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Unit – IV

TOPIC: RNA Interference

Dr. A. S. VIJAYAKUMAR
Guest lecturer
Department of Biomedical Science

RNA Interference:

Mechanism, Delivery and Preclinical applications

What is RNA Interference?

RNA stands for ribonucleic acid, a nucleic acid present in all living cells. RNAi is short for “RNA interference” and it refers to a phenomenon where small pieces of RNA can shut down protein translation by binding to the messenger RNAs that code for those proteins.

RNA interference is a natural process with a role in the regulation of protein synthesis and in immunity. It’s also a potent tool for the exploration and manipulation of gene expression.

Introduction

RNA Interference has dramatically impacted basic biomedical research as laboratories worldwide adopted the technology shortly after its discovery.

The ability to relatively easily silence the expression of almost any given gene opened up new frontiers for research. In addition, the therapeutic potential of RNAi was quickly recognized, accelerating its clinical development.

In comparison to antisense and ribozyme strategies, RNAi is distinguished by its ease-of-use and high efficiency. It has thus become the most commonly used method of posttranscriptional gene silencing (PTGS).

Double-stranded RNA molecules trigger RNAi-mediated gene silencing. In the early 1990s, observations in plants indicated that the overexpression of genes that should have led to a specific pattern of flower coloring surprisingly led to an absence of color.

The mechanism of what was called “cosuppression” was not fully understood at that time. At the end of the 1990s, experiments using the nematode Caenorhabditis elegans first elucidated the phenomenon now known as RNA interference.

The American scientists Andrew Fire and Craig Mello demonstrated that double-stranded RNA molecules inhibit the expression of homologous genes (Figure 13.15).

Interestingly, silencing is transitive in C. elegans, that is, RNAi-mediated inhibition of gene expression spreads from a single cell to the whole organism and from the organism to its progeny.

Fire and Mello received a Nobel Prize for Medicine or Physiology in 2006 for their discovery.

Andrew Fire

Craig

In 2001, Thomas Tuschl and colleagues demonstrated that siRNAs specifically and efficiently inhibit the expression of a target gene in mammalian cells. Standard siRNAs are 21 nucleotides long: 19 nucleotides of each strand form a helix while the two on the 3′ end do not base pair.

After the introduction of siRNAs as RNAi triggers, it has been possible to determine the function of individual human genes and to develop novel therapeutic approaches for diseases hitherto deemed untreatable.

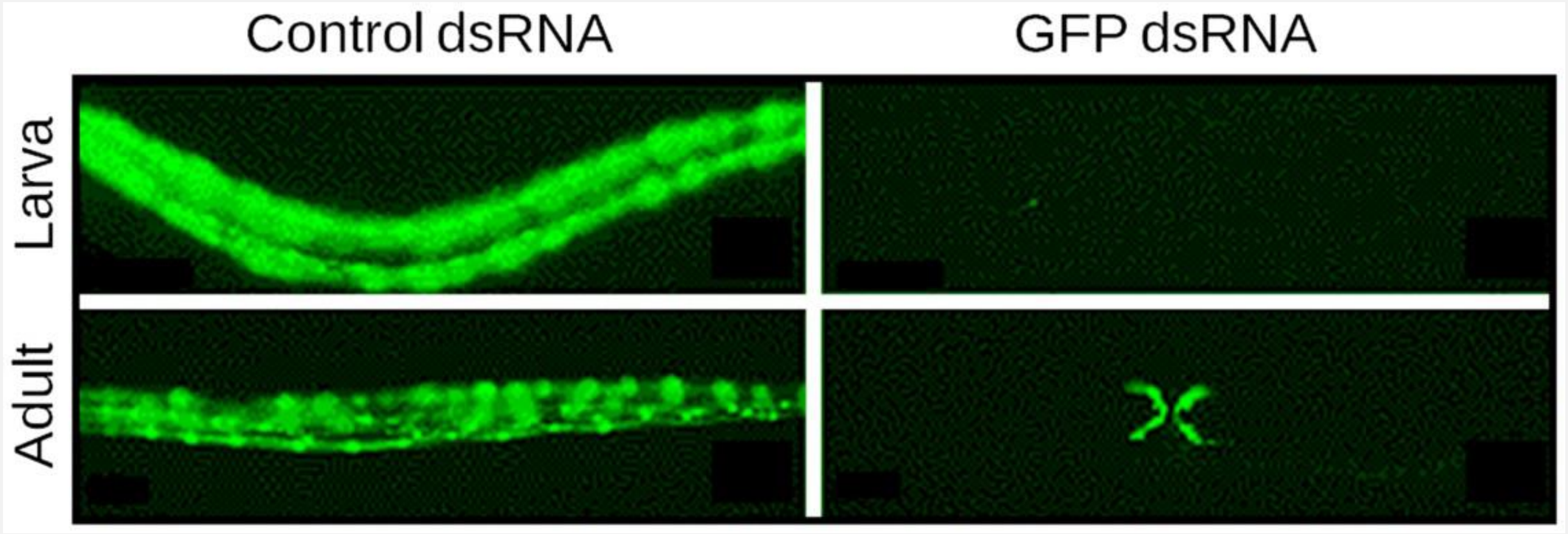

Fig. 13.15 Silencing of green fluorescent protein (GFP) in C. elegans. Due to a genetically engineered modification, the worms express highly fluorescent GFP. The progeny of animals injected with double-stranded RNA (dsRNA) molecules were analyzed by fluorescence microscopy. While adult animals and larva still exhibited strong fluorescence after administration of a control dsRNA (left), the fluorescent cell fraction markedly decreased after injection of dsRNA targeting GFP (right).

The natural function of RNAi is still not completely understood, but it appears to protect cells from viruses, particularly in plants and invertebrates.

However, the significance of RNAi as a mechanism of antiviral defence in mammals is still controversial since mammals possess an elaborate immune system.

RNAi may thus be a relic, retained from lower organisms throughout evolutionary development.

Nevertheless, RNAi appears to be important in higher eukaryotes to guarantee genetic stability and to keep mobile genetic elements, such as transposons, under control.

Mechanism of RNA Interference

As already mentioned above, dsRNA molecules trigger RNAi. In lower eukaryotes such as the nematode C. elegans, dsRNAs are several hundred base pairs in length.

An endonuclease called Dicer processes them into siRNAs that are approximately 21 nucleotides in length (Figure 13.16).

The siRNAs are loaded into the actual effector complex, called RISC (RNA-induced silencing complex) with the aid of the RISC-loading complex (RLC).

In the process of this loading and activation, one of the strands is presumably cleaved. The active strand found in RISC is termed the antisense or guide strand, and the discarded strand the sense or passenger strand.

The antisense strand directs RISC to the target mRNA, to which it hybridizes by Watson–Crick base pairing. An important component of RISC, the protein Argonaute 2 (Ago2), possesses slicer activity and cleaves the target RNA.

This creates two free ends unprotected by a cap or a poly A-tail that cellular nucleases rapidly degrade.

RISC is released from the degraded target RNA and can cleave further target molecules in a multiple turnover process.

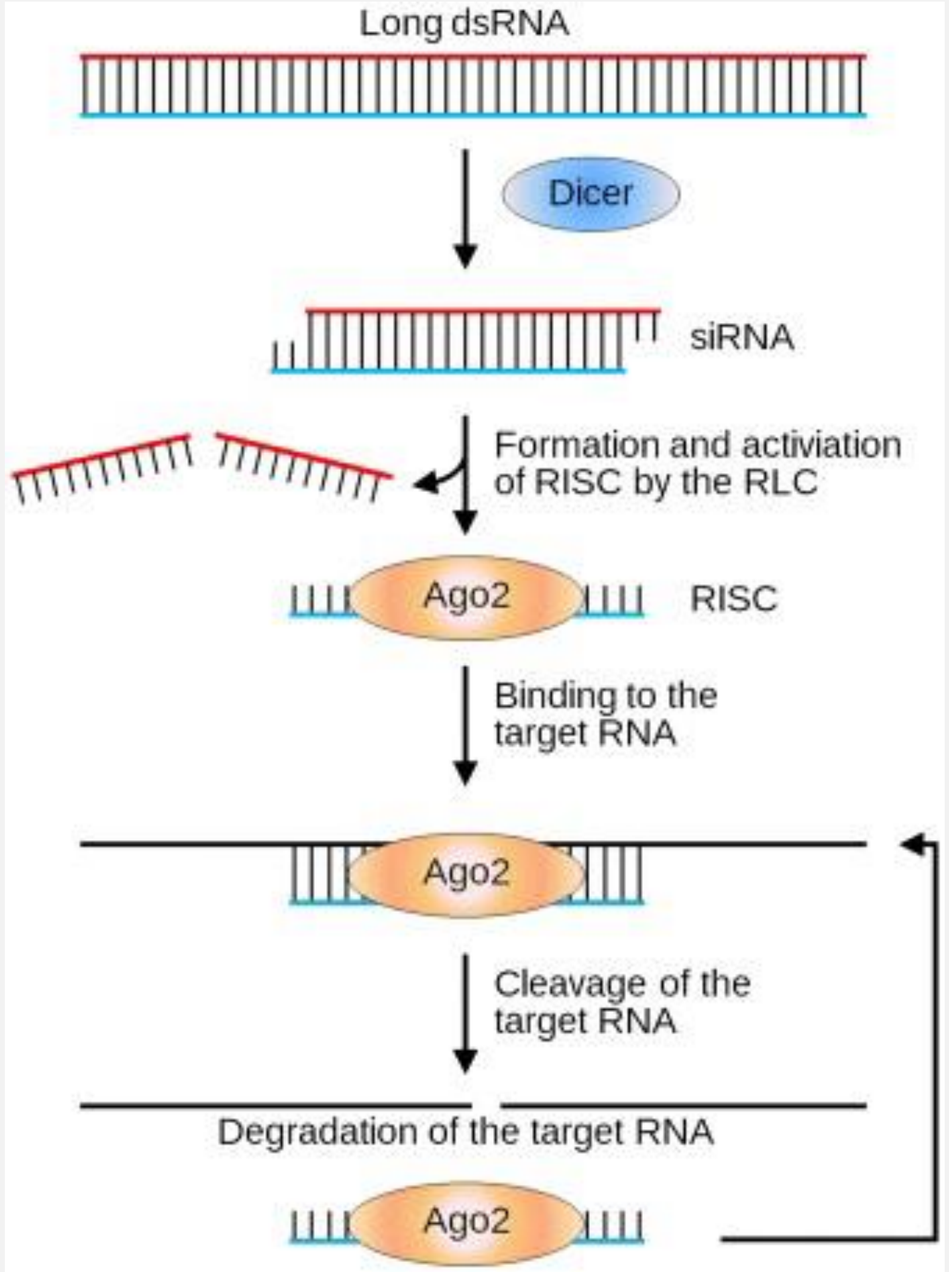

Fig. 13.16 Mechanism of RNAi. The nuclease Dicer processes the long double-stranded RNA into 21mer siRNAs. One strand complexes with RISC while the other is degraded. The remaining strand directs RISC to the target mRNA, which is cleaved and degraded. RISC can then cleave additional target molecules.
dsRNA: double-stranded RNA;
siRNA: small interfering RNA;
RISC: RNA-induced silencing complex;
red: passenger strand of the siRNA;
blue: guide strand of the siRNA.

The use of RNAi as a genetic tool was restricted to lower organisms in the first few years after its discovery, since in mammals long double-stranded RNA molecules trigger a strong interferon response.

Subsequently, two enzymes become activated, leading to a global termination of protein synthesis: Protein kinase R activation completely blocks translation and nonspecific RNase L activity destroys all RNAs.

Protein synthesis is completely shut down and inhibition of individual target genes is no longer possible.

A careful elucidation of the RNAi pathway enabled critical improvements in the technology, allowing its use in mammalian cells.

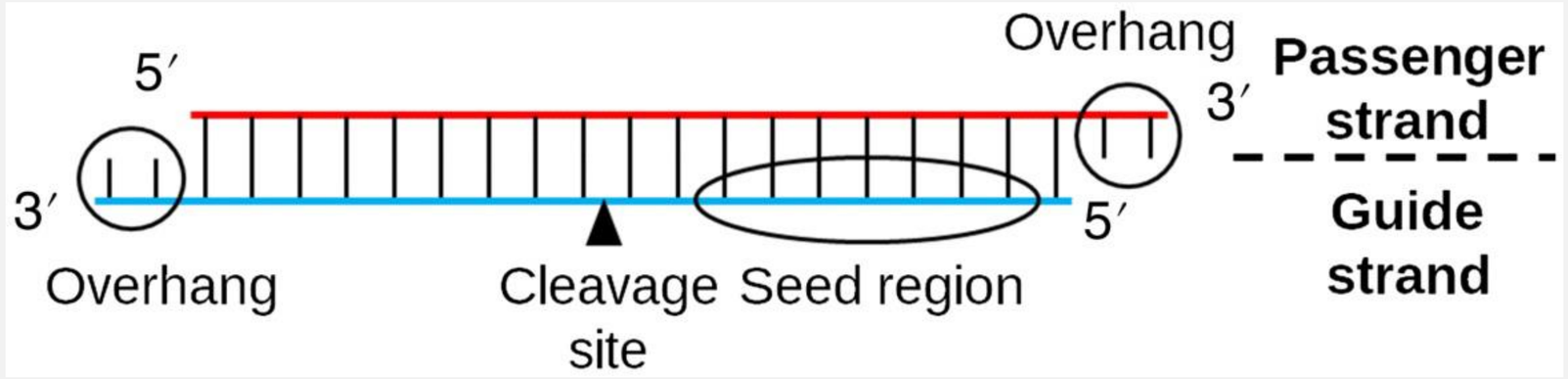

Fig. 13.17 Design of a small interfering RNA (siRNA). Standard siRNAs consist of two RNA strands, which are each 21 nucleotides long. Nineteen nucleotides form a double helix, while two nucleotides on each 3′ end form an overhang. Other designs also exist: siRNAs may be longer or have blunt ends. The passenger, or sense strand of the siRNA is discarded when RISC is loaded, while the guide or antisense strand directs RISC to the target mRNA and hybridizes with it, beginning with the seed region. RISC then cleaves the target RNA in the center of the duplex, 10 nucleotides from the 5′ end of the siRNA strand.

RNAi occurs in the cytoplasm. This is an important difference between the RNAi and antisense technologies: While AS ONs induce the degradation of the target RNA by RNase H in the nucleus (although more recent experiments have also demonstrated cytoplasmic antisense activity), the RNAi machinery is located in the cytoplasm.

Colocalization studies have shown that the Argonaute proteins, which cleave the targeted mRNAs, are found in the processing bodies (P-bodies).

These distinct foci within the cytoplasm of the eukaryotic cell play fundamental roles in mRNA turnover.

The different locations of silencing activity have implications for delivery: For siRNAs, it is sufficient that they pass through the cell membrane and enter the cytoplasm. AS ONs, however, may need to penetrate the nuclear membrane to reach their location of action.

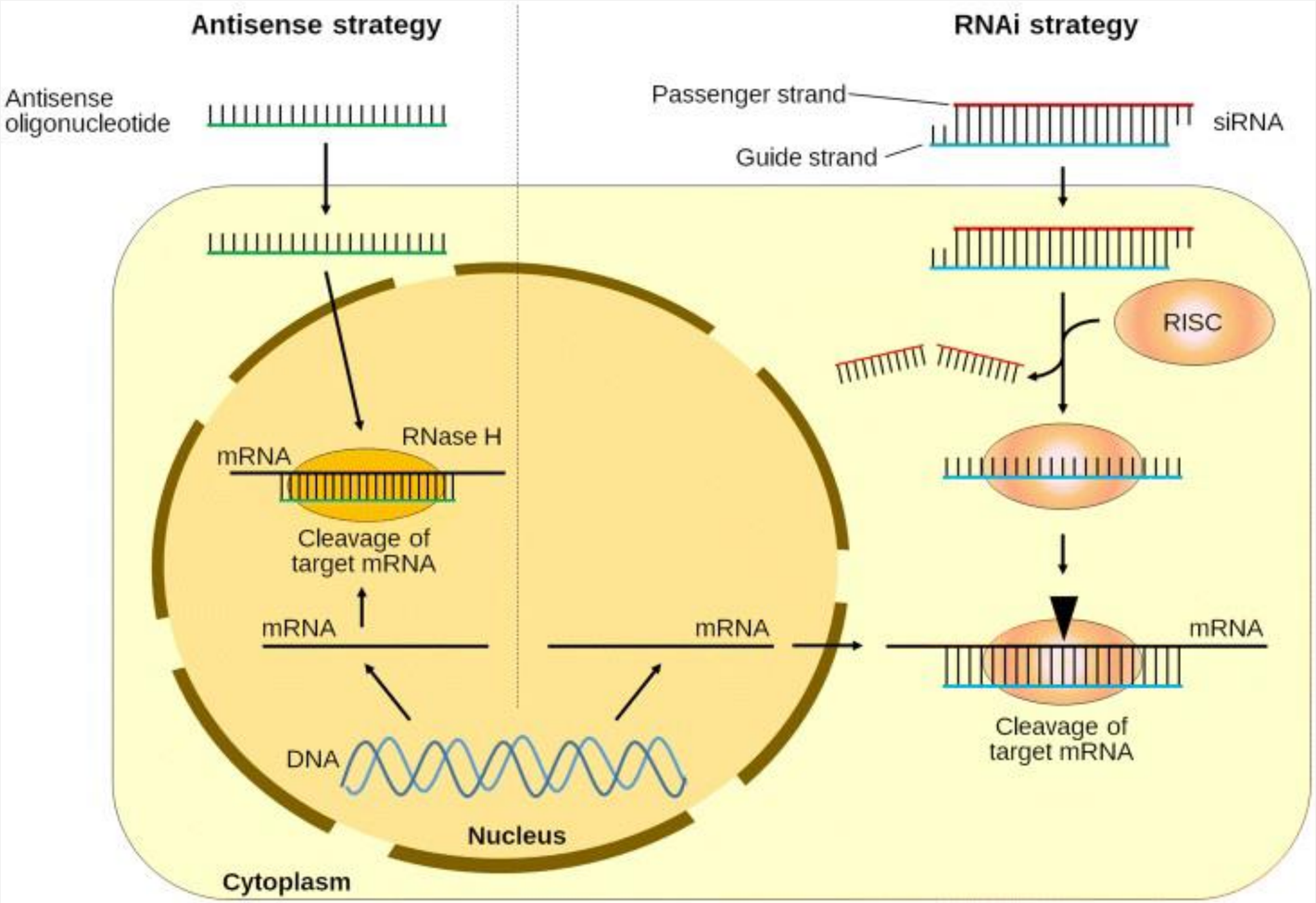

Fig. 13.18 Comparison of the mechanisms of antisense and RNAi technologies. As described in Section 13.1, most AS ONs activate RNase H. This enzyme is located in the nucleus. In contrast, the machinery of RNAi is located in the cytoplasm, where RNA cleavage occurs.

The silencing activity of chemically synthesized siRNAs is transient. Depending on the rate of turnover of the target proteins, the inhibitory effect begins about 2 days after siRNA treatment and may persist for several days before protein levels returns to baseline.

The target is usually only partially inhibited, which means that RNAi, like the antisense and ribozyme technologies, is a method to knockdown, or reduce, protein expression.

This contrasts with the complete knockout of gene expression by homologous recombination, which is used for the generation of transgenic animals.

An important advance in RNAi technology has been the development of strategies for the intracellular expression of dsRNAs.

Expression systems permit the extension of the duration of RNAi-mediated silencing, the regulation of RNAi by reversible or irreversible inducers, and the use of viral transduction vectors.

In the expression approach, the dsRNA is usually transcribed in the nucleus as a self-complementary short hairpin RNA (shRNA), which is then exported into the cytoplasm (Figure 13.19).

The Dicer enzyme then processes the shRNA to siRNA, which follows the previously described pathway.

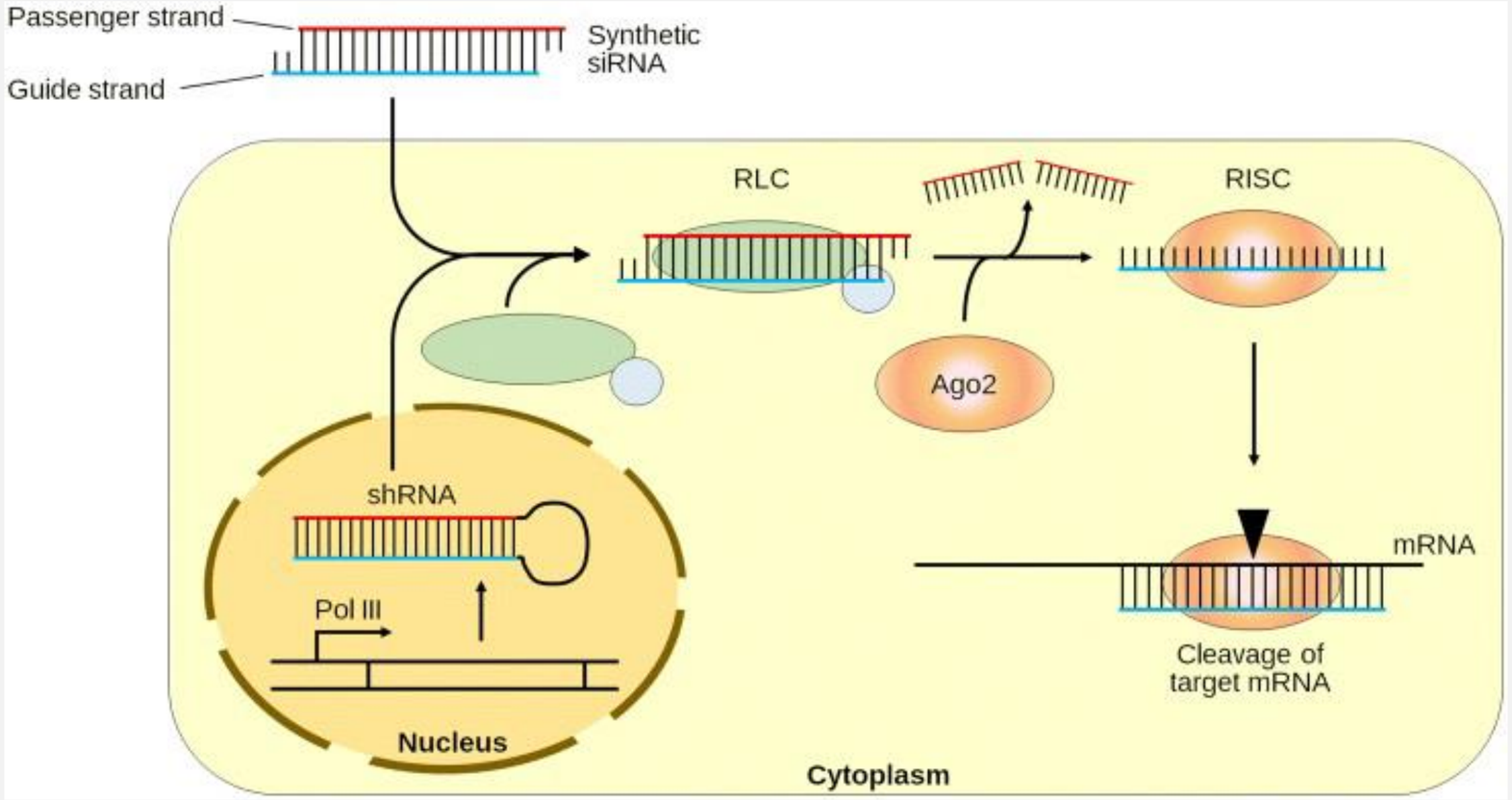

Fig. 13.19 Small interfering (siRNA)- and short hairpin (shRNA)-induced RNA interference (RNAi). Delivery agents can introduce chemically synthesized siRNAs into target cells. There they are loaded into RISC by RLC. The guide strand leads RISC to the target RNA and results in its cleavage by the Ago2 protein. Alternatively, an shRNA can be intracellularly expressed. After export from the nucleus into the cytoplasm, Dicer processes shRNAs into siRNAs so they can enter into the RNAi pathway. RISC: RNA-induced silencing complex; RLC: RISC-loading complex; Ago: Argonaute protein.

Delivery of RNA Interference

There are two basic strategies for RNAi delivery:
(i) the delivery of chemically synthesized siRNAs and
(ii) the transfer of shRNA expression cassettes with the help of viral vectors.

The delivery of siRNAs can be improved by targeting specific cell types with antibodies, aptamers, or receptor ligands.

Unmodified siRNAs are very sensitive to nucleolytic degradation; incorporation of modified nucleotides is usually necessary to render them sufficiently resistant to nucleases for in vivo use.

Cationic lipids are the most frequently used carriers for the transfer of siRNAs into cells. The positive charges of the lipids associate with the negatively charged siRNAs, forming what is referred to as a lipoplex (Figure 13.22a).

Cationic lipids also interact with negatively charged groups on the surface of cells, which take up the complexes by endocytosis.

For in vivo uses, less toxic liposomes are often used. These enclose the siRNAs in an inner vesicle (Figure 13.22b). Several clinical studies have used a class of liposomes known as “stable nucleic acid-lipid particles” (SNALPs).

An alternative strategy is to couple lipophilic molecules directly to the siRNA. A successful example is the use of siRNAs that carry cholesterol on the 3′ end of the sense strand (Figure 13.22c). Due in part to the lipophilic group, siRNAs injected intravenously efficiently reach the liver and the jejunum.

The targeted delivery of siRNAs allows the use of smaller doses and avoids side effects in nontargeted tissues.

One of the first approaches to attempt the cell-type specific transfer of siRNAs employed antibodies recognizing specific proteins on the surface of the target cells. The antigen-binding fragments of the antibody (Fab) were fused to protamine, which bound several siRNAs noncovalently through ionic interactions (Figure 13.22d). The siRNAs entered only into cells that expressed the protein targeted by the antibody on their surface.

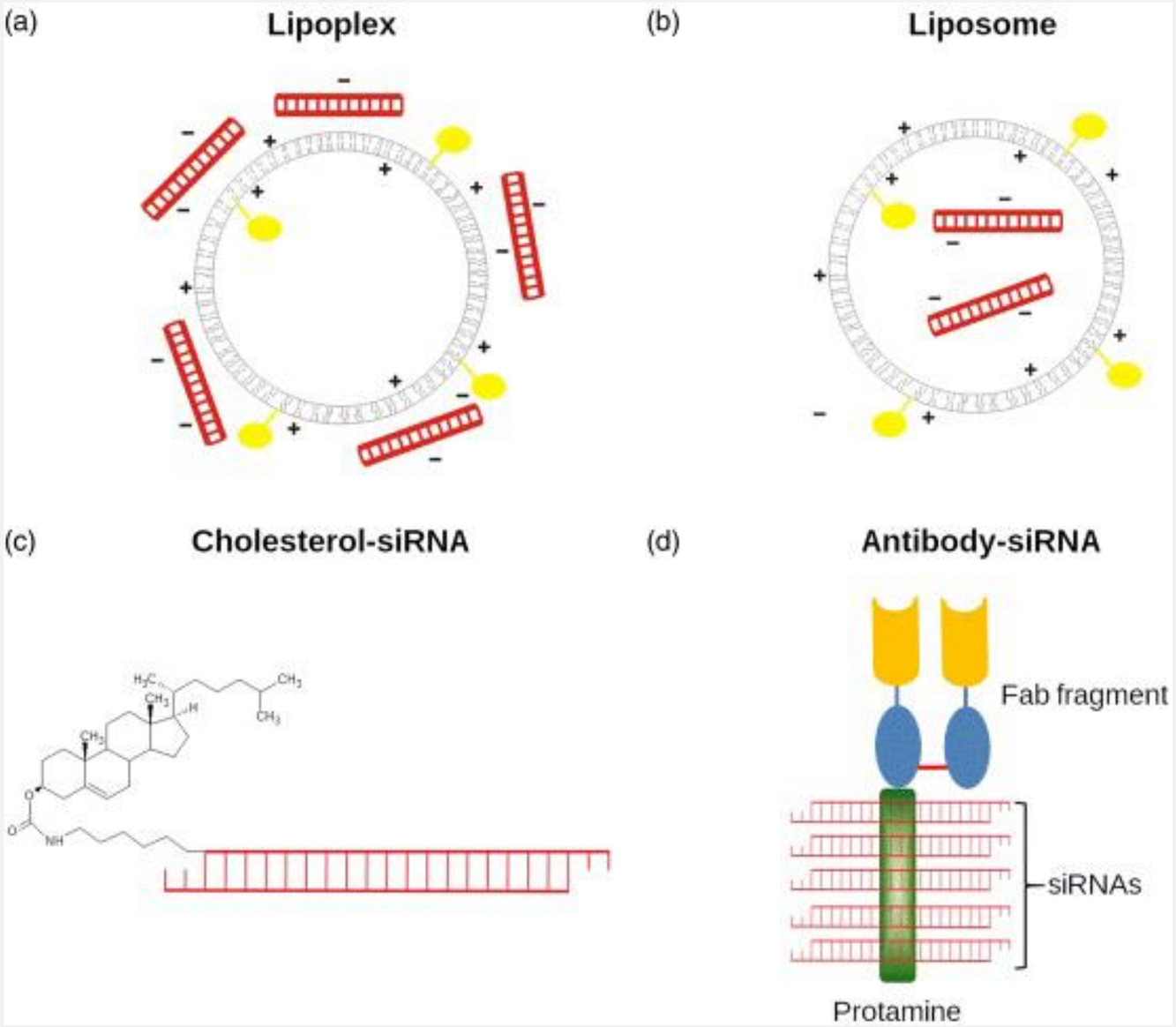

Fig. 13.22 Nonviral delivery of small interfering RNAs (siRNAs).
(a)Lipoplex: Cationic lipids form complexes with negatively charged siRNAs. PEG (yellow) is frequently attached to improve pharmacokinetic characteristics.
(b) Liposomes in which cationic lipids encapsulate the siRNA
(c)siRNA coupled to cholesterol to increase its lipophilicity.
(d) Specific delivery by the coupling of siRNAs to the antigen-binding fragment of an antibody through positively charged protamine.

Vectors based on lenti-, adeno-, and adeno-associated viruses are commonly employed for the viral delivery of shRNA expression cassettes (Figure 13.23).

Lentiviral vectors integrate their genetic material into the host genome and lead to long-term expression of the shRNA. Adenoviral and AAV vectors remain episomal and are, therefore, suitable for transient RNAi. The choice of suitable serotypes can facilitate the transfer of the shRNA expression cassettes into specific target cells.

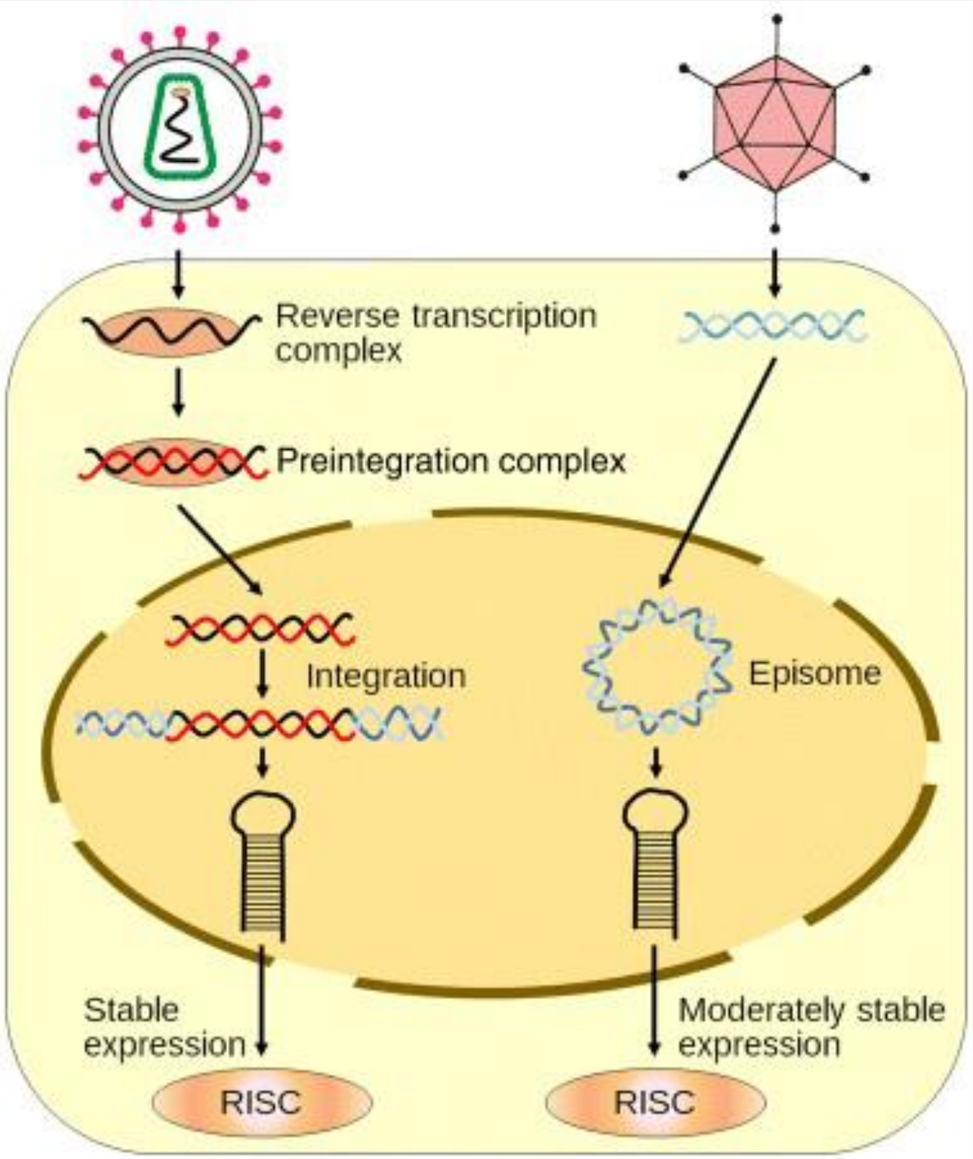

Fig. 13.23 Viral delivery of short hairpin RNA (shRNA) expression cassettes.
Lentiviral vectors integrate into the host genome and allow long-term expression of the shRNA, while adenoviral and AAV vectors remain episomal and induce transient silencing of the target gene.

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THANK YOU