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RNA Interference—A Silent but an Efficient Therapeutic Tool

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Abstract RNA interference (RNAi) is an evolutionary conserved gene regulation pathway that has emerged as an important discovery in the field of molecular biology. One of the important advantages of RNAi in therapy is that it brings about efficient downregulation of gene expression by targeting complementary transcripts in comparison with other antisense-based techniques. RNAi can be can be achieved by introducing chemically synthesized small interfering RNAs (siRNAs) into a cell system. A more stable knockdown effect can be brought about by the use of plasmid or viral vectors encoding the siRNA. RNAi has been used in reverse genetics to understand the function of specific genes and also as a therapeutic tool in treating human diseases. This review provides a brief insight into the therapeutic applications of RNAi against debilitating diseases.

Keywords RNAi · siRNA · shRNA · miRNA · Cancer · Ocular diseases · Cardiovascular diseases · Diabetes mellitus · Viral diseases · Gene silencing · Therapeutic tool

Introduction

The basic principle underlying RNA interference (RNAi) involves the disruption of mRNA by the use of homologous double stranded RNA (dsRNA). Small interfering RNAs (siRNAs) are generated intracellularly from endogenous and exogenous dsRNA molecules by cleavage activity of Dicer, a ribonuclease III-type protein [1–4]. Dicer contains an RNA helicase domain at the N-terminus, an RNA-binding Piwi/Argonaute (AGO)/Zwille (PAZ) domain, two RNAse III domains and a double-stranded RNA-binding domain (dsRBD) [5, 6]. This Dicer cuts the dsRNA into short 19–21 duplexes having two symmetric nucleotide overhangs at the 3′ end and a 5′ phosphate along with a 3′ hydroxyl group that are referred to as siRNAs.

The siRNAs get incorporated into a nuclease-containing multiprotein complex referred to as the RNA-induced silencing complex (RISC) [7]. The phosphorylation of the siRNA at the 5′ end is required for its entry into the RISC [8]. The helicase domain of the RISC assembly binds to one end of the duplex and unwinds the siRNA in an ATP-dependent manner. The

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RISC gets activated as soon as the antisense strand attaches to the complex. The activated RISC containing the antisense strand reaches the target mRNA and induces an endonucleolytic cleavage of the mRNA within the target site [9]. This cleavage leads to degradation of the entire mRNA molecule, while the RISC is recovered for further cleavage cycles. This mechanism clearly highlights the fact that intracellular presence of siRNAs complementary to the target mRNA is important for the induction of RNAi.

Types of RNA Interference Inducers

Small Interfering RNAs

The dsRNAs are processed by a combination of RNAse III Dicer, TAR RNA-binding protein (TRBP), and PACT into siRNAs about 20–24 mer nucleotides in length with a phosphate group at both the 5′ ends along with hydroxyl groups and two nucleotide overhangs at both the 3′ ends [3, 4, 7]. The siRNAs, thus, processed consist of a sense strand and a complementary antisense strand. The siRNAs unwind due to helicase activity of the pre-RISC domain and the help of an AGO protein [10] that later cleaves the sense strand [11–14] and then the mature RISC containing the antisense strand along with the AGO protein attaches and cleaves the complementary mRNA as shown in Fig. 1 [2].

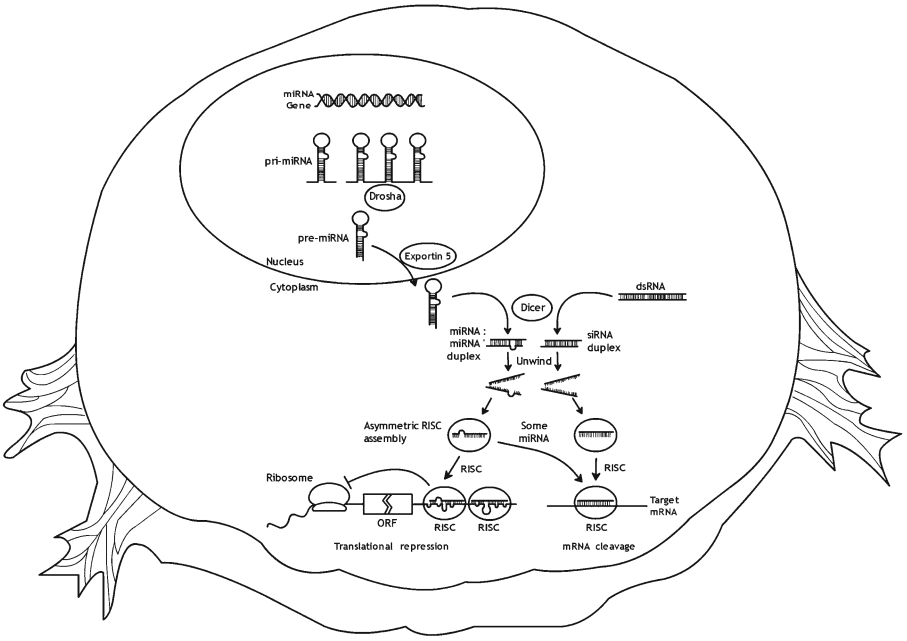

Fig. 1 siRNA and miRNA biogenesis. The dsRNA molecules are processed by Dicer into short interfering RNA (siRNA) duplexes. The antisense strand of the siRNA duplex is assembled into the RNA-RISC and results in the cleavage of the target mRNA. The primary micro-RNA (pri-miRNA) transcripts get processed by Drosha into 70-nucleotide precursor micro-RNAs (pre-miRNAs). These pre-miRNAs get transported from the nucleus to the cytoplasm with the help of exportin-5 and get processed by Dicer to form dsRNAs. The antisense strand of this duplex gets incorporated to the RISC complex and results in translational repression or cleavage of the target mRNA

Designing siRNAs that are fully complementary to their target mRNA is of utmost importance. Initially, investigators followed a few empirical guidelines like the GC content and the region of mRNA to be targeted to carry out efficient gene silencing [4]. Guidelines for designing successful siRNAs have been explained in Fig. 2.

Short Hairpin RNAs

Short hairpin RNAs (shRNAs) are a class of siRNAs that are expressed using U6, H1, or tRNA promoter [15–17]. In comparison to siRNA, these shRNAs have many advantages like long-lasting silencing effects, cost effectiveness as well as easy delivery methods. Generally, shRNA is transcribed in cells from a DNA template as a single-stranded RNA molecule (about 50–100 bases). The complementary regions are spaced by a hairpin loop, thereby getting the name "short hairpin" RNA [3].

Micro RNAs (miRNAs)

The micro RNAs (miRNAs) are a class of RNAi inducers that bring about post-transcriptional gene silencing and are processed from endogenously expressed transcripts. The primary miRNAs (pri-miRNAs) are approximately >100-nucleotide long, with one or more micro-RNA stem loops that are the initial products of micro-RNA genes. The pri-miRNAs are transcribed by RNA polymerases [18–20] and are processed by the microprocessor complex that consists of an enzyme Drosha and a subunit DCGR8 into ~70 nucleotide precursors, called precursor miRNAs (pre-miRNAs) [21–23]. The pre-miRNAs are

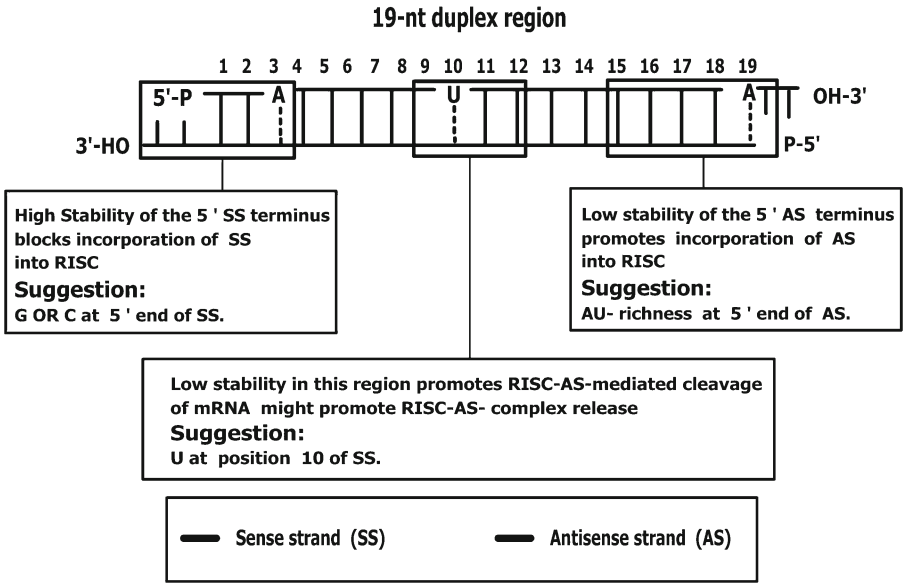

Fig. 2 Criteria for efficient siRNA. A short interfering RNA consists of a 19-nt duplexed region with symmetric two to three nucleotide overhangs along with hydroxyl (–OH) group at the 3′ end and a phosphate group at the 5′ end. Based on design criteria, high stability at the 5′ terminus of the sense strand and low stability at the 5′ terminus of the antisense strand increases the efficiency of the siRNA. The presence of an A at position 3 as well as position 19 and a U at position 10 in the sense strand is of utmost importance to obtain an effective siRNA

exported to the cytoplasm with the help of a protein called exportin 5 [24, 25], where a complex that contains the enzyme Dicer, TRBP, and PACT converts the pre-miRNAs into miRNA–miRNA* duplexes (sense and antisense strands) [26, 27]. The duplex associates with an AGO protein within the precursor RNAi-RISC (pre-RISC). One strand of the duplex (the antisense strand) is removed. The mature RISC contains the antisense strand, which directs the complex to the target mRNA and the miRNA antisense strand binds to the 3′ UTR resulting in translational repression (Fig. 1) [28–30].

Delivery of Small Interfering RNA

The major challenge in the use of siRNA as therapy lies in the delivery of these biomolecules into the desired cell, tissue, or organ. Successful delivery of chemically synthesized siRNAs or in vitro transcribed siRNAs in mice models can be brought about by a cationic liposome-based system [31–33]. A number of innovative delivery methods have been developed by combining siRNAs with other molecules like conjugation of cholesterol to the sense strand of the siRNA [34], siRNAs in combination with antibody–protamine fusion [35], and siRNAs complexed with cyclodextrin nanoparticles, exosome nanoparticles [36, 37], and aptamer-conjugated siRNAs [38, 39].

Among the varied methods of delivery, the use of scaffolds is in the forefront of current research [40]. A scaffold is composed of a natural or synthetic biodegradable material or a composite that provides structural support; it must be capable of cellular infiltration and also ensure safe release of encapsulated biomolecules. Scaffolds have been used in the delivery of RNAi molecules and they are more effective than conventional delivery systems [41, 42]. Scaffold-mediated delivery has the capacity to maintain bioactivity of siRNAs for longer time duration, thereby increasing the chances of transfection. A major advantage in using a scaffold is that it protects the siRNA from immune response as well as degradation by serum nucleases and proteases [43]. Most of these approaches resulted in cell- or tissue-specific targeting, thereby ensuring efficient in vivo siRNA delivery (Fig. 3).

Delivery of Short Hairpin RNA

Apart from chemically synthesized siRNAs, it is also possible to generate DNA-based expression cassettes that express shRNA or separate sense and antisense 21 mers from pol III promoters [16, 44–46]. A conventional shRNA expression cassette includes a pol III promoter along with 19 nucleotides of sense strand of the target sequence, a four- to ten-nucleotide base loop followed by the complementary antisense strand of the target sequence and finally, a group of four to six uridines as the terminator. It is also possible to incorporate a dual promoter system, whereby the separately expressed RNA strands hybridize to produce functional siRNAs [45, 47]. Once the shRNAs reach the cytoplasm, they get processed by Dicer to yield functional siRNA duplexes that will follow the conventional RNAi machinery to degrade the target mRNA.

Expression systems that use pol III promoters (Fig. 2) produce long-term silencing in cell culture systems that are less favorable for therapeutic applications. The expression can be modulated by using inducible systems. Inducible systems are normally controlled by the inducer with dose-dependent and reversible regulation of transcription. A number of pol III-based systems have been regulated by inducers like tetracycline or ecdysone. The use of doxycycline, a tetracycline analog, has successfully been applied for in vivo study in mice [48]. The use of ecdysone has been successful in a cell culture system [49].

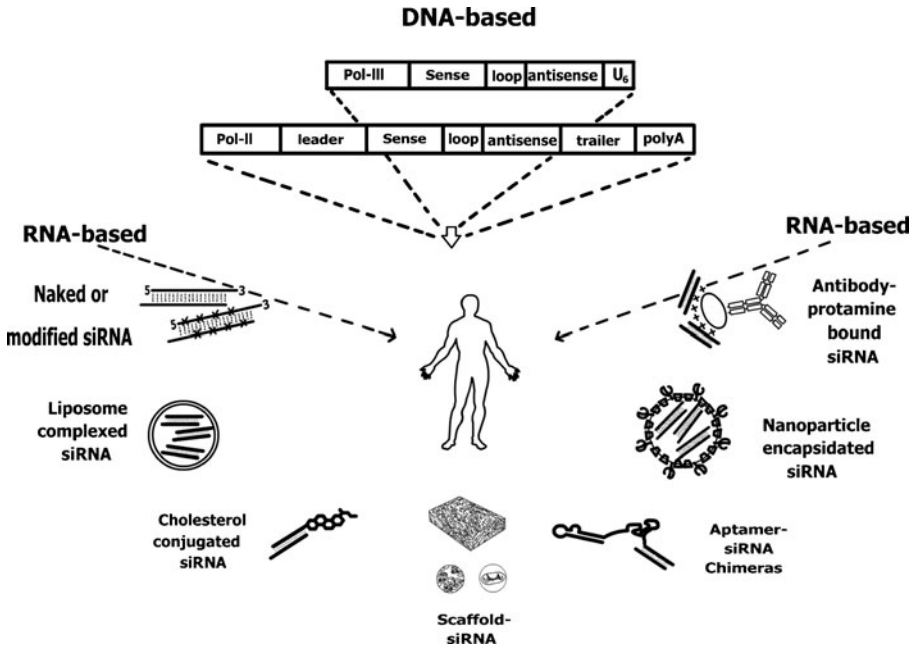

Fig. 3 Delivery methods for RNAi inducers. Different in vivo delivery methods for RNA-based RNAi inducers as well as DNA-based pol-III or pol-II promoter shRNA expression cassettes. These innovative delivery methods ensure efficient and effective delivery of siRNAs as well as shRNAs to the target tissues

Viral promoters like cytomegalovirus (CMV) promoters are incorporated in pol II-based expression systems that produce 5′-capped and 3′-polyadenylated transcripts but lack efficient transcription start sites when compared to pol III-based promoters [50]. Many pol II-based miRNA systems have been developed and they are being assessed for their in vivo applicability [51–53].

Viral vector-based shRNAs that use adenovirus and adeno-associated virus (AAV) have been found to be efficient delivery vehicles to carry out transient shRNA expression [54–57]. Retro viral vectors like murine leukemia virus (MLV) can bring about silencing of target genes by stable transfection as the virus-encoded proteins get integrated into the host cell chromosomal DNA [58].

Lentiviral vectors like human immunodeficiency virus (HIV-1), feline immunodeficiency virus (FIV), or equine infectious anemia virus (EIAV) have been successfully used in a number of studies and have proven to be quite promising [59–62]. RNAi technology can indeed offer therapeutic approaches to treat a number of chronic infections.

Delivery of micro RNA Cassettes (miRNA Cassettes)

In order to bring about stable gene silencing, shRNA or miRNA constructs can be developed. It is possible to use miRNA expression cassettes to explore the fundamental pathways involved in the biological system or to develop therapeutic strategies for debilitating diseases. Viral vectors have been successfully employed in the delivery of miRNAs to target specific genes and many viral vectors have been developed to carry out basic research as well as gene therapy experiments [63].

Adenoviral vectors are commonly used in the delivery of miRNA expression cassettes for in vitro studies. An in vivo study using adenoviral vectors harboring miRNAs showed successful targeting of genes in the brain as well as the liver [50]. A number of in vivo studies have been carried out using AAV vector-borne RNAi biomolecules to treat muscular dystrophies, cancers, metabolic diseases, cardiac diseases as well as neurodegenerative diseases [64, 65].

Studies by Mclaughlin et al. [66] showed that lentiviral vectors could be successfully used to deliver miRNAs to inhibit BCR-ABL oncogene, thereby preventing regrowth of leukemic cells both in vitro as well as in vivo. An important issue that needs to be taken into consideration when targeting genes using miRNA expression cassettes is that these miRNA systems must not, in any way, hinder the endogenous RNAi machinery that can disrupt the function of cellular miRNAs that regulate their natural targets. It is necessary to carry out further studies to elucidate whether these RNAi-based techniques can be safely applied as therapeutic tools in medicine.

RNA Interference in Cancer Therapy

The use of chemotherapy has been detrimental to both cancer cells as well as normal cells, since it lacks the selectivity to distinguish tumor cells from normal cells. RNAi therapy can be used to specifically target cancer cells. The use of siRNAs to inhibit proliferation of cancer cells has been reported in a number of in vivo and in vitro studies. In vitro studies highlighting chromosomal translocations in lymphomas and leukemias result in oncogenic gene fusions that have been inhibited by RNAi [67]. Allele-specific targeting by RNAi-based approaches on single nucleotide mutant forms of tumor suppressor genes such as p53 and ras have been successful in different types of cancer [68–71].

Aptamer–siRNA combinations were successfully used to bring about tumor regression in prostate cancer cells by targeting a surface-expressed tumor cell marker, namely, a prostate-specific membrane antigen (PSMA) [39]. Another successful study involving siRNAs complexed in atelocollagen was successfully used to limit angiogenesis tumor growth in vivo by silencing vascular endothelial growth factor (VEGF) [72].The use of cyclodextrin nanoparticles to deliver anti-Ews-Fli1 siRNAs was effective in blocking metastasis in Ewing's tumors [73, 74]. RNAi has been effectively used to inhibit AKT2 kinase, a class of protein kinase that is over expressed in malignant gliomas and confers chemotherapeutic resistance to the malignant cells [75].

ATP-binding cassette subfamily C member 4 (ABCC4) is highly expressed in pancreatic cancer tissues. RNAi was successfully used to downregulate ABCC4 expression in pancreatic cancer cell lines like Panc-1 and BxPC-3 cells, which in turn, resulted in inhibition of proliferation and cell cycle arrest [76].

Glycogen synthase kinase-3 beta (GSK-3b), a protein kinase, plays an important role in tumor formation in different cancers. Knockdown of the GSK-3b gene using sequence specific siRNAs in pancreatic cancer xenograft mice models resulted in inhibition of tumor and angiogenesis [77].

RNAi has also been used as a combinational gene therapy to target human telomerase reverse transcriptase (TERT) and epidermal growth factor receptor (EGFR), resulting in effective apoptosis in hepatocellular carcinoma cell lines as well as tumor growth inhibition in xenograft mice models [78]. Further reviews on the potential use of RNAi technology as an effective anticancer therapeutic tool have been discussed in detail by Mocellin et al. and by Wang et al. [79, 80].

RNA Interference Against Viral Diseases

RNAi-based therapy can be successfully used to inhibit different types of viruses. It is possible to inhibit the expression of numerous viruses both in vitro and in vivo by introducing virus-specific siRNAs [81]. Extensive research carried out in hepatitis B virus (HBV) mouse models demonstrated that in vivo hydrodynamic transfection of HBV expression plasmids and HBV-specific siRNAs (or shRNA expression vectors) to the mouse liver prevented the induction of HBV gene expression and replication [82–84].

Shlomai et al. [85] described an innovative strategy by using a recombinant HBV as a "Trojan horse" vector for effective delivery and expression of anti-HBV siRNAs in the liver. The recombinant virus uses the resident HBV for its own propagation and targets the resident virus by incessant production of anti-HBV shRNAs.

Infections from different kinds of viruses such as influenza virus [86], coxsackie virus [87], and respiratory syncytial virus (RSV) [88, 89] have been successfully inhibited by the delivery of siRNAs in mice. Further studies have been carried out in Huh-7 cells to study viral replication of hepatitis C virus (HCV), since subgenomic and full-length HCV replicons replicate and express HCV proteins in stably transfected human hepatoma cell-derived Huh-7 cells [90–95].

RNAi-based techniques have been found to be quite effective in controlling replicon function of HCV in vitro [94, 95]. Specific siRNAs that target the mRNAs of internal ribosome entry site (IRES) and nonstructural proteins NS3 and NS5b were shown to inhibit the replicon function of HCV in cell culture [95]. In vivo studies by McCaffrey et al. [96] ascertained that hydrodynamic tail vein injections of synthetic as well as pol III promoter expressed anti-HCV siRNAs that resulted in cleavage of HCV sequences in a HCV luciferase fusion construct in mouse hepatocytes. Most of the regions of HCV can be effectively targeted using RNAi-based techniques [97].

Antisense strategies using siRNAs can also be used to reduce the severity of certain diseases by targeting the inflammatory response pathway. Studies in mice using hydrodynamically injected siRNAs to treat fulminant hepatitis induced by an agonistic Fas-specific antibody resulted in survival of majority of the treated mice up to a longer time period when compared to control [35].

RNAi techniques have been used in targeting components involved in the life cycle of HIV. Specific siRNAs have been used to target a number of HIV-encoded RNAs like Tat [45, 98, 99], Rev [45, 98], Gag [100, 101], Env [101], Vif, Nef [102], and cellular cofactor NF-κB [99]. HIV receptors like CD4 [100] and coreceptors like CXCR4 and CCR5 are potential targets, as the inhibition of these factors block initial viral entry into cells [103]. Inhibition of viral replication in the case of HIV has been successfully carried out in a number of human cell lines [103–106]. Clinical application of RNAi-mediated inhibition of HIV is quite a challenging task due to the high mutation rate of the virus [107].

The diversity of HIV-1 makes it quite difficult to develop an effective vaccine and poses great difficulties in current retroviral therapies as well as new therapies based on RNAi. It is necessary to target specific genes in conserved gene regions by using sequence-specific siRNAs/shRNAs along with second-generation shRNAs to target genes that prevent HIV-1 escape mutations. RNAi therapy should include a concoction of siRNAs to limit viral escape within the targeted gene region [108]. Future prospects in the direction of human clinical trials are likely to happen in the case of HIV-1 therapy using RNAi technology.

Use of RNA Interference in Ocular Diseases

Some of the ocular diseases that occur as a result of angiogenesis include diabetic retinopathy, age-related macular degeneration (AMD), and herpetic stromal keratitis [109]. It has

been observed that VEGF plays an important role in destructive vascularization associated with these ocular diseases.

Successful reduction of VEGF-dependent vascular invasion of the eye by using VEGF-specific siRNAs has been demonstrated in animal models [110–112]. RNAi therapy using VEGF siRNAs is at the phase I stage of clinical trials carried out in AMD patients [113]. RNAi-based targeting of immune pathways improved therapy against macular degeneration-related blindness [114].

RNA Interference in Bone-Related Diseases

Of late, a number of studies have highlighted the potential of RNAi-based techniques in bone-related diseases. Previous studies have shown that cathepsin B is a cytokine that is responsible for cartilage destruction in osteoarthritis and pathological proteolysis in rheumatoid arthritis. In vitro targeting of cathepsin B in T/C-28a2 chondrocytes by sequence-specific siRNAs resulted in a 70 % reduction in expression; hence, cathepsin B can be marked as a potential target in the therapeutic management of osteoarthritis [115].

A bone tumor disease called Ewing's sarcoma occurs in children as well as young adults. This sarcoma has a translocation t (11; 22) (q24; q12) that produces a EWS/FLI fusion protein that plays a major part in the growth and development of Ewing's sarcoma. This protein was clearly identified as a potential target for RNAi-based therapy. Studies by Kovar et al. [116] and Owen and Lessnick [117] have confirmed this by successfully using retroviral shRNA to target EWS/FLI gene in Ewing's sarcoma cell line. Silencing of EWS/FLI gene changed the tumor cell phenotypes and restored the osteoblastic differentiating potential. In the near future, RNAi techniques can be harnessed for its therapeutic potential in the treatment of Ewing's sarcoma.

Rheumatoid arthritis is an autoimmune inflammatory disease that leads to gradual destruction of cartilage and bone within affected joints. Targeting NF-κB by intraarticular injection of sequence-specific siRNAs into collagen-induced arthritic rats resulted in amelioration of joint destruction [118, 119].

Targeting of tumor necrosis factor-alpha (TNF-α) by electroporation of specific siRNAs reversed the joint inflammation symptoms in collagen-induced arthritic rats, thereby proving that TNF-α can also be considered as a potential therapeutic target [120].

RNAi has been quite effective in controlling osteoclast-mediated excessive bone reabsorption and has been considered as an efficient tool in treating connective tissue disorders [121]. These studies indicate that it is feasible to use RNAi-based technology to target factors that play an important role in the pathological processes leading to bone-related diseases.

RNA Interference in Cardiovascular Diseases

A common pathophysiological response in cardiovascular diseases like hypertension, ischemic heart disease, and heart valve disorders is cardiac hypertrophy. A number of miRNAs are dysregulated during cardiac hypertrophy [122]. Studies by Van Rooij et al. [123] reported that overexpression of miR-195, an miRNA, results in hypertrophy in cultured rat cardiomyocytes as well as cardiac failure in mice. The miR-195 targets cyclin D1 and CDK6; both these cyclins play an important role in the cell cycle [124]. In vivo silencing of another miRNA, miR-21, inhibits interstitial fibrosis and arrests heart dysfunction as miR-21 causes heart attacks by stimulating mitogen-activated protein (MAP) kinase signaling [125].

The miRNAs play a major role in cardiac diseases like arrhythmias and ischemic heart disease. The overexpression of another class of miRNAs like miR-133 or miR-1 that regulate cardiac conductance inhibits hypertrophy in cardiomyocytes [126, 127]. Elevated expression levels of miR-1 are found in infarcted rats and also in human hearts affected with coronary heart disease [128]. These studies indicate that miRNAs play an important role in heart-related diseases and by further studies of miRNA regulation, it is possible to understand human diseases that will pave the way for better understanding as well as therapy.

Role of RNA Interference in Diabetes Mellitus

Diabetes mellitus is a common metabolic disorder due to an alteration in glucose homeostasis. Pancreatic β cells are unable to secrete suitable amounts of insulin to regulate glucose level in the blood. Many studies have indicated that miRNAs are involved in the pathogenesis of diabetes. Lynn et al. [129] reported that miRNAs play an important role in the development of pancreatic islets by using a mice model that lacked miRNA processing Dicer in the pancreas. An miRNA, miR-375, one of the most abundant miRNAs present in islet cells was found to be involved in the control of insulin gene expression [130]. The importance of this group of miRNA in blood glucose homeostasis has been confirmed in miR-375 knockout mice models [131]. Hyperglycemia is a condition in both type 1 as well as type 2 diabetes. In vitro studies in MIN6 pancreatic β cell line showed that prolonged exposure to high glucose levels resulted in changes in the expression levels of large sets of miRNAs [132]. A group of miRNAs, namely, miR-124a, miR-107, and miR-30d, were upregulated in hyperglycaemic conditions, whereas miR-296, miR-484, and miR-690 were downregulated. Overexpression of miR-30d caused reduction in insulin gene expression confirming its involvement in defective insulin biosynthesis during diabetic condition [130].

Type 1 and type 2 diabetes are associated with alterations in the level of several miRNAs in insulin-secreting cells as well as in insulin-target tissues. An attractive therapeutic strategy in diabetes is restoration of miRNA functions to normal levels. AAV vectors have shown promising results in gene therapy. Rehman et al. [133] used an AAV8 vector with an insulin promoter to deliver IL-4 to pancreatic β cells, thereby delaying the appearance of diabetes in nonobese diabetic (NOD) mice. RNAi-based techniques can be used for in vivo delivery of miRNA mimics

Table 1 Potential therapeutic applications of RNA interference based techniques (siRNAs/shRNAs) in clinical trials

Disease	Company	Target gene	Phase	Reference
Ocular diseases AMD (age-related macular degeneration	Quark/Pfizer	RTP801	II	[135]
Non-arteritic anterior ischemic optic neuropathy (*NAION*)	Quark Pharma	Caspase2	I	[136]
Colon cancer	Marina Biotech	β-Catenin	I	[137]
Solid tumors	Tekmira	PLK1	I	[138]
Solid tumours	Calando Pharma	RRM2	I	[139]
Advanced solid tumours	Silence Therapeutics	PKN3	I	[140]
Chronic myeloid leukaemia	University Duisburg	BCR-ABL	I	[141]
Metastatic melanoma	Duke University	LMP2, LMP7, MECL1	I	[142]
Respiratory syncytial virus	Alnylam	RSV nucleocapsid	IIb	[143]

or anti-miRs in pancreatic β cells and insulin target tissues to correct the level of important miRNAs under diabetic conditions, thereby leading to new avenues to treat diabetes.

Conclusion

The past era has witnessed the development of RNAi at an interesting pace. RNAi has been regarded as one of the major breakthroughs in the field of molecular medicine. The high point of using RNAi as tools in therapy mainly depends on the effective delivery methods and the stability of these biomolecules inside the cell system. Recent studies point out that chemical synthesis and modifications of RNAi molecules ensure successful delivery of these biomolecules. Current research involves the prevention of off-target effects, increasing resistance to nuclease degradation, nonspecific activation of immune system by siRNAs including the TLR3 as well as the TLR7 pathways, toll-like immunity, and the induction of α, β interferons. A number of studies have also been carried out using RNAi to knockdown certain genes to determine the fate of stem cells resulting in enhanced stem cell differentiation [134]. RNA-based therapeutics that are currently under clinical investigation for various diseases ranging from genetic disorders to various cancers have been shown in Table 1. The potential of use of RNAi as a tool for the treatment of many debilitating diseases has aroused a great deal of interest among scientists, thereby encouraging research in the clinical applications of RNAi.

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