RNA Activation

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Abstract The ability to manipulate gene expression is invaluable for understanding the molecular pathogenesis of disease as well as for developing novel therapeutics. RNA interference provides a robust platform for the knockdown of a specific gene at the post-transcriptional level, but activation of specific genes traditionally has been limited to ligand-mediated activation of signal transduction pathways or introduction of exogenous transgenes from expression vectors. Recent work has shown that small RNA molecules targeted to the promoter region of a gene can activate gene expression. This phenomenon, called RNA activation, provides a tool for specific activation of endogenous genes, and introduces a new role for noncoding RNAs in the regulation of gene expression. These small RNAs are typically 21-nucleotide duplexes, and have been shown to activate a wide variety of genes in many cell types and across species. The application of this technology will prove invaluable for basic research through gain-of-function studies and potentially targeted gene activation for disease intervention. This chapter will cover what is currently defined on the mechanism of RNA activation, and will explore the possible application of this technology for novel therapeutics.

Keywords RNA activation • Non-coding RNA • Small RNA • Gene regulation • Regenerative medicine • RNA therapeutics

Introduction

Noncoding RNA (ncRNA) gained popularity as tools for loss-of-function studies by their ability to cause target transcript degradation through small inhibitory RNAs (siRNAs). The full compendium of noncoding RNAs now includes miRNA, PIWI-interacting RNA (piRNA), and long noncoding RNA (lncRNA) to carry out

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crucial roles such as transcriptional, post-transcriptional, and chromatin-modifying regulators. Although ncRNAs were predominantly seen as inhibitors of gene expression, we now recognize that these small RNA strands have much more complex functions including strong transcriptional upregulation; concepts which ultimately may have implications in the etiology of cancer [1, 2].

Small ncRNAs are classified into three main categories, all of which modulate gene expression by targeting transcripts within the cytoplasm. These include microRNAs (miRNAs), which inhibit protein translation by base-pairing with imperfect complementarity to target sequences within mRNA molecules; PIWI-interacting RNAs (piRNAs), which are involved in gene silencing by targeting transposons in animal germ cells; and small interfering RNAs (siRNAs) or RNA interference (RNAi), which target homologous mRNA sequences to degrade transcripts, leading to post-transcriptional gene silencing [3–5]. A new addition can be made to this category, in the form of activating RNAs, the discovery of which was made serendipitously [6]. While attempting to silence E-cadherin gene transcription, using small double-stranded RNAs (dsRNAs) in human cells, Li et al. [7] observed that several promoter-targeted dsRNA molecules were able to activate gene and protein expression. A similar event was also confirmed independently by Janowski *et al*. [8] who demonstrated that expression of the progesterone receptor (PR) could be activated by specific dsRNAs interacting upstream of the transcription start site (TSS) within the promoter region of mammalian cells and even beyond the 3' terminus region of the PR mRNA [9]. This phenomenon of activating RNA has now adopted numerous nomenclatures across the scientific community, including RNA activation (RNAa), small activating RNAs or short-activating RNAs (saRNAs, which will be used for this chapter), and antigene RNAs (agRNAs). To date, the number of characterized saRNA targets, which include E-cadherin, PR, p21, KLF4, and p53 amongst others, demonstrate that this non-integrative approach to selectively activate target genes will undoubtedly make an invaluable contribution to gain-of-function studies in translational medicine. The future of RNA therapy will entail the ability to synthesise saRNA duplexes for desired gene targets and the loading of these therapeutic agents into appropriate carrier particles for biodistribution to target organs. This chapter will provide an overview on saRNAs and their potential introduction into therapeutic applications.

Molecular Mechanism

saRNA-Associated Proteins

Argonaute Proteins

Despite entering a decade since the first report of activating RNA was made to the scientific community, a clear and complete mechanistic understanding of how these molecules work is still being sought after. The accumulation of work over this time

has highlighted important components that demonstrate how saRNA molecules might function. These include the orchestration of numerous RNA-binding enzymes, epigenetic changes, and recruitment of the transcriptional activation machinery. The most accepted mechanism of action currently includes the association of saRNAs with the Argonaute (Ago) protein family. Mammalian cells contain four variants of Ago. These are Ago1, Ago2, Ago3, and Ago4, and are classed as catalytic proteins which exert important biological roles in embryonic development and cell differentiation. The Ago proteins are particularly well characterized for their contribution in gene-silencing pathways guided by small RNA molecules [10]. Human Ago1, Ago3, and Ago4 are clustered on chromosome 1, whereas Ago2 is located on chromosome 8. Although the clustering might not be indicative of independent function, Ago2 demonstrates a more prominent role in siRNA-mediated gene silencing through its ability to cleave mRNA strands [11]. During the canonical RNA interference process, siRNAs are recruited by Ago2 for the formation of RNA-induced silencing complex (RISC). Now it appears that Ago2 is also implicated in molecular events that orchestrate RNA-dependent gene activation. Its exact role has yet to be fully defined; however, it is believed that Ago2 cleaves out the passenger strand from saRNA duplexes, after which the Ago2/saRNA guide strand translocates to the nucleus [7, 12, 13]. The Ago2/saRNA complex is then believed to recognize complementary sequences on either the promoter or enhancer regions of target genes or even on nascent promoter RNA [14, 15]. The guide strand also recruits chromatin-modifying enzymes to the cis-regulatory elements of target genes to modulate epigenetic changes and allow transcriptional activation of target genes. This multi-complex protein module regulates and stabilizes histone modification processes, which, as a result, leads to a longer duration of gene regulation when saRNA effects are compared with the more transient dynamics of siRNA [10, 16].

Heterogeneous Nuclear RNA-Binding Proteins

The saRNA guide strand mediates the interaction of functional proteins that form part of the ribonucleoprotein complex. Historically, the role of heterogeneous nuclear proteins (hnRNPs) has been known to be crucial in regulating gene transcription and other processes such as DNA and RNA strand stability and subcellular shuttling of mRNA [17–19]. HnRNPs are now known to be intricately implicated in modulating saRNA activity as demonstrated by saRNA-induced recruitment of hnRNP-K. Recently, Jia *et al.* discovered that hnRNPs (A1, A2/B1, and C1/C2) were involved in promoter-associated saRNA. HnRNPA2/B1, in particular, was crucially important for saRNA-induced activation of target genes in both the *in vitro* and *in vivo* settings [20–22]. Whether additional proteins are also involved is still unknown; however, defining the role of each component will undoubtedly bring us closer to understanding the nuclear mechanism of saRNA-dependent gene activation.

Histone Modification Enzymes

The kinetics of saRNA appear to be distinct from classical RNAi. While siRNA-induced post-transcriptional gene silencing can be observed within hours after transfection and lasts for up to 7 days; the dynamics of saRNA-induced gene activation, on the other hand, reveal a typical delay of up to 48 h following transfection into mammalian cells [12, 16, 23, 24]. For example, Place *et al.* [16] noticed that MOF or E2F1 silencing by siRNA began to emerge 6 h following transfection, with levels subsiding by 24 h. In contrast, E-cadherin and p21 activation by saRNA was observed at 48 h, with levels almost maximally increasing by 72 h. In some cell lines, saRNA-induced gene upregulation is seen to be preserved for almost 2 weeks. These discoveries, accompanied by the observation of Janowski's team, suggest that the delayed response of saRNAs can be attributed to the more complex orchestration of events that need to occur for targeted gene activation.

saRNA initially requires shuttling into the nucleus to its intended target. The interaction of Ago2 with saRNA, as mentioned above, and subsequent cleavage of the guide strand are believed to be the key initiation processes. Once the Ago2/saRNA guide complex accesses the gene target site together with their association with hnRNPs, a series of histone modifications is thought to occur. This observation originates from studies where agents that block histone modification appeared to suppress the effects of saRNAs, while agents that decrease acetylation of histone (H3K4), H3K14, and H3K9 appeared to upregulate saRNA-targeted upregulation of the PR gene and E-cadherin [25–29]. DNA-bound Ago2/saRNA guide might then regulate changes in DNA methylation. Since the prolonged duration of saRNA-induced gene activation occurs for up to 7 days, this suggests that major regulatory modifications ensue. This presents an attractive feature of saRNA for therapeutic use [16, 25].

Target Site Selection of saRNA

The recognition that RNA duplexes strongly activate transcription of target genes has unfortunately made very slow progress toward exploitation for therapeutic use. The main hurdle on this front has been the lack of a mechanistic understanding of how saRNAs induce targeted transcriptional activation, and whether the same process occurs systematically across all variants of activating RNAs or whether it refers to RNAa, agRNA, or saRNA. On the basis of existing concepts, activating RNA appears to involve components of the transcriptional and epigenetic machinery to exert their effects on target genes. However, the genomic target site of saRNA still holds subjective observations, which are sometimes contradictory. saRNAs generally demonstrate strand pattern preference, and this is guided by just one strand (either the sense or the antisense strand) [10]. The interaction of the single 'guide' strand with Ago2 is thought to be drawn toward either complementary sense strand or, as seen with the PR and low-density lipoprotein receptor (LDLR), this can

be with the antisense transcript where the saRNA–Ago complex interacts with heterochromatic protein 1-γ (HP1γ) and RNA polymerase II (RNAPII) [30–33].

Some have suggested that saRNAs target sequences downstream from the 3′ untranslated region (3′-UTR) of the intended target gene. Others have suggested that saRNAs demonstrate optimal function when they overlap the TSS of the intended target gene or are within a 200–1,200 bp region upstream of the TSS [9, 25, 34].

To further add to the complicated mechanism of how saRNAs recognize their targets, the efficacy of its activity is also dependent on the selection method of the guide strand and the systematic understanding of how, in conjunction with its interaction with Ago2, it can mediate specificity. This is an important feature, as the effects of RNA activation changes significantly in response to subtle modifications in the sequence of the activating RNA. Moreover, the specific sequence of the saRNA is also thought to strongly influence its subcellular localization, as this is clearly seen with miRNAs where their distribution in the cytoplasm or nucleus is affected on the basis of the sequence they contain. Again, these features are yet to be fully characterized with saRNA duplexes [35, 36].

Since Ago2 recruitment of saRNA initiates a strong transcriptional activation effect in mammalian cells, it is thought that target genes must be accessible to the Ago2/saRNA complex. Therefore, the effectiveness of the saRNA would be dependent on epigenetic alterations. [7]. This concept, again, is subjective, as there is evidence to show that transcription factors that are able to drive pluripotency can be induced by saRNAs in human mesenchymal stem cells [37]. The Ago2 processing unit has also created some dispute, where it is thought that Ago2-induced degradation of the strand does not emerge on the noncoding transcript of the target gene. Others have suggested that Ago2-induced antisense cleavage is directly influenced by the saRNA sequence [38–40]. This also remains to be defined. Despite these mechanistic oversights, the existence of iterative approaches to design and select saRNA duplexes for any desired target gene, and the established potential of their use in medical research, provide certainty that there will be strong drive to decipher the mechanism of action of saRNAs. During this time many promising targets for gain-of-function therapies will be at the ready.

Application of saRNA

Stem Cell Differentiation

RNA activation presents itself as a powerful biological tool to selectively enhance the transcriptional expression of target genes. Recently, it has been demonstrated that once the process of target gene site selection is optimized, synthetic double-stranded saRNA can be synthesized and used appropriately as a workflow to achieve a variety of desired biological effects. For regenerative medicine, the focus on inducible pluripotent stem cells or transdifferentiation of adult hematopoietic stem

cells toward surrogate organ cell phenotypes could be greatly impacted by the use of saRNAs. Driving CD34+ hematopoietic stem cells toward an insulin-expressing phenotype is currently an attractive 'autologous therapy' for patients with type 1 diabetes. While numerous methods, which include changes in the growth factor and cytokine microenvironment of stem cells, have paved the way to achieving this, the introduction of saRNA targeting the islet-β cell master transcription factor (MAFA) has bypassed many hurdles. saRNAs targeting MAFA have significantly reduced the duration of transdifferentiation and greatly enhanced maturation of adult hematopoietic CD34+ stem cells toward an insulin-secreting phenotype that responds to changes in the glucose gradient [41]. Although this strategy may not completely eliminate the demand for pharmacological insulin replacement, it might offer a baseline to reduce the risk of non-compliance in many patient groups with diabetes who are entirely dependent on insulin replacement therapy.

The ability to modulate pluripotent reprogramming factors, such as Kruppel-like factor 4 (KFL4) and c-Myc, by synthetic saRNA is likely to accelerate stem cell research [37]. Combined expression of OCT3/4, SOX2, KLF4, and c-Myc already allows reprogramming of adult fibroblasts into induced pluripotent stem cells for a myriad of applications [42]. Achieving this with synthetic saRNA that can be used safely across different platforms will undoubtedly improve the scope for using this technology as a clinically safe and efficient alternative to reprogram genes for regenerative medicine.

Clinical Therapy (Regenerative Medicine)

Over the past decade, a number of clinical trials have been initiated to evaluate the safety and efficacy of a variety of innovative RNA-based therapeutic strategies. Although most of these systems inhibit gene expression, block protein function, or induce potent immune responses of cytotoxic T lymphocytes to control tumor growth, RNA therapeutics are now increasingly being validated toward gain-of-function studies. saRNA that enhances transcription of C/EBPα, a member of the C/EBP family of transcription factors, in hepatocellular carcinoma (HCC-HepG2) cells was shown to have a powerful anti-proliferation role in hepatocytes by differentially regulating tumor suppressors [24]. It was demonstrated that peripheral injection of the same saRNA formulated with a clinically relevant synthetic carrier molecule, such as PAMAM dendrimers, into an animal model of liver failure and hepatocellular carcinoma drastically improved both liver function and tumor burden [24, 43]. While the current of list of saRNA targets continue to grow, the prospect of gain-of-function therapy with tumor suppressor genes relevant to liver, prostate, breast, and bladder cancer gives great promise for oncology [44–47]. SaRNA-induced activation of p21 and Wilm's tumor 1 gene (WT1) has been shown to inhibit cell proliferation as well as accelerating apoptosis in HCC (HepG2) and suppression of cell invasion in breast cancer cells (MCF7) [48–51]. It has also been shown that saRNA-induced p21 activation causes cellular senescence to repress or

terminate androgen-independent prostate cancer cell (PC3) proliferation [52–54]. Additionally, the most challenging issue of innate or acquire resistance to chemotherapy can now potentially be addressed, since it has been demonstrated that saRNA-based intervention targeting p21 enhances the chemosensitivity of cisplatin in A549 non-small-cell lung carcinoma [55]. With new formulations of clinically efficient carrier molecules on the horizon for organ-specific biodistribution, this alone offers a promising and optimistic future for cancer survival rates.

Concluding Remarks

It should be noted that the reports and reviews cited in this chapter have only come to light over the past 5 years. Just as it took over two decades for siRNAs to be fully appreciated and integrated as a powerful tool for loss-of-function studies in clinical research, the future of saRNA is undoubtedly promising. Its potential to regulate gene expression endogenously in living cells—and, as a result, its ability to selectively modulate signaling networks for known diseases or for stem cell reprogramming—will likely attract the attention of the pharmaceutical industry [56]. This alone sets the momentum for more mechanistic work to prevail in deciphering the complicated but truly fascinating concept of gene activation via man-made RNA duplexes. saRNA-based therapeutics will hopefully become integrated into 'personalized' medicine for more precise patient care.

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