therapeutics. Modification to the sense and antisense backbones (i.e. 2'-OMe, 2'-flouro, etc.) in saRNA duplexes may also improve therapeutic application by increasing endonuclease resistance and serum stability, much as they are utilized to stabilize siRNAs, as long the passenger strand is devoid of inhibitory modifications. Tethering small molecules (i.e. cholesterol) to the 3'-ends of saRNAs could also be used to improve systemic delivery of RNAa-based drugs. Conjugation of other compounds (i.e. flurogenic labels) to the 3'-termini may be effective at providing visual confirmation of saRNA uptake into target cells or tissue, as well [52].

Recently, the therapeutic potential of RNAa in prostate cancer xenografts by using lipidoid-based formulation is investigate to facilitate *in vivo* delivery. Lipidoid-encapsulated nanoparticle (LNP) formulation is validated as a delivery vehicle to mediate p21 induction and inhibit growth of prostate tumor xenografts grown in nude mice following intra-tumoral injection. It is provide insight into the stepwise creation and analysis of a putative RNAa-based therapeutic with antitumor activity. The results of this study provide proof-of principle that RNAa in conjunction with lipidioids may represent a novel approach for stimulating gene expression *in vivo* to treat disease [60].

Perhaps the most important take-home message from the silencing and activation studies is that we are on the tip of the iceberg with respect to our understanding of the multiple roles small RNAs can have in regulating gene expression. It seems that some of the most exciting times still lay ahead [19].

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