Chapter 4

RNAi Towards Functional Genomics Studies

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Additional information is available at the end of the chapter

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1. Introduction

RNA interference is an evolutionarily conserved mechanism that uses short antisense RNAs that are generated by 'dicing' dsRNA precursors to target corresponding mRNAs for cleavage. Pioneering observations on RNAi were reported in plants, but later on RNAi-related events were described in almost all eukaryotic organisms, including protozoa, flies, nematodes, insects, parasites, and mouse and human cell lines [1, 2]. Called initial cosupression or PTGS (post-transcriptional gene silencing), RNAi was first discovered in transgenic petunia plants [3]. In order to increase the pigmentation the chalcone synthase (CHS) gene was over-expressed in petunia plants and instead of enhancing in the flower pigmentation an opposite effect was observed. Some of the flowers were completely lacked of pigmentation and others showed different degrees of pigmentation. It was shown that even though an extra copy of the transgene was present, the CHS mRNA levels were strongly reduced in the white sectors. It was suggested that interaction between transgenes and native transcripts triggers a mechanisms that leads to the destruction of both transcripts or to the obstruction of the translation process and to gene silencing. This phenomenon was called co-suppression because the extra copies of CHS transgene determined reduction of its own expression but also the endogenous gene expression.

Later on, other study in the field of virus resistance was being exploited in order to produce virus resistance plants. Using different viral systems it has been shown that the expression of viral genes in the target plant genome was not associated with resistance to that particular virus [4-6]. The virus resistance in the recovered plants correlated with reduction of transgene mRNA in the cytoplasm, these phenomena was also called co-suppression. The finding provided supporting evidence of plant natural response to viral infection that the recovered parts of this plants response to virus would not only be resistant to initially inoculated virus but also cross-protect the plants against other viruses carrying homologous sequences [7]. This phenomenon was later called VIGS (virus-induced gene silencing). Further work found that the transcripts produced from both loci have been degraded in the

cytoplasm. In this case, activation of PTGS was taught to be due to the production of aberrant dsRNA by the transgene, which results in the silencing of the mRNA [8].

In fungi *Neurospora crassa*, it was shown that an overexpressed transgene could induce gene silencing at the post-transcriptional level a phenomenon called "quelling"[9]. Few years later, the efficiency of injecting single-stranded anti-sense RNA as a method of gene silencing in the nematode *Caenorhabditis elegans* by using its ability to hybridize with endogenous mRNA and inhibits translation was investigated [10]. The discovery that introduction of a dsRNA was more effective at inhibiting the target gene led to the conclusion that both original single stranded sense and antisense samples, may have been initially were contaminated with dsRNA. However, similarities between plant and nematode were recognized and the term RNAi was adopted in both systems [11]. Initial experiments on RNAi were successfully in plants and nematodes by introduction of a dsRNA into the cytoplasm. In mammalian cells, however, similar techniques, results in the initiation of the interferon response and cell death before processing could occur. This happen till the group of Elbashir and co-workers [12] reported as alternative method by introduction of a siRNAs under 30 base pair length in the mammalian cells and the interferon response was avoided and activated the RISC (RNA interfering silencing complex) complex and the mRNA destruction.

The importance of the discovery of the RNAi by Fire and Mello was acknowledged in 2006 with the Nobel Prize in Physiology and Medicine. Shortly after this discovery, dsRNAs were found to induce similar gene silencing in a variety of other organisms: in the fruit fly *Drosophila* [13], zebrafish *Danio rerio* [14], *Hydra magnipapillata* (cnidarian) [15], and in some plant species [16-17]. Many experiments have shown that an intermediate in the RNAi process, called short-interfering RNAs (siRNA), might be effective in degrading mRNA in mammalian cells [18-19]. Nonetheless, it was still not believed that RNAi could work in humans, because long dsRNAs, larger than 30 base pairs in length, induce a cellular response (e.g. interferon response). The first evidence that RNAi functions in humans came from experiments performed by two groups of researchers. Kreutzer and Limmer (2000) [20] demonstrated that short fragments of dsRNA might mediate the RNAi response triggered by the long dsRNAs as observed by Fire and Mello. Despite the fact that these findings were not published, a key patent around this discovery and a company focused on the development and commercialization of RNAi therapeutics was established (www.huntington-assoc.com). In the same time, Elbashir and co-workers [21] found that synthetic short dsRNA molecules result in a potent RNAi gene silencing in mammalian cells without inducing interferon response.

The knowledge accumulated from RNAi studies opened an enormous potential for the use as a tool in functional genomics studies in both plant and animal systems. In recent years, numerous strategies have been developed for targeted gene silencing and a combination of approaches enhanced the manipulation of gene silencing for functional genomics studies.

2. The molecular mechanism of RNA interference in eukaryote system

RNA silencing mechanism was first recognized as antiviral mechanism that protects organisms from RNA viruses, or prevents random integration of transposable elements [22-

25, 26]. In the last few years, important insights have been gained in elucidating the molecular mechanism of RNAi by identification and characterization of the central players of the core RNAi pathway. Extensive genetic and biochemical studies in various species have yielded a common model of RNA silencing in which trigger dsRNA. Using genetic screening analyses performed in several organisms, such as the fungus *Neurospora crassa*, the alga *Chlamydomonas reinhardtii*, the nematode *Caenorhabditis elegans*, and the plant *Arabidopsis thaliana* several host-encoded proteins involved in gene silencing as well as the essential enzymes or factors common in this process has been identified [27-28]. The molecular mechanism of RNA silencing involves several steps and a key step of silencing is the processing of dsRNAs precursors into short RNA duplexes [29-30].

2.1. The core RNAi mechanism

2.1.1. Processing the dsRNA precursors

In the initiator step, the enzyme Dicer (RNAse III-like enzyme) chops dsRNA into small pieces called short interfering RNA (siRNA), which are around 21-24 nucleotide in length [12, 21, 26, 31-33]. Dicer or Drosha, proteins known for their catalytic RNAseIII and dsRNA-binding domains, catalyzes the maturation of small RNAs. miRNAs are transcribed as long primary transcripts, which are processed by Drosha in the nucleus. Nuclear transport occurs through nuclear pore complexes, which are large proteinaceous channels deposited in the nuclear membrane. The miRNA precursor in further transported to the cytoplasm by means of the nuclear export receptor, exportin-5. Following their export from the nucleus, pre-miRNAs are subsequently processed by the cytoplasmic Dicer that yields RNAs duplexes of 21 nucleotides in length, with 5′ phosphates and 2-nucleotide 3′ overhangs. Numerous Dicer proteins have been identified in plants as well as animal system and each Dicer is preferentially processing dsRNAs, which comes from different sources [26].

2.1.2. RNA silencing effector complex assembly

siRNAs are loaded into the effector protein complex to form an RNA-induced gene silencing complex, called RISC-complex. Subsequently, the siRNA within RISC unzips, exposing anticodons and thus activating the RISC. Usually, effector complexes containing siRNAs are known as a RISC, while those containing miRNAs are known as miRNPs. For example, in *Arabidopsis thaliana* the rasiRNA-containing effector complexes are known as RITSs. All RISCs or miRNPs have a member of the Argonaute (Ago) family of proteins attached to them. RISCs and miRNPs differ in size and composition, based on the provenience organism. Further studies for identification of more specific and active siRNA duplexes for guidance of cleavage of mRNA, revealed that the sequence of the siRNA duplex had a significant impact on the ratio of sense and antisense siRNAs that were entering the RISC complex [26]. There have been different numbers of Ago proteins identified in different organisms. *Arabidopsis thaliana* has ten members, *D. melanogaster* has five members, and humans have eight members of the Ago protein family. Only a small number of these proteins have actually had their function characterized [26].

2.1.3. mRNA cleavage and repression of translation

After the formation of the RISC complex, the siRNAs in the RISC complex guide degradation that is sequence-specific, of the complementary or near complementary mRNAs [26]. The RISC complex cleaved the mRNA in the middle of its complementary region. The cleavage does not require the presence of ATP, however multiple cleavages are more efficient in the presence of ATP. RISC and miRNP complexes work by catalyzing hydrolysis of the phosphodiester linkage of the target RNA [26]. It is not fully understood the mechanism by which repression of translation guided by miRNA (micro-RNA) as well as the mechanism by which mRNA cleavage are working. The first evidence that this occurs was described in *C. elegans* mutants, where specifically targeted miRNAs reduced synthesis of proteins without affecting the levels of mRNA. It has been suggested that miRNAs affect translation termination or elongation rather than actual initiation of the process. In addition, it has been shown that miRNAs can act as siRNAs and vice versa. Further investigations suggested that mRNA degradation and translational regulation guided by miRNAs could be used as simultaneous mechanisms for natural regulation [26]. Notably, siRNAs can also move from cell to cell and systematically spread and deliver the silencing signal to the entire organism [34].

2.2. RNA silencing pathways in mammals

The 21-nucleotide miRNAs derive from dsRNA-like hairpin regions of 70 nucleotides within primary transcript [35]. Firstly, cleavage of the pri-miRNA in the nucleus by the RNAse III enzyme Drosha releases the stem-loop (or hairpin), and this precursor (pre-miRNA) is subsequently exported to the cytoplasm. The end of the stem of the pre-miRNA has the same characteristic 5'and 3'termini as siRNAs. In the cytoplasm a Dicer enzyme makes a pair of cuts that liberates a 21–nucleotide RNA duplex. Similar to siRNA duplexes, the strand whose 5'end is less stably paired will be used as guide/miRNA strand [36]. miRNA and RNAi pathways share the same core machinery, but in various animal species exist different specialization. MicroRNAs and non-coding RNAs are a major breakthrough in epigenetic of the last years, and have been found to contribute to almost all biological pathways, including gametogenesis, early development and cell signaling. While, this RNA gene silencing pathway is used by both siRNAs and miRNAs, there exist some important differences.

Comparison of *Drosophila, C. elegans* and humans has revealed that homologous Drosha enzymes catalyze the first processing step of their miRNA pathway [35]. Nonetheless, these three species show more variation in respect to the functional roles of Dicer enzymes. In *Drosophila,* two distinct enzymes are responsible for pre-miRNA cleavage and siRNA production [26]. In *C. elegans* and humans only one Dicer enzyme is present, having both cleavage functions [26].

In humans and other vertebrates, the main RNA silencing pathway seems to involved miRNA because of the existence of an immune response against long dsRNA, suggesting that the processing pathway for this type of RNAi trigger would be less important [37]. In humans, four Ago proteins (ago 1-4) have been identified [38]. A study of the assembly of

human RISC has revealed that miRNA processing and Ago2-mediated target-RNA cleavage are functionally coupled [39]. The demonstration of physical and functional coupling of pre-miRNA processing and target-RNA cleavage provides an explanation for earlier observations that 27 nucleotide dsRNA and short hairpin RNA (shRNA) are considerably more potent triggers of RNAi than duplex siRNA [40-41].

Animal miRNAs may act combinatorial, several miRNAs could binding a single transcript [40]. Also, experiments performed by Doench and his co-workers (2003) [42] suggested that multiple miRNAs could act cooperatively, reducing mRNA translation by more than the sum of their individual effect. The high number of putative target genes indicates that miRNAs function in a broad range of biological processes. Until now, the function of only a few miRNA have been analyzed in animals, but these studies have already revealed important roles of miRNAs in control of cell division, differentiation, apoptosis [43] in several developmental processes such as morphogenesis, neurogenesis and developmental timing [44, 45]. Nonetheless, antisense RNA has been implicated in imprinting and X inactivation.

Studies of miRNA processing have also provided information that could improve scientists ability to design more efficient RNAi inducing RNA molecules for experimental and therapeutic applications [46]. Krutzfeldt and co-workers [47] identified a novel class of chemically engineered oligonucleotides named “antagomirs” which silenced miRNA in murine model. Antagomirs are cholesterol-conjugated single-stranded RNA molecules 21-23nt in length and complementary to the mature target miRNA [48]. These oligonucleotides can be designed to specifically bind to the miRNA-RISC complex and they inhibit its function. Antagomirs down-regulated the proteins translation by the silencing miRNA [48]. It has been shown that these molecules are very stable *in vivo* and after one intravenous injection only, and can silence target miRNA in the liver, lung, intestine, heart, skin and bone marrow for more than a week.

2.3. RNA silencing pathways in plants

RNA gene silencing was discovered in plants as a mechanism whereby invading nucleic acids, such as transgenes and viruses, are silenced through the action of small (20–26nt) homologous RNA molecules [3]. Crucial to understanding the gene silencing mechanism is to know how to trigger it from the theoretical perspective of understanding a remarkable biological response to the practical use of silencing as an experimental tool.

This process is initially triggered by dsRNA, which can be introduced experimentally or arise from endogenous transposons, replicating RNA viruses, or the transcription of the transgenes as shown in Figure 1. In brief, double-stranded RNAs generated through aberrant gene expression from a foreign gene, virus infection or tandem repeats sequences due to insertion of transposons / retrotransposon are digested into 21-25 nucleotide long siRNAs by Dicer. This siRNA functioned as a template for the targeted degradation of mRNA in RISC and acts also as the primer for RdRP to amplify the secondary dsRNA [49]. As mentioned in the mammals system numerous components of RNAi machinery were

identified and characterized in plants. For example, Argonaute proteins played an important role in RNA silencing in plants because they are components of the silencing effector complexes that bind to siRNAs and miRNAs. Dicer proteins are required for miRNAs biogenesis. Unlike the animal system, miRNAs in plants are more paired to their target RNA and use RNA cleavage rather than translational suppression as the primary silencing mechanism [35].

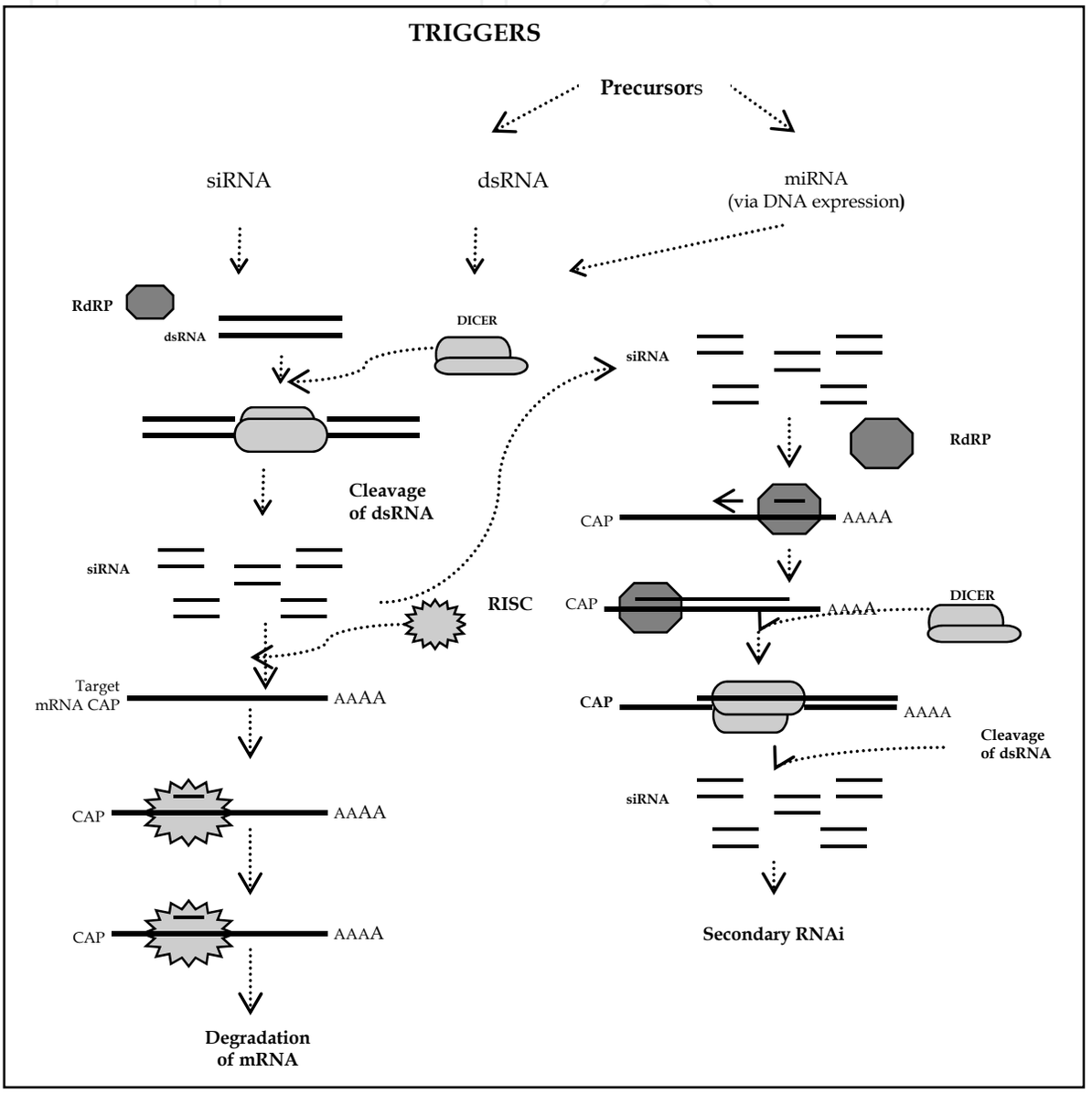

Figure 1. RNA mediated gene -silencing pathway. Double stranded RNA is digested in siRNAs by Dicer; this siRNA function as a template for the target degradation of mRNA in RISC. siRNA acts as the primer for RdRp to amplify the secondary dsRNA. RISC (RNA inducing silencing complex); Dicer (RNaseIII –like RNase); RdRP (RNA-dependent RNA polymerase)

In plants RNAi process engages the participation of numerous pathways [32]. Diverse biological roles of these pathways have been established including the mechanism of viral defense, regulation of gene expression and the condensation of chromatin into

heterochromatin. The first pathway of RNA silencing, called cytoplasmic siRNA silencing, is a mechanism by which the dsRNA could be a replication intermediate or a secondary-structure feature of single-stranded viral RNA and maybe important for virus-infected plant cells. The source of dsRNAs includes replication intermediates of plant RNA viruses, transgenic inverted repeats, and products of RNA-dependent RNA polymerases (RdRPs). The dsRNA may be form by annealing of overlapping complementary transcripts [30].

Silencing of endogenous messenger RNAs by miRNAs is a second pathway of silencing in plants. These miRNAs negatively regulate gene expression by base pairing to specific mRNAs, resulting in either RNA cleavage or arrest of protein translation. Like siRNAs, the miRNAs are short 21-24-nucleotide RNAs derived by Dicer cleavage of a precursor [32]. miRNAs downregulate gene expression through base-pairing to target mRNAs, leading to either the degradation of mRNAs or the inhibition of translation or both. In plants, the prototype miRNAs were identified as a subset of the short RNA population, and are derived from an inverted repeat precursor RNA with partially double-stranded regions, and they target a complementary single-stranded mRNA.

The third pathway of RNA silencing in plants is associated with DNA methylation and suppression of transcription**.** This type of silencing was evidenced in plants by the discovery that the transgene and viral RNAs guide DNA methylation to specific nucleotide sequences. More recently, these findings have been extended by the observations that siRNA-directed DNA methylation in plants is linked to histone modification [30]. An important role of RNA silencing at the chromatin level is probably protecting the genome against damage caused by transposons.

2.4. Types of small RNAs in eukaryote

2.4.1. siRNAs

Small interfering RNAs (siRNAs) are 22nt fragments, which bind to the complementary portion of their target mRNA and tag it for degradation. siRNA have a role in conferring viral resistance and secures genome stability by preventing transposon hopping. RNAi mechanisms, in which siRNAs are involved keep chromatin condensed and suppress transcription, repress protein synthesis and regulate the development of organisms.

2.4.2. miRNAs

miRNAs are integral components of the genetic program that account for approximately 5% of the predicted genes in plants, worms and vertebrates. Loci encoding these miRNA are localised in the introns of protein-coding genes or in the noncoding region of the genome [50]. miRNAs help regulate gene expression, particularly during development [51]. The phenomenon of RNA interference, broadly defined, includes the endogenously induced gene silencing effects of miRNA as well as silencing triggered by foreign dsRNA. Mature miRNAs are structurally similar to siRNAs produced from exogenous dsRNA, but before reaching maturity, miRNAs must first undergo extensive post-transcriptional modification.

miRNA is express from a longer RNA coding locus as a primary transcript called pri-miRNA which is processed in the nucleus, to a 70-nucleotide hairpin structure known as pre-miRNA. The small miRNAs are processed from larger hairpin precursors by an RNAi-like machinery.

The first miRNA, lin-4, was discovered in *C. elegans* five years prior to the demonstration of dsRNA as an inducer or RNAi [52]. Short non-coding transcript from lin-4 represses the expression of the nuclear protein encoding gene lin-14 as part of the control of developmental timing. The existence of partial complementarity between the small lin-4 RNA and several elements in the 3′ untranslated region (UTR) of the lin-14 mRNA suggested a mechanism of translational inhibition via an antisense RNA-RNA interaction. In this context, miRNAs were shown to compose a large class of ribo-regulators [36, 53-54]. In the same time, was demonstrated that Dicer converts pre-miRNA into mature miRNAs of approximately the same length as single-stranded siRNAs, establishing a formal connection between miRNAs and siRNAs [55-56]. Other studies have revealed the complete pathway of miRNA processing in animals, which is based on two steps catalysed by the RNase III enzymes Drosha and Dicer. The mature miRNAs of animals generally regulate their target genes by translational repression, but some cases of target mRNA cleavage have also been reported [35, 57]. This is in contrast with the situation in plants, in which target mRNA cleavage appears to be the main mechanism [58].

With the discovery of the first miRNA lin-4, interest in the role of miRNA in the regulation of fundamental biological processes has rapidly emerged. Now, more than 18226 entries representing hairpin precursor miRNAs, expressing 21643 mature miRNA products, in 168 species are tabulated in the miRNA registry (http://microrna.sanger.ac.uk). Among them, more than 300 miRNA have been discovered in humans [46]. In mammals, about one-half of the know miRNA are located within the transcription units of other genes and share a single primary transcript [59-60]. These miRNAs generally reside in the introns or in exon sequences that are not protein coding. The expression pattern of the miRNA varied. While some *C. elegans* and *Drosophila* miRNAs were expressed in all cells and at all developmental stages, other had a more restricted spatial and temporal expression pattern. This suggested that such miRNAs might be involved in post-transcriptional regulation of developmental genes [18].

In plants, as in animal systems, miRNAs, are generated as single-stranded 20-24-nucleotide species, by several proteins such Dicer and Argonaute (Ago). Ago proteins are components of the silencing effector complexes that bind the siRNAs and miRNAs. miRNAs act in *trans* on cellular target transcripts to induce their degradation via cleavage, or to attenuate protein production. Based on a computational genome analyses in *Arabidopsis*, it has been estimated that there are about 100 miRNA loci and some of them are conserved between *Arabidopsis* and *Lotus, Medicago* or *Populus* but not founded in rice [30]. Currently, there are numerous known plant miRNAs, and, in several cases, the target mRNA has been experimentally validated by expression of a miRNA-resistant target gene with silent mutations in the putative miRNA complementary region [30]. In *Arabidopsis* many miRNAs have been identified and correspond to the mRNAs for transcription factors and other proteins

involved in gene regulation [61-63]. For example, *miR159* and its putative target transcription factor MYB33vmRNA, has been regulated by the hormone gibberellic acid [27]. Gibberellic acid stimulus could lend to an increase in MYB33 mRNA that would initiate flowering, and, directly or indirectly, to an increase in *miR159*. A similar mechanism has been identified for miR177 that target a transcription factor in GRAS mRNA [62].

2.4.3. Other molecules involved in RNAi processing

In addition to endogenous miRNAs and exogenous siRNAs, several other classes of siRNAs such as: *trans*-acting siRNAs (tasiRNAs), repeat-associated siRNAs (rasiRNAs), small-scan (scn)RNAs and Piwi-interacting (pi)RNAs have been identified. tasiRNAs are small (~21nt) RNAs that have been reported in plants, and they are encoded in intergenic regions that correspond to both the sense and antisense strands [64-65]. In *Arabidopsis thaliana*, tasiRNAs require components of the miRNA machinery and cleave their target mRNAs in *trans* [64-65]. These siRNAs represses the gene expression though post-transcriptional gene silencing in plants and it is transcribed from the genome to form a polyadenylated, double-stranded precursor. rasiRNAs that match sense and antisense sequences could be involved in transcriptional gene silencing in *Schizosaccharomyces pombe* and *A. thaliana* [66-68]. scnRNAs are ~28-nt RNAs that have been found in *Tetrahymena thermophila* and that might be involved in scanning DNA sequences in order to induce genome rearrangement [69]. piRNAs are different from miRNAs and are possibly important in mammalian gametogenesis [70]. They are small (~26–31-nt) RNAs that bind to MILI and MIWI proteins, a subgroup of Argonaute proteins that belong to the Piwi family and that are essential for spermatogenesis in mice.

2.5. Factors and proteins involved silencing

2.5.1. Dicers

Members of Dicer family which showed specificity for cleavage of dcRNAs, played central role in the processing of the dsRNAs precursors: miRNA and siRNA. Processing of dsRNAs by Dicer yields RNA duplexes of 21 nucleotides with 3′ overhangs of 2 to 3 nucleotides and 5′-phosphate and 3′-hydroxyl termini [12]. Dicer namely DCR (in *Drosophila*) / DCL (*Arabidopsis*), has four distinct domains: an amino terminal helicase domain, dual RNase III motifs, a dsRNA binding domain, and a PAZ domain (a 110-amino-acid domain present in proteins like Piwi, Argo, and Zwille/Pinhead), which it shares with the RDE1/QDE2/Argonaute family of proteins that has been genetically linked to RNAi by independent studies [71-72]. Cleavage by Dicer is thought to be catalyzed by its tandem RNase III domains. Some DCR proteins, including the one from *D. melanogaster*, contain an ATP-binding motif along with the DEAD box RNA helicase domain. In *Arabidopsis thaliana*, four Dicer-like proteins (DCL1, DCL2, DCL3 and DCL4) have been identified and are involved in the processing of several dsRNAs coming from different sources [73]. For example, DCL2 is required for production of siRNA from plant viruses while DCL3 is involved in production of rasiRNA [73]. On the other hand, in *C. elegans* and mammals one single Dicer gene, DCR-1 has been identified.

2.5.2. RISC complex

RICS complex, the effector of RNAi silencing is a multi-protein complex of which several components were identified. One of the proteins identified in almost all organisms is AGO protein that is essential for mRNA silencing activity [74]. In plants 10 AGO member proteins have been identified. For example, AGO1 mutant plants have been found to develop distinctive developmental defects. miRNAs are accumulated in these mutants but the cleavage of target mRNA not longer occur [75]. AGO4 has role in the production of long siRNAs of 24bp and it was early reported that AGO4 is involved in long siRNA mediated chromatin modifications (histone methylation and non-CpG DNA methylation) [76]. In addition to AGO family members, several other proteins associated with RISC complex have been identified in vertebrate and invertebrate models. For example, the *Drosophila* homologue of the fragile X mental retardation protein (FMRP); R2D2, found in *Drosophila* and thought to facilitate the passage of the Dicer substrate to the RISC; members of the mammalian Gemin family, some of which are thought to have helicase activity [44].

2.5.3. RNA-directed RNA polymerase (RdRP)

In both plants and *C. elegans*, RNAi/PTGS requires proteins similar in sequence to a tomato RNA-directed RNA polymerase [77]. In *Arabidopsis*, RdRP homologue SDE1/SGS2 is required for transgene silencing, but not for virally induced gene silencing [78]. This may suggest that SDE1/SGS2 act as an RdRP, since viral replicases could substitute for this function in VIGS. In *Neurospora*, RdRP homologue QDE-1 is required for efficient quelling [79]. EGO-1, one of the *C. elegans* RdRP, is essential for RNAi in the germline of the worm [80], and another RdRP homologue, RRF-1/RDE-9, is required for silencing in the soma. All RdRP proteins could be involved in amplifying the RNAi signal. However, only the tomato and *Neurospora* enzymes have been demonstrated to posses RNA polymerase activity, and biochemical studies are required to establish definitively the role of these proteins in RNAi [81].

2.5.4. Putative helicase

Other proteins, helicases have been identified in plants (e.g *sde3* in *Arabidopsis*) [78]. Although possible roles in RNAi for some of these proteins were proposed, e.g. MUT6 might involved in the degradation of misprocessed aberrant RNAs [81], their functions are mostly unknown and further biochemical experiments are needed to reveal their exact roles in RNAi. The quelling-defective mutant in *Neurospora*, *qde3*, was cloned and the sequence encodes a 1,955-amino acid protein. This protein shows homology with the family of RecQ DNA helicases, which includes the human proteins for Bloom syndrome and Werner syndrome.

3. Applications of RNAi in plant systems

RNAi has been used as new tool to reduce the expression of a particular gene in mammalian and plant cell systems, to analyze the effect that gene has on cellular function, and also it has the potential to be exploited therapeutically and clinical trials. However, by using RNAi,

scientists can quickly and easily reduce the expression of a particular gene in mammalian and plant cell systems, often by 90% or greater, to analyze the effect that gene has on cellular function [18, 49, 82].

3.1. Development of efficient RNAi vector cassettes

In plants, many efforts were concentrated on the improvement of the nutritional content using the classical breeding approaches such as selection of the natural or induced genetic variations, or by genetic engineering of transgenic plants [83]. Genetic engineering technologies have advantages over classical breeding not only because they increase the scope of genes and the types of mutation that can be manipulated, but also because they have the ability to control the spatial and temporal expression patterns of the genes of interest [84]. The delivery of siRNAs in plants has been always achieved by expressing hairpin RNAs that fold back to create a double-strand region that will be recognized by the Dicer-like enzyme. Figure 2 depicted an typically RNAi construct in plants with the promoter region, the inverted repeats of the target gene with the appropriate orientation, the spacer region which separate the two inverted repeats sequences and the terminator region.

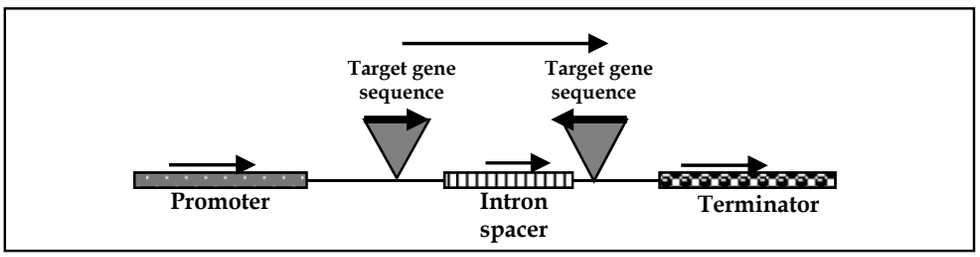

Figure 2. A schematic representation of a RNAi vector cassette with the promoter/ terminator region, target inverted repeats, intron spacer region; the arrows represent the direction of transcription

The double stranded RNA generated through aberrant gene expression from a foreign gene, viral infection, tandem repeat sequences formed due to insertion of a transposon/retrotransposon, are recognized by Dicer and digested in small interfering RNAs, which functioned as template for the targeted degradation of mRNA in RISC. This siRNA functioned also as primer for RdRP to amplify secondary dsRNA. On the other hand, in plants, RNAi is both systemic and heritable and siRNAs move between cells through channels in cell walls, thus enabling communication and transport throughout the plant. In addition, methylation of promoters targeted by RNAi confers heritability, as the new methylation pattern is copied in each new generation of the cell [85].

Genetic transformation via *Agrobacterium* or by particle bombardment or by infecting plants with viruses that can express the dsRNAs, or the infiltration of *Agrobacterium* harboring the hairpin cassette for transient gene silencing are the common methods for inducing gene silencing in plant system. The transgene expression should be evaluated as soon as possible for each transgenic event, and over multiple generations to insure that each line is stable-silencing its target. Many transgenic events should be generated and analyzed and the lines with active transgenes that are effectively inducing silencing cal be selected and maintained.

Currently, several vectors used for RNAi silencing that make use of *Agrobacterium* mediated delivery or artificially introduced dsRNA and/or VIGS into plants has been reported. For example, in 2007, an *Arabidopsis* genomic RNAi knock-out line analysis consortium was lunched out (AGRIKOLA) which is using the PCR products to generate gene-specific RNAi constructs for each *Arabidopsis* gene used in large scale gene silencing studies [86-87]; other consortium called CATMA (Complete *Arabidopsis* Transcriptome MicroArray), is generating gene sequence tags (GSTs) representing each *Arabidopsis* gene, designed so that they will hybridize on *Arabidopsis* cDNA microarrays in a gene-specific manner; the *Medicago truncatula* RNAi database (https://mtrnai.msi.umn.edu/) is a NSF-funded project planning to silence 1500 genes involved in symbiosis in this model legume; amiRNAi Central (http://www.agrikola.org) a NSF project funded to provide a comprehensive resource for knockdown of *Arabidopsis* genes.

Moreover, a set of binary vectors, called ChromDB's RNAi vectors, were designed for producing dominant negative RNAi mutants using a target sequence cloning strategy that is based on the inclusion of two restriction enzyme cleavage sites in each of two primers used to amplify gene-specific fragments from cDNA. This design minimizes the number of PCR primers and results in the placement of unique restriction enzyme recognition sites to allow for flexibility in future manipulations of the plasmid, *e.g.*, moving the inverted repeat target sequence to a different vector (Chrom database). These vectors are based on pCAMBIA binary vectors, a set of plasmids developed by the Center for Application of Molecular Biology to International Agriculture (CAMBIA).

The pHELLSGATE, high-throughput gene silencing vector and a high throughput tobacco rattle virus (TRV) based VIGS vector are binary vectors developed by Invitrogen are used for expression of GUS and GFP proteins. These vectors are base on Gateway recombination-based technology, which replaced the conventional cloning strategy. It is based on the phage lambda system of recombination. It enables segments of DNA to be transferred between different vectors while orientation and reading frame are maintained. It can also be used for transfer of PCR products. It saves valuable time, because once the DNA has been cloned into a Gateway vector, it can be used as many genome function analysis systems as is required. In this way, the use of vectors in the process of plant functional genomics has been made much easier, while the process has also been made faster. This allows for higher throughput analysis to occur [88].

3.2. RNAi and functional genomic studies

An important application of RNAi for functional genomics studies is to generate lines that are deficient for the activity of a subset of genes and then test the knockdown lines for a specific phenotype. The assessing of a specific phenotype requires the presence of a specific allele of marker genes and several generation of crosses are necessary for selecting a specific mutant allele for specific genotype. RNAi technology for functional genomics has advantage that a specific gene can be silenced if the target sequence is better chosen. However, since RNAi is a homology-dependent process a carefully selection of a unique or conserved

region of the target gene ensures that a specific member of a multiples gene family can be silenced. For example, RNAi can down-regulate specific target sequences when 3'UTR region is used as a trigger sequence [89-90]. It has been also shown that RNAi facilitates the generation of dominant loss-of-function mutation in polyploidy plants, even with short dsRNAs of a 37 nucleotide long [91].

Nowadays, numerous projects are lunched to produce siRNAs that will silence essential genes in insects, nematodes and pathogens using an approach called hdRNAi (host-delivered RNAi) [92-94] based on the partial sequences similarities between plant and animal genes. There is also a limitation of this approach because, some unexpected genes can be silenced with consequences on the organisms itself but also environment.

RNAi has been also used for the improvement of nutritional value of some important crops. For example, to decrease the levels of natural toxins in food plants a stable, heritable and distinct siRNA against the toxin could be used. Cottonseeds are rich in dietary proteins but unpalatable by humans as they contain a natural toxic terpenoid item, called gossypol. RNAi mechanism has been used to minimize the levels of delta-cadinene synthase, an enzyme crucial for the production of gossypol [95].

RNAi technology has been also applied to barley for developing varieties resistant to BYDV (barley yellow dwarf virus) [96]. In rice, RNAi has been used to reduce the level of glutein and produce rice varieties with low-glutein content [97]. Soybeans can be engineered to produce oil with low levels of polyunsaturated fatty acids through a reduction of FAD2, a fatty acyl Δ12 desaturase. This enzyme converts the monounsaturated fatty acid oleic acid (18:1Δ9) to linoleic acid (18:2Δ9, Δ12), which can be subsequently desaturated to α-linolenic acid (18:3Δ9, Δ12, Δ15) by FAD3 [98]. The reduced polyunsaturated fatty acid levels from >65% of the total oil content in normal soybean oil to less than 5% was observed [99]. In an attempt to specifically target FAD2-1, and not related family members, the soybean FAD2-1A intron was tested as an RNAi trigger, resulting in a reduction in polyunsaturated fatty acids in the seeds to about 20% [100]. This result was surprising, given that intron sequences are removed from precursor mRNAs (pre-mRNAs) by splicing in the nucleus and spatially separated from the cytoplasm where mature mRNAs are presumed to be targeted by the PTGS machinery [101].

There are also some limitations of using RNAi in functional genomics studies. Unlike the insertional mutagenesis, for the use of RNAi the exact sequence of the target gene is required. Secondly, the methods to delivery RNAi are very important, some species are easily transformable and some not. Nonetheless, further improvement of the delivery methods and vectors that can be used safely and reliably are needed. There have also been some reports that revealed the difficulty to detect mutants with subtle changes in gene expression. However, in plants, numerous marker genes are being developed that will indicate if a change in gene expression occurs [102].

3.3. RNAi and viral infections

RNA silencing in plants prevents viral accumulation and accordingly, viruses have evolved several strategies to counteract the defense mechanism. A viral protein, HC-Pro (helper

component proteinase) was shown to mediate one class of viral synergism disease [103] and expression of this protein in transgenic plants allows the accumulation of heterologous viruses beyond the normal level suggesting that HC-Pro blocked the target plant defence mechanism [104]. There are several methods known to identify viral suppressor proteins, such as transient expression assay, the reversal of silencing assay and stable expression assay.

A well known used method to study the transient expression is co-infiltration method using *Agrobacterium* strains, one strain used for inducing of RNA silencing of a reporter gene such as GFP (green fluorescent protein) and one strain that will express the candidate suppressor gene. Both strains will be infiltrated in a plant tissues such tobacco leaves, which are suited for production of a higher amount of protein in response to agro-infiltration. However, if the local silencing is triggered three days after infiltration the effect can be evaluated under UV light. If the candidate suppressor expressed from the co-infiltrated *Agrobacterium* interferes with RNA silencing, the tissue will remain bright green and in case not, the tissue will turn red [105]. In the case of reversal approach the candidates that may suppress silencing are identified. Several studies have shown that the viral suppressor proteins play an important role in this defense mechanism [106]. For two suppressor proteins, p21 encoded by beet western yellow virus [107] and p19 encoded by the tomato bushy stunt virus (TBSV) group [108] the molecular mechanism was identified.

Stable expression assay approach, a stable RNAi line expressing a suppressor candidate is crossed with several lines silenced for a repressor gene [109-110]. This method is also advantageous because provide information about the molecular mechanisms of the suppression and is also suited to investigate the role of suppression in systemic silencing using grafting [111].

However, the findings that certain viral proteins suppress RNA silencing open a new tool for biotechnologies applications. With silencing under control, many transgenic plants can be generated to produce desired plant traits or very higher level of expression to use the plant as a factor for producing pharmaceutical compounds, vaccines and other gene-products.

4. Application of RNAi in animal systems

4.1. RNAi and medicine

The ability to trigger RNAi in mammals was first demonstrated by microinjection of long dsRNA into mouse oocytes and one-cell stage embryos [19]. In this case was demonstrated that the antiviral interferon response to long dsRNAs is not yet functional in early mouse embryos. It was discovered rather quickly that chemically synthesized siRNAs could trigger sequence-specific silencing in cultured mammalian cells without inducing the interferon response [21]. Starting from this important breakthrough, RNAi has emerged as a powerful experimental tool for analyzing mammalian systems.

The ability of RNAi to determine ablation of gene expression has open up the possibility of using collections of siRNAs to analyze the role of hundreds or thousands of different genes

whose expression is know to be up-regulated in a disease, given an appropriate tissue culture model of that disease. The libraries of RNAi reagents can be used in one of two ways. One is in a high throughput manner, in which each gene in the genome is knocked down one at a time. The other approach is to use large pools of RNA interference viral vectors and apply a selective pressure that only cells with the desired change in behavior can survive [112]. Rapid progress in the application of RNAi to mammalian cells, including neurons, muscle cells, offers new approaches to drug target identification. Advances in targeted delivery of RNAi-inducing molecules has raised the possibility of using RNAi directly as a therapy for a variety of human genetic disorders.

4.2. RNAi and therapy

Considering the gene-specific features of RNAi, it is conceivable that this method it will be very useful for therapeutic applications. Direct transfection of siRNAs into cells, creating an expression construct in which a promoter drives the production of both the sense and antisense siRNAs which then hybridize in the cell to produce the double stranded siRNA and using viral vectors to infect cells with an expression construct are the methods used nowadays for RNAi-based therapy.

Nonetheless, this hypothesis is based on the assumption that the effect of exogenous siRNA applications will remain gene specific and do not show nonspecific side effects relating to mismatches off-target hybridization or protein binding to nucleic acids. For example, several research groups have explored the use of RNAi to limit infection by viruses in cultured cells. There is a huge potential for using RNAi for the treatment of viral diseases such as those caused by the human immunodeficiency virus (HIV) and the hepatitis C virus.

RNAi strategy includes multiple targets to neutralize HIV. For example, directed siRNAs against several regions of the HIV-1 genome, including the viral long terminal repeat (LTR) and the accessory genes, *vif* and *nef* [113-115]. Using Magi cells (CD4-positive HeLa cells) as a model system, they demonstrated a sequence specific reduction of >95% in viral infection after cotransfection of siRNAs with an HIV-1 proviral DNA. When the same assay was done in primary peripheral blood lymphocytes, which are natural targets for HIV-1, the frequency of infected cells was also substantially reduced. These could be targets that block entry into the cell and disrupts the virus reproduction cycle inside the cells. This technology will help researchers dissect the biology of HIV infection and design drugs based on this molecular information [116]. Researchers from Hope Cancer Center in Duarte have developed a DNA-based delivery system in which human cells are generated to produce siRNA against REV protein, which is important in causing AIDS [117].

The delivery of siRNA to HIV-infected T lymphocytes, monocytes and macrophages is a challenge. As synthetic siRNAs do not persist for long periods in cells, they would have to be delivered repeatedly for years to treat the infection. Systemic delivery of siRNAs to lymphocytes is not feasible owning to the huge number of these cells. Therefore, the preferred method is to isolate T cells from patients. In clinical trial T cells from HIV-infected individuals are transduced ex vivo with a lentiviral vector that encodes an anti-HIV antisense RNA, and then reinfused into patients [118]. In other study, it was reported that

the GFP siRNA induced gene silencing of transient or stably expressed GFP mRNA was highly specific in the human embryonic kidney (HEK) 293 cell background [119]. Further study, in human non-cell lung carcinoma cell line H1299 have shown that specific siRNAs corresponding to *akt1*, *rb1*, and *plk1* could be used as highly specific tools for targeted gene knockdown and can be used in high-throughput approaches and drug target validation.

RNAi is also utilized as an antiviral therapy against diseases caused by herpes simplex virus type 2, hepatits A and hepatits B. Early RNAi studies noted that RNA silencing was prominent in the liver, which made this organ an attractive target for therapeutic approaches. Vaccine against HBV is used only for prevention and there is no vaccine for HCV. Mc Caffrey and his co-workers (2003) [120] demonstrated that a significant knockdown of the HBV core antigen in liver hepatocytes could be achieved by the siRNA, providing an important proof of principle for future antiviral applications of RNAi. They developed a transient model of HBV infection in which a plasmid containing approximately 1-3 copies of the HBV genome (pTHBV2) was introduced into the livers of mice by hydrodynamic transfection. This results in production of all four families of viral mRNAs, including the pregenomic RNA. The pregenomic RNA is the template for the viral reverse transcriptase, which replicates new viral DNA. All four viral proteins are also made. Transient viral replication occured in the mouse liver for about 1 week. In addition, RNAi has achieved regression of clinical traits in neurodegenerative disease model [121] but its potential for use in pharmaceutical target validation and as a therapeutic tool is still ongoing.

The ability to induce RNAi across mucosal surfaces was also investigated as a means for treating de sexually transmitted disorders [122]. siRNA targeting tumor necrosis factor alpha was injected into the joints of mice with collagen induced arthritis (CIA) and the development of arthritis was scored by assessing the inflammation of joints in the mouse paw, and the mice with CIA, joint inflammation was successfully inhibited [123]. Antiviral RNAi therapeutics have already entered human clinical trials and will hopefully prove to be safe and efficacious.

4.3. RNAi and cancer

The discovery of RNAi led to the realization that the RNAi machinery is also involved in normal gene regulation through the action of a class of small RNAs known as microRNAs. There is experimental evidence that miRNAs regulate cell division, differentiation, cell fate decisions, development, oncogenesis, apoptosis, and many other processes [35]. miRNA levels are also dramatically shifted in various cancers, and miRNAs can act as oncogenes [35]. It is now clear that miRNAs represent a gene regulatory network of enormous significance. The expression profile of miRNA is highly specific for a particular type of tissue and cell stage of cell differentiation [124]. Impaired miRNA functioning, which occurs during tumor transformation, can be evaluated as a consequence rather than the cause of loss of cell identity. However, detection of chromosomal rearrangement like deletions, local amplifications and chromosomal breackpoints in the region of miRNA genes (causing impairments in miRNA expression during cancerogenesis) is a good demonstration of direct role of miRNA in these processes.

Defects in miRNA expression could cause the development of cancer associated with impaired formation of oncoproteins or tumor supressors regulated by these miRNAs. One of the first identified miRNAs, let-7 found in *C. elegans* as well as in humans, is a tumor suppressor decreasing formation of the oncoproteins Ras and HMGA2 (High Mobility Group protein A2). In patients with lung cancer was a inverse correlation between expression of let-7 and Ras/HMGA2 [125]. miR-155 is an exemple of miRNA exhibiting oncogenic properties. This miRNA is required for B- and T- cells functioning [126]. Increased expression of miR-155 was observed in paediatric Burkitt's lymphoma, diffuse large cell lymphoma, Hodgkin's disease, lung, breast and pancreatic cancers [124]. In some tumor cells miR-21 and mi-R 24 act as oncogenes, and in others they act as tumor supressors. In HeLa cells, inhibition of miR-21 or miR-24 activity by modified anti-miR oligonucleotides accelerated proliferation [127]. Analysis of bioinformatic predictions of putative targets suggests that proto- and anti-oncogenic activity is typical for various miRNA [128].

Defects in miRNA expression associated with carcinogens are caused not only by the chromosomal rearrangements, but also due to impairments in the machinery responsible for miRNA formation and processing. Inhibition of expression of ribonucleases Dicer and Drosha by complementary siRNA caused acceleration of growth of lung adenocarcinoma cells in murine model [129]. Tumor transformation may be also determined by primary impairments in regulation of expression of a single miRNA, which is then accompanied by imbalance in the entire miRNA network [124]. Impairments in miRNA functioning seen in cancerogenesis can be used for detection of miRNA expression for diagnostics of tumor origin. Each type of cancer is characterized by a certain pattern of miRNA expression [124]. Moreover, the evaluation of miRNA profiles can be used for prognosis of the development of tumors [130].

The importance of epigenomic modifications of chromatin structure for the development of tumors has been recognized [13]. Methylation of cytosine in DNA followed by formation of 5-methylCytozine occurs in dinucleotide sequence CpG. Methylation can cause suppression of transcription by proteins recognizing methylated CpG attraction. Excesive methylation of CpG island of miRNA genes has been found in cancer cells [131]. The authors attribute the effect of this methylation to suppression of tumor suppressor genes represented by miRNA genes. Processes of DNA methylation may be closely associated with modification of chromatin histones. Some histone modifications like acetylation, methylation and phosphorylation of specific aminoacides residues are involved in gene expression. Impairments of histone modifications in tumor cells have been found in many studies [132]. Processes of epigenomic silencing may involve another type of non-coding RNAs, short siRNAs. This observation demonstrates the existence of nuclear RNA-interference, which is based on suppression of mRNA translation. Some experimental data suggest that siRNA plays a certain role in gene silencing at the level of chromatin in human cancer cells. There are some examples of involvement of short RNAs in epigenomic silencing, which is coupled to DNA methylation, histone modification of target genes, and attraction of the heterochromatin HP1 protein to them. All these chromatin modifications typically occur in the cancer epigenome [124]. The role of RNAi is recognized not only in silencing of proto-oncogenes or

tumor suppressors, but also in maintenance of heterochromatin structure of centromeric region in mammalian cells [133].

4.4. Challenges for RNAi as a tool for diseases inverstigations

One of the advantages of RNAi over gene knockout is the ability to restrict gene knockdown to specific tissues or even cell types. This is important when a disease is a result of a mutation in an essential gene. The versatility of the technique has led to many applications. RNAi can be used in drug target validation and RNAi can target specific spliced exons, enabling the investigation of the functional roles of alternatively spliced forms of a gene [134]. An important opportunity is the use of RNAi in identification of all candidate genes involved in certain physiological processes using genome-wide RNAi screening [135].

RNAi can be applied to genetic model organisms such *Drosophila*, *C. elegans* and mouse in order to investigate and/or to treat some human disorders. Several models of human neural and neuromuscular disorders are available in this three experimental models. *C. elegans* is model for RNAi knockdown of genes to mimic loss of in order to elucidate the mechanisms of a number of muscle wasting diseases like: Duchenne muscular dystrophy [136], X-linked form of Emery-Dreifuss muscular distrophy [137], spinal muscular atrophy [137], fragile X-syndrome [138], Alzheimer's disease [139]. RNAi has been used in combination with overexpression studies to study the role of Parkin, an E3 ubiquitin ligase, in dopamine neuron degeneration in *Drosophila* in order to investigate the molecular mechanisms underlying Parkinson's disease. Overexpression of Parkin was shown to degrade its substrate (Pael-R) and suppress its toxicity, whereas interfering with endigenous Parkin promoted substrate accumulation and augmented its neurotoxicity [140].

Other experiment uses *Drosophila* in order to investigate human neurodegenerative disorders that are caused by expansion of CAG trinucleotide repeat. RNAi has been performed on two such diseases: Huntington's disease and spinobulbar muscular atrophy. *Drosophila* S2 cells were engineered to express a portion of human ar gene with CAG tracs of 26, 43 or 106 repeats tagged by green fluorescent protein (GFP) [141]. Cells carrying CAG repeats of 43 and 106 developed GFP aggregates. Using RNAi directed against AR protein, a loos of AR-GFP aggregates by 80% in co-transfected S2 cells has been observed. Therefore, RNAi could have considerable therapeutic potential in neurodegenerative disorders [134]. Murine model was used to investigate the potential of RNAi as therapeutic tool in neurodegenerative disorders. Spinocerebellar ataxia type 1 (SCA1) has been successfully suppressed by RNAi in mouse model of this disease [121].

RNAi might also allow future treatments of human disease such as Lou Gehrig's disease (amyotrophic lateral sclerosis, ALS), a genetically dominant inherited disease. This pattern of inheritance allowed identification of a specific gene that is linked to the death of Betz cells in some families, the gene for an enzyme called superoxide dismutase. Superoxide dismutase can protect cells from molecular damage caused by free radicals of oxygen. Mutant forms of superoxide dismutase can lead to cell death [142].

The Nobel Prize-winning research on RNA inhibition might lead to new treatments for patients with this disease due to dominant mutations in the superoxide dismutase gene. Reduction in the level of the superoxide dismutase enzyme coded for by the mutant gene has been studied in animal models [143]. Working with laboratory mice as an experimental model system for the human disease ALS, Miller and coworkers showed that loss of muscle function could be slowed using RNA interference [144]. This result was obtained by using a virus to induce RNA interference in neurons. These results from laboratory experiments suggest that if RNA-induced inhibition of mutant superoxide dismutase can be induced in the correct cells of the brain and spinal cord, it might be possible to slow progression of *Lou Gehrig's disease* in humans.

A high number of pharmaceutical and biotechnology companies have declared an interest in or have an active drug development program already underway in RNAi-based therapeutics to silence disease associated genes. This web (www.rnaiweb.com) collects together the latest research covering the development of RNAi based tools for drug target and gene function analysis. These include Sirna Therapeutics (Colorado) for macular degeneration; Avocel (Sunnyvale, California) for hepatits C; Alnylam Pharmaceuticals (Cambridge) for Parkinson's disease; CytRx (Los Angeles, California) for obesity, type II diabetes and ALS etc. But the major challenge in turning RNAi into an effective therapeutic strategy is the delivery of the RNAi agents, whether they are synthetic short double stranded RNAs or viral vectors directing production of double stranded RNA. During diseases, changes in the pattern of microRNAs will occur with some being indicative of treatment outcome and disease progression. More exciting then diagnostic value is the evidence that directly involves miRNAs in a number of diseases (cancer, imprinting impairments etc). In this context there is an increased interest in manipulations miRNAs for therapeutic purposes. In the loos of miRNA function, one approch is to mimic miRNA activity by introducing microRNA „mimics"with the same genetic information as the natural miRNA. For exemple, by adding more of a microRNA named let-t, it has been possible to halt cancer cells from further multiplying [145].

Another complementary approach to using miRNAs for therapy is to inhibit the activity of disease-associated miRNAs. This can be achieved by employing antisense oligonucleotides that, based on sequence complementarity, will bind to inactivate miRNA function. Esau end colleagues demonstrated that the inhibition of miRNA may be a potential therapeutic approach to the treatment of disease [146]. They inhibited miR-122 expression with antagomirs, which resulted in reduced plasma cholesterol levels and a decrease in hepatic fatty aceid and cholesterol synthesis rates in normal mice and in diet-induced obese mice. Targeting miR-122 with antagomirs resulted in inhibition of disease development.

RNA interference has much promise in laboratory. In principle, RNAi might be used to treat any disease that is linked to expression of an identified gene [112]. The most important challenge in turning RNA interference into an effective therapeutic strategy is the delivery of the RNA interference agents. Given sufficient research into delivery methods, some of these diseases will probably be treated effectively by RNAi based therapeutics.

5. Conclusions

The study of RNAi has led to a revolution in the understanding of gene expression and the examples in plants, animal and mammalian system as reviewed here, showed the diversity and the potential of RNAi as new approach to replaces the classical genetic technologies and manipulation.

Today, the RNAi strategies as a new tool for cheap screen of gene function in organisms for which, a genetic approach was not developed yet. However, after 11 years of extensive research, RNAi has now been demonstrated to function in mammalian cells to alter gene expression and used as a means for genetic discovery as well as a possible strategy for genetic correction and genetic therapy in cancer and other diseases.

Finally, RNAi represent a significant tool for the accomplishment of these goals, and will undoubtedly be used to address many other challenges in eukaryote functional genomics.

In future, a combination of RNAi and whole genome sequencing can contribute to the enhancement of the drug development success rates through better targets and RNAi platform efficiencies and keeping waste to a minimum by only treating people genetically predicted to respond to the therapeutic.

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