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Small RNA, Big Impact and Robust Hope: Biochemical Basis for RNA Interference

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Published Date: March 24, 2017

SUMMARY

RNA interference (RNAi) has initially emerged as a strategy for innate immunity in plants against about 90% RNA viruses. The complete biochemical characterization of RNAi proteins is a pre-requisite for realization of the unique potential of this mechanism-including the explicit behaviour of each protein involved, and in identifying the target genes and it in-depth regulation. The inherent biological and technological applications of RNAi were determined by the biochemical studies that unravelled novel biological roles of RNAi factors. The process is interfere specific gene expression, ruling out the possibility of off-target effects. This chapter highlights the potential of RNAi as a unique and powerful experimental tool wherein small synthetic regulatory RNA incorporations regulate different critical process of life, including the development and characterization of embryonic stem cells. Thus, the biochemical role of RNAi will unravel the orchestrated and harmonized functions, and help devise newer technologies, alleviating several ailments, benefitting mankind.

Keywords: Biochemistry; RNAi; Dicer; siRNA; miRNA; piRNA; Ping-pong

INTRODUCTION

RNAi or RNA interference, the ready-made machinery that knocks down specific gene expression, is a preferable initiative for realization of in-depth and prompt studies pertaining to functional genomics and gene therapy. The transgenic experiments related to co-suppression performed successfully in animal models [1] laid the foundation for RNAi. Subsequent to the discovery of the RNAi mechanism, an extensive genome-wide genetic screening identified a handful of genes, required for transcriptional and post-transcriptional gene silencing in different eukaryotes. In *Drosophila,* several genes simulataneously interplay between the transcriptional and post transcriptional silencing mechanism and act as common targets for understanding the different gene silencing mechanisms [2]. The molecular characterizations of these genes define the mechanistic pathway of the various processes of RNAi. The major direction of such analysis is the critical reconstitution of the artificially synthesized components in every step of the interference pathway. It also associated with the particular function of the every structural domain of constituted synthetic proteins. The detail biochemical description of RNAi is a prerequisite for full consideration of the tremendous ability of the artificial machinery. The extraordinary target identification and functional efficacy in controlling the target expression occurs by a surprisingly diverse array of mechanisms.

The in-built biochemical technicalities of the RNAi mechanism have implications in improving both biological and synthetic technology. In fact, the processing enzymes and their functional mechanism unravel novel biological roles of the respective pathway. Biochemical studies have been important in the development of RNAi as a artificial tool specifically in mammals, where each intermediate steps in the RNAi triggers interference process without instigating non sequence–specific effects. Biochemical studies accommodated in this report, enhance our ability to harness the RNAi mechanism as a routine experimental tool in mammalian cells, for different cell lines, embryonic stem cells characterization and to discover unique development stages of animal embryogenesis. No doubt, the diversified biochemical studies of RNAi will continue to have an exceptional contribution in further honing this adept gene silencing tool in differentiated as well as non-differentiated cells.

The biochemistry underlying RNAi

The premier discovery that RNA molecules help in mRNA transcripts degradation with a decreased gene expression proved to be a new trendsetter shifting the existing dogma in the field of molecular biology. For the first time, silencing of a transgene in plant cells [3-5] was discovered to occur in both the levels of transcription and post-transcription, in either of which, short RNA moieties were involved. RNAi, as a technique was first discovered in *Caenorhabditis elegans* in 1994 [6] and in the later years established to occur in a large array of eukarotes including *Drosophila,Schizosaccharomyces pombe*, *Dictyostelium*, *Neurospora,* plants, mice and humans [7-9]. The prime active ingredient for the achievement of RNAi was indeed a double stranded RNA

or dsRNA [10]. The source of this molecule may be from endogenous heterochromatic repeats or may be artificially introduced, as one commonly cloned from 500–700 bp inverted repeats to produce a dsRNA hairpin–loop [11]. This hairpin undergoes cleavage by enzymes, assembled onto multi-protein complex, specifically cleaving mRNA stretches having complementarity with the dsRNA fragments.

The RNAi is a strategic measure adopted by the cell to preserve its genomic integrity against the invasion by viruses and aberrant transposition by invading transposable elements [12,13]. But with recent advances, its role has been extrapolated to the maintenance of heterochromatin formed at the centromeres [14,15] and in the dividing germline stem cells [8]. This immense potential of RNAi can be therefore harnessed in studies involving gene silencing and for designing powerful therapeutics which can elicit a targeted yet effective biological action [16].

Mechanism of knock-down *in vivo*

The knock-down of gene expression, postulated to occur through dsRNAs were an initial beacon to the devise new RNAi-dependent methods that continued to revolutionize functional genomics because of the ease of use and the possibility to use the approach of reverse genetics across all model systems, including mammals. The sequencing of genome sequences of *C. elegans* [17-19], rat [20] and *Drosophila* comprehensively identified all the genes that made reverse genetics even more popular and exploited its benefits to the utmost. The genome-wide RNAi screens of the *Drosophila* cells [20] made RNAi, a well-established tool for studies pertaining to tissue culture. Presently, high throughput screening of Drosophila and mammalian cells has become a routine aspect in the molecular biology laboratories, thanks to the advancements made in RNAi, particularly found helpful in characterization of pleiotropic gene functions for whose identification genetic tools were not very established.

The discovery of mRNA degradation in a nematode, *Caenorhabditis elegans* via introduction of a dsRNA [10] (Fire et al. 1998) paved a way for new ventures in in vivo RNAi. The RNAi exhibited in *C. elegans* is both systemic and transitive, where dsRNA injected into a tissue, can cause the gene to be silenced in tissues elsewhere [10,21]. When genetically analysed, this effect was found to be as a result of expression of genes like the trans-membrane multiple spanning protein, SID-1, that ensured the uptake of dsRNA [22]. A transitive nature of RNAi means that the amplification step of RNAi requires a RNA-dependent RNA polymerase (RdRP) and siRNAs are formed in the upstream of the native dsRNA [23]. Introduction of dsRNAs into the nematode is done either by soaking in a concentrated dsRNA solution, feeding food rich in bacteria expressing long dsRNAs, by micro-injecting dsRNA, or producing transgenic worms expressing hairpin. These RNAi screens have answered a variety of biological questions in the field of developmental biology, cell signaling, aging, metabolic regulation, and neurodegenerative diseases. Amongst all the methods of dsRNA introduction, feeding the worms with dsRNAs is the one most preferred alternative [24], owing to its simplicity and a completely documented and known repository of over 20,000 characterised genes in this organism [25-27].

The dsRNA in the range of 200-2000 bps, and siRNAs of 21–22 can be successfully delivered in *Drosophila* by micro-injection into the pre-cellular blastoderm embryo [28-30]. Using such a method, the somatic muscle forming role of the Myo-D related gene, nautilus in embryos were discovered [29], and the role of the receptors, Frizzled1 and Frizzled2 in Wingless signalling were exposed [28]. Such extensive screening has revealed more than 5000 genes for cardiogenesis and embryonic nervous system. To screen the heart, embryos were injected with the transgene, D-mef2-lacZ , while to screen the nervous system, the 22C10 antibody was utilised that detected the peripheral nervous system and a section of central nervous system.

RISC: an impetus to silencing

The RNA induced Silencing Complex or RISC is a multi-protein complex of 150 to 200 kDs that houses multiple components that play a role in assembly, target cleavage and formation of a distinct effector complex for undergoing targeted mRNA degradation. The Complex from Drosophila embryonic lysates after purification, were found to comprise the factors like Ago2, dFXR (Drosophila ortholog of fragile X mental retardation protein), VIG (vasa intronic gene), Tudor-SN (a nuclease with a tudor domain and bearing five nuclease domains homologous to the Staphylococcus nuclease domain), R2D2 (a dsRNA binding protein with two dsRNA binding domains), Aubergine (an Ago family protein) and Armitage–RNA helicase.

The assembly of an active RISC takes place by sequential loading of the siRNA. The siRNA duplex synthesized has an unstable 5' end enter the RISC [31] via the concomitant action of Dcr2-R2D2, where binding of R2D2 to the 5' end of the passenger strand results in its cleavage by Ago2 and gets discarded, while the 3' end of the guide strand remains associated with the Dcr-2 via its PAZ domain and hence the siRNA guide the RISC to its target mRNA, binds to it and averts the mRNA expression. Thus, Dicer here plays a dual role, by simultaneously generating siRNA and delivering it to RISC. The assembly of RISC begins with the binding of R2D2-Dicer-2 heterodimer to the siRNA duplex, together referred to as RDI (R2D2–Dicer-2 initiator) complex , independent of the ATP. It has a transient complex similar to the RISC loading complex that cleaves the passenger strand with the help of AGO2 and leads the entry of the guide strand to the holo-RISC, having other proteins like Tudor-SN [32], responsible for the catalytic activity for the 80S- holo-RISC (Figure 1).

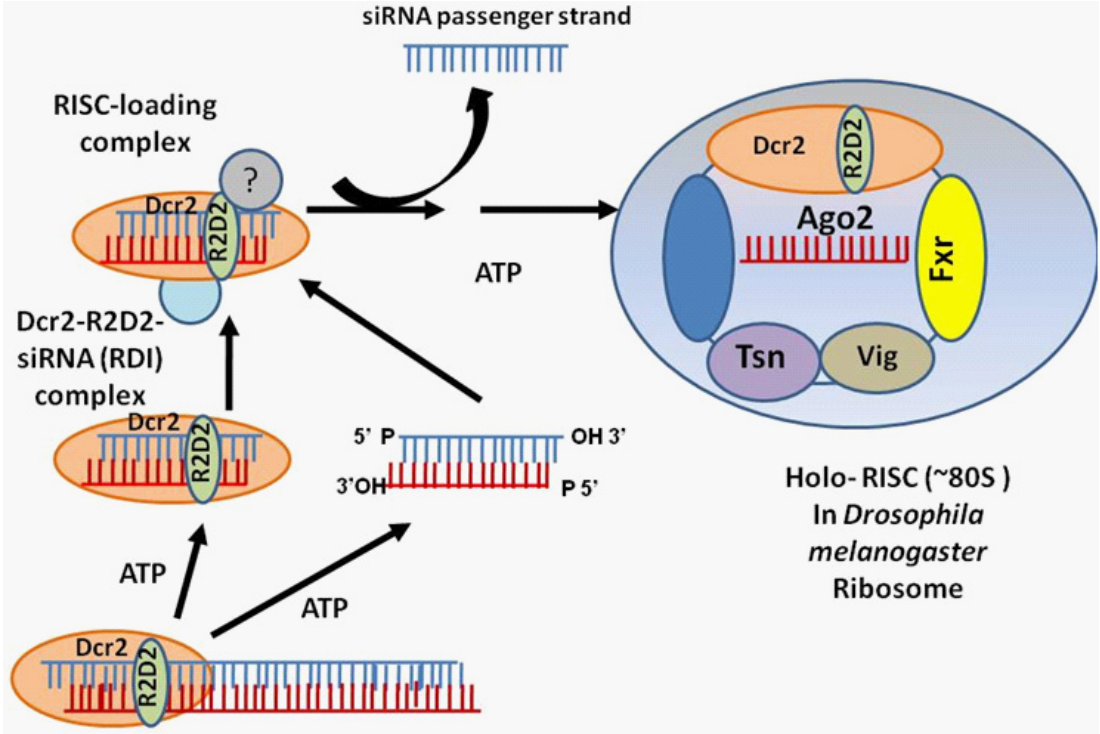

Figure 1: The functioning of RISC.

A biochemical characterization of the equi-length 5′-PO_4 and 3′-OH cleavage products of RISC deems it to be an endonuclease that gets produced from the 5′- phosphor-monoester (Meister et al. 2004) that can cleave a target of length 15nt. The 5′-PO_4 guides the siRNA of RISC is capable of attacking the target RNA at the 10^{th} and the 11^{th} nucleotides.

siRNAs are synthesized chemically or processed from long starnds of dsRNA by a protein, Dicer forming a heterodimer with dsRNA binding protein, R2D2. Once that a dsRNA is processed by this complex it remains bound to the dsRNA or may be released. Such a complex of siRNA and Dcr2-R2D2 is called the RISC Dicer inducer complex. The siRNAs are then taken up by the RISC loading Complex along with other factors to form RISC , utilising ATP to unwind siRNA forming a passenger strand and a guide strand that recognizes the target mRNAs and cleaves it. In *D. melanogaster* embryo lysates, RISC activity resides in an ~80S holo-RISC assembly that might include a ribosome.

If there is any mutation in the central part of the substrate that pairs with the centrally placed 13-nt of the guide siRNA, the target cleavage's probability reduces relatively. The presence of Mg^{+2} incites the functioning of RISC by binding to the phosphodiester bond between the targets RNA's 11^{th} and 12^{th} nucleotide. On the other hand, adding EDTA or ethylenediamine tetraacetate or bulky moieties as 2′-O-methyl groups diminishes the cleavage of the target RNA [33]. Thus, the steric hindrance and transformation of conformation with respect to the residual active site act

as the rate limiting factor for RISC. The RISC in *Drosophila* acts as a classical Michaelis–Menten enzyme when ATP is present as ATP enhances the turnover reaction [34]. When RISC is in excess, the presence ATP however stands no impact on the rate of cleavage of the target. The nucleotides present at the 5'end of the guide siRNA increases the Km of the RISC while the nucleotides in the center and the 3'end is mainly responsible for its catalytic efficiency. The PAZ domain of AGO2 binds to the 3' end that checks its binding to mRNA and the energy expenditure for binding is subdued by that which is required in releasing the 3'end of the siRNA to PAZ domain which highlights the importance of the 3'end nucleotides for target cleavage but not for target binding.

Generation of siRNA by dicer

Dicer is a ribonuclease II enzyme that triggers the targeted cleavage of mRNA molecules to cause the RNAi to successfully occur. It bears a molecular weight of approximate 200KDa and bear orthologs and paralogs across all strata of organisms. The fruitfly, *Drosophila* bears two paralogs of Dicer, namely, dcr-1 and dcr-2, plants are found to possess about four paralogs of Dicer- dcl-1,-2,-3, and -4 but humans possess a single variant of this gene [35]. The action of Dicer on the dsRNA is the most critical step for RNA-dependent silencing mechanisms and its efficiency rests upon the structural integrity of the siRNA molecule. The variations in thermodynamic stability come into play when identifying the actual strand participating in the process. Once that the siRNA unwinds, it give rise to the "guide" strand which binds to the RISC and targets the specific mRNA based on sequence complementarity and the "passenger" strand on the other hand, gets degraded. Thus, the accomplishment of mRNA degradation is the sequential interaction of a number of RNAi machinery factors, right from its generation to the recognition of the target (Figure 2). The maintenance of an optimum helical structure and functional group of the siRNA is a cruicial requisition for the enhancement of the RNAi mechanism. The siRNA that gets generated possesses about 21-23 nucleotides with 5'-PO_4 and 3'-OH with a 3'end 2-nt overhang [36,37]. The 5'-PO_4 and 3'-OH are the indicator of a ribonuclease II catalytic activity similar to that shown by Dicer. A variation in the number of nucleotides at the 3'overhang had a tremendous impact on the efficiency of silencing. The 5-PO_4 'provides stability and determines the exact site for cleavage on the target RNA [38], hence the siRNA duplex lacking these group on both the sides can not initiate RNAi, in vivo. Replacing the antisense strand with the 2'-O- methyl group is found to completely disrupt the phenomenon of RNAi. Recent studies have also proved that chemically modifying the A-form of the helical structure between the siRNA and target RNA can drastically alter the ability for RNA interference [39]. Therefore, such modifications can be really instrumental if one considers it while making a potent synthetic siRNA for *in vivo* studies.

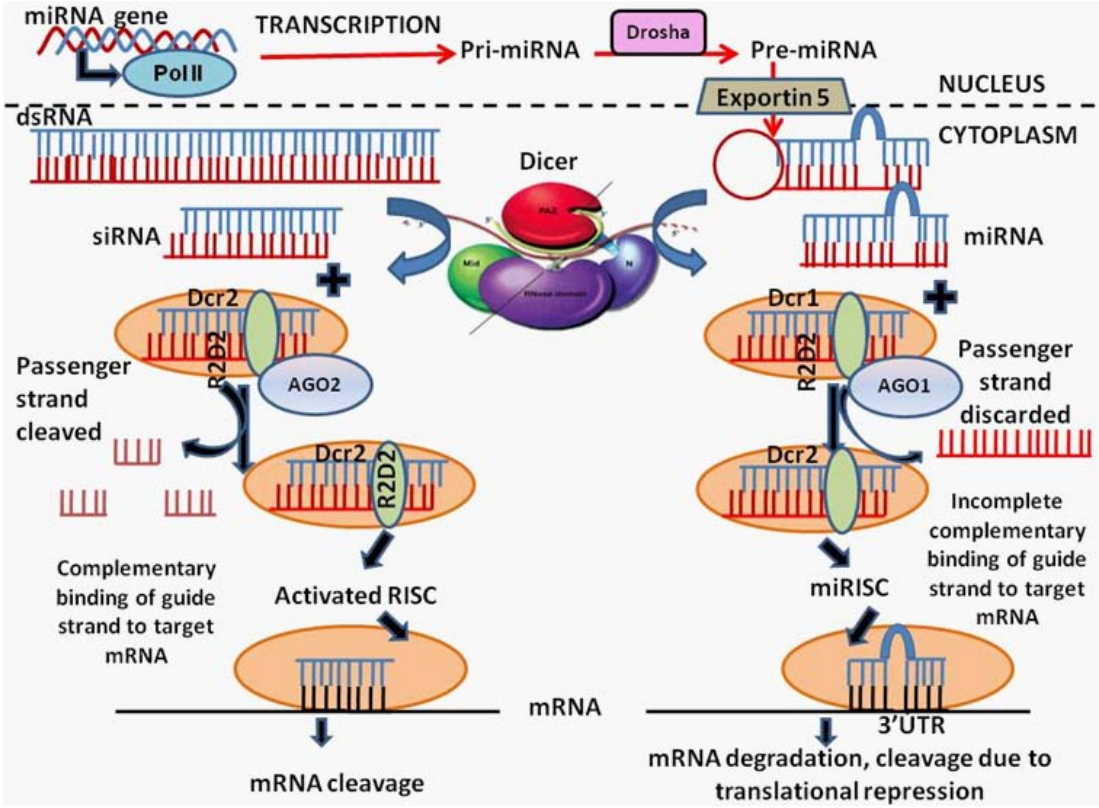

Figure 2: The mechanism of gene silencing by siRNA and miRNA.

In case of the siRNA, the dsRNA is processed into siRNA by Dicer, and is loaded into the RNA Induced Silencing Complex., It is then cleaved by AGO2, a component of RISC to produce a passenger strand of siRNA. The other strand, called the guide strand then dictates the activated RISC to the target mRNA, where once the guide strand shows complete complementarity, with the target mRNA the cleavage of the mRNA occurs. In case of the miRNA, RNA polymerase II in the nucleus transcribes the gene of miRNA to produce pri-miRNA, which further forms pre-miRNA due to cleaved by Drosha. The nuclear pre-miRNA is then carried by Exportin 5 to the cytoplasm, where Dicer processes to miRNA. When the miRNA is loaded into the RISC, the passenger strand gets discarded, and the guide strand is guided by miRISC to the bind to the partially complementary target mRNA, that leads to repression of translation

Dicer and the discovery of microRNAs

Micro RNAs represents a unique cadre of small RNAs that are partially complementary to their target mRNA that suppress the translation of the target mRNA rather than cleaving them, as in the case of siRNA. Dicer, a ribonuclease III is found to cleave pre-miRNAs, which bear hairpin structures with single stranded stretch of about 50-70 nt [40]. The human variant of Dicer possesses a weight of 220-kDa with an amino terminal helicase domain that bears homology to DExD/H-box

helicases, two RNase III domains of RNase IIIa and RNase IIIb, a DUF283 domain, a PAZ (Piwi-ArgonauteZwille) domain, and a dsRNA-binding domain (dsRBD) [35,40-43]. According to the first proposition of Dicer ribonuclease activity by Filipowicz et al. [43], the precursors of miRNAs and siRNAs are identified by the PAZ domain that gets attached to the the substrate depending upon the presence of an overhang of 2 nt [43-47]. This enzyme makes a cut on both the strands of the dsRNA precursor at 20 bp away from their termini.

The dsRNA binding domain acts only in binding the substrate and executing the cleavage. The RNase IIIa and RNase IIIb domains form the site of cleavage where dicer makes cuts on both the strands of dsRNA precursors at 20 bp from the termini. Recently it was shown that dicer possesses two binding pockets, at the 3'end, located at the PAZ domain and the other at the 5'end, bearing a platform domain. Docking to the 5'end occurs only when substrate ends are unstably paired, which is a characteristics of precursor miRNA. The fact that the two fragments, DUF283 and PAZ domains at the N-terminal and the RNase III and the dsRBD domains at the C-terminal produce 22-bp products, even if lacking the linker connecting the PAZ and RNase III domains [45] can make us conclude that the interaction of PAZ and RNase III domain determine the lengths of Dicer-generated products produced through a RNA substrate, directly or indirectly. The presence of Dicer throughout eukaryotes and its absence in bacteria and archaea prove the independent diversification of Dicer in the animal, plant and fungal lineages [45,48]. Dicer is lost in some protozoan parasites like the *Leishmania major* and *Trypanosoma cruzi* and from some fungi like the *Saccharomyces cerevisiae* [49,50]. This can suggest that the gene, Dicer goes through duplication at the early animal and plant evolution producing two different classes of dicer in animals. Studies have also highlighted the importance of Dicer-2 in defending against RNA viral infection in *Drosophila* [51]. Any mutations in the gene, Dicer produce an array of phenotypes, correlating with various cancer, therefore they demand a further introspection.

piRNA synthesis: ping pong loop

piRNAs or Piwi interacting RNAs were discovered by the seminal study in *Drosophila* where small RNAs expressed in testis, as a derivative of the *Su(Ste)* locus were found to target and silence the *Stellate* transcripts, that facilitated the occurrence of spermiogenesis [52]. When small RNAs were catalogued from various tissues and developmental stages, they were found to be 23 to 30 nt long, matching with transposable elements and repeats [53]. Surprisingly, such RNA species, native to the tissues of the male and female reproductive system were found across all taxa like flies, fish and mammals [54-59]. The piRNAs upon maturation show similarity with Piwi proteins, with respect to their interaction, and a length of 26 and 30 nt in case of *D. melanogaster* and vertebrates, possessing preferrably a 5′ uracil, and a sugar with 2′-O-methylation [60-62]. Contrary to it, the piRNAs in *C. elegans* have 21 nt but have similar features at 5′ and 3′ of piRNAs as in other organisms The piRNA biogenesis pathway is distinctly variable from those producing of miRNAs or siRNAs as it needs [63]. no double-stranded RNA precursor or Dicer for synthesis [54,62,64].

In the primary pathway of piRNA biogenesis, the precursors undergoes cleavage by Zucchini (Zuc) [65-67] to form cleaved fragments .These fragments then get incorporated into Piwi or Aubergine (Aub) with the help of proteins, Shutdown (Shu) and Heat shock protein 83 (Hsp83, which is the fly homologue of HSP90) [68]. The fragments having a 5'U stand a high chance for selection. After loading into the Piwi proteins, the Trimmer enzyme trims the 3'end, making room for the Piwi protein which gets methylated by the protein Hen1 at the 3'end thereby completing the primary biogenesis of the piRNA.

Unlike the primary pathway, the ping-pong cycle was a culmination of the idea that Piwi and Aub bound sequences have a prominent tendency to ensue with 1U, which explains the biasness of the primary piRNA in insects and pachytene piRNAs in mouse towards a uridine at 5', whereas piRNAs associated with Ago3 possess an adenine at position 10. The ping-pong cycle of piRNA biogenesis is the PTGS mechanism mediated by the piRNA. In such a process, Aub gets associated with the primary piRNA derived from the cluster and slices the active transcript of the transposon producing 5' ends of the new piRNA in sense to the transposon. Subsequent to the loading into Ago3 and trimming by a nuclease, the Ago3-piRNA complex identifies and cleaves transcripts to produce anti-sense piRNAs, identical to primary small RNA. Intermediates associated with Aub mature when cleaved by Zuc, producing phased piRNAs downstream, which are predominantly associated with Piwi to allow the target to adapt (Figure 3).

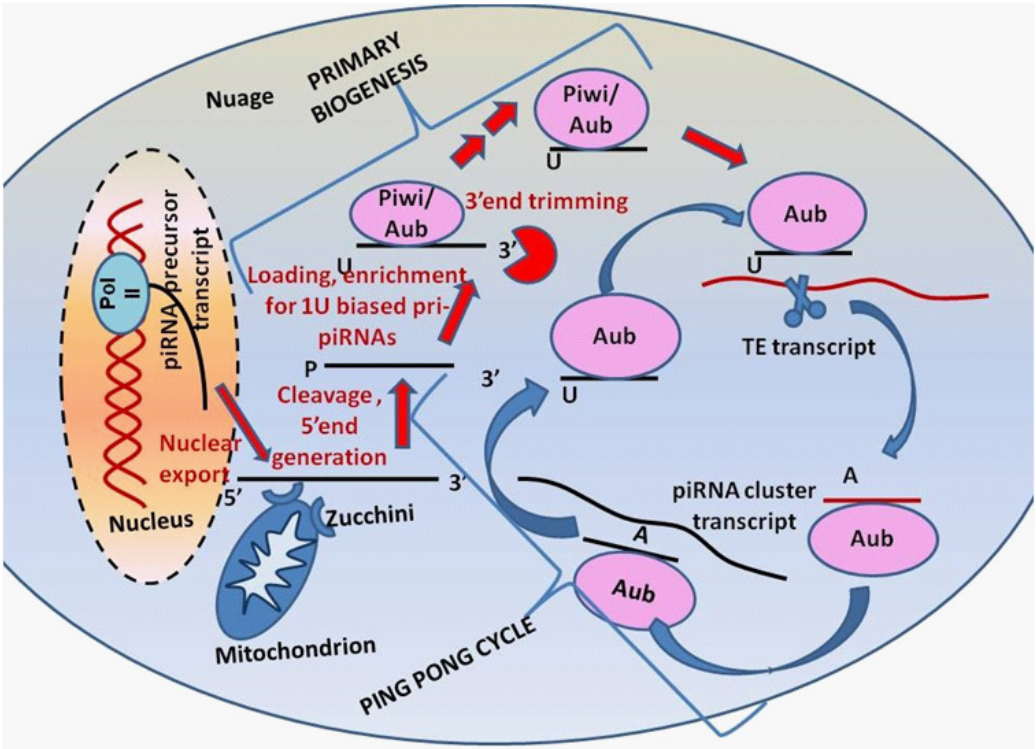

Figure 3: The biogenesis of piRNA.

The unique aspect of ping-pong amplification is its coupling of piRNA biogenesis with silencing of the targets. In the *Drosophila* germ cells, the primary piRNA derived from the cluster associate with Aub, then gets identified and slices across the transposon transcripts producing piRNAs with 5'ends in sense orientation to transposons. The cluster derived from the pericentomeric and telomeric heterochromatin transcripts [56] are trimmed by an unknown nuclease or the Zuc-dependent 3'end formation that leads to the Ago3-piRNA complex identification, followed by the generation of multiple anti-sense piRNA copies, identical to the primary triggers of the small RNA. Zucchini mediates a cleavage of the Aub-associated intermediates produce phased piRNAs downstream to the transcript with mainly associate with Piwi to adapt to the target via diversification of the sequence. The ping-pong amplification depends upon the presence of a number of factors like the DEAD-box RNA helicase- Vasa, and a group of Tudor-domain proteins, like the Spindle-E (Spn-E), Krimper, Tejas, and Tapas. The Tudor domains act as scaffolding agents binding symmetric Dimethyl-Arginines (sDMAs), added by the methyltransferase, Capsuleen and its co-factor, Valois to the PIWI proteins [69,70].

There are mainly two pathways- the primary piRNA biogenesis that occurs in the follicular cells and the Ping-Pong cycle operating in the germ cells in *Drosophila*. Long RNAs transcribed from piRNA cluster regions produce long RNAs by transcription in the nucleus and get exported from the nucleus to nuage granules. The nuage granules house many piRNA –related components and piRNA biogenesis mainly occur here. The precursors of the long piRNA, in a primary piRNA biogenesis, undergo cleavage by an endo-nuclease, Zucchini, having a mitochondrial localization to generate the 5′ end of the prospective piRNA. The transcript produced after cleavage is loaded onto Piwi proteins (Piwi and Aub) and trimmed from the 3′ end by a nuclease nuclease to produce a transcript of final length. In the Ping-Pong cycle, complementary RNAs of transposon mRNAs are cleaved after recognition by Aub to produce 5'end of a new piRNA. This new piRNA can in turn get loaded into Ago3 to induce the cleavage of the complementary RNA. The result of such cleavage is a new piRNA that looks identical to the piRNA that started the cycle [71-73].

The piRNA biogenesis however poses some handicaps which are firstly, its complex nature compared to other small RNA pathways , mainly the miRNA and siRNA and secondly, the limited occurrence of the piRNA system to gonadal tissues that prevents a systematic analysis *ex vivo*. However, the introduction and detailed characterization of cell lines containing primary or secondary piRNA pathways will greatly enhance studies of piRNA biogenesis and silencing [74-77]. Indeed, the first *in vitro* systems that replicates the aspects of the pathway have been successfully established [78,79]. Furthermore, recently published large-scale screens have likely revealed the complete catalogue of factors involved in piRNA-mediated genome defense [80-82], ther by, allowing each pathway protein to be systematically annotated. Booming technical advances at present and the near future, in the field of proteomics and microscopy with CRISPR/Cas9-mediated genome editing will aid in dissecting the various molecular steps generating piRNAs and will provide detailed insight into transcriptional and post-transcriptional silencing by the PIWI-piRNA complexes.

Biochemical aspect of mammalian RNAi

RNAi has successfully unravelled the basis of gene function in the nematode*, C. elegans* since about one third of its genome has been functionally analysed by RNAi [25,83-86]. It is only in the very recent years that mammalian cells have become feasible for RNAi via techniques like the in vitro transcription, that produced long dsRNAs, greater than 30 bp, that knockdown of the gene expression via abrogation of protein synthesis through mRNA degradation [87]. RNA interference has worked perfectly in case of early mouse development [88,89], but still remains far from application in mammalian cells [89,90]. If RNA duplexes of 21-nt length with 2-nt 3′ overhang are introduced by transfection into human and other mammalian cultured cells, they can specifically interfere with gene expression [37], which is similar to the functioning of a siRNA [36]. The siRNA is capable of silencing lamin A/C in HeLa cells and so this approach is also adopted for lamin B1 and the nuclear mitotic apparatus protein NuMA [37]. Recent studies on mammalian tissue culture cells have shown that RNAi can silence many cellular proteins such as actin and vimentin. This has added to the knowledge about various essential genes like the lamin B1, lamin B2, NUP153, GAS41, ARC21, cytoplasmic dynein and the mitotic protein kinase cdk1. Thus, RNAi is useful in studying organotypic cultures such as polarized epithelia and muscle cultures, which otherwise need a long time to observe. If improvements in the transfection efficiencies are made, then the silenced cultures can act as starting material for analysing biochemical parameters. This can therefore find great use in analysing genome wide gene functions and can help in target validation of various therapeutic genes. A combination with modern screening methods such as high throughput screening based on siRNAs can explore the functional genomics of cultured mammalian cells, so that siRNAs become a widely used tool for cell biologists to study mammalian gene function, and various general cell biological mechanisms such as mitosis, processing and traffic of RNA transcripts, the formation of cellular junctions and membrane trafficking.

RNAi in remediating human diseases

In contrary, to the method of targeting a downstream target of the molecular cascade for a disease through a small molecule, RNAi can specifically target an inhibition of the protein expression that lack an enzyme-binding pocket for the drug binding and therefore, promise a targeted recovery. Till now, unmodified siRNAs have been successfully administered locally to the eyes and few other organs. To avert a degradation of siRNA therapeutic before it reaches the target through bloodstream, they were encased in lipid nanoparticles as in case of siRNA transport to the liver. The highly vascularised liver is fenestrated with multiple pores of about 100 nm that allow the 70 to 80 nm LNPs to enter to the hepatocytes. In this way it can be a promising treatment for several ailments of the liver, including, liver scarring, liver failure and liver cancer.

RNAi can be a promising dream weapon in combating cancer, against the currently undruggable oncogenes like *MYC,* since it has the potential to specifically inhibit the mutant proteins that drive a tumor's growth. Several methods of siRNA delivery and administration are now under

trials. siRNA embedded in a nanoparticle, built up from a polymer containing cyclodextrin sugar molecules, CALAA-01, can be deposited in solid tumors to block the translation of its target, RRM2, the M2 subunit of the enzyme ribonucleotide reductase, that regulate cell division, but it is still in its clinical trial. Hepatocellular cancer may be treated with TKM-PLK1, which inhibits expression of *polo-like kinase 1*(*PLK1*), which is a potential target in oncology.

The Alzhemier's disease is characterized by two primary pathological marks: the presence of senile plaques which contain beta amyloid derived from APP and neurofibrillary tangles caused by a filamentous protein, Tau [91]. Tau and Amyloid Precursor Protein (APP) are the key proteins responsible for the pathogenesis of sporadic and inherited Alzheimer's disease. Thus, an inhibition in production of these proteins by selectively silencing mutant alleles can treat inherited dementias and other dominantly inherited disorders. The tau and APP acts as model targets because of their role in inherited and acquired forms of age-related dementia, like the Alzheimer's disease [91-95]. These targets produce siRNA against any functional region of the gene. This strategy has been used in case of siRNAs that show allele-specific silencing against an established tau mutation called V337M and the predominantly studied APP mutation, APPsw. RNA duplexes specific to these alleles can be used to produce short hairpin RNA (shRNA) plasmids that potentially silence the expression of the mutant tau or APP alleles. A major challenge in applying siRNA therapy to the nervous system is achieving sustained, effective delivery of siRNA to the correct target cells in the brain. These data, combined with *in vivo* results from other groups [96,97], suggest that siRNA will effectively suppress the expression of the targeted gene, provided that it can be delivered efficiently to the appropriate neurons.

Osteoarthritis (OA) is an auto-immune disease caused by a number of cytokines, mainly the IL-1 and TNF-α, which cause joint damage [98], hence are potentially targeted for therapy. The transcription factor, NF-κB regulates the inflammatory pathway, and hence methods to inhibit NF-κB induction are employed using antisense oligonucleotides and adenoviral constructs of mutated IkB [99]. Recently, a study of OA in animal model has shown the efficacy of siRNA specific for NF-κB p65 subunit [100]. RNAi has been found useful in inhibiting the expression of genes related to NF-κB signaling pathway, such as genes of cyclooxygenase-2 , nitric oxide synthase-2, and matrix metalloproteinase-9 which are linked with the initial progression of joint damage in osteoarthritis patients. This therefore proves that NF-κB p65 specific siRNA can be a promising candidate in remediating an early stage OA.

Systemic Lupus Erythematosus (SLE) diseases models have also depicted the benefit of RNAi in gene therapy where homozygous mutation in the gene, Roquin in mice enhanced the number of receptor based expression of Inducible Co-Stimulating T cells (ICOS), consequently accumulating lymphocytes and developing a syndrome similar to SLE [101]. The protein, Roquin possesses an RNA binding domain and is associated with stress granules and GW-bodies, which means that it can be able to cause some mRNA to decay by miRNA . The miR-101 primarily suppresses the expression of ICOS, so lessening of the expression of miR-101 can reduce the levels of co-

stimulatory receptor. Thus, many studies highlight the importance of proteins in regulating repression by the miRNA, including a large number of auto-antibodies, against Argonaut and Dicer [102]. In autoimmune diseases as systemic sclerosis, TGF-β1 is a cytokine that plays an important role in fibrosis pathophysiology of various diseases, therefore the use of RNAi targeting in this molecule may prevent fibrosis development or even improvement of this condition (2005) of glomerulonephritis. siRNA against TGF-β1 can therefore significantly improve the fibrotic matrix progression and expansion [103]. siRNAs give the opportunity of performing modular pharmacology by swapping in targeting ligands specifically at certain cell types and RNA sequences causing specific mutations. Hence, RNAi has successfully emerged out of the experimental realm into the clinical realm. But in spite of a high degree of specificity, off-target effects and other potential complications cripple some efforts. Sometimes, siRNA may induce post-translational suppression of unwanted gene products. Such an effect is observed by miRNAs activity, acting on different gene products, only in cases where the concentration of non-specific siRNA used is low and has partial complementarity with target gene.

CONCLUSION

In the past two decades, we have made remarkable and extra-ordinary progress in understanding the mechanism of RNAi and related gene silencing phenomena. In 1998, the mechanism of gene silencing has been incorporated as a unique tool for gene knock down process in which small double-stranded RNA could be used as ingredient of sequence specific gene silencing. The mystery of gene silencing has been revealed through the co-suppression phenomenon in animals because earlier it was established that co-suppression occurred only in plants as core innate immunity mechanisms against different virus infection. In this report, we have described the outlines of the integrated model of the RNA interference process and have identified many of the components involved therein. Still many central questions remain unresolved, particularly the biochemical integration of dsRNA that cause heritable changes in gene expression. We have identified different complex enzymes; however their mode of action is not fully explored. The effector enzymes function particularly in preference to the target, the catalyzing activity and cleavage events are still superficially known. Truly one of major outcome is the biochemical analysis of RNAi mechanism which will unravel the remarkable complexity, with respect to the pathway determination and the proteins comprises the RNAi system. The unexpected biochemical complexity which is described here deserves a marvellous appreciation for the roles played by dsRNA- induced silencing process.

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