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RNA Interference – A Hallmark of Cellular Function and Gene Manipulation

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Abstract

The discovery of RNA interference (RNAi) and its utilization in downregulation of specific target transcripts have revolutionized gene function analysis and elucidation of many key biochemical/genetic pathways. The insights into gene function, combined with a technology that made silencing of gene function possible using the potent, highly specific and selective RNAi approaches, provided the solution to longstanding complex obstacles in targeted crop improvements for agriculture, and disease therapies for medicine. In this introductory chapter, I aim briefly to cover the basics and peculiarities of RNAi and the advances made in understanding the mechanisms, components, function, evolution, application, safety and risk assessment of RNAi, while at the same time highlighting the related chapters of this book.

Keywords: Gene silencing, RNA interference, RNAi inducers and delivery, RNAi-based disease therapy, biosafety

1. Introduction

The “central dogma” of genetics as first presented by Francis Crick is that genes, packed inside the cell as the deoxyribonucleic acid (DNA) molecule, are transcribed into messenger ribonucleic acids (mRNA), which are subsequently translated into proteins (or enzymes). These final protein products provide all life functions, and together with DNA and RNA, constitute the molecules of life. Therefore, if there is a disruption (interference) of a gene function, messenger RNA synthesis, or protein translation, normal life processes get altered or even stopped. “No gene-no messenger”, or “no messenger-no protein”, has been the basis of understanding biological processes. One of the easy-to-access points in cellular processes is messenger RNA due to its cytoplasmic location, “naked” structure, comparatively short half-life, and temporal existence between transcription and translation. Further, mRNA is in between the chain of
events from DNA to protein; it has the universal chemical structure, consisting of only four nucleotides, regardless of the encoded message. In contrast, proteins are chemically much more variable, consisting of combinations of 21 different amino acids, with side chains that vary from very hydrophilic to highly hydrophobic. If mRNA is altered or eliminated before translation, there is no functional gene product, which results in changing the cellular process from the native state. This is the entire rationale of RNA interference (RNAi).

RNA interference is a process in eukaryotic cells in which double stranded endogenous or exogenous RNA molecules trigger a cytoplasmic response, which involves sequence specific target identification and destruction. This may include native messenger RNAs (mRNAs) that code vitally important proteins [1]. Any type of double-stranded RNA (dsRNA) molecules can activate RNAi where long dsRNAs, microRNAs (miRNAs) and small interfering RNAs (siRNAs) and their various forms and modifications are considered the main players/inducers [1]. Let us take a look at RNAi discovery history.

Plant scientists in the 1990s first used targeted gene silencing by introducing an antisense gene into plants. The first example was silencing of a nopaline synthase (NOS) gene, for which the silencing was only visible by loss of a band on a Norther blot and loss of NOS activity [2]. The second antisense gene used in plants targeted the petunia chalcone synthase (CHS) gene, encoding the first step in floral pigment production, and the result was visible in the loss of petal pigmentation [3]. Curiously, attempts to create dark pigmented petunia flowers by overexpression of the same CHS gene resulted in similar colorless petunia petals [4, 5]. It was thought that such a phenotype was “due to post-transcriptional inhibition of gene expression via an increased rate of mRNA degradation” [6]. The observed phenomenon was named as “co-suppression” of gene expression and the molecular mechanism behind “co-suppression” remained unknown for many years [7]. Later, a transient gene inactivation of the carotenogenic *albino-3* (*AL-3*) and *albino-1* (*AL-1*) genes was reported after transformation with homologous sequences in *Neurospora crassa* [8]. This phenomenon, named as gene “*quelling*”, was observed to be severely destructive but spontaneously and progressively reversible and monodirectional, resulting in mutant, intermediate, and wild-type phenotypes [8]. In the years to follow, the co-suppression phenomenon were attributed to inverted repeat T-DNA insertions, which result in RNA transcripts with internal complementary sequences that can fold back on themselves, generating double-stranded RNA and can seed the now well-known Argonaute/dicer silencing system.

Following these seminal discoveries, similar phenomena were discovered in other organisms including the nematode (*Caenorhabditis elegans*) and insects (*Drosophila melanogaster*) from studying the function of a PAR-1 gene (required for establishing embryo polarity) in the former and alcohol dehydrogenase in the latter (*ADH*) [9, 10]. These studies not only demonstrated a wide range of functionality of “co-suppression” phenomenon but also prompted an intense effort to understand the exact mechanism causing this process. In one experiment, injection of dsRNAs associated with muscle protein production into nematodes successfully silenced the targeted gene. The effect on muscle production was not observed using either mRNA or antisense RNA [11]. With this work, for the first time, the agent directly responsible for “co-suppression” was identified and formally named as “RNA interference” or RNAi. This work was later recognized with the 2006 Nobel Prize.
In plants, the suppression of targeted genes during viral infections was discovered [12] and subsequently developed into a system by which plant gene function may be studied through inhibition by infection with viruses bearing a short sequence targeted against plant mRNAs [13]. This phenomenon was termed as "virus-induced gene silencing" (VIGS) and is often used to study gene function in plant species that are recalcitrant to transformation or just take a very long time to regenerate.

Over the past decade, RNAi has been demonstrated in many eukaryotes including humans as well as some prokaryotic life forms [14] and has been recognized to form an integral part of many gene regulatory networks during development. This revolutionary breakthrough in biological science has become a valuable in vitro, in vivo, and ex vivo manipulation of gene expression, allowing for large-scale studies of gene function. It is now a routine laboratory practice to introduce the desired gene-specific dsRNA inducers into cells and selectively, robustly, and systematically silence the targeted sequence signature revealing its cellular function. In addition, RNAi has become an efficient tool for agricultural biotechnology to improve production [15] and combat disease pests as well as for medicine and molecular pharmacology to cure complex infectious, inflammatory, and hereditary diseases [16].

Figure 1. Dynamics of scientific publications devoted to the RNA interference for the past three decades. Source: PubMed [18] data sorted by the year of publications, which were retrieved by the search with the unquoted keyword "RNA interference".

RNAi research has rapidly advanced and expanded over the past decade, evidenced by increasing numbers of publications, research projects, and practical applications in both agriculture and medicine. For example, searching Google Scholar [17] with the unquoted keyword “RNA interference” retrieved over 1 million (1,110,000) documents. Repeating the same search with “organism-specified RNA interference” in PubMed database [18] on the same date returned a total of 50,824 indexed scientific documents with a major pick after 2002 reaching to over 1,000 scientific publications per year (Figure 1). The distribution of specified search results revealed a number of PubMed-indexed, RNAi-related publications for human (32,007), plant (3,701), animal (27,751), insect (4,145), fungal (690), and prokaryotic (119).
organisms. Moreover, the therapeutic application of RNAi is also expanding rapidly with 9,953 articles related to this topic and found in PubMed searching with "RNA interference therapy" keyword. In this brief introductory chapter, I aim to cover the basic understanding behind RNAi and an update knowledge on its applications, limitations, safety, and risks, highlighting and discussing some of the key points presented in this book.

2. Components, mechanism, and function

The principle mechanism of RNAi is complex, but very straightforward and easy to understand. RNAi is induced by the introduction of specific exogenous dsRNAs either by virus genome RNAs, injection of synthetic dsRNAs or, in plants, is mediated by Agrobacterium. RNAi is also part of the normal development and dsRNAs are produced by endogenous genes encoding miRNA precursors or other long dsRNA molecules. In either case, the dsRNAs are recognized by the enzyme dicer and cleaved into short, double-stranded fragments of ~19-25 base pair long siRNAs [1]. These siRNAs are separated into two single-stranded RNAs (ssRNAs), which are referred to as the "passenger" and the "guide" strands. The passenger strand is degraded, while the guide strand is picked up by the RNA-induced silencing complex (RISC) that has enzymatic digestion activity and contains the key components of Argonaute (AGO) and P-element induced wimpy testis (PIWI) proteins [1]. The RISC proteins perform the unwinding of the guide and passenger strands in ATP-independent manner [19, 20]; however, ATP is required to unwind and remove the cleaved mRNA strand from the RISC complex after catalysis [21]. There are effector proteins such as RDE-4 (nematodes) and R2D2 (insects) that recognize exogenous dsRNAs and stimulate dicer activity. R2D2 also has a differentiating function for siRNA strands by stably binding to 5’ end of the passenger strand, thus directing the guide strand to the RISC [22]. Here, it should be noted that the 5′ end of the guide strand is involved in matching and binding the target mRNA while the 3′ end physically arranges target mRNAs into the cleavage-favorable site of the RISC complex [21]. AGO/PIWI proteins localize within the specific P-body regions in the cytoplasm, considered to be a critical site for RNAi [23–25].

It is not clear as yet how the guide strand-bound active RISC complex finds mRNA targets within the cell, but it is known that this process is sequence-specific. Once the target mRNA is identified and captured though RNAi machinery, RISC cleaves the target mRNA rendering it untranslatable [1]. In most cases, the entire process is triggered by amplification of the cleavage process through synthesis of additional dsRNAs from primarily digested fragments of mRNA. Upon annealing to the mRNA target, the guide RNA may also be extended by RNA-dependent RNA polymerase (RdRP), resulting in extended “secondary” dsRNAs which in turn may lead to the formation of new siRNAs that enhance and further systematically spread the degradation of the target mRNA in cytoplasm [26, 27].

Although the pathways toward RNAi from exogenous and endogenous dsRNA converge at the RISC and use the same downstream RNAi machinery, there are also some clear differences in their processing and handling [1]. Endogenous dsRNAs cleaved by dicer (1) produce 20–25 bp fragments with a two-nucleotide overhang at the 3’end of siRNA duplex [1], while the length of exogenous dsRNAs-derived siRNAs, required for specificity, is unknown. Exoge-
nous dsRNAs are distinctly (2) handled by the above-mentioned effector proteins, RDE-4 or R2D2 [26, 27], whereas siRNA derived from endogenous dsRNAs (i.e., miRNA precursors) are handled by double-stranded miRNA precursor-binding DGCR8 and Drosha proteins with RNAse III enzyme activity. Plants do not have Drosha homologs, instead, processing of miRNA to siRNAs is carried out by one of four dicer-like proteins. Endogenous miRNAs (3), except some plant miRNAs, typically have several mismatches to the target sequence, while siRNAs derived from exogenous dsRNAs usually are designed to have a perfect match to the target. Most importantly, (4) endogenous dsRNA-derived miRNAs are capable of mildly inhibiting the translation of hundreds of mRNAs [28–30], while exogenous dsRNA-derived ones usually silence only single specific target [31]. Depending on organisms, for instance in C. elegans and D. melanogaster, (5) distinct Argonaute proteins and dicer enzymes [32, 33] process miRNAs and exogenous siRNAs. Furthermore, endogenously processed miRNAs prevalently (6) interact with miRNA response elements (MREs) located within the 3'-UTRs region of target mRNAs. Upon binding to MREs, miRNAs can decrease the gene expression of various mRNAs by either inhibiting translation (in animals) or directly causing degradation of the transcript (in plants). In contrast, exogenous dsRNA-derived siRNAs may interact with any complementary sequence region of the target mRNA, causing direct cleavage of the transcript [1]. miRNAs may actually regulate translation of target mRNAs in dual ways, as translation regulation by miRNAs oscillates between repression and activation during the cell cycle through a yet unknown mechanism [34].

The main biological function of RNAi is regulation of gene activity of cells at the post-transcriptional level (PTGS) either by the inhibition of translation of mRNA or by direct degradation of the mRNA. In addition to PTGS, RNAi pathway components may contribute to maintenance of genome organization and structure, mediated by RNA-induced histone modification. Histone modification in turn affects heterochromatin formation and may silence gene activity at the pre-transcriptional level [35]. This process is referred to as “RNA-induced gene silencing (RITS) and requires dicer, siRNA and RISC component proteins such as AGO and R2D2 [36]. In addition, RNAi components and inducers (siRNA/dicer/AGO) may also possibly upregulate expression of genes in binding into a promoter region and through histone demethylation, a process dubbed RNA activation [37, 38].

Because of sequence-specific recognition, regulatory properties, and the possibility of systemic spreading of dsRNAs, RNAi is the key “sterilizing agent” of cells and tissues, and it functions as potent immune response against foreign nucleic acids from viruses, transposons, or transformation events which can invade and harm the genome and its stability [39]. The chapters presented in Section 2 of this book have a more detailed coverage of the history of the RNAi discovery, mechanism, and functional components and on the biological role of RNAi including natural small RNAs/microRNAs as well as long noncoding RNAs in gene regulations.

3. Differences among organisms

Although the RNAi pathway is a universal process in eukaryotic cells, and it consists of similar component(s), mechanisms, and functions as described above, there are some variations
among organisms in both up-take of exogenous dsRNAs and induction of RNAi. First, RNAi is systemic and heritable in plants and C. elegans. The systemic spreading of RNAi in plants occurs because of transfer of siRNAs between cells through plasmodesmata and the phloem [40]. Second, in plants, RNAi induces epigenetic silencing of genes through methylation of promoters of targeted genes which may be passed to the next generation [41], while in Drosophila and mammals this is not the case. Third, plant miRNAs have perfect or nearly perfect complementary to their target genes and directly cleave and degrade targeted mRNA. In contrast, animal miRNAs have one or more mismatches to target sequence and halt the translation process [42].

RNAi is not found in some eukaryotic protozoa (e.g., Leishmania major and Trypanosoma cruzi) [43, 44]. Some fungi (e.g., Saccharomyces cerevisiae) lack specific RNAi component(s) and the reintroduction of these missing components can recover RNAi [45, 46]. Further, prokaryotic organisms have distinctive RNA-dependent gene regulation system controlled by RNA products of translation-inhibiting genes. These regulatory RNAs are not processed by dicer enzymes, differentiating them from eukaryotic RNAi [47]. However, recently, the clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) interference system has been characterized in prokaryotes, which is a gene silencing pathway analogous to eukaryotic RNAi systems [14]. The CRISPR interference system has its specific components, advantages, and limitations that are well described in the literature [48, 49], but will not be presented here. The chapter by Dr. Devi Singh and his colleagues in Section 2 of this book has a detailed coverage of RNAi in various organisms. RNAi in various organisms is also discussed in the chapter by Galay et al. presented in Section 6 of this book, highlighting the specifics of RNAi in ticks while Dr. Tayota and his colleagues present an interesting methodological paper on RNAi in the water flea in Section 3.

4. Evolution

Studies on components, mechanisms, and functions of RNAi have demonstrated variations among organisms, differences in eukaryotes and prokaryotes, and indicate that RNAi is derived from an ancestral immune defense function against transcripts of transposons and viruses [50, 51]. Although some eukaryotes might have lost RNAi components or, even, the entire pathway following the emergence of the Eukaryota, parsimony-based phylogenetic analyses suggest that an ancestral lineage of all eukaryotes possibly had a primitive RNAi capability including relevant components for some key functions such as histone modification [50]. Phylogenetic studies also indicate that miRNAs of plants and animals may have evolved independently, but the conservation of some key proteins involved in RNAi also indicate that the last common ancestor of modern eukaryotes already possessed an siRNA-based gene silencing system. The RNAi-like defense system of prokaryotes is functionally similar, but structurally distinct from the eukaryotic RNAi system [52]. It seems likely that a proto-RNAi system possessed at least some form of dicer-like, AGO, PIWI, and RdRP proteins. These basic components were shared by major eukaryotic lineages and functioned within an RNA degradation exosome complex [53].
5. Applications

Since its first discovery as anti-sense gene suppression, co-suppression or quelling phenomenon, the sequence specificity, efficiency, and systemic spreading (in some organisms) characteristics of RNAi to suppress target gene expression have caught researchers’ attention and soon became an attractive and powerful tool for gene function discovery in life sciences [1]. By full or partial suppression of target gene expression using RNAi, the change in cell physiology and/or developmental phenotype helps to reveal the function of the target gene. Therefore, a utilization of RNAi has revolutionized the annotation of cellular functions of many unknown and unique genes, adding to our understanding the complex genetic/biochemical pathways and their interactions. Thanks to its partial silencing effect, RNAi also helped to discover the function of genes when complete knockout would cause lethality [57]. Moreover, by targeting homologous sequences within a gene family, a single RNAi construct can suppress the expression of multiple members of a gene family, and thus reveal phenotypes that would have been missed in a single mutant due to redundancy in gene function.

The results of the functional genomics studies, advances in the understanding of the RNAi mechanism, improved design of trait-specific RNAi inducers (such as miRNAs), selection of target gene sequences combined with the development of proper delivery systems, as well as screens for “off-target” and cross reactivity have brought the practical applications of RNAi far beyond its initial experimental reach.

Agricultural application of RNAi through tissue culture-derived genetic modifications and transgenic research in a wide range of technical, food, and horticulture crops have been particularly successful and have solved many problems. Examples include, but are not limited to, crop yield and quality improvements [15, 58], food/nutrient quality improvements and fortification [59–62], decreasing the harmful precursors and carcinogens [63, 64], and improvement of plant pest and disease resistance [65–66]. Many of these applications are now evaluated for commercialization or are already in commercial production [67]. In this context, targeting far red (FR) photoreceptor gene (PHYA1) using RNAi approach [15], our team succeed to develop the world’s first RNAi cotton cultivars with improved fiber quality and other key agronomic traits without adversely affecting the yield, which successfully passed multi-environmental large field trials and have been approved for cotton farming in Uzbekistan.
Therapeutic application of RNAi has also been successful in medicine and molecular pharmacology with examples in inflammatory and infectious disease [68-71], cancer [72-75], as well as hereditary and neurodegenerative diseases [76]. Indeed, for many other disorders RNAi may have great potential. To highlight advances made on this field, in Section 4 of this book, we present several relevant chapters on advances of RNAi application in key human diseases of blood, ocular, nervous, kidney, and oncogenic origin. In addition, Section 5 chapters discuss RNAi utilization in various immune and infectious diseases. Section 6 chapters present the latest advances of RNAi application in studies of insects and parasitic pests such as ticks. All of these chapters highlight various aspects of RNAi and add interesting insights to the present RNAi discussions.

6. Safety and risk assessment

Manipulation of the organisms’ own genetic sequence signature(s) (cis-genesis) is usually considered safer compared to “trans-genesis” that utilizes “foreign” genetic material to create genetically modified (GM) crops and its products [77]. However, for RNAi, when broken down to ~21 nucleotides this quickly may lose its meaning, as a trans-RNAi will only work if it has sufficient homology to an endogenous target transcript. Chemically, RNA is “generally recognized as safe (GRAS)” or it is “rarely formally considered in risk assessment” [67]. Despite this and many other examples of successful application of RNAi technology in agriculture and medicine, there may be risks associated with high or repeated dosages of dsRNA, which inadvertently may interfere with unintended target sequences. A growing body of evidences suggests that testing for the safety and assessing possible risks associated with the use of RNAi-derived products sound practical, in particular, evidence of the remarkable stability of dsRNAs in the environment, their survival and resistance in the acidic conditions of the digestive tracts of higher organisms, and consequent transmissibility of dsRNA from foods to humans/animals. Further, production of possibly harmful “secondary” dsRNAs [67] by primary RNAi inducers raised an early warning signal regarding the GRAS signature of any RNA molecule and the possibility of risks for human health and environment.

Safety concerns about RNAi-based drugs are exemplified by the lethality of 23 out of 49 distinct RNAi therapy experiments in mice because of potential “off-target” effects that could shut down non-targeted gene(s) with sequence similarity to therapeutic RNAi inducer [78]. This observed lethality, however, could be due to “oversaturation” of the dsRNA pathway and delivery issues of short hairpin RNAs [79] that needs to be optimized for harmless therapeutic applications. There are several suggested approaches to minimize or eliminate such “off-target”, “oversaturation” or delivery issues, in particular through the use of (1) comprehensive in silico target and off-target analyses [80], (2) modified designing of RNAi inducers with improved target selectivity, and (3) efficient delivery systems.

There may also be concerns about the uptake of intact plant miRNA by consumers through plant diet. Plant microRNAs and some long dsRNA molecules, with sequence complimentary and perfect matches to endogenous human genes, were demonstrated to survive the digestive
tract of humans and can freely and routinely enter the blood system [67, 81]. In vitro human cell culture experiments further showed that such plant siRNA entered into human blood system could silence endogenous human genes due to sequence complementarity. While this may require attention of regulatory systems on one hand, on the other hand, human consumption of food crops with natural occurring siRNAs is considered safe and so far has not caused any dramatic biohazards or risk [81]. The chapters in Section 3 of this book also present updated information on RNAi delivery methods (e.g. Tayota et al.); synthesis, chemical and structural modifications, and designing for high specificity and selectivity of RNAi inducers (see Gvozdeva and Chernolovskaya), and limitations of RNAi and possible alternative technology such as ER-targeted intrabodies for gene silencing (see chapter by Backhaus and Böldicke).

Risk assessment and available protocols/guidelines are in the early stages of development. Some suggest that dsRNA-derived products must be subject to risk assessment studies [67]. Other findings indirectly support the safety of RNAi [81, 82], provided its use is within specific dosage ranges, the correct delivery system is in place and RNAi inducers without possible off-target effects, unintended gene silencing and secondary dsRNA production can be designed. However, it is always advisable to admit to possible risks of any novel genomic technology, including RNAi, and consider potential biohazards and evaluate risks for environmental health, before release of a new product [58, 81–85]. To accomplish this, Heinemann et al. [67] proposed the following five-step guidance: (1) to perform detailed in silico comparative bioinformatics analyses for targets of designed dsRNA and identify possible “off-targets” in key consumers; (2) to experimentally quantify designed dsRNAs, and the processing of any other unknown sequence signatures or secondary dsRNA as a result of introducing intended RNAi inducer into recipient or its product; (3) to test possible biohazards and risks due to exposure of RNAi product in animal and human cell/tissue culture; (4) to conduct animal feeding experiments for the long-term physiological and toxicological patterns and possible chronic effects; and (5) to perform clinical trials of RNAi-derived products in humans.

7. Conclusions and future perspectives

Thus, being a revolutionizing discovery in genome biology to characterize functions of any desired unknown genetic sequences, the discovery of RNAi has significantly widened our knowledge on core cellular processes. This knowledge has created opportunities and solutions to longstanding obstacles in conventional agriculture and medicine, offering a bright future to curing complex human and animal diseases, improve crop production and protection, and a sustained global food security through proper manipulation of key genes with agricultural or medicinal importance. Although key issues on specificity, selectivity, and delivery of RNAi inducing structures still exist, and some safety risks associated with the use of RNAi products have been recognized, the general believe is that RNAi is a safer technology than trans-genomics utilizing “foreign” genetic information. Safe applications, however, require proper designing, dosage and delivery of RNAi inducers, and before its delivery for wide consumer market, the safety risks should be assessed. Addressing the advances made over the past three decades in RNAi research and commercialization, in this book, we have compiled and
presented a diverse collection of chapters contributed by the science research communities. We all believe that RNAi, in combination with the rapidly expanding genomic information in key organisms and novel genome editing tools, will become even more powerful and efficient, and that we will all enjoy its benefits far into the future.

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