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Future of RNAi in Medicine: A Review

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Abstract: The discovery of 21-23 nucleotide RNA duplexes, called small interference RNA (siRNA) may well be one of the transforming events in biology in the past decade. RNAi can result in gene silencing or even in the expulsion of sequences from the genome. Efforts to understand its mode of action have revealed a central role in gene regulation and host defense. The specificity, efficiency and potency of RNAi make it an attractive tool for analyzing the function of genes. RNA interference can be exploited artificially to inhibit the expression of any gene of interest. RNA interference systems could be used clinically to suppress gene expression as a therapeutic strategy in many diseases characterized by elevated gene function. Finally, as a therapeutic tool, it has shown enormous promise in the control of a large array of diseases. This review focuses on the potential therapeutic use of RNAi for various diseases, the current understanding of RNAi biology and how RNAi has been utilized to study the role of different genes in the pathogenesis of cancer, HIV, infectious diseases, HBV, cardiovascular and cerebral diseases, neurogenerative diseases, malaria, etc.

Key words: RNA interference · cancer · HIV · hepatitis B · neurogenerative diseases · malaria · infectious diseases

INTRODUCTION

Reverse genetics is the most effective way to assess the function of a gene, but so far there has been no general method for reverse genetics other than gene targeting, which is slow and costly. Antisense approaches, such as antisense oligonucleotide and Ribozyme technologies, have also been useful in reverse genetics, but only a limited degree. By contrast, the promise of small interfering RNA (siRNA) technology to 'knock down' the expression of any gene in vertebrate cells is set to revolutionize reverse genetic approaches. In 1998 and at the time of the completion of the *Caenorhabditis elegans* genome project andrew Fire and Craig Mello described a new technology that was based on the silencing of specific genes by double stranded RNA (dsRNA); a technology they called RNA interference (RNAi).

The discovery of 21-23 nucleotide RNA duplexes, called small interference RNA (siRNA) may well be one of the transforming events in biology in the past decade. Production of small interfering RNAs (siRNAs) that bind to and induce the degradation of specific endogenous mRNAs is now recognized as a mechanism that is widely employed by eukaryotic cells to inhibit protein production at a posttranscriptional. RNAi can result in gene silencing or even in the expulsion of sequences from the genome. In animal's micro RNA are transcribed as long primary transcripts (pri-iRNAs) by RNA polymerase II enzyme (Fig. 1). They are cropped into hairpin-shaped pre-iRNAs by the nuclear RNase III Drosha-DGCR8 complex. The processing intermediates, pre-iRNAs are exported out of the nucleus by exportin-5 (Exp-5) and subsequently cleaved by the cytoplasmic RNase III Dicer into ~22 nucleotides duplexes [1, 2]. Human dicer preferentially cleaves the dsRNA at their termini without a requirement of ATP [3]. One strand of the duplex (the mature micro RNA) is incorporated into the RISC (RNA -induced Silencing Complex) or iRNP complex, a protein complex that, when bound to the micro RNA is responsible for the post-transcriptional regulation of the corresponding protein [1, 4]. The RISC-iRNA combination can mediate down regulate of the target mRNA activate by two modes of action. 1) Translation inhibition, in case where micro RNA only partially complements it corresponding mRNA and

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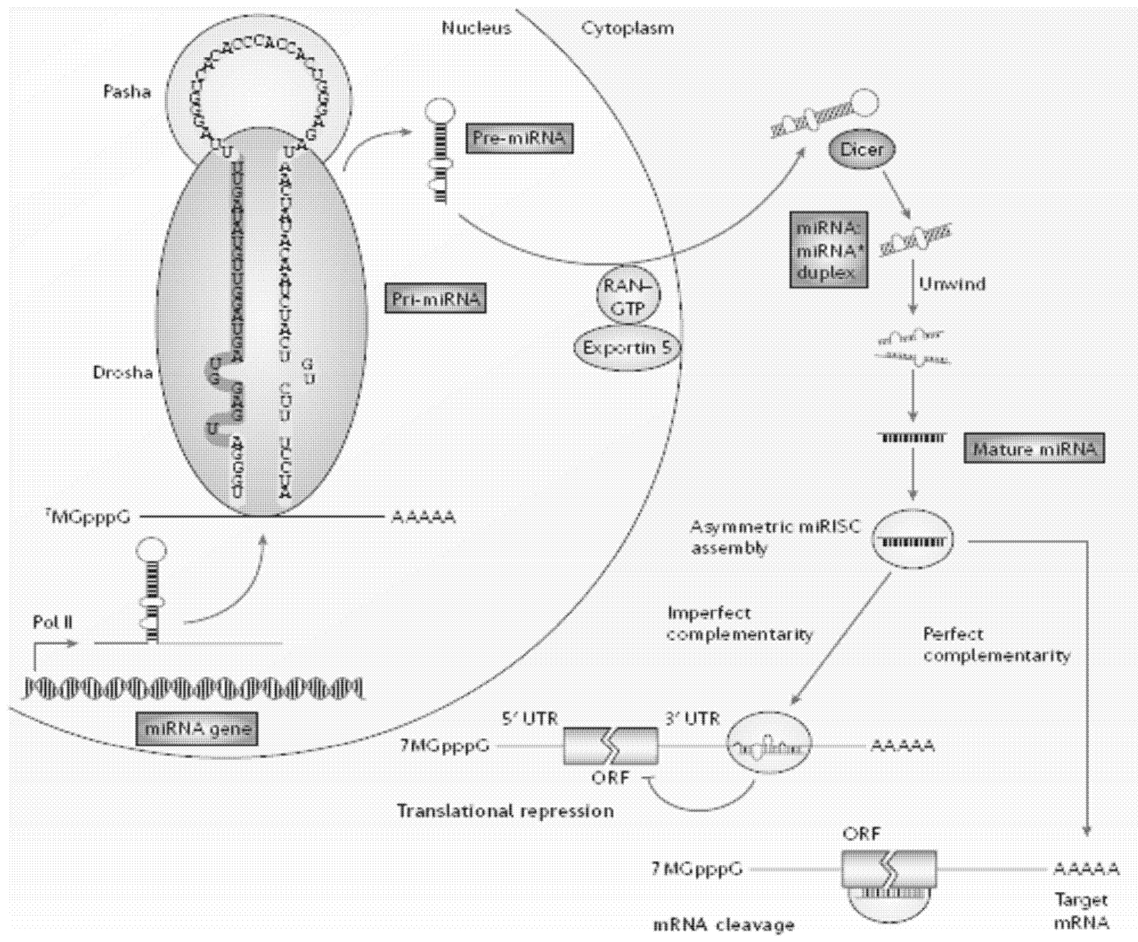

Fig. 1: The biogenesis of microRNAs. MicroRNA (miRNA) genes are generally transcribed by RNA Polymerase II (Pol II) in the nucleus to form large pri-miRNA transcripts, which are capped (7MGpppG) and polyadenylated (AAAAA). These pri-miRNA transcripts are processed by the RNase III enzyme Drosha and its co-factor, Pasha, to release the ~70-nucleotide pre-miRNA precursor product. (Note that the human let-7a-1 miRNA is shown here as an example of a pre-miRNA hairpin sequence. The mature miRNA sequence is shown in red.) RAN–GTP and exportin 5 transport the pre-miRNA into the cytoplasm. Subsequently, another RNase III enzyme, Dicer, processes the pre-miRNA to generate a transient ~22-nucleotide miRNA:miRNA* duplex. This duplex is then loaded into the miRNA-associated multiprotein RNA-induced silencing complex (miRISC) (light blue), which includes the Argonaute proteins, and the mature single-stranded miRNA (red) is preferentially retained in this complex. The mature miRNA then binds to complementary sites in the mRNA target to negatively regulate gene expression in one of two ways that depend on the degree of complementarity between the miRNA and its target. miRNAs that bind to mRNA targets with imperfect complementarity block target gene expression at the level of protein translation (lower left). However, recent evidence indicates that miRNAs might also affect mRNA stability (not shown). Complementary sites for miRNAs using this mechanism are generally found in the 3' untranslated regions (3' UTRs) of the target mRNA genes. miRNAs that bind to their mRNA targets with perfect (or nearly perfect) complementarity induce target-mRNA cleavage (lower right). miRNAs using this mechanism bind to miRNA complementary sites that are generally found in the coding sequence or open reading frame (ORF) of the mRNA target

2) Target mRNA cleavage, in case where there is a near perfect complement between the iRNA and the mRNA.

Salient features of RNAi:

- Double stranded RNA rather than single-stranded antisense RNA is the interfering agent.
- High degree of specific gene silencing with less effort.
- Highly potent and effective.
- Silencing can be introduced in different developmental stages.
- Systemic silencing.
- Avoids problems with abnormalities caused by a knocked out gene in early stages
- Silencing effects passed through generations.

THERAPEUTIC APPLICATIONS OF RNA INTERFERENCE

RNAi was described by the journal *Science* as the "Break through of the Year" in 2002 having the potential to become a powerful therapeutic drug. RNAi-based therapeutics has potentially significant advantages over traditional approaches to treating diseases, including broad applicability, therapeutic precision and selectivity avoiding side effects. This widespread applicability, coupled with relative ease of synthesis and low cost of production make siRNAs an attractive new class of small-molecule drugs. RNAi-based drugs are designed to destroy the target RNA and therefore stop the associated undesirable protein production required for disease progression. Finally, as a therapeutic tool, it has shown enormous promise in the control of a large array of diseases. This review focuses on the potential therapeutic use of RNAi for various diseases, the current understanding of RNAi biology and how RNAi has been utilized to study the role of different genes in the pathogenesis of cancer, HIV, infectious diseases, HBV, cardiovascular and cerebral vascular diseases, neurogenerative diseases, malaria, diabetes and obesity.

We will focus on seven different types of diseases that are very common and for which RNAi approaches are currently being tested in preclinical studies.

Cancer: In cancer cells, proto-oncogenes have frequently been activated by various mechanisms, producing oncogenes that act in a dominant fashion. In epithelial tumors, point mutations are predominant whereas hematological malignancies often show gene fusions that result from chromosomal translocations [5]. There are two general abnormalities in cancer cells- they exhibit dysregulation of the cell cycle resulting in uncontrolled growth and they are resistant to death as a result of abnormalities in one or more proteins that mediate apoptosis [6]. The goals for RNAi approaches for cancer therapy are therefore to knock out the expression of a cell cycle gene and/or an anti-apoptotic gene in the cancer cells thereby stopping tumor growth and killing the cancer cells. To selectively eliminate cancer cells without damaging normal cells, the RNAi would be targeted to a gene specifically involved in the growth or survival of the cancer cell, or the siRNAs would be selectively delivered into the cancer cells. Interference RNA can function as tumor suppressor and oncogenes and they are therefore referred as "Oncomirs" [7]. Recent evidence has shown that miRNA mutations or mis-expression correlate with various human cancers and indicates that miRNAs can function as tumour suppressors and oncogenes. miRNAs have been shown to repress the expression of important cancer-related genes and might prove useful in the diagnosis and treatment of cancer [7-10]. miRNA expression correlates with various cancers and these genes are thought to function as both tumour suppressors and oncogenes (Fig. 2). A recent study showed that about 50% of annotated human miRNAs are located in areas of the genome, known as fragile sites that are associated with cancer. This indicates that miRNAs might have a crucial function in cancer progression.

The differential expression of certain miRNAs in various tumours might become a powerful tool to aid in the diagnosis and treatment of cancer. Northern-blot analyses and miRNA microarrays have been useful in determining tissue-specific 'signatures' of miRNA genes in humans [7, 11-23]. Researchers are now using miRNA-expression signatures to classify cancers and to define miRNA markers that might predict favorable prognosis [24-30]. Gene therapies that use miRNAs might be an effective approach to blocking tumour progression. miRNAs such as let-7, which has been shown to negatively regulate the Ras oncogenes and miR-15 and miR-16, which negatively regulate BCL2, are promising candidates for cancer treatment.

Infectious diseases: Diseases caused by viruses and bacteria continue to be major causes of death worldwide and are an increasing concern because of the emergence of resistant strains and the potential use of infectious pathogens by terrorists. The HIV and other prominent

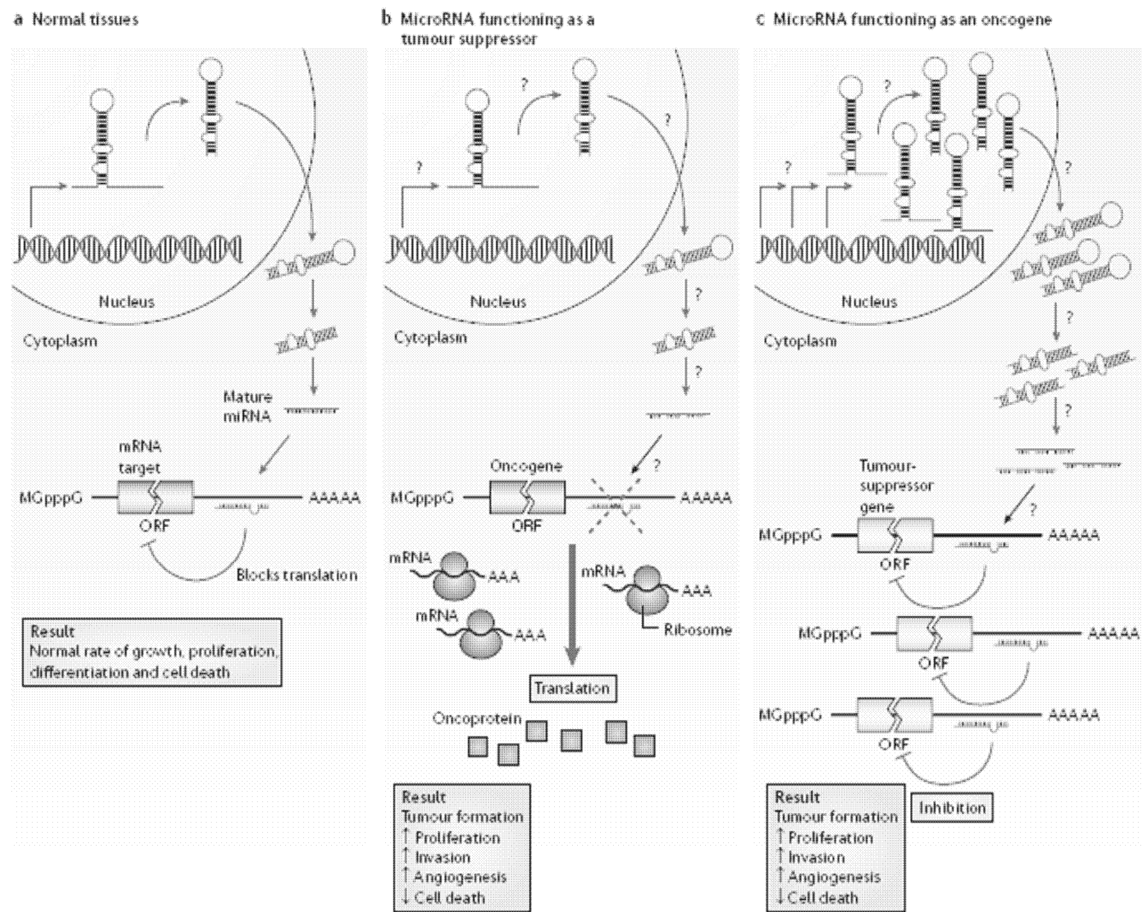

Fig. 2: MicroRNAs can function as tumour suppressors and oncogenes. a: In normal tissues, proper microRNA (miRNA) transcription, processing and binding to complementary sequences on the target mRNA results in the repression of target-gene expression through a block in protein translation or altered mRNA stability (not shown). The overall result is normal rates of cellular growth, proliferation, differentiation and cell death. b | The reduction or deletion of a miRNA that functions as a tumour suppressor leads to tumour formation. A reduction in or elimination of mature miRNA levels can occur because of defects at any stage of miRNA biogenesis (indicated by question marks) and ultimately leads to the inappropriate expression of the miRNA-target oncoprotein (purple squares). The overall outcome might involve increased proliferation, invasiveness or angiogenesis, decreased levels of apoptosis, or undifferentiated or de-differentiated tissue, ultimately leading to tumour formation. c | The amplification or overexpression of a miRNA that has an oncogenic role would also result in tumour formation. In this situation, increased amounts of a miRNA, which might be produced at inappropriate times or in the wrong tissues, would eliminate the expression of a miRNA-target tumour-suppressor gene (pink) and lead to cancer progression. Increased levels of mature miRNA might occur because of amplification of the miRNA gene, a constitutively active promoter, increased efficiency in miRNA processing or increased stability of the miRNA (indicated by question marks). ORF, open reading frame

infectious diseases include Influenza, Hepatitis and West Nile virus. Many deaths also result from bacterial infections, with pneumonia and sepsis being prominent examples. The ability of RNAi to inhibit the replication or cellular uptake of viruses and other infectious agents has been clearly demonstrated in cell culture studies and therefore, holds promise for the treatment of human patients. Transfection of human cells with siRNAs directed against different genes in the poliovirus genome resulted in resistance of the cells to infection with poliovirus [31]. The ability of siRNAs targeting the gene encoding the death receptor Fas to protect mice from liver failure and fibrosis in two models of autoimmune hepatitis was tested by Song and colleagues [32]. Intravenous injection of Fas siRNA specifically reduced Fas protein levels in the livers of mice during a 10-day period. Fas siRNA treatment abrogated hepatocyte necrosis and inflammatory infiltration and markedly reduced serum concentrations of transaminases demonstrating a clear hepatoprotective effect of the siRNA therapy.

RNAi as a treatment for HIV: HIV was the first infectious agent targeted by RNAi, perhaps because the lifecycle and pattern of gene expression of HIV is well understood. Synthetic siRNAs and expressed shRNAs have been used to target several early and late HIV-encoded RNAs in cell lines and in primary haematopoietic cells including the TAR element [33], tat [34, 35], rev [1, 34], gag [36, 37], env [37], vif [33], nef [33] and reverse transcriptase [35]. 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 [38]. Therefore RNAi-mediated downregulation of the cellular cofactors required for HIV infection is an attractive alternative or complementary approach. Cellular cofactors such as NF-B [35], the HIV receptor CD4 [36] and the co-receptors CXCR4 and CCR5 [39] have been successfully downregulated by RNAi, resulting in the inhibition of HIV replication in numerous human cell lines and in primary cells including T lymphocytes and haematopoietic -stem-cell-derived macrophages [1, 33, 34, 36, 39-42]. There are drawbacks in targeting cellular HIV cofactors because non-infected cells will inevitably be targeted as well, leading to toxicities that are similar to those observed with the current anti-retroviral drugs. Viral targets will need to be included in any successful strategy using RNAi. These targets should be sequences that are highly conserved throughout the various clades to ensure efficacy against all viral strains.

An approach to relying solely upon RNAi as an anti-HIV approach is mixing a single shRNA with other antiviral genes to provide a potent combinatorial approach. This has been successfully accomplished by co-expressing an anti-tat/rev shRNA, a nucleolar-localizing TAR decoy and an anti-CCR5 ribozyme in a single vector backbone [43]. An example of how HIV-1 can be targeted by this approach is shown in Fig. 3. A somewhat different combination used a shRNA with a dominant negative Rev M10 protein in a co-expression system [44]. Perhaps other, more potent combinations of iRNAs with mixtures of noniRNA antivirals will be developed in the near future for testing in preclinical settings.

The delivery of iRNAs to HIV-infected cells is also a challenge. The target cells are primarily T lymphocytes, monocytes and macrophages. As synthetic siRNAs do not persist for long periods in cells, they would have to be delivered repeatedly for years to effectively treat the infection. Systemic delivery of siRNAs to T lymphocytes is probably not feasible owing to the immense number of these cells. Using viral vectors to deliver anti-HIV-encoding short hairpin RNA (shRNA) genes is also problematic and systemic delivery is not yet practicable because the immunogenicity of the vectors themselves precludes performing multiple injections. Therefore the preferred method is to isolate T cells from patients; these T cells are then transduced, expanded and re-infused into the same patients. In a continuing clinical trial, T lymphocytes from HIV-infected individuals are transduced *ex vivo* with a lentiviral vector that encodes an anti-HIV antisense RNA. The transduced cells are subsequently expanded and reinfused into patients [46]. This type of therapeutic approach would also be applicable to vectors harbouring genes that encode siRNAs. A different approach is to transduce isolated haematopoietic progenitor or stem cells with vectors harbouring the therapeutic genes. These cells give rise to all the haematopoietic cells capable of being infected by the virus. Haematopoietic stem cells are mobilized from the patient and transduced *ex vivo* before reinfusion (Fig. 4). Two clinical trials in which retroviral vectors expressing ribozymes were transduced into haematopoietic stem cells have demonstrated the feasibility of this approach [41, 45]. Because RNAi is more potent than ribozyme or antisense approaches,

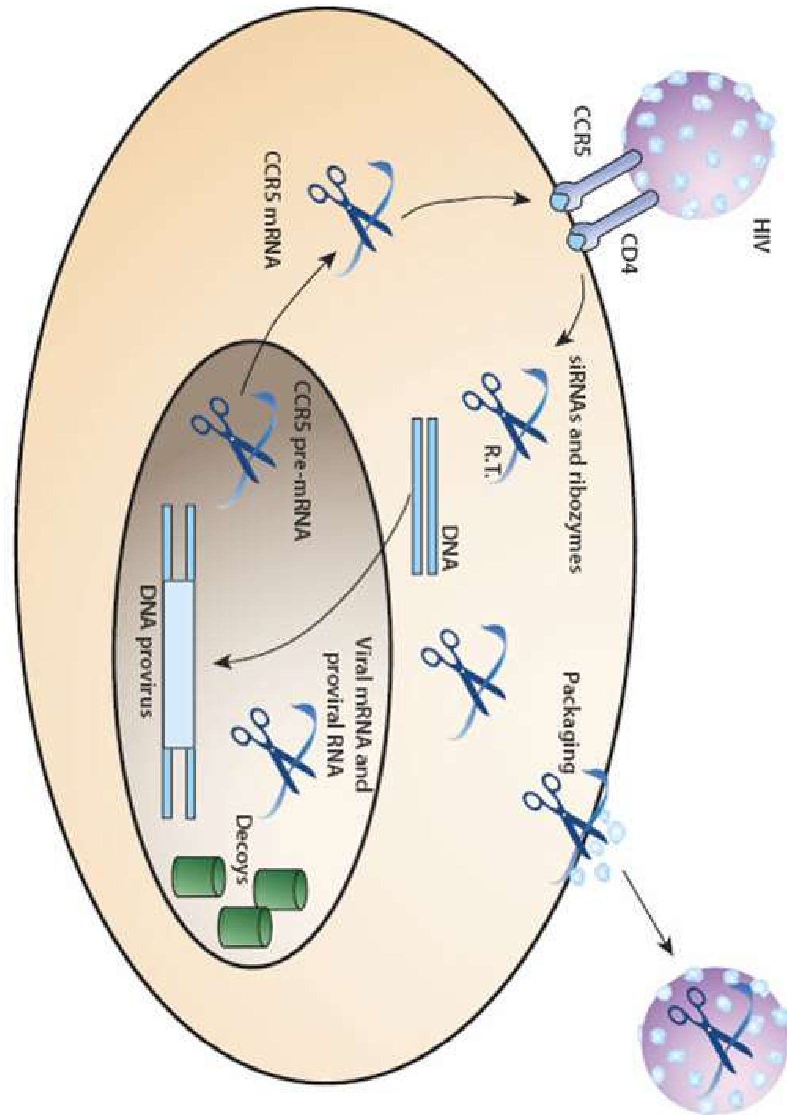

Fig. 3: Proposed multiplexing RNA interference (RNAi) with ribozymes and decoys for the treatment of human immunodeficiency virus (HIV) infection. HIV-1 binds to the CD4 receptor and CCR5 co-receptor, which triggers entry and uncoating. The proviral RNA is reverse transcribed into DNA, which integrates randomly in the host chromosomes. Different stages of the HIV-1 replicative cycle can be attacked using RNAi and combinations of other RNA-based inhibitors. The scissors represent either small interfering RNAs (siRNAs) or ribozymes, and the decoy for binding viral Tat or Rev is depicted as a barrel in the nucleus. The virus may be exposed to RNAi or ribozymes at the preintegration step blocking proviral DNA formation and integration. Postintegration, siRNAs targeting all classes of HIV transcripts can be used. The CCR5 co-receptor RNA is targeted by either an siRNA or a ribozyme in this depiction. mRNA, messenger RNA

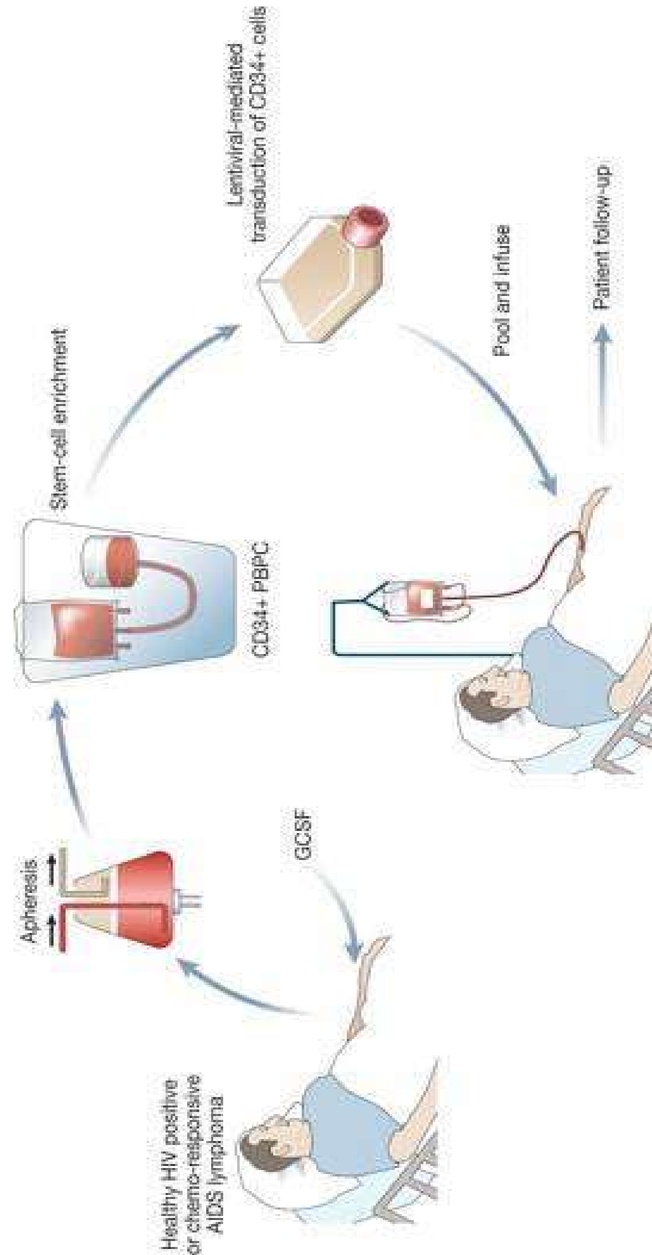

Fig. 4: Patients are given several injections of granulocyte colony stimulation factor (GCSF), which mobilizes haematopoietic stem cells into the patients' peripheral circulation. Haematopoietic stem cells expressing the CD34 antigen are collected by affinity columns (apheresis) and transduced with a lentiviral vector harbouring the anti-HIV shRNA genes. The cells are then re-infused into patients. Depending on the population, the patient will have been pretreated with no, or with one, or with more than one marrow-chemoablative agent. Following stem-cell engraftment, patients are monitored for a period of several years for HIV loads, CD4+ T-lymphocytes and shRNA gene expression. This overall scheme follows that described by Michienzi *et al*. **0000**. PBPC, peripheral blood progenitor cells

movement of this technology to a human clinical trial for HIV treatment is expected to take place in the next year or two.

HBV (Hepatitis-B virus) as an RNAi target: Although a vaccine is available for hepatitis A and B virus, treatment options for chronically infected patients are limited and particularly ineffective in case of hepatitis C virus (HCV) infection. A promising new avenue currently being explored is to harness the power of RNA interference for development of an antiviral therapy. The timing to pursue this particular approach is excellent, with the first *in vivo* animal models for HCV infection becoming available and the technology for liver-specific expression of short hairpin RNAs advancing at a rapid pace. HBV makes extensive use of overlapping reading frames within its DNA genome, suggesting that while the viral DNA itself cannot be targeted; the multiple HBV RNAs will make the virus highly susceptible for RNAi. HBV is in fact an excellent candidate for therapeutic RNAi, as its unusually compact genome with lack of redundancy results in very limited sequence plasticity and prevents the virus from evading RNAi by mutation. Thus, ideally, a single iRNA can potentially target multiple viral transcripts simultaneously and efficiently inhibit not only viral gene expression, but also DNA replication, because HBV amplifies through RNA intermediate [47].

Most noteworthy findings were obtained by McCaffrey *et al.* [48], Shlomai and Shaul [49] and Uprichard *et al*. [50] who independently assessed anti-HBV shRNAs in different *in vivo* models and found high efficiencies of their constructs, albeit for only relatively short periods (up to 26 days) [50]. An important consistent finding in many studies was that inhibition of viral gene expression did not require active viral replication, suggesting that RNAi strategies are excellent options as adjutants to conventional anti-HBV therapies, for example with inhibitors of the viral reverse transcriptase.

Cardiovascular and cerebral vascular diseases: Cardiovascular disease is the leading cause of death in the United States and many other industrialized countries. It most commonly results from the progressive occlusion of arteries in a process called Atherosclerosis, which can ultimately culminate in a myocardial infarction or stroke. Atherosclerosis involves damage to vascular endothelial cells, local production of inflammatory cytokines and the recruitment of macrophages to the site forming foam cells; in addition, apoptosis of foam cells and vascular smooth muscle cells occurs [51]. The severe irritability that occurs in heart or brain cells during a myocardial infarction or stroke results in the death of cardiac muscle cells or neurons. Although some of the cells die rapidly by necrosis, many other cells die more slowly by apoptosis; data from animal studies suggest that such cardiac myocytes and brain neurons that die by apoptosis can be saved [52, 53]. It may be possible to use RNAi technology to intervene in the process of atherosclerosis or to reduce the damage to heart tissue and brain cells that patients suffer following a myocardial infarction or stroke. A key step in the process of atherosclerosis is the up-regulation of cell adhesion molecules in vascular endothelial cells, which play an essential role in the recruitment of macrophages to the site of endothelial damage. The production of cell adhesion molecules can be selectively suppressed in cultured cells [54].

Neurodegenerative disorders: Recent rapid progress in the application of RNAi to mammalian cells, including neurons and muscle cells, offers new approaches to drug target identification and validation. Advances in targeted delivery of RNAi-inducing molecules has raised the possibility of using RNAi directly as a therapy for a variety of human genetic and other neural and neuromuscular disorders. RNA interference (RNAi) is a powerful new gene knockdown technique that permits tissue-specific, temporally controlled suppression of gene expression. Alzheimer’s disease, Parkinson’s disease, Huntington’s disease and Amyotrophic lateral sclerosis (ALS) are examples of relatively common age-related neurodegenerative disorders that are increasing as average life expectancy increases. Each disorder is characterized by the dysfunction and death of specific populations of neurons: Hippocampal and cortical neurons involved in learning and memory processes in Alzheimer’s disease, dopamine-producing neurons in the substantia nigra that control body movements in Parkinson’s disease and spinal cord motor neurons in amyotrophic lateral sclerosis. Specific genetic mutations are responsible for a small percentage of cases of Alzheimer’s and Parkinson’s disease and Amyotrophic lateral sclerosis [55], whereas all cases of Huntington’s disease result from mutations (polyglutamine expansions) in the Huntington protein [56]. Studies of patients and of animal and cell culture models of each disease, have revealed shared biochemical cascades that result in neuronal death. Those cascades include increased oxidative stress, dysregulation of cellular calcium

homeostasis and apoptosis [52]. There have therefore been two different strategies for preventative and therapeutic interventions in neurodegenerative disorders. One strategy is to block the disease-specific events that are believed to initiate the neurodegenerative process, whereas the second strategy targets downstream events in the neurodegenerative cascade. Recent studies have shown that cultured neurons can be efficiently transfected with siRNAs and that the targeted genes are effectively silenced. In one study it was shown that cultured neurons can be depleted of the p75 neurotrophin receptor, a protein in the TNF receptor family that has been implicated in neuronal apoptosis in certain settings [57]. Pro-apoptotic members of the Bcl-2 family [58] and caspases [60] have been effectively targeted and neuronal death prevented, using RNAi methods. Caplen and colleagues [61] performed studies aimed at determining whether RNAi could be used to target the pathogenic process in inherited neurodegenerative disorders caused by polyglutamine expansions. Thus, now it is possible, at least in cell culture, to selectively silence a transcript associated with an important group of genetic diseases by RNAi.

Antiproliferative/Proapoptotic miRNAs: Interference RNA with antiproliferative and proapoptotic activity are likely to function as tumour suppressor genes and thus may be under expressed in cancer cells. The family of let-7 miRNAs represents a clear example of this. The let-7 family consists of a group of highly conserved miRNAs in multiple species including *C. elegans*, Drosophila and vertebrates. let-60/RAS was recently identified as a target of let-7 in *C. elegans* [62]. The regulation appears to be conserved in humans where three RAS genes, known to be potent oncogenes, have also been demonstrated to be directly regulated by human let-7. Since RAS dysregulation is a key oncogenic event in lung cancer, loss of let-7 may contribute to pathogenesis in this disorder. Indeed, let-7 is generally expressed at low levels in cancerous lung tissue compared to normal tissue and low expression of this miRNA correlates with shorter postoperative lung cancer survival [62, 63]. miR-15a and miR-16-1 represent additional miRNAs with likely tumour suppressing activity. The miRNAs are located on human chromosome 13 in a region frequently deleted in B-CLL. Cimmino *et al*. [26] identified a conserved target site for miR-15a and miR-16-1 in the 3' UTR of BCL2, a potent inhibitor of cell death. Consistent with a regulatory interaction between these miRNAs and this gene, the levels of miR-15a and miR-16-1 are inversely correlated with BCL2 protein levels in samples from CLL patients. Furthermore, over expression of miR-15a and miR-16-1 in a leukaemic cell line results in decreased Bcl2 protein expression, activation of the intrinsic apoptosis pathway and ultimately cell death.

Malaria: Despite intense efforts, malaria remains a leading cause of morbidity and mortality worldwide. The World Health Organization estimates that nearly half a billion clinical cases of malaria occur each year, with over one million deaths [79, 80]. Recent evidence strongly suggests that RNAi can play a key role in identifying the genetic factors shape the vector parasite relationship may be crucial to identifying new genetic means of controlling mosquito-borne diseases [64, 65]. RNAi can and indeed has been made inheritable in *Anopheles* mosquitoes by stably transforming the mosquito with a transgene that contains two copies of the target gene arranged in an inverted repeat configuration [65]. Hairpin RNA is expressed *in vivo* whenever the inverted repeat is transcribed from an upstream promoter. By placing dsRNA expression under the control of a tissue- and time-specific promoter, dsRNA expression can be tailored to coincide spatially and temporally with the journey of the parasite through the mosquito. Both parasite receptors and immune components protective of the parasite are putative targets for engineering parasite resistance through RNAi and, in principle, mosquito strains that have been rendered refractory to malaria transmission could be released in the field to replace wild-type, permissive populations and achieve malaria eradication. Only few publications to date have all described characteristic RNAi-like silencing phenomena [43, 66-69]. In the sole *in vivo* study published to date, injection of *P. berghei* - infected mice with siRNAs targeting the *P. berghei* cysteine protease berghepain resulted in its specific down-regulation [69]. Injections resulted in only approximately 0.01% of the siRNA being internalized into the parasite and the observed 40-50% reduction in berghepain mRNA levels did not alter the parasitemia of the siRNA-treated mice. More studies will be needed to elucidate the mechanisms of gene silencing observed in *Plasmodium* and to assess the therapeutic potential of RNAi and urgent need to develop new therapeutics for malaria control warrants a thorough assessment of the possible application of novel technologies like this.

Metabolic diseases: RNAi has arrived as a novel and essential biological process, as well as a powerful experimental tool and a potential therapeutic strategy.

RNAi can be used to silence endogenous genes involved in the cause or pathway of metabolic diseases and holds considerable promise as a therapeutic approach to silence disease-causing genes, particularly those that encode so-called "non-drugable" targets. In addition, the high potency, specificity and chemical structure of siRNAs may eliminate the toxicity and adverse events commonly seen with small molecule and other oligonucleotide approaches. Several studies have demonstrated efficient *in vivo* delivery of siRNAs and therapeutic benefit in mice. One group developed an adenovirus-mediated RNAi technique that utilizes shRNAs to substantially and stably knock down insulin receptor substrates; IRS-1 and IRS-2, expression specifically in the livers of mice to better understand the roles of these proteins in hepatic insulin action [70]. By knocking down IRS-1 and IRS-2 separately and together in liver, they showed that IRS-1 signaling may be more closely linked to the regulation of genes involved in glucose homeostasis, whereas IRS-2 signaling may have specific roles in the regulation of hepatic lipid metabolism. Vector-based RNAi approach was used to induce posttranscriptional gene silencing of hepatic PEPCK using nonviral gene delivery. PEPCK is the rate-controlling enzyme in gluconeogenesis and altered rates of gluconeogenesis are responsible for increased hepatic glucose output and sustained hyperglycemia [71]. Treatment of diabetic mice with PEPCK siRNA caused a 50% decrease in hepatic PEPCK protein content and was sufficient to lower blood glucose and to improve glucose tolerance. These data reinforce the significance of PEPCK in sustaining diabetes-induced hyperglycemia and validate liver-specific intervention at the level of PEPCK for diabetes gene therapy. RNAi holds promise for the development of novel therapeutic strategies for disorders that are yet difficult to treat and might be beneficial for the treatment of diseases, such as obesity, neuropathic pain and depression. After the in vitro success in down-regulating gene expression in neurons, chemically synthesized siRNAs were tested for their *in vivo* gene knockdown ability in the brain [72].

Sirna Therapeutics, Inc., an RNAi therapeutics company, has selected a systemically delivered, chemically modified siRNA compound as its candidate for advancement to human clinical testing against the hepatitis C virus [73]. The selection of this clinical candidate reflects two major advancements. The first advancement is the design, chemical modification and synthesis of a stable and potent siRNA compound and the second advancement is the development of a nanoparticle delivery technology capable of efficient and specific delivery of the siRNA compound to hepatocytes. This delivery technology enables siRNA Therapeutics to address other liver-associated disease indications such as diabetes. This group also claimed that they had promising results for the application of RNAi technology in the treatment of diabetes using systemically delivered siRNA to reduce phosphatase 1B (PTP-1B) in liver, a validated target in diabetes associated with insulin resistance [74].

The future of RNA interference in biology and medicine: Even at this early stage of understanding the molecular mechanisms of RNAi and in the development of methods for the use of RNAi technology for selective gene silencing, it is clear that RNAi will be a widely used tool for establishing the functions of genes. Improvements on the currently available protocols for RNAi are being made and the methods are being applied by thousands of investigators in diverse fields. With the advent of these methods has come an explosion of studies that have employed RNAi. The current status of RNAi as an experimental tool is such that many investigators are now aware of the technology, but most have not yet implemented it in their own studies. The development of RNAi kits by several companies will facilitate the implementation of RNAi methods by essentially any investigator, regardless of their knowledge of RNAi mechanisms.

As for uses of RNAi in medicine, its potential remains to be established. The application of gene therapy approaches for the treatment of specific diseases has progressed much more slowly than initially anticipate. There are, of course, many potential gene targets for therapeutic intervention using RNAi. Studies that employ RNAi to counteract a disease process *in vivo* are emerging. For example, RNAi that targeted the Fas gene [32] or the Hepatitis C virus genome [75] protected mice from hepatitis. One might expect that the next demonstrations of successful treatment of disease in mice will come from models of cancer and neurodegenerative disorders. Ongoing and future preclinical studies in animal models will hopefully help optimize RNAi therapeutics for applications in humans. Although development of RNAi-based therapeutics for diabetes is in its infancy, early clinical studies are soon to begin assessing the use of this new class of therapeutics to tackle metabolic diseases and other diseases. Because of the potential of RNAi for therapeutic intervention, major efforts should be placed on preclinical studies using this technology.

Challenges for RNAi as a therapy: Two key challenges in developing RNAi as a therapy are avoiding off-target effects and ensuring efficient delivery. One potential risk for side effects emerges from the feature that distinguishes RNAi from other antisense technologies - the use of cellular machinery for directing sequence-specific silencing. Using iRNAs to target specific cellular or viral transcripts in essence hijacks the endogenous RNAi machinery and we know little about the potential for saturating the RNAi pathway in primary cells, although saturation of RISC is demonstrable in cultured cells [76]. So, endogenous RNAi pathways could potentially be affected by siRNAs. It will be important to pay close attention to basic research studies on off-target effects of siRNAs and on the design of effective iRNAs [77, 78]. A better understanding of the mechanisms that lead to nonspecific effects of short dsRNAs is essential before the use of siRNAs or shRNAs can be tested in patient trials.

The issue of delivery has restricted the antisense field for almost two decades. Targeted delivery to specific cell or tissue types is still not a practical reality for oligonucleotide-based therapeutics. The alternative approach is viral-vector-mediated delivery of therapeutic shRNA genes. Because this type of delivery results in gene therapy, there are several associated safety concerns and systemic delivery of viral vectors is still a major hurdle. Nevertheless, the potency and potential general therapeutic utility of RNAi is prompting renewed vigour into delivery-related research. It remains to be determined whether backbone-modified, nuclease-resistant siRNAs will move to the clinic more quickly than synthetic deoxyoligonucleotides.

CONCLUSIONS

The ability to target a specific gene or genes using siRNAs or vector-mediated RNA expression methods, suggests the potential of RNAi to block the disease process or relieve symptoms of the disease. Depletion of proteins critical for the cell cycle, such as cyclins, cyclin-dependent kinases, or telomerase, might be effective in treating cancers and some neurodegenerative disorders. Blocking the production of anti-apoptotic proteins such as Bcl-2, inhibitor of apoptosis proteins and antioxidant enzymes may be used to kill cancer cells. Conversely, RNAi-mediated suppression of expression of apoptotic proteins (Bax, Par-4, p53, AIF and caspases, for example) may slow or stop the degenerative processes in degenerative myocardial, neurological and autoimmune disorders. Ligands, receptors and downstream signal transduction proteins critical for a specific disease process might also be targeted. Genes that encode proteins involved in oxidative stress and inflammation might be targeted in autoimmune and infectious or inflammatory diseases. Viral and bacterial genes are obvious targets for RNAi-based therapeutic intervention in infectious diseases.

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