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Can siRNA Technology Provide the Tools for Gene Therapy of the Future?

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Abstract: A new era in genetics has started 15 years ago, when co-suppression in petunia has been discovered. Later, co-suppression was identified as RNA interference (RNAi) in many plant and lower eukaryote animals. Although an ancient antiviral host defense mechanism in plants, the physiologic role of RNAi in mammals is still not completely understood. RNAi is directed by short interfering RNAs (siRNAs), one subtype of short double stranded RNAs. In this review we summarize the history and mechanisms of RNAi. We also aim to highlight the correlation between structure and efficacy of siRNAs. Delivery is the most important obstacle for siRNA based gene therapy. Viral and nonviral deliveries are discussed. *In vivo* delivery is the next obstacle to clinical trials with siRNAs. Although hydrodynamic treatment is effective in animals, it cannot be used in human therapy. One possibility is organ selective catheterization. The known side effects of synthesized siRNAs are also discussed. Although there are many problems to face in this new field of gene therapy, successful *in vitro* and *in vivo* experiments raise hope for treating human disease with siRNA.

Keywords: RNA interference (RNAi), short interfering RNA (siRNA), delivery, human gene therapy.

INTRODUCTION

The possibility of gene therapy has grown into one of the most interesting issues today. On the fiftiethanniversary of the discovery of the double helix structure of DNA, Watson declared to Time magazine that fifty years ago it had been the most exciting project to reveal the structure of the genetic material [1]. Since, gene therapy has emerged from science-fiction to a realistic choice of clinical therapy. Although the first clinical trials were banned because of serious adverse events: two of ten patients developed T-cell leukemia after retroviral gene therapy aimed to treat X-linked severe combined immune deficiency (X-SCID) [2]. Nevertheless, the Food and Drug Administration keeps on file more than 169 clinical gene therapy trials in different phases [3]. RNA interference (RNAi), which is carried out by the use of small interfering RNAs (siRNAs), has emerged as one of the most promising fields of gene therapy [4]. The possibility of using siRNAs might range from infectious and autoimmune diseases to malignancies and genetic disorders [5].

HISTORY AND PHYSIOLOGICAL FUNCTION OF GENE SILENCING WITH RNA INTERFERENCE

RNA interference is a type of gene silencing first described in plants [6] and in invertebrates [7]. In plants, RNAi protects the genome from viruses and insertable elements, but its role in the antiviral response of mammalian cells is still not completely understood.

RNA interference was first described in petunia [8]. Jorgensen and colleagues showed that externally administered molecules are capable of changing the expression of host's genes. This process was named as "cosuppression". Later on, Mello *et al.* used antisense RNAs (asRNAs) in the worm *Caenorhabditis elegans* which was the first animal model of gene silencing [9]. They found that introducing long dsRNA into C. elegans led to the targeted degradation of homologous messenger RNA (mRNA). On the other hand, administering the sense and antisense RNA strands of dsRNA together led to as much as ten times more effective silencing than using one strand alone. This process was termed Post-Transcriptional Gene Silencing (PTGS). After all, PTGS was thought to be related to cosuppression that was already observed in plants, funghi and Drosophila [10]. After recognition of RNAi in lower eukaryotes, attention of biomedical research has been drawn to RNAi by the discovery of its occurrence in mammalian cells. Tuschl and colleagues showed that RNAi could be induced by siRNAs in mammalian culture cells [11]. Until today, siRNAs have already been successfully used for gene silencing in numerous animal models, such as Trypanosoma [12], nematodes [13], Drosophila [14], hydra [15], honey bee [16], zebrafish [17] mouse [18,19] and rat [20].

The *in vivo* function of RNA interference is the host's protection from viruses and foreign genes, as double stranded RNAs (dsRNAs) are often produced during the life cycle of viruses [21]. Recognition and cleavage of foreign RNA sequences results in inhibition of viral infection. Although RNAi is an ancient antiviral defense in plants, it has not yet been ascertained that this kind of PTGS mechanism plays a role in the natural antiviral defense of mammalian cells.

Besides gene silencing, RNAi might be involved in other phenomena of gene regulation. It appears that RNAi can also have a function in methylating cytosine as well as CpG sequences. If the target sequence shares homology with a promoter, transcriptional silencing may occur *via* methylation [22].

BIOCHEMICAL MECHANISMS OF RNAi

Long dsRNAs are processed into siRNAs by an enzyme called Dicer, which belongs to the RNase III family of

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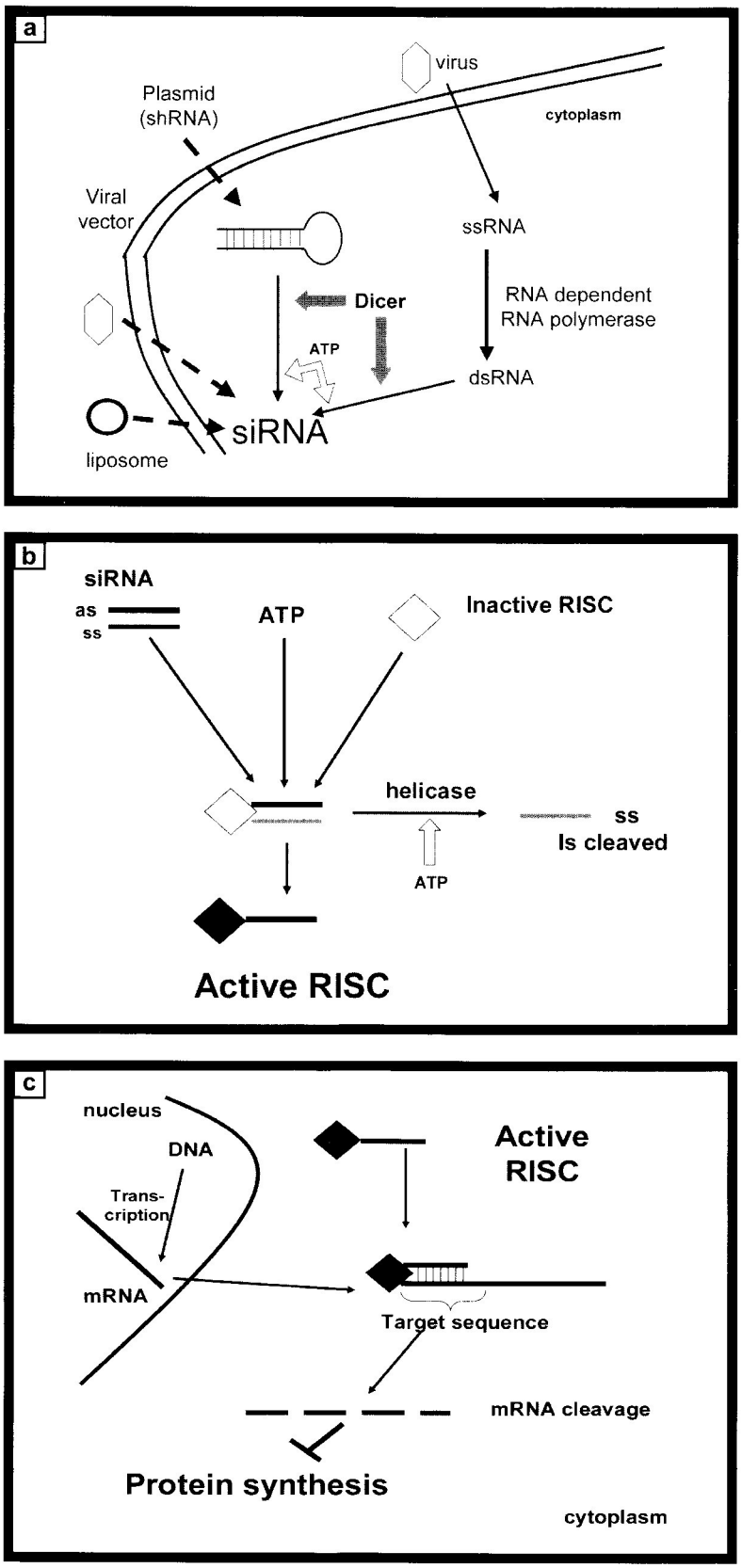

Fig. (1). (**a**) External and internal routes that may account for the presence of inhibitory siRNA inside the cell: (arrows on the right: physiologic role of RNAi, dashed arrows (left): direct introduction of siRNA into cells with therapeutic aims). (**1b**) Activation of RISC complex: (as = antisense strand, ss = sense strand, ATP = adenosine triphosphate, RISC = RNA Induced Silencing Complex). (**1c**) The silencing mechanism: RNAi can also be initiated by introducing chemically synthesized siRNAs into cells. These molecules are 19-21 base pair-RNAs that are homologous to a target sequence.

ribonucleases [23]. siRNAs have a free 5'-phosphate end followed by 19 base pairs (bp) of paired RNA with two nucleotide unpaired overhang at the 3' end [24]. siRNAs are recognized and incorporated into a multisubunit ribonucleoprotein complex called RNA Induced Silencing Complex (RISC). siRNAs are unwinded in RISC into sense and antisense strands. The antisense strand directs target mRNA recognition and cleavage [25]. The activated RISC contains the antisense RNA strand, a protein homologous to the product of the *Argonaute* gene family (protein products of which are RNA-binding proteins involved in RNAi) in plants (*rde1* in *C. elegans*, AGO2 (Slicer) in Drosophila and in mammals, GERp95 in human cells) and other uncharacterized factors including an endonuclease responsible for cleaving the target RNA [26]. Due to the cleavage of mRNAs, the whole process of transcription and translation is interrupted. In other words, protein synthesis is inhibited without any effect on the genome (Fig. (**1**)).

ATP Requirements

The RNAi mechanism can be divided into 4 consecutive biochemical steps:

- processing dsRNA into siRNAs,
- incorporation of siRNA into the inactive RISC,
- unwinding the siRNA duplex,
- recognition and cleavage of the RNA target

ATP is needed for dsRNA cleavage into siRNA, siRNA unwinding and the 5'-phosphorylation of siRNA. The 5'-phosphate of the siRNA may serve as a key point from which the cleavage site is measured. 5'-Phosphorylation of synthetic siRNA duplexes in the *Drosophila* embryo lysate is catalyzed by an endogenous kinase to maintain the 5'-phosphate end *in vivo* [27].

The Duration of RNAi

RNAi-mediated silencing is incomplete, which means that the result is a "knockdown" and not "knockout". Transfection of siRNAs into rapidly dividing cells has a maximal effect 2-3 days posttransfection, with silencing lasting up to maximal one week. The loss of effective silencing is presumably due to dilution of siRNA with each cell division. In end-stage differentiated cells, such as macrophages, *in vivo* RNAi effects persist for several days after a single dose delivery [28].

STRUCTURE AND EFFECTIVENESS

Up to now, several aspects of siRNA effectiveness have been described. It has been demonstrated already, that phosphorylation of the 5'-hydroxyl terminus of the antisense strand controls targeted RNA cleavage [29]. If the 5'-hydroxyl group can not be phosphorylated, the RNAi activity is abolished. This can be seen when the 5'-end is blocked by chemical modifications [30]. The 5' end is therefore more sensitive to mismatches with the target RNA. On the other hand, two nucleotide (TT or UU) overhangs on the 3'-end also seem to have a crucial role: it is responsible for target recognition and binding [31]. Up to 4 deoxynucleotide modifications at the 3'-end were found to support RNAi activity.

Structure of Highly Effective siRNAs

Tei and colleagues have demonstrated that highly effective siRNAs must have a special structure. These specifications are:

- Adenine (A)/Uracil (U) at the 5' antisense strand end
- Guanine (G)/Cytosine (C) at the 5' sense strand end
- At least 5 A/U residues in the 5' terminal one-third of the antisense strand
- The absence of any G/C stretch of >9 nt in length [32].

Moreover, there are some other observations in connection with the position of possible mismatches and RNAi activity. Although a single mismatch in the middle of the siRNA duplex is able to prevent target RNA cleavage, more changes are tolerated in the 3' end [33]. The centre of the siRNA molecule also has a crucial role in effectivity. This has been demonstrated with the use of scrambled siRNAs. Scrambled siRNAs are used as negative controls for siRNA experiments with the same length and nucleotide composition as the investigated siRNA, with at least 4-5 mismatches so they have no homology to any gene in the organism of interest. Thus, scrambled siRNA do not affecte protein synthesis [34].

siRNAs *vs* Oligonucleotides

The great advantage of siRNAs over oligonucleotides is that siRNAs are much more stable than naked oligonucleotide sequences. The better bioavailability is due to their resistance to nucleases in the plasma. Activity of siRNAs and oligonucleotides in mice was observed by Bertrand *et al.* but antisense oligonucleotides could not be measured after administering *in vivo*. The absence of efficiency of antisense oligonucleotides is due to their low resistance to nuclease degradation [35]. With the aid of chemical modifications [36] the loss and degradation of siRNAs can be further reduced.

CHEMICAL MODIFICATIONS

To increase the possibility of clinical application, improvements in the stability of the siRNA against serum nucleases should be made to strengthen the in-vivo effect of synthetic siRNA [36]. Two chemical modifications are now being intensively evaluated [37]:

1. phosphorothioate modification and
2. modifications to the 2'-carbon of the ribose, such as 2'-O-methylation and 2'-O-allylation.

Internal 2'-*O*-methyl modification on ribonucleotides at all positions is a commonly used modification to increase stability of RNA-containing molecules [36]. siRNAs with internal 2'-O-methylation showed extreme resistance against degradation in the serum. Since extensive modification of

the backbone may abrogate the silencing activity of siRNA, it is essential to balance the beneficial effect of chemical modification on stability and the reduction of RNAi activity. To overcome this problem, partial modifications have to be made with up to six nucleotides in the form of either thiolation or methylation [33]. Alternating modifications on every second nucleotide significantly enhances resistance of siRNA against serum-derived nucleases without loss of RNAi activities in case of avoidance of modifications at the 5'-hydroxyl group of the antisense strand. In addition, 2-fluoro pyrimidines [38] and locked nucleic acids [39] (LNA) have been described to enhance stability and efficiency over unmodified siRNA.

Besides these modifications aiming to increase resistance to degradation, special modifications may be used to target tissues specifically. In the liver, for instance, different modification strategies help the uptake of oligonucleotides by different subpopulations of liver cells. Intravenous administration of phosphorotioate antisense oligonucleotides resulted in accumulation of the reagent in both Kupffer and endothelial cells. Scavenger receptors are responsible for the uptake of nucleotides [40]. Therefore, coupling cholesterol, cationic lipids to oligonucleotides enhanced its bioavailability and antisense activity in the liver [41]. Another possibility is to entrap oligonucleotides into liposomes, which are captured by cells of the hepatic reticuloendothelial system [42]. Although being able to complex and deliver nucleic acids into cells, polycation-containing vectors (such as polyethyleneimine (PEI), or poly-L-lysin (PLL)) turned out to be too stable to release mRNA molecules to enable efficient translation. A study has recently demonstrated efficient siRNA delivery using linear PEI (JetPEI) in a mouse tumor model [43].

DELIVERY STRATEGIES

Delivery remains a major obstacle for RNAi based therapy, because siRNAs do not cross the mammalian cell membrane unaided and most of the transfection methods used for *in vitro* studies cannot be used *in vivo*. Furthermore, due to rapid renal clearance, the *in vivo* half-life of siRNAs is extremely short (about seconds to minutes). One solution is to express siRNA precursors from viral vectors, the other is to deliver synthetic siRNAs by complexing or linking with delivery lipids or proteins. Coupling siRNAs to basic peptides has been proven to enhance the transport of siRNAs across the cell membrane [44]. Cell-specific delivery by linking siRNAs to cell surface receptor ligands or antibodies could reduce potential toxicity both in viral and non-viral vector systems [45].

Innumerable viral and non-viral expression systems make us possible to deliver siRNAs into cells, such as adenoviral, adeno-associated viral, oncoretroviral, lentiviral vectors, liposomes, nanoparticles or peptide-lipid transfection protocols.

VIRAL DELIVERY STRATEGIES

In recent viral RNAi-mediated gene therapy trials several virus vectors have been used, the most common of which (adenoviral, adeno-associated, retroviral and lentiviral vectors) are reviewed in this paragraph.

Adenoviral vectors (AdVs) are often used in gene therapy experiments, even in clinical trials. AdVs have many advantages;

- they are easy to produce in high titers,
- have relatively large size of insert (AdVs can transduce cells *in vivo* with size of transgene more than 30 kb),
- have wide spectrum of cell tropism,
- they do not integrate into the host cell genome,
- yield high levels of gene transfer,
- AdVs have low pathogenicity in humans [46].

Following systemic administration, adenoviruses accumulate in the liver within minutes due to the fenestration of the sinusoidal wall in the liver. Type 5 AdVs (Ad5), for instance, are efficiently taken up by both hepatocytes and Kupffer cells [47].

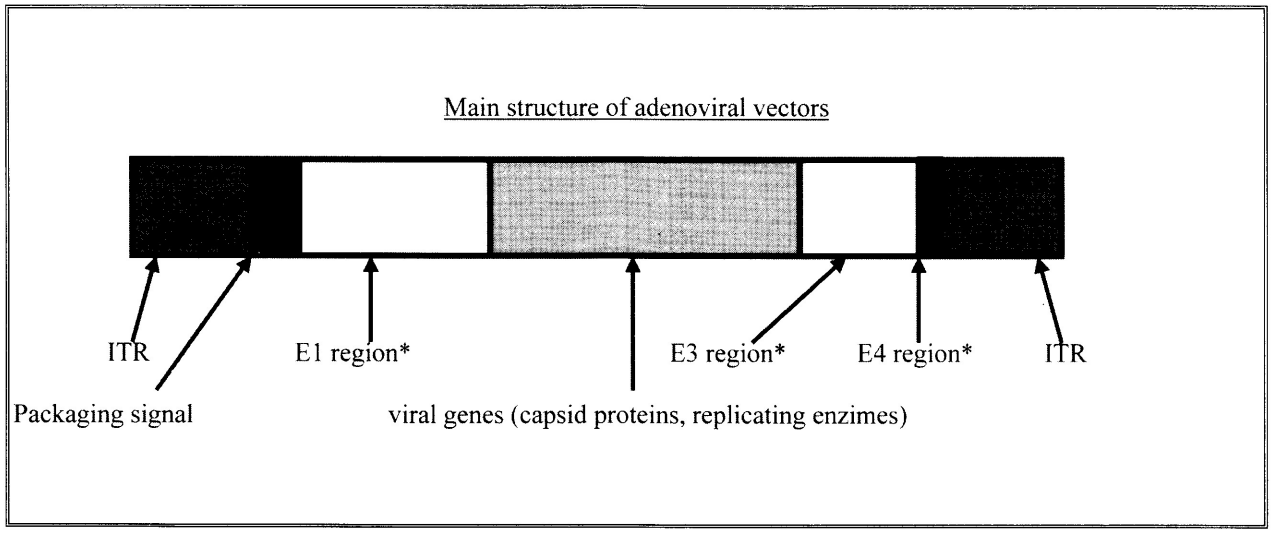

Fig. (2). Structure of adenoviral vectors: (ITR= inverted terminal repeat, * = delectable and/or insertable regions).

The main drawback of the use of AdVs is the inflammatory reaction induced by viral proteins expressed by the vectors. These viral proteins stimulate the innate-, immune system shortly after application. Furthermore, 2-7 days after application vector-encoded MHC-presented proteins trigger the adaptive immune response, usually after the production of antibodies against viral capsid antigenes by host cells [48].

Another disadvantage of AdVs is the shortness of transgene expression (from a few days to some weeks[1] which can be prolonged by special promoters (for example, by the use of U6 promoter) [49].

Recombinant adenoviral vectors can be constructed by replacing viral genes with therapeutic sequences into the E1, E2 and E3 regions or between the E4 region and the end of the viral genome (Fig. (**2**)).

One of the most commonly applied adenoviral vectors is type 5 adenoviruses (Ad5). The first and second generation of adenoviral vectors, and the so-called high-capacity or "gutless" vectors are derived from Ad5. While in the first generation vectors the E1 region is deleted, in the second-generation vectors the E2 and/ or the E4 are also deleted besides E1. First-generation vectors can have an additional deletion in the E3 region, which is responsible for viral replication in cell culture. High-capacity (HC) or "gutless" Ad vectors are devoid of all coding viral genes, but contain the Inverted Terminal Repeats (ITRs) and the packaging signal [50]. Lack of viral genes has been shown to reduce toxicity and immunogenicity *in vivo* [51]. Replication-competent (RC) Ad vectors have deletions in E1A and E1B regions, protein products (E1A, E1B) of which normally bind and inactivate Rb and p53, thereby activating the cell cycle. Thus, RC-Ad vectors could be used in p53 or Rb-negative tumors [52].

For effective adenoviral transfection, presence of the coxsackie-and adenovirus-receptor (CAR) is necessary. The "Knob" region of the Ad capsid fibers is responsible for virus attachment to CAR, which triggers the cellular uptake of AdVs by clathrin-dependent receptor-mediated endocytosis [53,54].

Adeno-Associated Virus Vectors

Members of the *Dependovirus* genus, the adeno-associated viruses (AAV) need a so-called helper virus such as an adenovirus to replicate [55]. AAV2 virions bind to heparin sulphate proteoglycans, and internalization is aided by the co-receptors $\alpha v\beta 5$ integrin heterodimers [56]-both are ligands for which receptors can be engineered to manipulate vector uptake of the host cell.

Once being in the host cell nucleus, AAV can have two ways of lifecycle: the lytic and the lysogenic pathway. The former develops in cells infected with the helper virus, while in the case of the lysogenic pathway, there is no need for helper virus and AAV genome integrates into the long arm of chromosome 19 and remains there in a latent form [57].

[1]Parpala-Sparman, T. Surgical organ perfusion method for somatic gene transfer. An experimental study on gene transfer into the kidney, spleen, lung and mammary gland, Academic dissertation, Faculty of Medicine, University of Oulu, 2000.

Due to this integration into the host genome, transgene expression from AAV vectors may last as long as 18 months (see later). This longevity of expression can be harnessed in RNAi if long-term expression of shRNAs is necessary.

AAV vectors have the advantages of both the adenoviral and retroviral vectors, which are;

- transferring genes to different cell types, even dividing and non-dividing cells [58],
- broad host range,
- low level of immune response,
- longevity of gene expression (in retinal pigmental epithelial cells (RPE) RPE65 protein expression was detected up to 18 months after injection of rAAV *RPE65, [59], while a major limitation of adeno*-associated viral vectors is the low efficiency of host cell infection [60].

Clinical trials using AAV vectors are in phase I and phase II. Parkinson's disease [61], haemophilia B [62], cystic fibrosis [63], and $\alpha 1$-antitrypsin [64] deficiency are some diseases that have already been treated with AAV vectors.

Retroviral vector mediated gene transfer is attractive to biomedical research, as Retroviruses have several advantages over other viruses. The retroviral virion is about 80-100nm in diameter enclosed into a lipid-bilayer derived from the host cell plasma membrane [65]. One of the most important advantages of retroviruses is that retroviruses have the ability to transform their single stranded RNA genome into the host genome. This stable integration into the target cell genome results in a permanent gene transfer up to 8-9 kb of foreign DNA. Retroviral vectors can also be used to express short hairpin RNA (shRNA) under the control of an RNA polymerase III (Pol III) promoter, such as U6 or H1 (see later).

Replication-defective retroviruses lack all viral genes and require packaging cell lines or a helper virus for virus production. A great advantage of replication-defective retroviral vectors is the bigger size of the transgene and milder response of the immune system. One of the main drawbacks of retroviral gene therapy trials is insertional mutagenesis. Integration of a retroviral genome into actively transcribed genes and/ or protooncogenes may lead to malignancies, as in two out of ten patients, after treating X-linked Severe Combined Immune Deficiency (X-SCID) with retroviral-based gene therapy [66]. The potency of retroviruses to revert to replication-competent retrovirus (RCR) means the possibility of occurrence of neoplasms; and oncogene activation due to random retroviral integration into the host genome (insertional mutagenesis) [67].

Cell division is necessary for many types of viruses for effective infection, thus, non-dividing cells are often difficult to transduce. One subclass of retroviruses, the family *Lentiviridae*, can infect nondividing cells and are able to maintain long-term expression of transgenes. Brain [68], muscle [69], liver cell lines [70], haematopoietic stem cells [71], T cells [72], primary macrophages [73], keratinocytes [76], and neurons [74] have already been successfully transfected with lentiviral vectors. Lentiviral vectors integrate desired genes into quiescent cells because the so-

called viral preintegration complex (a large nucleoprotein complex containing viral cDNA, viral and host cell proteins [75] can be transported through the nuclear membrane [76], thus, once integrated, a lentiviral provirus becomes transmissible-a great opportunity for gene therapy. The ability of stable integration into the host genome makes lentiviral vectors popular in gene therapy experiments. In comparison with adenoviral transgene expression of 6 weeks, lentiviral expression may be expanded up to 6 months.

As far as application of enveloped viral vectors is concerned, viral entrance into target cells can only be achieved by interaction of viral glycoproteins with specific cell surface receptors. This glycoprotein-receptor binding catalyzes the fusion of the viral and the host membrane. Specific alterations of glycoproteins with foreign viral glycoproteins (for instance vesicular stomatitis virus), named as *pseudotyping*, may have a crucial role in transfection efficiency and specificity [77].

Summarizing gene therapy with the aid of viral vectors, all the above mentioned viral vectors have already been harnessed for RNAi. Choosing the proper vector system is the main point in each experiment. Due to the wide spectrum of tissue-specific promoters and viral antigenes directed to specific receptors on the surface of target cells, almost all cell types are able to be transduced by viral vectors. Moreover, viral vectors are easy to produce and to use. If stable transgene expression is needed retroviral and AAV vectors systems should be used. Expression from all the other vectors is transient. One of the major drawbacks of viral vectors is the immune response of the host and the production of virus neutralizing antibodies shortly after the first administration of viral vector, thus preventing the second use of the same serotype of the viral vector [78]. Changes in viral antigenes may help to solve this problem. Pseudotyping and coat modifying changes can be achieved to broaden or narrow host tropism.

NONVIRAL DELIVERY STRATEGIES

As already seen above, in many cases viral vectors cannot be used. In these cases nonviral delivery methods have to be evaluated. There are many strategies to achieve efficient nonviral delivery, both *in vitro* and *in vivo*.

Therapeutic benefit from *in vivo* delivery of siRNAs has been demonstrated in mice. Synthetic siRNAs can be delivered *in vivo* using a modified *"hydrodynamic transfection method"* which is a high-pressure injection method originally developed to deliver antisense oligonucleotides and plasmid DNA. In rodents, the veins of the tail or the penis can be used for hydrodynamic treatment. When siRNAs are rapidly (within seconds) injected intravenously in large volumes (50-100 % of the circulating blood volume of the animal), fluid backs up in the venous system of the vena cava, establishing a venous and capillary pressure that presses siRNAs through the endothelial layer in parenchymal organs with high bloodflow (liver, kidney etc.) [79]. This way, siRNAs were taken up by ~90% of hepatocytes and silenced both Fas mRNA and protein in the liver by ~80-90% [18].

Although effective in mice, hydrodynamic treatment is not applicable in human therapy. Regional delivery of siRNAs in smaller volumes of injection into tissues or catheterization of regional veins may be the right alternative. It has already been proven in many experiments, that siRNAs can be delivered into the central nervous system [80], the subretinal area [81], and the peritoneal cavity [82].

Delivering siRNAs into cells can be achieved by constructing short hairpin RNA (shRNA) expressing ***plasmids***. When successfully transfected, cells generate the coded shRNAs. shRNAs are short RNAs with special structure: sense strand-hairpin (5-9 bp)-and a complementary antisense strand. Following synthesis of the shRNA, the hairpin is cut by Dicer and the result is a siRNA (Fig. (**1**)). As this process happens inside the cells and shRNA constructs (shRNA coding plasmids) are much more stable in biological fluids than siRNAs [83], silencing efficiency is enhanced [84]. The expression of shRNAs in particular tissues or cell types can be achieved by expressing shRNAs in plasmids with tissue-specific or inducible promoters.

POSSIBLE SIDE EFFECTS OF GENE SILENCING WITH siRNAs

Application of siRNAs in high concentrations may lead to the activation of the interferon system or to off target silencing.

Interferon Response

Duplex RNA molecules in the cytoplasm of cells may trigger a profound physiologic reaction. A single molecule of dsRNA (formed during most viral infections) is sufficient to induce the synthesis of interferons (IFNs). Interferons are multifunctional cytokines that modulate host immunological functions and can inhibit tumor cell growth and virus multiplication. Most dsRNAs or virus infections induce type I IFNs, which include IFN-α and IFN-β.

Cytoplasmatic dsRNA activates the dsRNA-activated protein kinase-R (PKR) by binding to it. The binding of dsRNA to PKR leads to autophosphorylation of PKR. Upon activation, 2 pathways are known downstream of PKR.

Along the first pathway, activated PKR phosphorylates IκB, to be released from the nuclear transcription factor NF-κB. The thus activated NF-κB translocates to the nucleus and activates transcription of genes with NF-κB binding sites in their promoter region [85]. The most common activated gene is IFN-β [86].

The second known pathway from the active PKR is the phosphorylation of the α subunit of the translation Elongation Initiation Factor (EIF). Phosphorylation of EIFα leads to the inhibition of translation initiation and by doing so a non-selective blockade of the cell's protein synthesis. As part of the antiviral response, dsRNAs activate the inactive form of 2',5'-OligoAdenylate Synthetase (OAS) that catalyzes the conversion of ATP into long oligoadenylate chains which in turn activate RiboNuclease L (RNaseL). Active RNaseL degrade nonspecific cellular RNAs to suppress viral infection.

A PKR independent way of inducing IFN response is that, the dsRNA can also activate the expression of IFNs directly by activating Interferon Responsible Factor 1 (IRF-

1), which can bind to the Positive Regulatory Element I (PRD I) on the promoters of both IFN-α and IFN-β genes and may thus stimulate their expression [87]. Following synthesis, IFNs are secreted to neighboring cells, where IFNs induce IFN-Stimulated Genes (ISGs) [88]. The activation of ISGs depends upon the presence of a promoter sequence called the IFN-Stimulated Responsive Element (ISRE). The ISG factors (ISGFs) bind to ISRE and activate transcription from genes possessing ISRE in their promoter regions.

In the first years of siRNA experiments it was thought that siRNAs are too small to induce interferon response. Sledz and colleagues discovered that siRNAs designed against different targets activate the interferon response *in vitro* [89]. Fish *et al.* observed that in cells, infected with lentiviral vectors expressing 21 nt long shRNA sequences, the OAS1 gene was induced [90].

Off Target Silencing

Due to perfect base pairing between siRNA and target mRNA, silencing occurs in cells and gene expression is reduced. In some experiments [91, 92] high concentrations of siRNAs have been shown to trigger the so-called off target silencing effect. Off target silencing means siRNA binding to unintended, partially complementary sequences. Scacheri *et al.* studied gene silencing of the tumorsuppressor MEN1 gene. HeLa cells were transfected with 10 various, sequence specific siRNAs. Silencing effect of the target gene and expression of p53 and p21 gene was observed. Of the four most efficient siRNAs of ten targeted against the MEN1 gene, one upregulated p53 and p21, another siRNA downregulated p21 and the two other caused no significant change in p53 or p21 levels relative to mock transfected cells. Titration of siRNAs to 10nM reduced silencing of MEN1 gene, but did not abolish non-specific effects on p53 and p21 [93]. Consequently, off-target effect can be induced even by low concentrations of siRNA. This problem could be overcome by siRNA design and/or testing different siRNA sequences to choose the one with the best target-specific/off-target profile.

Sledz *et al.* observed that long, 500 bp dsRNAs (that are found in Drosophila and nematodes) induce non-specific suppression by activation of PKR (non-specific suppression in PKR deficient cells was at 25% *vs* in wild phenotypic cells 75%) [89].

Jackson and colleagues transfected cultured mammalian cells with 16 different siRNAs targeting Insulin-like Growth Factor 1 receptor (IGF1R) gene and 8 other targeting Mitogen Activated Protein Kinase 14 (MAPK14) gene. A thousand fold decrease of –the siRNA concentration did not inhibit the off-target effect completely. Furthermore, silencing of nontargeted genes containing as few as eleven contiguous nucleotides of siRNA is enough to evoke the off-target effect [94]. This problem may be defeated by careful testing of different sequences in animal models.

THERAPEUTIC USE OF RNAi

Any change in the genetic material is a target for gene silencing. Besides genetic alterations, infections (both acute and chronic), neurodegenerative diseases and malignancies could also be treated with RNAi (see later). Reducing fulminant hepatitis [18], kidney injury following ischaemia-reperfusion [19], inflammation [82] or septic shock caused by bacterial agents [95] can also be inhibited with siRNAs as seen above.

Treating viral infections with the aid of RNAi-based therapy includes hepatitis C virus [96] -induced liver disease, severe acute respiratory syndrome [97], human papilloma virus-related tumorigenesis [103], virus-induced influenza [98], HIV-1 [99, 100] and many others. Efficiency of RNAi has been demonstrated against other intracellular pathogens, such as Mycobacterium [101] and Trypanosoma [102].

RNAi activity can be harnessed to slow or prevent tumor growth by local injection of siRNAs [103], or to overcome the problem of metastases or to prevent the vascularization of cancer [60]. RNAi has been successfully used against malignancies both *in vitro* and *in vivo*. Animal models using si RNAs and shRNAs have been applied in cases of brain [104], breast [105], and ovarian [106] cancer.

Neurodegenerative diseases may also become therapeutic targets of RNAi. Silencing of mutant genes may slow the development of many progressive neurodegenerative diseases, such as Alzheimer's [107] or Huntington's disease [108].

RNAi-based therapy has been growing enormously. Today's question remains the delivery of siRNAs. Future clinical trials may come to treat infections, hereditary, neurodegenerative diseases or cancer with siRNAs.

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