REVIEW
ARTICLE

Therapeutic opportunities of small interfering RNA

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INTRODUCTION

RNA interference [also called ‘RNA-mediated interference’ (RNAi)] is a mechanism for RNA-guided regulation of gene expression in which double-stranded ribonucleic acid (dsRNA) inhibits the expression of genes with complementary nucleotide sequences. The functional mediator of RNAi is small interfering RNAs (siRNA). The dsRNA has been shown to be a potent sequence-specific inhibitor of gene function in the nematode Caenorhabditis elegans [1]. RNAi has been described in several eukaryotic unicellular organisms like Trypanosoma brucei [2] and also in multicellular organisms like C. elegans [1], Drosophila [3], Planaria [4,5], Hydra [6], plants [7], zebrafish [8], mice [9–11] and humans [12,13]. Several observations suggest that RNAi involves some active processes. For example, siRNA delivered by microinjection into the intestine exerts interference effects in tissues in both the injected animal and its progeny [1], suggesting the presence of activities that transport and perhaps amplify the interfering agent. This technology is an extremely useful tool for identifying gene functions and evaluating potential therapeutic targets. So, an increasing number of biotechnological and pharmaceutical companies are attempting to develop RNAi-based drugs for the prevention and treatment of human disease such as cardiovascular diseases, neurological diseases, viral infections, cancer, etc. [14–16]. Currently, for many diseases like heart failure and cancer, surgery remains the only option because the drugs for treating the diseases are not available. Although siRNA-based therapy is still in infancy, it holds tremendous promise for use in routine clinical practice as an adjunct to existing procedures as it can help to overcome the limitations associated with current therapeutic regimens [17,18]. In the present review, we have discussed the basics of siRNA and the therapeutic applications of this technology for various disorders such as cardiovascular diseases, neurological disorders, infectious diseases, cancer, human immunodeficiency virus (HIV), hepatic disorders, etc. The present review gives an overview of the applications of siRNA and their potential for treating many hitherto untreatable diseases.

MECHANISM OF RNAi

RNAi works in two steps. The first step, referred to as the ‘RNAi-initiating step’, involves binding of the RNA nucleases to a large dsRNA and its cleavage into discrete 21- to 25-nucleotide RNA fragments called siRNA. In the second step, these siRNAs join a multinuclease complex, which degrades the homologous single-stranded mRNAs. The delivery of siRNA involves viral- and non-viral-mediated delivery systems; the approaches for chemical modifications have also been developed. It has various therapeutic applications for disorders like cardiovascular diseases, central nervous system (CNS) disorders, cancer, human immunodeficiency virus (HIV), hepatic disorders, etc. The present review gives an overview of the applications of siRNA and their potential for treating many hitherto untreatable diseases.

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the second step, these siRNAs (also called ‘guide RNAs’) join a multinuclease complex, the ‘RNA-induced silencing complex’, which degrades the homologous single-stranded mRNAs.

**Step 1: processing of dsRNA into siRNAs**

First, the dsRNA is cleaved into 21- to 23-nucleotide-long fragments by Dicer or a Dicer homologue. Processing starts from the ends of the blunt-ended dsRNA or dsRNAs with short 3’ overhangs and proceeds in 21–23 nucleotide steps. The resulting fragments (siRNAs) are bound by RNAi-specific enzymes possibly still including Dicer and could be incorporated into a distinct nuclease complex, the ‘RNA-induced silencing complex’. This complex comprising of siRNAs, RNAi-specific enzyme (including Dicer) and nuclease, targets mRNA for the process of degradation. In this complex, siRNAs pair with the target mRNA and cleave the mRNA in the center of the region recognized by the siRNA, whereby the mRNA cleavage boundaries are determined by the sequence of the dsRNA (Figure 1). Either the same ribonuclease that cleaves the dsRNA or another ribonuclease that has to be recruited cleaves the target RNA. The ribonuclease temporarily displaces the passive siRNA strand not used for target recognition. The dsRNA-processing proteins or a subset of them remains associated with the siRNA duplex after the processing reaction. The orientation of the siRNA duplex relative to these proteins determines which of the two complementary strands functions in guiding target RNA degradation. (Chemically synthesized siRNA duplexes guide cleavage of sense as well as antisense target RNA, as they are able to associate with protein components in either possible orientation.) The antisense strand could, for instance, interact directly with the target RNA, whereas the sense strand could more indirectly participate in RNAi, e.g. by protecting the antisense strand against ribonucleases [19–22].

**Step 2: amplification of siRNAs**

One of the many intriguing features of RNA interference is the apparent catalytic nature of the phenomenon. A few molecules of dsRNA are sufficient to degrade a continuously transcribed target mRNA for a long period of time. Although the conversion of long dsRNA into many small siRNAs results in some degree of amplification, it is not sufficient to bring about such continuous mRNA degradation. Recent studies have provided convincing biochemical and genetic evidence that RNA-dependent RNA polymerase (RdRP), which was first identified in plants [23], indeed plays a critical role in amplifying RNAi effects [24,25] in some insects. An RdRp such as that encoded by the ego-1 gene in *C. elegans* or the qde-1 gene in *Neurospora* might amplify the dsRNA signal, producing long-lasting post-transcriptional gene silencing in the absence of the dsRNA that initiated the effect. Thereby, RdRp might convert an aberrant single-stranded RNA population into dsRNA; repeatedly copy the dsRNA to produce a population of single-stranded RNAs that could then interact with target RNA; or copy copies of the trigger thus generating a ‘self-replicating’ trigger population. The absence of identified RdRP homologues in *Drosophila* and mammals suggests either that other RNA copying enzymes are used or that the primary siRNAs may be sufficient to produce detectable interference response. Regardless of whether the dsRNA signal is amplified in nature by an RdRp activity or not, it has been shown that RNAi works better when more dsRNA is used to initiate the process.

**DELIVERY OF siRNA**

The delivery of siRNA poses a major challenge as far as its therapeutic use is concerned. However, various approaches have been considered (Table I) for the efficient delivery of siRNA, which can be classified as follows:

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**Figure 1** Mechanism of RNAi: Processing of dsRNA into siRNAs.
Chemical modifications
Small interfering RNA are sensitive to nucleases, have poor tissue distribution and poor membrane permeability. Hence, chemical modifications of these would help in the proper delivery of siRNA. The following are the methods followed for chemical modification.

Increasing nuclease resistance
Resistance of siRNA to nucleases can be increased by chemically modifying the nucleobases, sugars and the phosphate ester backbone of siRNA [26–28]. Furthermore, boranophosphates [29], 4¢-thioribonucleosides, phosphorothioates, 2‘-deoxy-2‘-fluorouridine, 2‘-O-methyl, 2‘-O-(2-methoxyethyl) and locked nucleotides are some other chemically modified forms for decreasing the sensitivity of siRNA to nucleases [30–35].

Increasing intracellular uptake
Poly-2‘-O-(2,4-dinitrophenyl)-modified siRNA [36] and membrane-permeant peptides (penetratin and transportin) coupled to siRNA [37] are some of the approaches in which the chemically modified system results in increasing the intracellular uptake of siRNA. In another approach, siRNAs with partial phosphorothioate backbone modifications and 2‘-O-methyl sugar variations on the sense and antisense strands promoted nuclease resistance, while cholesterol was conjugated to the 3‘-end of the sense strand using a pyrrolidine linker to change tissue distribution [38].

Increasing specificity
Second-generation siRNA, known as ‘stealth RNAi’ are developed in which chemical modifications are designed to increase the specificity of RNAi effects by allowing only the antisense strand to efficiently enter the RNAi pathway and eliminating induction of interferon-related pathways [39,40].

Viral nucleic acid delivery
Viruses bind to the cell and then deliver their nucleic acid payload intracellularly, proficiently along with nuclear localization. Usually, virus-mediated delivery methods are based on delivery of genes encoding short hairpin RNA (shRNA). However, few approaches used viruses to deliver chemically synthesized siRNA in vivo [41,42].

Delivery of chemically synthesized siRNA
Small interfering RNAs are encapsulated in reconstituted viral envolops derived from influenza virus. The reconstituted membrane vesicles contain the influenza virus spike protein hemagglutinin, which is responsible for binding to and fusion with cellular membranes and additionally added cationic lipids. The siRNA-loaded vesicles are taken up by receptor-mediated endocytosis, and are able to escape endosomal degradation by fusion with the endosomal membrane. Functional siRNA delivery was demonstrated in vitro, while in vivo uptake by macrophages in the peritoneal cavity was demonstrated after intraperitoneal injection. A similar approach described siRNA delivery by simian virus SV40-based particles in vitro in lymphoblastoid cells [41]. Difficulties of repeated administration and limited control over transduced cell type are the major drawbacks of these systems.

Delivery of DNA-encoding siRNA/shRNA
A number of studies investigated the use of DNA encoding for shRNA delivered by viruses for gene silencing in vivo. Almost complete inhibition of viral

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**Table 1** Delivery of siRNA by chemically modifying siRNA or by viral delivery systems: benefits and examples.

| Sr. No. | Approach                      | Benefits                                      | Examples                                      |
|---------|-------------------------------|----------------------------------------------|----------------------------------------------|
| 1       | Chemical modification         | Increased nuclease resistance                 | Boranophosphates, 4¢-thioribonucleosides,    |
|         |                               |                                               | phosphorothioates, 2‘-deoxy-2‘-fluorouridine,|
|         |                               |                                               | 2‘-O-methyl, 2‘-O-(2-methoxyethyl)           |
|         |                               | Increased intracellular uptake                | Poly-2‘-O-(2,4-dinitrophenyl) modified siRNA,|
|         |                               |                                               | penetratin and transportin coupled to siRNA  |
| 2       | Viral delivery of siRNA       | Direct delivery of the siRNA                 | Influenza virus                              |
| 3       | Viral delivery of DNA encoding siRNA | Efficient binding and transduction of cells, increased nuclease resistance | Adenovirus, Lentivirus, |
protein production was observed by intravenous injection of $5 \times 10^9$ plaque-forming units recombinant adenovirus expressing shRNA-targeting hepatitis B virus transcripts in mice with active replication of the hepatitis B virus [43]. This in turn led to the arrest of viral replication on day 17 after viral infection. The inhibitory effect persisted for at least 10 days. Uchida et al. used expression of two separate siRNA strands against survivin by adenoviral transduction to inhibit tumor growth [44]. Survivin is a protein that inhibits cancer cell apoptosis. Mice bearing subcutaneous U251 glioma tumors were treated with intratumoral injections of 1010 viral particles on three consecutive days every 20 days, ultimately leading to fourfold smaller tumors on day 48 after start of treatment when compared with empty adenoviral vector and adenoviral vector expressing irrelevant siRNA [44].

Intracranial delivery of lentivirus-produced shRNA for inhibition of reporter gene expression in cortical neurons [45], intraperitoneal delivery of lentivirus-produced shRNA for inhibition of viral cyclin to prevent primary effusion lymphoma in mice [46], intramuscular or intraspinal delivery of lentivirus-produced shRNA for inhibition of mutant SOD1 in amyotrophic lateral sclerosis [47,48], and ex vivo delivery of lentivirus-produced shRNA for inhibition of CC-chemokine receptor 2 in hematopoietic cells in mice [49] are other approaches for delivering DNA-encoding si/shRNA for gene silencing. Taken together, the viral DNA-based sh/shRNA delivery process is very efficient: binding to the target cell surface and subsequent transduction, carrier stability, and protection against nucleases appear satisfactory [50–53]. However, viruses usually lack selectivity for the target cell type. To improve specificity, the natural tropism of viruses for certain cell types may be used.

Redirecting the natural preferred cell type of viruses towards therapeutically interesting receptors on the surface of target cells is one of the newer approaches being studied these days. Examples include the retargeting of murine coronavirus towards the human epidermal growth factor receptor [54], directing adenovirus via fibroblast growth factor ligand towards its associated receptor for delivery to glioma, or adenoviral delivery to angiogenic endothelium via RGD-peptides (arginine-glycine-aspartate sequence peptides) binding alpha $\gamma$-integrins [55]. However, such approaches have not been tried as yet in combination with RNAi-mediated gene silencing in vivo.

The efficient transduction of cells is the main strength of the viral delivery approach. However, major drawbacks are the control over transduced cell type, especially after systemic administration, inflammatory reactions, immunogenicity, and oncogenic transformations [56,57].

**Non-viral nucleic acid delivery**

Non-viral vectors possess several advantages over viral vectors. Lack of immunogenicity, low frequency of integration and ease of large-scale production are the major advantages of synthetic vector systems. In addition, they can accommodate a wide variety of nucleic acid sizes and allow easy modification. However, efficiency of transfection becomes a problem and to overcome this, many functional groups need to be incorporated. A cationic functional group is usually required to bind and condense the nucleic acid, thereby protecting it against nucleases and increasing the apparent molecular weight above the renal clearance cut-off. In addition, some cationic compounds are being used as endosomal escape enhancers. Because of the resulting positive charge, complexes tend to form aggregates by binding in the blood stream to negatively charged biomolecules. As a result, their clearance is usually rapid. Moreover, such cationic complexes possess a propensity to interact with virtually any cell type they encounter, creating a need to insulate the interactive surface of the particle to promote specificity. For that purpose, shielding groups can be added to enhance colloidal stability and reduce surface charge, thereby avoiding non-specific cell uptake. To restore cell interaction in a target-specific manner, targeting ligands can be coupled to induce site-specific binding and uptake. In the case of delivery of DNA encoding for shRNA by non-viral delivery systems, nuclear translocation of the DNA is often inadequate. As such, the cytoplasmic site of activity of chemically synthesized siRNA provides an important advantage.

**Delivery system based on RNA**

A system consisting completely of RNA is based on the packaging RNA of the DNA-packaging motor of bacteriophage phi29, which can spontaneously form dimers via interlocking right- and left-hand loops. By attaching the siRNA to one loop and an RNA aptamer to CD4 to the other, a cancer cell-targeted system was created that could silence survivin gene expression in vitro. Alternatively, the system could also be targeted by folate [58].
Cationic delivery systems
Unshielded, untargeted complexes of siRNA with cationic polymers or lipids can provide local or systemic transfection of a sufficient number in target cells for therapeutic effects. Tumor necrosis factor (TNF)-alpha in intraperitoneal macrophages after intraperitoneal administration [10], delta opioid receptor in spinal cord and dorsal root ganglia after intrathecal administration [59], polo-like kinase-1 in bladder cancer after intravesical administration, and c-raf-1 in prostate cancer cells after intravenous administration [60] are some examples employing cationic lipids to complex siRNA. The major drawback with this approach is the non-specific effects that can be induced by cationic lipids themselves and in particular in combination with dsRNA, which may severely hamper therapeutic application [61,62]. A variety of other cationic compounds have also been investigated for siRNA-delivery purposes. A linear low-molecular-weight form of the cationic polymer poly(ethylene imine) was used for treatment of (subcutaneously implanted) ovarian carcinoma in mice [63]. After intraperitoneal administration, complexed siRNA was primarily recovered from muscle, liver, kidney, and tumor and silencing of Her-2 with these polyplexes inhibited ovarian carcinoma growth in vivo.

A highly purified type-I collagen of calf dermis digested by a pepsin named ‘atelocollagen’ was shown to be a suitable vehicle for local delivery of siRNA [64,65]. On intravenous administration, atelocollagen–siRNA localized at sites of tumor metastases and inhibited metastasis outgrowth [66]. More specifically, tumor levels increased sixfold when compared with levels after ‘naked’ siRNA administration and delivery of these levels of siRNA, silencing EZH2 (enhancer of zest homologue-2, a gene overexpressed in hormone-refractory metastatic prostate cancer) or p110-α (a phosphatidylinositol 3-kinase regulating cell survival, proliferation, and migration), resulting in strong inhibition of growth of bone metastases of prostate cancer cells. Importantly, siRNA–atelocollagen complexes failed to induce non-specific proinflammatory responses like secretion of interferon (IFN)-α and interleukin (IL)-12.

Targeted cationic delivery systems
A targeted amino acid-based system was based on the cationic peptide protamine [67]. To the system’s protamine block the C-terminus of the heavy-chain Fab fragment of an HIV-1 envelope antibody was coupled to form a protein construct known as ‘F105-P’. This system was highly efficient in binding to and transfection of cells expressing the HIV-envelope protein, although it is unclear why the HIV-envelope protein would be internalized. Importantly, expression of interferon-β, 2’,5’-oligoadenylate synthetase, and Stat-1, as indicators of non-specific effects, was not increased upon siRNA transfection of HIV-envelope-expressing melanoma cells. In addition, when these cells formed subcutaneous tumors in vivo, 30% of cells took up fluorescent siRNA when delivered by F105-P after intravenous administration. Naked siRNA was not taken up, nor was F105-P-siRNA delivered to cells that were envelope protein-negative. Delivery of a combination of siRNAs against c-myc, Mdm2 p53 binding protein homologue (MDM2) and vascular endothelial growth factor (VEGF) strongly inhibited tumor growth in vivo when delivered using the F105-P system. This combination of siRNAs attacking the tumor at multiple fronts is an important advantage of the siRNA technology as it allows simultaneous interference with a number of different pathways, while the delivery problem for each individual drug molecule (siRNA) remains the same. The versatility of this targeted system was demonstrated by exchanging the HIV-envelope antibody for an ErbB2 antibody, thus changing the specificity of the system to ErbB2-positive breast carcinoma cells.

A transferrin receptor single-chain Fv region antibody fragment was coupled to the surface of cationic DOTAP:DOPE complexes containing siRNA [68]. The study evaluated the targeting potential of these systems in different murine tumor models, i.e. an orthotopically implanted pancreatic carcinoma (that produced spontaneous metastases), an orthotopically implanted prostate carcinoma, and intravenously administered breast carcinoma cells giving rise to metastases in the lung. In all these models, specific accumulation of fluorescently labeled siRNA complexed to the targeted cationic lipid particles at the site of the malignancy could be demonstrated when compared with surrounding normal tissue and liver.

Shielded targeted cationic delivery systems
Shielding of the cationic surface may enhance target cell specificity by reducing non-target tissue uptake and may additionally increase the colloidal stability of the siRNA complexes. In a study, the cationic polymer poly(ethylene imine) was coupled to poly ethylene glycol (PEG) which acted as a shielding polymer. To the distal end of the PEG chain a cyclic RGD peptide was coupled, which is a high-affinity ligand for alpha γ-integrins that are overexpressed on angiogenic endothelial surfaces [69]. It was observed that injection of ‘naked’ siRNA did not
produce appreciable tumor levels, but rather rapid clearance into the urine in in vivo tissue distribution study involving administration of fluorescently labeled siRNA in subcutaneous neuroblastoma-bearing mice. Poly(ethylene imine) (PEI)–siRNA complexes also lacked the production of high fluorescence in the tumor, but they increased liver and lung levels. When the PEG-shielded, targeted nanoparticles were used, a higher level of specificity for the tumor and lower levels of fluorescence in the lung and liver were observed. In another study, siRNA against murine VEGF receptor-2 was used and delivery to host tumor endothelium is required to inhibit tumor proliferation. Efficacy studies with VEG-FR2-specific siRNA complexed in RGD–PEG–PEI nanoparticles resulted in strong inhibition of subcutaneous neuroblastoma growth rate, which was sequence-specific. These experiments suggest that the targeted shielded nanoparticles indeed deliver the siRNA to the angiogenic endothelial cells. In line with these findings, the reduced tumor growth rate was paralleled by a reduction in blood vessels in the periphery of the tumor and changes in vascular morphology of remaining vessels, supporting an antiangiogenic mechanism of action. These results were supported by studies in a model of pathological angiogenesis in the eye [70], again demonstrating vasculature-specific delivery and inhibition of angiogenesis leading to therapeutic effects. Targeted cationic systems have the important advantage that they possess a recognition signal for specific interaction with the target cell type.

**THERAPEUTIC APPLICATIONS**

In spite of the development of the siRNA in a span of just 10 years, it has wide potential in applications for cardiovascular diseases (CVD), central nervous system (CNS) diseases, cancer, etc. The applications and targets of siRNA are depicted in Figure 2.

**CARDIOVASCULAR DISEASES**

Various CVD like hypertension, hyperlipidemia and cardiac hypertrophy can be treated using siRNA by modification of the specific target gene. They have been discussed at length as follows.

**Hypertension**

Hypertension increases the risk of sudden cardiac death, mainly through its causal association with left ventricular hypertrophy [71]. Hypertension is one of the most important risk factors for stroke, congestive heart failure, myocardial infarction, and peripheral vascular disease. The renin–angiotensin system plays an important role in the development and progression of devastating disorders in patients with hypertension [72]. Angiotensin II, a physiologically active major substance of the renin–angiotensin system, acts as a vasopressor by inducing vasoconstriction and elicits water and sodium absorption in the proximal renal tubule by stimulating the secretion of aldosterone [73]. It binds to two distinct receptor subtypes, namely type 1 (AT_1) and type 2 (AT_2). AT_1_ is the primary subtype accountable for angiotensin II actions in mice [74]. Vazquez et al. selected AT_1_R as the target gene to design corresponding dsRNAs, and then transfected them into Chinese hamster ovary cells (CHO), which express rat AT_1_R, and found an 80% decrease in the level of AT_1_R mRNA [75]. Furthermore, there was a dose-dependent decrease in the specific binding of angiotensin II to AT_1_R-expressing CHO cells. To determine whether the decrease in AT_1_R-specific binding was associated with a reduction in functional AT_1_R, they examined the effects of dsRNA transfection on angiotensin II-stimulated calcium uptake. In AT_1_R dsRNA transfected cells, the angiotensin II-induced increase in calcium uptake was completely abolished. It has been found that knocking down the expression of heat shock factor-1 with RNAi technology exacerbated angiotensin II-induced inflammatory injury by causing significantly higher activation of nuclear factor kappa \( \beta \).
(NF-κB) in vascular smooth muscle cells (VSMCs) [76]. In another research into the mechanism of hypertension, it was revealed that the RhoA–Rho kinase pathway was the significant pathway behind the pathogenesis of the abnormal contraction of the VSMCs in cardiovascular diseases [77]. Knockdown of RhoA by RNAi decreases the level of RhoA mRNA and the contractility of the cultured VSMCs [78], which indicates that the expression level of RhoA played a critical role in the regulation of contractility in the de-differentiated VSMC, and that RhoA could be a new therapy target for hypertension. Thus, these findings suggest that RNAi might have potential as an alternative to drug therapy for hypertension.

Atherosclerosis
Atherosclerosis is a chronic inflammatory disease of the arterial intima, resulting from a concerted action of multiple factors [79]. Many studies have shown that macrophages and T cells play critical roles in the multiple aspects of the pathogenesis of the disease. Adiponectin has been found to enhance Akt phosphorylation [80]. Pretreatment with Akt siRNA transfection blocked the inhibitory effect of adiponectin on TNF-α-induced IL-8 synthesis in porcine coronary artery SMC, suggesting that Akt activation might inhibit IL-8 synthesis, a pro-inflammatory chemokine that plays a role in atherogenesis [80]. Molecular analysis indicates that the NF-κB plays a prominent role in the formation of atherosclerosis because of its ability to adhere to elements in the promoters of key inflammatory and atherosclerosis genes [81]. siRNA targeting NF-κB has been synthesized by the use of a rapid polymerase chain reaction (PCR)-based approach that generates sense and antisense siRNA separated by a hairpin loop downstream of the U6 promoter [82]. This was then transfected into the VSMC derived from 12/15-Loko mice. The mRNA and protein of NF-κB and NF-κB-dependent transcriptional responses were reduced markedly by the siRNA. Some experiments have shown that excessive apoptosis of VSMCs plays a key role in the progression of atherosclerotic lesions, resulting in many cardiovascular events [83]. GADD153, a member of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors, has been linked to apoptosis in VSMCs and inhibition of GADD153 by siRNAs reduces C-reactive protein-induced GADD153 mRNA expression and apoptosis [84]. In another study, it has been demonstrated that Omi/HtrA2, a serine protease, could be released into the cytosol from mitochondria and promote caspase-dependent apoptosis after an apoptotic insult. Removal of Omi/HtrA2 by RNA interference has been shown to reduce UV light-induced apoptosis in U2OS cells [85]. These observations may suggest new options for the treatment of atherosclerosis.

Cardiac hypertrophy
Cardiac hypertrophy is a compensatory response to a variety of physiological or pathological stimuli, and prolonged hypertrophic responses may eventually lead to arrhythmia, heart failure and sudden death [86]. A novel human gene, myofibrillogenesis regulator-1 (MR-1) from a human skeletal muscle cDNA library that interacts with contractile proteins and exists in human myocardial myofibrils has been identified [87]. A hypertrophy model in which hypertrophic cell growth can be induced by angiotensin II incubation in cultured neonatal rat cardiomyocytes is being established. By transfecting neonatal cardiomyocytes with a pSi-1 targeting the MR-1 sequence, it was found that the MR-1 mRNA and protein expression were greatly decreased. Furthermore, compared with the angiotensin II-treated group, the MR-1 RNAi + Ang II group showed a decrease in the surface area of cells by 36% [87]. In another study, it is reported that the translocation of the hypertrophic transcription factor, NF-AT, to the nucleus of the cardiomyocyte and the enhancement of NF-AT transcriptional activity induced by angiotensin II could be prevented by 17b-estradiol [88]. Angiotensin II also stimulated the activation of protein kinase C, contributing to cardiac hypertrophy. 17b-estradiol inhibited these pathways, related to the stimulation of atrial natriuretic peptide production and secretion. These observations were further supported by the evidence that siRNA against the MCIP1 gene significantly reversed both the 17b-estradiol restraint of protein synthesis and the inhibition of angiotensin II-induced calcineurin activity. Accordingly, their findings may provide a better understanding of the mechanism of cardiac remodeling and new insights into the development of novel therapeutic strategies in cardiac hypertrophy.

Myocarditis
Viral myocarditis is an important cause of heart failure and dilated cardiomyopathy [89]. More effective approaches are needed to treat viral infection. If genes responsible for affective disorders are identified, gene silencing could be an alternative therapeutic tool, especially for cases of drug therapy resistance. Coxsackie virus B3 (CVB3) has been identified as the most...
common causal agent of viral myocarditis, but existing drug therapies are of limited value [90]. Many studies have shown that RNAi can control viral infection by targeting viral genes. Recently, Schubert et al. found that two independent siRNA targeting the 3D RNA-dependent RNA polymerase were able to reduce virus titer by 80% and 90%, respectively [91]. Their results demonstrate the enormous potential of the RNAi approach. An siRNA double-expression vector (SiDEx) is constructed to achieve simultaneous expression of both siRNA from one plasmid [91]. Compared with conventional expression vectors, SiDEx showed substantial gene regulation of the mutated target RNA. Hence it is believed that SiDEx may be a helpful tool to achieve sustained silencing of viruses, ultimately reducing the risk of emergence of viable mutants. Yuan et al. reported that the siRNA targeting the viral protease 2A displayed a 92% inhibition of CVB3 replication and a 90% protection of the siRNA-pretreated cells in HeLa cells and murine cardiomyocytes [92]. Moreover, they found that administration of the siRNA after viral infection could effectively inhibit CVB3 replication, indicating its therapeutic potential. These findings imply that siRNA-based gene drugs may be an effective therapy for viral myocarditis.

Heart failure
Studies show that heart failure is a common lethal condition associated with various CVD and remains the leading cause of morbidity and mortality. One of the important features of heart failure is a decreased Ca\textsuperscript{2+} uptake into the sarcoplasmic reticulum (SR) by the sarcoplasmic/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase 2 (SERCA2), which is negatively regulated by phospholamban (PLB), a key regulator of cardiac calcium homeostasis [93]. Recent findings demonstrated that the development of severe heart failure in the genetic muscle LIM protein (MLP) (−/−) animal model could be abolished completely by the targeted ablation of PLB. PLB has been considered as the potential therapeutic target for the improvement of SR Ca\textsuperscript{2+} uptake and cardiac function. Twenty-one nucleotide–siRNA duplexes with symmetric 2-nucleotide 3’-overhangs targeting PLB mRNA were synthesized and introduced into neonatal rat cardiac myocytes by the use of the hemagglutinating virus of Japan (HJV) envelope vector [94]. It was found that PLB siRNA resulted in a significant decrease in the levels of both PLB mRNA and protein, while the mRNA and protein of SERCA2 calsequestrin were not affected. The affinity of SERCA2 for Ca\textsuperscript{2+} was also increased. In order to determine the effect of PLB RNAi on the cardiac myocytes in which Ca\textsuperscript{2+} handling was impaired, the myocytes were exposed to H\textsubscript{2}O\textsubscript{2}, a reactive oxygen species. The same result with decreased PLB mRNA and protein levels was achieved. So Watanabe’s strategy used for the PLB ablation may be considered as a novel and attractive candidate for clinical therapy in heart failure. Table II summarizes application and target genes of siRNA for CVDs.

**NEUROLOGICAL DISORDERS**

**Muscular dystrophy**
These are degenerative disorders marked by progressive paralysis of body muscle ending in premature death. The age of patients with Duchenne muscular dystrophy (DMD) – the major muscular dystrophy in children, with a mean death age of 25.3 years – can be shortened further because of cardiomyopathy complications [95]. DMD is associated with mutations in the dystrophin gene, which encodes key components of a muscle complex comprising transmembrane and cytoplasmic proteins. A number of these proteins have orthologues in C. elegans [96]. RNAi knockdown of the conserved orthologues resulted in phenotypes similar to that seen in dystrophin gene-1. RNAi can be applied in the context of a positive transgenic background to provide bidirectional control of gene expression. For instance, a protein-coding gene and an shRNA directed against that gene, each under the control of different inducible promoters, can be transfected into the same cell. This approach has been applied to C. elegans to enable bidirectional control of transmembrane calcium flux in an attempt to unravel the poorly understood involvement of elevated intracellular calcium in DMD [97]. egl-19 is a C. elegans calcium channel, the gain-of-function mutation of which was used to enhance the signal and RNAi knockdown of egl-19 to diminish it. Enhancing the calcium signal resulted in enhanced muscle degeneration, and this was blocked by RNAi knockdown of egl-19 [97]. Thus, there is considerable potential for the application of RNAi to worm and fly models of this disease. In humans, the X-linked form of Emery-Dreifuss muscular dystrophy is caused by the loss of function of emerin, a nuclear membrane LEM-domain protein. Emerin is a member of the lamin class of proteins that form networks of filaments in the inner nuclear envelope and are essential for maintaining nuclear shape, DNA replication and transcription [98]. Although the C. elegans equivalent of emerin, EMR-1, is homologous to its vertebrate counterpart, RNAi knockdown of emr-1 produced no detect-
able phenotype in C. elegans at any stage of development [99]. This suggested that functional loss of EMR-1 was being compensated for by another pathway. To test the hypothesis that EMR-1 overlaps functionally with the structurally similar MAN-1, for which no function was known, it was established that RNAi knockdown of MAN-1 alone was lethal in 15% of embryos, but MAN-1 knockdown in the absence of EMR-1 was lethal to all embryos [100]. Thus, applying RNAi approaches using the worm model suggests that EMR-1 and MAN-1 overlap functionally, raising hopes for a gene replacement strategy as a therapy for Emery-Dreifuss muscular dystrophy.

Spinal muscular atrophy

Spinal muscular atrophy (SMA) is associated with lower motor neuron loss in the spinal cord caused by the mutations in the survival motor neuron protein, SMN. However, it is not clear how the loss of SMN is linked to the death of neurons. C. elegans also has an orthologue of SMN, which is ubiquitously expressed [101]. Knockdown of the worm SMN protein using RNAi results in poor embryonic viability and severely uncoordinated locomotion [98]. It is suggested that some C. elegans SMN protein interactions are similar to those of human equivalents [98]. Drosophila has an orthologue of SMN (dSMN) [102] and has been used as a model of this disease. A Drosophila SMN gene point mutation results in a phenotype that includes abnormal motor behavior, functionally impaired neurons and altered synaptic transmission [102]. RNAi suppression of SMN in Drosophila S2 cells resulted in a significant increase in apoptosis through a pathway involving the caspases DRONC and DRICE. The effect was reversed by the caspase inhibitor, Z-VAD-fmk, suggesting a possible target for novel therapies.

Alzheimer’s disease

Alzheimer’s disease (AD) is associated with the accumulation of insoluble plaques of amyloid protein (Aβ) in the CNS, as well as intracellular microfibrillar tangles, along with loss of cholinergic neurons projecting from the basal forebrain into the hippocampus and amygdala. Plaque deposition is a result of overproduction of Aβ through the activity of γ-secretase, of which the presenilins are thought to be a component. C. elegans model was used in microarray studies to identify the genes upregulated and downregulated as a result of the heterologous expression of Aβ [104], and should also prove useful in global RNAi approaches designed to identify genes
regulating the secretion of Aβ. In the worm AD model, the Aβ is expressed only in muscle. Clearly, there is a need for improved invertebrate models of AD, particularly for a C. elegans model in which human Ab is expressed in neurons and is secreted. A Drosophila model of AD has been generated that expresses the amyloid precursor protein (APP) [105], but it is not known if the APP is processed into Aβ. More recent models of AD have been reported in which Aβ is expressed in the CNS, resulting in neurodegeneration and shortened lifespan [106,107]. These models offer exciting possibilities for RNAi-based studies. However, RNAi applied to Drosophila cell lines is a potentially powerful alternative approach in determining the pathways involved in the development of Alzheimer’s, as the Drosophila equivalent to human presenilins PS1 and PS2 (PSN) appears to be involved in pathways similar to its vertebrate counterparts [108]. Early-onset familial Alzheimer’s, for example, is associated with mutations in presenilin genes PS1 and PS2 which lead to oversecretion of Aβ. RNAi knockdown of PSN results in a blockage of γ-secretase activity [108], providing confirmation of this gene’s role in the γ-secretase pathway. Another study [109] used RNAi to reduce expression of two genes, aph-1 and pen-2, in Drosophila S2 cells. Knockdown of these genes resulted in reduced proteolytic cleavage of Aβ precursor protein and Notch substrates and reduced production of processed presenilin, suggesting that they are required for the action of, and the accumulation of, the γ-secretase. The increased levels of Aβ in AD caused by the γ-secretase is thought to be counteracted by α-secretases that cleave the precursor protein in the middle. The identity of the α-secretase is uncertain, but a recent study [110] showed that RNAi knockdown of each of three ADAM (α disintegrin and metalloprotease) proteins, ADAM9, ADAM10 and ADAM17, in human glioblastoma A172 cells, which have elevated endogenous α-secretase, indicates that all three of these proteins are involved in the α-secretase activity. An extension of this approach in mammalian systems will also enable potential gene targets for therapeutic intervention to be identified. For example, siRNAs targeting the β-secretase, β-site of APP cleaving enzyme, were shown to reduce APP production in mouse cortical neurons, offering a potential therapeutic approach for AD [111].

Poly(Q) diseases
There are at least nine human neurodegenerative disorders that are caused by expansion of the CAG trinucleotide repeat. RNAi has recently been performed on two prevalent poly(Q) diseases, Huntington’s disease (HD) and spinobulbar muscular atrophy (SBMA). HD is an autosomal dominant hereditary brain disorder that is progressive and fatal. Mutations in the Huntingtin gene result in involuntary movement (chorea), cognitive impairment and psychiatric problems such as depression and anxiety [112]. HD is caused by expansion of a CAG trinucleotide repeat in exon-1 of the Huntingtin gene. This expansion elongates the N-terminal poly(Q) stretch of the protein, resulting in aggregation and the formation of neuronal intranuclear inclusions. A number of treatment strategies have been proposed that target Huntingtin proteolysis, aggregation and transcription [113]. SBMA is an X-linked motor neuron disease that occurs in adulthood [114]. This progressive disease results in the loss of motor neurons in the lower spinal cord and the brain stem and is caused by CAG trinucleotide expansion in the first exon of androgen receptor (AR). Unaffected individuals have between 11 and 35 poly(Q) repeats as opposed to 38 and 62 repeats in SBMA individuals [115]. This poly(Q) expansion results in intranuclear aggregate formation in tissues where the AR gene is normally expressed. One laboratory [116] synthesized transgenic strains that expressed poly(Q35) expansion fused with yellow fluorescent protein and used it in a global RNAi screen. This screen identified 186 genes that resulted in premature appearance of protein aggregates. The genes identified are mainly involved in RNA metabolism, protein synthesis, protein folding, protein degradation and protein trafficking. It would be interesting to see whether the same genes control other protein aggregation diseases such as Alzheimer’s. Drosophila S2 cells were engineered to express a portion of human AR gene with CAG tracts of 26, 43 or 106 repeats tagged by green fluorescent protein [116]. Cells carrying the CAG repeats of 43 and 106 developed green fluorescent protein aggregates and aggresomes, with 106 aggregates being formed much faster than 43 CAG repeats. Using RNAi directed against AR protein, Caplen et al. showed loss of AR green fluorescent protein aggregates by 80% in cotransfected S2 cells [117]. Therefore, RNA interference could have considerable therapeutic potential in poly(Q) neurodegenerative disorders.

Parkinson’s disease
Where a disorder results from a reduction in gene expression, RNAi can be used to mimic gene function loss. Parkinson’s disease, for example, is associated with loss of dopaminergic neurons, and as with SMA, RNAi will be an important approach for exploring the molecular mechanisms underlying Parkinson’s disease.
Recently, this approach has been used in combination with overexpression studies to explore the role of Parkin, an E3 ubiquitin ligase, in dopamine neuron degeneration in Drosophila. Overexpression of Parkin was shown to degrade its substrate (Pael-R) and suppress its toxicity, whereas interfering with endogenous Parkin promoted substrate accumulation and augmented its neurotoxicity [118]. Viral-mediated RNAi has been used [119] to block dopamine synthesis in mid-brain neurons of adult mice. This study used an adeno-associated virus vector in which a U6 promoter drove the expression of shRNA directed against tyrosine hydroxylase, an enzyme required for the production of dopamine. This was injected stereotactically into the substantia nigra of one side of the brain and a similar vector promoting the expression of a randomized shRNA injected into the other side. GFP expression was observed in both halves of the brain, but dopamine staining was reduced only in the side of the brain into which the anti-tyrosine hydroxylase shRNA had been injected. The resultant behavioral deficits included loss of motor performance; bilateral shRNA knockdown of dopamine synthesis resulted in reduced motor activity in response to amphetamine (a well-established dopamine-dependent behavior) and poorer performance in the rotarod test.

Machado–Joseph disease

The polyglutamine disorder spinocerebellar ataxia type 3 is also known as Machado–Joseph disease. This disease offers a compelling example of how a linked polymorphism can be exploited. Miller et al. took advantage of a single-nucleotide polymorphism (G → C) that exists in tight linkage disequilibrium with the CAG repeat expansion to silence expression of the mutant disease protein, ataxin-3, without altering levels of normal ataxin-3 [120]. About 70% of MJD1 disease alleles have a ‘C’ at this polymorphism, which sits immediately 3’ to the repeat, while most normal alleles have a ‘G’ [121]. Miller and co-workers were able to generate siRNAs that selectively reduced expression of either the mutant or the normal allele, depending on the specific nucleotide present at this site in the complementary guide strand and the targeted mRNA [110]. Some researchers have performed studies aimed at determining whether RNAi could be used to target the pathogenic process in inherited neurodegenerative disorders caused by polyglutamine expansions [117]. They used Drosophila and human tissue culture models of spinobulbar muscular atrophy, a disease caused by CAG expansions in the gene encoding the androgen receptor. They assessed the abilities of different siRNAs to selectively inhibit expression of transcripts that included a truncated human androgen receptor gene containing different CAG repeat lengths (16–112 repeats). They found that RNA duplexes containing CAG repeat tracts only induced gene-specific inhibition when flanking androgen receptor sequences were included. Sequence-specific small dsRNAs of 22 nucleotides rescued the toxicity and caspase-3 activation induced by plasmids expressing a transcript encoding an expanded polyglutamine tract [117].

Pain

The study of the purinergic P2X receptor family is difficult because no small-molecule compounds are available for differentiating between the closely related receptors. Antisense and RNAi approaches are therefore a suitable strategy to address these medically relevant pain-related genes. An siRNA that efficiently inhibits the mRNA of the purinergic P2X3 receptor was generated [122]. Dorn et al. have reported that RNAi can block a pathophysiological pain response and provide relief from neuropathic pain in a rat disease model [123]. Rats, intrathecally infused with a 21-nucleotide siRNA, showed diminished pain responses compared with nonsense (MS) siRNA-treated and -untreated controls in models of both agonist-evoked pain and chronic neuropathic pain [123]. G proteins are important mediators of opioid receptor-induced signaling, and the $G_\alpha$ subunit is involved in extracellular signal-regulated kinase activity that is induced by the $\delta$-opioid receptor [124]. Decreasing $G_\alpha$ expression by RNAi significantly blocks this signaling pathway in mouse neural cells. Kinase activity could subsequently be restored by exogenously expressed human $G_\alpha_1$ and $G_\alpha_2$ subunits that were not silenced by the siRNA [124]. The vanilloid receptor VR1 is another new target for therapeutic approaches against chronic pain. Grünweller et al. revealed that an siRNA targeting VR1 mRNA was the most potent antisense molecule and was 1000-fold more efficient than a phosphorothioate antisense oligonucleotide and sixfold more active than a locked nucleic acid gapmer [125].

Developmental disorders

RNAi knockdown removes mRNA, leaving the parent gene intact. This can be an advantage over gene knockout studies, where the absence of a functional gene may induce compensatory expression. This may explain some contradictory findings in research into double cortical syndrome. This neurological disorder is associated with the doublecortin (DCX) gene, but gene deletion
approaches had failed to mimic the syndrome. Mice in which the DCX gene is deleted develop normal cortices. However, directing plasmid-mediated dsRNA against DCX caused disruption of radial migration of developing cortical neurons [126], suggesting that neurons lacking the DCX gene select alternative migration mechanisms. The DCX knockdown model might therefore serve as a useful experimental model of double cortical syndrome.

**Addiction**

The neural and genetic basis of ethanol addiction is poorly understood, but is a complex phenomenon involving several receptor types [127]. RNAi offers a complementary approach to dissecting the roles of these receptors in ethanol addiction. \(\gamma\)-amino butyric acid (GABA) receptors have a possible role in addiction, and RNAi reduction in expression of the R1 subtype of the GABA receptor in *D. melanogaster* reduced the behavior-impairing effects of alcohol [128]. Nicotine addiction, despite its well-known health hazards, is common around the world [129]. RNAi is a promising approach for unravelling the gene networks known to be involved in invertebrate models of nicotine tolerance [130].

**CANCER**

Many studies have used siRNAs as an experimental tool to dissect the cellular pathways that lead to uncontrolled cell proliferation and cancer. Moreover, RNAi has been proposed as a potential treatment for cancer [131–133]. The former systemically delivered antisense oligonucleotide for the treatment of cancer, which targets the anti-apoptotic gene *BCL2*, has shown promise in clinical trials for metastatic melanoma when used in combination with conventional chemotherapeutics [134]. RNAi targeted against the oncogenic fusion gene BCR/ABL mRNA in K562 cells has offered promising results. Effective downregulation of mRNA and BCR/ABL oncoprotein has been demonstrated by multiple investigators through quantitative RT-PCR and Western blot analysis, respectively [135,136]. Along similar lines, the AML1/MTG8 (acute myeloid leukemia1/myeloid transforming gene8) t(8:21) translocation has been targeted using RNAi in the Kasumi-1 and SKNO-1 human leukemic cell lines [137]. In a study, siRNA was shown to be more potent than antisense DNA in suppressing gene expression in human hepatoma and pancreatic cancer cell lines [138]. In another study, four different myeloid leukemia cell lines (HL-60, U937, THP-1, and K562) were transfected with dsRNA duplexes corresponding to the endogenous c-raf and bcl-2 genes [139]. Levels of Raf-1 and Bcl-2 proteins were markedly decreased in each of the transfected cell lines; combined RNAi for c-raf and bcl-2 induced apoptosis in HL-60, U937, and THP-1 cells and increased their sensitivity to the DNA-damaging agent etoposide. Activation of TNF receptors and related death receptors can induce death of some cancer cells, but may simultaneously activate pathways that promote cell survival; one protein that inhibits the TNF cell death pathway is called FLIP [FADD-like IL-1-converting enzyme (FLICE)-like inhibitory protein]. When FLIP expression was suppressed in cancer cells using siRNAs, the cells were more sensitive to being killed when death receptors were activated [140]. Several other studies have demonstrated efficacy of liposome- or viral vector-mediated transfection of cancer cells in suppressing their growth and/or inducing their death [141]. The next step in the development of RNAi technology for cancer therapy will be to establish methods for targeting tumor cells in vivo. Another approach might be to target genes that promote angiogenesis. Tumor cells require a rich supply of blood and achieve this by stimulating the process of angiogenesis; it may therefore be possible to inhibit tumor growth by targeting the vascular endothelial cells involved in angiogenesis. As evidence, it was shown that depletion of the crk adaptor protein using RNAi inhibited the migration of cultured vascular endothelial cells [142]. In another study, changes in cell shape, reduction in clonogenicity, and an increased susceptibility to transforming growth factor (TGF)b1/vitamin D3-induced differentiation were noted when AML1/MTG8 expression was inhibited using siRNA. Elucidating the role of AML1/MTG8 in preventing differentiation is an example of how targeting fusion genes that result in oncoproteins has become a novel and exciting approach towards killing tumor cells [143].

Further applications of RNAi in the field of tumor therapy have targeted the commonly mutated Ras oncogene after phase I/II studies with antisense DNA oligonucleotides have failed. Ras genes are frequently mutated in human cancers, making it a difficult oncogene to target [144,145]. Brummelkamp et al. report success inhibiting K-RAS (Kirsten-rat sarcoma 2 viral oncogene) expression in human pancreatic carcinoma cells using an RNAi retroviral system which led to the loss of anchorage-independent growth and tumorigenicity [145]. Furthermore, selective inhibition of mutant K-RAS and not wild-type exemplifies the specificity of RNAi and its potential in gene therapy. Complementary to studies on Ras, recent RNAi-related studies on the
tumor-suppressor gene promyelocytic leukemia (PML) gene have revealed no regulatory relationship between PML and major histocompatibility (MHC) class I expression. By inhibiting PML, the proposed downregulation of MHC class I was not seen suggesting an alternative method employed by acute promyelocytic leukemia cells to evade the host immune system [146]. Viral oncoproteins are another potential target for RNAi-mediated tumor therapy. Selectively inhibiting cells that are infected with cancer-causing viruses has been shown to be effective through study of human papilloma virus (HPV)-positive human cervical carcinoma cells [147]. It is known that defects in repair of DNA double-strand breaks make cells hypersensitive to ionizing radiation. Protein kinase, DNA-activated, catalytic polypeptide (Prkdc) is a gene that encodes for the catalytic subunit of the DNA-dependent protein kinase DNA-PKcs which is integral to DNA repair. Peng et al. used RNAi to target Prkdc in human fibroblasts and found that radiosensitivity was increased [148]. RNAi is also being used to study transcription factors implicated in aberrant signaling pathways responsible for oncologic disease. An example is the successful RNAi-mediated inhibition of the nuclear transcription factor cyclic adenosine monophosphate response element binding protein, which has been implicated to have a role in leukemogenesis by virtue of its overexpression in the bone marrow of patients with active leukemia [149].

**HUMAN IMMUNODEFICIENCY VIRAL INFECTION**

Human immunodeficiency viral infection, a dreaded disease, requires attention as therapeutic drugs are not available to treat it. HIV was the first infectious agent targeted by RNAi, perhaps because the life cycle and pattern of gene expression of HIV is well understood [150]. HIV messenger RNAs (mRNAs) and the viral genome are known, which can be degraded by RNA interference [151–153]. Novina et al. targeted the HIV gag gene, which is expressed during the later stages of HIV replication and encodes the Gag precursor protein that is proteolytically cleaved into p24 and other polypeptides; p24 forms the core of HIV and functions in uncoating and packaging of viral RNA. When cells transfected with anti-gag siRNA were exposed to HIV, in vitro production of p24 decreased [154]. An important finding was that anti-gag siRNA also inhibited the production of p24 in cells with stably integrated provirus. Despite the success of RNAi-mediated inhibition of HIV-encoded RNAs in cell culture, targeting the virus directly represents a substantial challenge for clinical applications because the high viral mutation rate will lead to mutants that can escape being targeted [154]. Therefore RNAi-mediated downregulation of the cellular cofactors required for HIV infection is an attractive alternative or complementary approach. After transfection of cultured T cells with siRNA against the mRNA for CD4, the principal HIV receptor [155,156], expression of CD4 on the surface of most cells was diminished and HIV production after exposure of the cells to the virus decreased substantially [153]. Coreceptor chemokine receptor 5 (CCR5) is another logical target for RNA interference, as homozygous mutations in the gene for this receptor have no deleterious effects on immune function yet confer a high level of resistance against HIV infection [157,158]. Some researchers have also successfully blocked HIV entry by targeting HIV-1 CCR5 in human peripheral blood T lymphocytes [159]. They used a lentiviral vector to transduce an anti-CCR5 shRNA in human lymphocytes. Downregulation of CCR5 resulted in a modest, but nevertheless significant three- to sevenfold reduction in viral infectivity relative to controls. Inhibition of HIV-1 replication has been successful with siRNA against the HIV-1 tat protein [160]. Other HIV proteins targeted by siRNA include the non-structural protein rev and gag genes. This may have the potential to manipulate stem cells that are virus-resistant for autologous transplantation [161].

**INFECTIOUS DISEASES**

Diseases caused by viruses and bacteria continue to be major causes of death worldwide and are an increasing cause of concern because of the emergence of resistant strains and the potential use of infectious pathogens by terrorists [162,163]. Respiratory syncytial virus and rotavirus are additional targets of RNAi-mediated inhibition with initial success in mammalian cell culture [164]. In children, both viruses are responsible for a significant proportion of acute respiratory infections and gastroenteritis, respectively. The influenza virus has been another recent target of RNAi as siRNA against influenza nucleocapsid and RNA transcriptase leads to inhibition of replication and blockade of other viral RNAs. This suggests the possible role for siRNAs in influenza prophylaxis and therapy [165] to be a very efficient and convenient approach. *Plasmodium falciparum* is an important pathogen in third world countries where it is endemic. Malhotra et al. applied RNAi technology against two
cysteine protease genes falcipain-1 and -2 as a possible approach to treat malaria [166]. Introduction of falciparum dsRNA produced morphologic changes in the food vacuoles of these parasites and also inhibited requisite hemoglobin degradation. These effects have helped investigators create a model for the pathogenesis of malaria. Another exciting application of RNAi has been the targeting of the pathogenic fungus Cryptococcus neoformans. With its propensity to modify exogenous DNA and high frequency of non-homologous recombination, gene replacement has been a challenge in C. neoformans. RNAi has proven to be a valuable tool for probing function of gene products potentially involved in the pathogenicity of this fungus. Using the capsular gene 59 (CAP59) and ADE2 (adenylosuccinate synthetase2) genes as initial targets, investigators have shown that RNAi works in fungus and can now apply this technology to study gene function, taking advantage of the rapidly accumulating genome sequence of C. neoformans [167].

HEPATIC DISORDERS

Hepatitis induced by hepatitis B virus (HBV) and hepatitis C virus (HCV) is a major health problem. In vivo application of siRNA against genes of HBV also led to an effective inhibition of virus replication [168]. Subgenomic and full-length HCV replicons that replicate and express HCV proteins in stably transfected human hepatoma-derived Huh-7 cells have been used to study the effects of various antiviral drugs [169–172]. siRNAs targeting the internal ribosome entry site or mRNAs encoding the viral non-structural proteins NS3 and NS5B inhibited HCV replicon function in cell culture [173]. Furthermore, anti-HCV siRNAs depleted Huh-7 cells of persistently replicating HCV replicons [174]. McCaffrey et al. performed hydrodynamic tail-vein injections of siRNAs or anti-HCV shRNAs to direct efficient cleavage of HCV sequences in an HCV-luciferase fusion construct in mouse hepatocytes in vivo [168]. Using this method, the silencing of either Fas receptor [66] or caspase-8 [175] resulted in a clearly measurable protection from severe Fas-induced liver damage. Anti-Fas siRNAs were hydrodynamically injected into the antibody-treated mice: 82% of the mice survived for 10 days of observation whereas all control mice died within 3 days [67].

MISCELLANEOUS

Endothelial di-hydrofolate reductase (DHFR) critically regulates endothelial tetrahydrobiopterin (H4B) and nitric oxide bioavailability under physiological and pathological conditions. By using angiotensin II as a model system, it has been found that a rapid and transient activation of endothelial NAD(P)H oxidases precedes H2O2-dependent downregulation of DHFR and eNOS uncoupling in the endothelium. This signaling cascade may represent a universal mechanism whereby eNOS uncouples under conditions associated with oxidant stress, such as atherosclerosis, hypertension, and diabetes, for which activation of vascular NAD(P)H oxidases has been documented. Specific inhibition of endothelial DHFR through RNA interference (RNAi) led to a marked reduction in endothelial H4B and NO bioavailability [176]. Evidence has confirmed that Fas-induced apoptosis can enlarge infarct size during reperfusion of ischemic tissue in multiple tissues such as the heart [177], kidney and brain. Hamar et al. found that siRNAs targeting Fas could inhibit Fas expression in the murine kidney in vivo and protect mice from postischemic acute renal failure [178].

CONCLUSIONS

Small interfering RNA has emerged as a novel target in the last decade and is still in its infancy. However, the developments in this area in the last decade have been remarkable and have revolutionized the basic research into gene functioning. Ongoing research worldwide has provided valuable clues regarding the precise mechanism of siRNA and therefore, siRNA have multi-faceted roles to play in the management of various disorders. Currently, there are many diseases like myocarditis, neurodegenerative diseases, cancer, HIV, etc. which are incurable. Conventional drugs provide only symptomatic relief as they are not able to modulate the cellular aberrations. With the advancements in understanding the altered cellular mechanism as well as the gene functioning in various pathological states, targeting these cellular components appears to be more promising. It may serve to be a breakthrough alternative molecular strategy for the treatment. Moreover, various approaches are being designed for the delivery of siRNAs including chemical modification, and viral- and non-viral-mediated delivery. Furthermore, apparently, the initial cost for development of this treatment option may seem to be high, but once developed may reduce the overall cost of disease therapy as well as the cost for the remission. This will serve to reduce the cost of morbidity and mortality on the society as a whole.
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