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Review

RNAi - Strategy to Control Viral Infections in Eukaryotic Organisms

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Abstract

Short double-stranded RNA molecules efficiently inhibit gene expression in eukaryotic cells. One class of these short molecules are the small interfering RNAs (siRNAs), which after introduction into cells elicit efficient induction of post-transcriptional gene silencing known as RNA interference (RNAi). They are incorporated into a multimeric protein complex named RNA-induced silencing complex (RISC). While one of the two RNA strands is discarded, the antisense strand guides RISC to the complementary target RNA and induces its endonucleolytic cleavage. RNA silencing by post-transcriptional gene silencing (PTGS) is a mechanism of gene regulation in eukaryotes. It is similar to classical humoral immunity, which protects eukaryotes against viruses and transposons. A major problem for the long term inhibition of viruses is the emergence of escape mutants. This limitation, which is well-known for conventional antiviral therapy with low molecular weight drugs, is applicable to RNAi approaches as well. Virus escape as a consequence of the accumulation of point mutations in or close to the siRNA target site has been observed for various types of viruses. To counter this problem a novel technology of production of dsRNAs is used, targeted to essential gene regions for viral replication. The technology is based on the replication complex of the bacteriophage phi 6. A pool of siRNAs is produced in this way, targeted to specific gene region, which overlaps and silences the target, whether a mutation or recombination appear.

Key words: RNAi, siRNAs, viruses

Резюме

Късите двРНКи ефективно инхибират генната експресия в еукариотните клетки. Един от класовете на тези къси молекули са малките интерфериращи РНКи (миРНКи), които след въвеждане в клетката предизвикват ефективно индуциране на пост-транскрипционно генно мълчание известно като РНК интерференция (RNAi). Те се включват в мултимерен протеинов комплекс наречен комплекс за индуциране на РНК мълчание (RISC). Сенс веригата на миРНК се отстранява, докато антисенс веригата насочва RISC комплекса към комплементарна РНК прицелна секвенция и по този начин индуцира ендонуклеотично срязване на прицелната секвенция. РНК заглушаването чрез пост-транскрипционно генно мълчание (PTGS) е механизъм на генна регулация при еукариотните организми. Процеса е подобен на класическия хуморален имунен отговор, който предпазва еукариотните организми от нашествието на вируси и транспозони. Основният проблем при продължителното инхибиране на вирусите е възникването на резистентни мутанти. Това ограничение, което е добре известно при традиционната антивирусна терапия с нискомолекулни съединения важи също така и за РНК интерференцията. Вирусната резистентност като последствие от натрупването на точкови мутации в прицелната секвенция или в близост до нея е наблюдавана при различни вируси. За разрешаването на проблема е използвана нова технология за получаване на специфични двРНКи, комплементарни на основни за вирусната репликация генетични региони. Технологията се базира на репликационния комплекс на бактериофага phi 6. По този начин се получава пул от миРНКи, насочени към специфичен генетичен регион, които припокриват и заглушават прицелната секвенция, без значение дали се е появила мутация или рекомбинация в нея.

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History of Gene Silencing

RNA gene silencing is a mechanism of gene regulation, that limits the transcript level by either suppressing transcription [transcriptional gene silencing (TGS)] or by activating a sequence-specific RNA degradation process [post transcriptional gene silencing (PTGS)]/RNA interference (RNAi). The evolutionary functions of RNAi and its related processes are designed for the protection of genome against invading mobile elements like viruses and transposons as well as for orchestrated functioning of developmental programs in eukaryotes. Prior to the discovery of RNAi, scientists applied various methods such as insertion of T-DNA elements and transposons, treatment with mutagens or irradiation and antisense RNA suppression to generate loss-of-function mutations. Antisense RNA technology was discovered in plants, when the inhibition of nopaline synthase gene was observed in tobacco cells, due to the expression of its corresponding antisense RNA (Rothstein *et al.,* 1987).

RNA silencing was first observed in plants, where attempts to overexpress endogenous genes by introducing transgenic copies of the endogenous gene, instead, resulted in blocked expression of both. It happened when scientists were trying to increase the purple pigmentation of petunia petals by sense overexpression of chalcone synthase, a gene related to anthocyanine pathway (Napoli *et al.,* 1990; Van der Krol *et al.,* 1990). The Ambrose Laboratory reported the first case of micro RNA (miRNA) in an attempt to silence heterochronic gene *lin*-4 of *C. elegans* (Lee *et al.,* 1993), and the Fire and Mello Laboratories described the gene silencing effect of double-stranded RNA (dsRNA) in *C. elegans* by injecting dsRNA that corresponds to *unc*22, responsible for body morphology. They took the antisense silencing approach a step further in *C. elegans* with simultaneous introduction of both the sense and antisense strands of the targeted mRNA, resulting in a tenfold higher potency in silencing of the targeted mRNA (Fire *et al.,* 1998).

In animals, RNA silencing was first reported when Guo and Kemphues (1995) injected RNA to block *par*-1 mRNA expression in *C. elegans*, but found that the *par*-1 mRNA itself also repressed *par*-1. Their paradoxical observations subsequently named as RNAi - inspired the experiment of Fire *et al*. (1998), who demonstrated that dsRNA was the trigger of gene silencing.

Three phenotypically different but similar by mechanism forms of gene silencing, co-suppression or PTGS in plants (Jorgensen, 2003), quelling in fungi (Cogoni *et al.,* 1996) and RNAi in the animal kingdom (Fire *et al*., 1998) have been described. More recently, miRNA formation (Pasquinelli, 2002; Bartel, 2004), promoter methylation (Matzke *et al.,* 2001; Wesley *et al.,* 2001), heterochromatinization (Schramke and Allshire, 2003), etc., have been revealed as other facets of naturally occurring RNAi processes in eukaryotic cells.

Different Classes of RNAs that Induce Gene Silencing

Ribosomal RNA (rRNA) is the most abundant type of RNAs inside the cell followed by transfer RNAs (tRNAs) and messenger RNAs (mRNAs). There are another four basic classes of RNAs, which take active parts in RNA silencing. These are hairpin RNAs (hpRNAs), double stranded RNAs (dsRNA), small interfering RNAs (siRNAs) and micro RNAs (miRNAs).

Double stranded RNA (dsRNA) is formed by the complementary base pairing of two single-stranded fragments of RNA and takes an active part in RNA silencing pathway (Agrawal *et al.,* 2003). The dsRNA found naturally in the cell and is derived generally from the replacement of transposons (Schramke and Allshire, 2003) or virus induction.

Small interfering RNA (siRNA), also known as short interfering RNA or silencing RNA, is a type of 20 to 25 nucleotide-long double-stranded RNA molecules with a 3' two nucleotide overhang, that take a variety of parts in the cell. This is formed from the long dsRNA by the cutting activity of a special enzyme called Dicer. The short interfering RNA is involved mainly in RNA interfering pathway, where it is involved in disrupting the function of a gene by interfering with the RNA expressed by that gene. siRNA was first discovered as a part of post-transcriptional gene silencing (PTGS) in plants by the group of David Baulcombe in Norwich, England (Hamilton and Baulcombe, 1999). Not much later in 2001, synthetic siRNAs have been reported to be involved in RNAi initiation in human cell line (Elbashir *et al.,* 2001). This discovery has been revolutionizing the field of gene-function analysis. Short interfering RNAs are believed to have an important role in viral resistance and in preventing transposons transposition (Lippman *et al.,* 2003).

Hairpin RNA (hpRNA) is another form of dsRNA. It is deduced from a long piece of single stranded RNA containing inverted repeat and connected by a hairpin (Wesley *et al.,* 2001). This long

piece of single stranded RNA is produced by a vector introduced in the cell. A constitutive promoter, U6 in animal cells and cauliflower mosaic virus 35S promoter (CaMV35S) in plants, is used to ensure the continuous expression of hpRNA in cell. Hairpin RNA is transcribed by RNA polymerase III in animal cell and by RNA polymerase II in plants miRNAs. These are small non-coding single stranded molecules of about 21-23 nucleotides in length, that negatively regulate gene expression, which were first discovered in the nematode *C. elegans*, while screening the genes that control developmental timing (Lee *et al.,* 1993), and hundreds of miRNAs have been identified in plants and animals, including the hundreds unique miRNA from *Arabidopsis* alone (Lee and Ambros, 2001; Llave *et al.,* 2002; Reinhart *et al.,* 2002; Millar and Waterhouse, 2005). Micro RNAs are formed from the precursor single-stranded RNA transcripts that have the ability to fold back onto themselves to produce imperfectly double-stranded stem loop precursor structures. The main function of miRNA is gene regulation (Grosshans and Slack, 2002).

Piwi-interacting RNA **(piRNA)** represents the largest class of small non-coding RNA molecules expressed in animal cells, deriving from a large variety of sources, including repetitive DNA and transposons (Klattenhoff *et al*., 2008). piRNAs appear to act both at post-transcriptional and chromatin levels. They are distinct from miRNA due to at least an increase in terms of size and complexity. Repeat associated small interfering RNA (**rasiRNAs**) are considered to be a subspecies of piRNA (Gunawardane *et al*., 2007).

Mechanism of Gene Silencing

Mechanism of PTGS is a two-step model for RNAi. The first step, referred to as the RNAi initiating step, involves binding of the RNA nucleases (Dicers) to a large dsRNA and its cleavage into discrete ~21 to ~25nt RNA fragments (siRNAs). In the second step, these siRNAs join a multinuclease complex (RISC), which degrades the homologous single stranded mRNAs. The first class of RNA taking an active role in RNAi is dsRNA, which is formed by complementary base pairing of two single-stranded fragments of RNA (Agrawal *et al.,* 2003). Long dsRNAs generally derived from such events as transposition of transposable elements (Schramke and Allshire, 2004) or virus induction (Rovere *et al.,* 2002), with which the PTGS process is initiated. The dsRNA alone cannot degrade mRNA, but requires the assistance of two enzymes - namely Dicer and RISC. Dicer, which was first discovered by Bernstein *et al.* (2001) in *Drosophila*, is a complex enzyme belonging to the RNase III family. It has four different domains with different functions. They are: (a) N-terminal helicase, (b) dual RNase III motifs, (c) C-terminal dsRNA binding domain, (d) PAZ (Piwi/Argonaute/Zwille) domain (Kuznetsov, 2003; Arenz and Schepers, 2003). The PAZ domain is believed to physically interact with the corresponding PAZ domain of the RISC complex. The dual RNase III motifs perform the actual cleavage of the dsRNA, hence the characteristic 5' phosphate and 3' hydroxyl residues on the resulting siRNAs. ARGONAUTE1 (AGO1) mediates the cleavage of miRNA-targeted mRNAs and maintenance of chromatin structure (Vaucheret, 2006). Experiments involving human Dicer showed that the cleavage mechanism of the enzyme is ATP independent (Kuznetsov, 2003). The helicase domain is also believed to take part in the process. The Dicer protein functions in two different pathways in silencing a gene by recognizing distinct types of precursor dsRNA. In the first pathway, Dicer cleaves long and perfect dsRNA structures that originated mainly from the protein-coding region to generate double stranded siRNAs, which guide the subsequent endonucleolytic cleavage of homologous RNAs with perfect base pairing interaction (Elbashir *et al.,* 2001). In the second pathway, Dicer can dice imperfect RNA duplexes predominantly derived from the regions between protein coding genes into short RNAs, which are subsequently recruited into a micro RNA ribonucleoprotein complex (miRNP) to further regulate translational inhibition or other PTGS effects (Hutvagner *et al.,* 2001; Voinnet, 2002). Accordingly, Dicer processes precursors dsRNAs to generate both siRNAs and miRNAs. Four types of Dicers involved in small interfering RNA biogenesis have been reported in *Arabidopsis* (Xie *et al*., 2004 and 2005), they are DCL1 (miRNA), DCL2 (viral RNA), DCL-3 (endogenous siRNA), and DCL-4 (exogenous siRNA). RISC is the component of the RNAi machinery that uses siRNAs to track down and degrade the target mRNAs. First discovered in *Drosophila* by Hammond *et al.* (2000), RISC consists of both protein and RNA. The protein component of the complex has ribonuclease activity with the ability to cleave RNA. In addition to ribonuclease activity, RISC also contains a PAZ domain involved in regulation of RNA interference (RNAi). Additional RISC components include two RNA binding proteins, intronic and dFMR protein (Arenz and Schepers, 2003).

The DCR-2/R2D2 complex also facilitates incorporation of siRNA into the RISC complex (Liu *et al*., 2006). RISC utilizes the siRNA and search for the complementary target mRNA. The sequence and structure of a siRNA determines which of its two strands is to participate in the RNA silencing pathway. Each siRNA dissociates from the Dicer active site soon after it is produced, its thermodynamics evaluated by the RNA silencing mechanisms and then, one strand is selectively loaded onto RISC and the other is destroyed. The degradation process is initiated once the successful location and cleavage of the complementary mRNA occurs by the siRNA-RISC complex. In case of translational repression pathway, small RNAs direct RISC bind to target mRNA and repress its translation process, rather than cleavage. Animal miRNAs typically, but not always, mediate translational repression rather than cleavage. Translational repression occurs at some stage after translational initiation, because the distribution of ribosomes along the length of the repressed mRNA undergoing active translation (Vauchert, 2006). Thus, these RNAs are not a single class of ~25nt, but instead are two distinct species with 21-22nt and 25nt in size. The 21-22nt siRNA represents the siRNA that guides the RISC ribonuclease to the target of PTGS (Elbashir *et al.,* 2001; Aigner, 2006). However, it is unlikely that the two classes of siRNA have the same function because they accumulate differently in locally and systematically silenced tissue. Lipardi *et al.,* (2001) found that the 3' hydroxyl group is required in order to direct RNAi *in vitro*. While, Dicer may incorporate siRNAs into RISC following their synthesis, they do not require the event to occur *in vivo*. RNA silencing pathways can be divided into those that require RNA dependent RNA polymerase (RdRPs) and those that do not. In *C. elegans* and *N. crassa*, RdRP is required for silencing (Sijen and Kooter, 2000). Two models have been proposed to explain the role of RdRPs. First, RdRPs might use primary siRNAs to prime the synthesis of dsRNA using the target mRNA itself as a template. The mechanism of in-trans silencing is most probably based on the presence of siRNAs that corresponds to the region of homology between silencing inducer and target RNA (Depicker and Montagu, 2003). Several studies on plants indicated that silencing could also spread to regions downstream of the target (5'-3' spreading; Braunstein *et al.,* 2002; Vaistij *et al.,* 2002). A remarkable feature of PTGS is that it is non-cell autonomous, i.e. it can be induced in tissue actively expressing a transgene and move as a mobile transmissible signal originating from tissue where the same transgene is silenced (Jorgensen *et al.,* 1998; Fagard and Vaucheret, 2000). The signal appears to be a sequence that is specific and move unidirectional from source to sink tissues (Voinnet and Baulcombe, 1997; Sonoda and Nishiguchi, 2000). The spread of silencing signal depends on the transcription of the target RNA (Baulcombe, 2002).

Dicers

Naturally occurring small RNAs can be: (1) endogenous siRNA; (2) miRNAs involved in gene regulation; (3) transposon-derived small RNAs; (4) virus-derived siRNAs. All siRNAs are the products of degradation of long dsRNA by RNase III-like enzyme family, first discovered in *Drosophila* (Bernstein *et al.,* 2001). This enzyme was named Dicer in animals or Dicer-like (DCL) in plants. Dicers are multifunctional proteins and contain one or more dsRNA binding domains. The number of Dicers may vary in different organisms. Many animals encode only a single Dicer, *Drosophila* encodes two (Lee *et al.,* 2004), and four DCL homologues (DCL1, DCL2, DCL3 and DCL4) have been identified in *Arabidopsis thaliana* that function differentially in siRNA and miRNA biogenesis (Schauer *et al.,* 2002). Other organisms (like *C. elegans* and humans) used only a single DCL protein to process both categories of silencing initiation. DCL1 is mainly responsible for the processing of miRNAs (Herr *et al.,* 2005). Some other factors, such as HEN1 and HYL1 (a dsRNA binding proteins) also help the DCL1 in the generation of miRNAs (Vazquez *et al.,* 2004; Xie *et al.,* 2004). HEN1 is also involved in some other functions like, natural virus resistance and transgene silencing (Boutet *et al.,* 2003). DCL2 was found to be involved in the production of viral-derived 22-nucleotide siRNAs and antiviral defence (Gasciolli *et al.,* 2005). This viral-derived siRNA production also required two RdRps (RdRp1 and RdRp6) depending on the kind of virus which infect the plant (Muangsan *et al.,* 2004; Xie *et al.,* 2004). DCL3 and RdRp2 play a role in the generation of longer class of endogenous siRNAs (24 nt) and RNA-dependent DNA methylation (Xie *et al.,* 2004). These endogenous siRNAs are involved in the initiation or maintenance of a heterochromatic state (Matzke *et al.,* 2004). DCL4 is involved in RNA silencing in plants and seems to produce 21-nucleotide siRNA component of the cell-to-cell silencing signal (Dunoyer *et al.,* 2005).

DCLs proteins have an interchangeable and overlapping role in both siRNA and miRNA pathways (Deleris *et al.,* 2006).

RISC Complex

RNA-induced silencing complex (RISC) is a multi-protein complex involved in different catalytic functions in the process of RNA silencing as mRNA cleavage and translational inhibition. The sequence specificity is provided by the small RNA molecules to RISC. Another proteins AGO have been found to be part with RISC in all organisms. The AGO proteins are essential for the slicing activity of mRNA. Ten members of the AGO family have been identified. AGO1 expression is regulated by a miRNA (miR168) indicating that the AGO1 protein regulates its own expression in a negative feedback loop (Vaucheret *et al.,* 2004). AGO1 involved in the slicing activity and process miRNA and certain classes of endogenous siRNAs but not the viral siRNAs (Baumberger and Baulcombe 2005). The role of AGO4 is in the production of the long siRNA of about 24 bp. Its involvement in long siRNA mediated chromatin alteration (histone methylation) also has been reported (Zilberman *et al.,* 2003). AGO2 is a part of RISC and it is essential for siRNA-directed RNA silencing, as reported in Drosophila. AGO2 has no role in the processing of miRNA, but the role of AGO1 was indicated (Okamura *et al*., 2004). Most of the AGO proteins are involved in different parts of RNA silencing (Baulcombe, 2004).

Development of PTGS Therapeutics

The target mRNA is enzymatically cleaved from any organism with PTGS, leading to decreased levels of gene expression of the targeted gene and the corresponding protein. The specificity of this mRNA silencing is controlled very precisely at the nucleotide level. RNAi (PTGS) is a fundamental cellular mechanism that can also be used to rapidly develop novel drugs against any disease target. RNAi was considered a novel option to treat viral infections. The reduction in expression of pathological proteins through RNAi is applicable to all classes of molecular targets, including those that have been traditionally difficult to target with either small molecules or proteins including monoclonal antibodies. RNAi therapeutics are under clinical investigation for age-related macular degeneration (AMD) and respiratory syncytial virus (RSV) infection, with numerous other drug candidates.

Application of PTGS

Until the end of 1980s, only modifications of DNA or protein that lead to transcriptional repression or activation were classified as epigenetic drugs. During the 1990s, a number of gene-silencing phenomena at the posttranscriptional level were discovered in plants, fungi, and animals, introducing the concept of PTGS or RNA silencing (Baulcombe, 2000; Matzke *et al.,* 2001). The application of dsRNA technology for large-scale investigation of gene function can be facilitated by making the critical experimental procedures fast and efficient (Wesley *et al.,* 2001; Brummell *et al.,* 2003). RNA silencing is an adaptive immune response that restricts accumulation or spread of viruses (Morris and Rossi, 2006). The transfection of mammalian cells with exogenous siRNAs has rapidly been adopted as a technology for targeted gene silencing (Elbashir *et al.,* 2001). Transgene intended to generate only sense or antisense RNA also silence gene expression, especially in plants (Jorgensen, 2003; Baulcombe, 2004).

A vector construct containing an inverted repeat of the 3' UTR of the nopaline synthase gene from *Agrobacteirum* has been developed to provide dsRNA region at the 3' end of the transcript (Brummell *et al.,* 2003). This method, which they termed as silencing by heterologous 3' UTRs (SHUTR), has been shown to operate effectively in *Arabidopsis* and *Lycopersicon*. Heterologous silencing was first reported by Hamilton *et al.* (2002), who used an inverted repeat of a 79bp fragment of the 5' UTR of tomato ACO1 transcript. Previous studies of transgene-induced PTGS in plants have suggested that gene silencing is initiated in the 3' region of the target gene (Sijen *et al.,* 2001). PTGS was induced in tobacco plants (Petrov and Stoyanova, 2011; Petrov, 2012) and potato plants cv. Agria by specific siRNAs for HC-Pro region of Potato virus Y (PVY) strain NTN, which effectively blocked the viral replication (Petrov *et al*., 2015a). PTGS was induced in potato plants cv. Arinda also by specific dsRNAs and siRNAs for HC-Pro region of PVY, which effectively reduced systemic spread of the virus. Reduction of the expression of the HC-Pro gene of PVY^N in newly grown leaves of potato plants, and, hence, the viral replication in all inoculated plants with the virus was established. The old treated and PVY inoculated leaves of the potato plants remain infected and later defoliate. All new leaves of potato plants cv. Arinda (not treated) grown after treatment with dsRNAs and siRNAs,

and PVY inoculation remain virus-free (Petrov *et al*., 2015b).

Successful RNAi applications have been reported for most classes of medically relevant viruses including HIV-1, HBV, HCV, RSV, SARS-coronavirus, influenza virus, and poliovirus (Haasnoot *et al*., 2007, Mescalchin *et al*., 2011; Leonard. and Schaffer, 2005). Some of these approaches have already reached the stage of clinical testing. While RNAi-mediated therapies against HIV-1 and HBV are based on shRNA expression systems (Haussecker, 2008), the most advanced clinical phase II trial make use of chemically synthesized siRNAs against RSV, which are administered by a nasal spray (DeVincenzo *et al*., 2010).

PTGS against HIV infections

HIV was the first infectious agent targeted by PTGS, because its life cycle and gene expression is well understood. Synthetic siRNAs and expressed shRNAs have been used to target all of the HIV encoded RNAs in cell lines (Coburn and Cullen, 2002). Despite the early successes of RNAi-mediated inhibition of HIV-encoded RNAs in cell lines, targeting the virus directly represents a substantial challenge for clinical applications because of the high viral mutation rate. Therefore, avoiding this problem is based on targeting cellular transcripts that encode functions required for HIV-1 entry and replication such as cellular cofactors - NF kappa beta, the HIV receptor CD4, and the co-receptors CCR5 and CXCR4 have all been down-regulated with the result of blocking viral replication or entry (Anderson and Akkina, 2005; Cordelier, *et al*., 2003; Surabhi and Gaynor, 2002).

PTGS against CVB3

PTGS has been evaluated as novel strategy to inhibit CVB3 (Schubert *et al*., 2005; Merl *et al*., 2005; Ahn *et al*., 2005; Yuan *et al*., 2005). In order to investigate the mechanism of RNAi-mediated inhibition of CVB3 in more detail, siRNAs were intentionally designed to target the viral plus strand, the minus strand, or both (Schubert *et al*., 2007).

Gene-Silencing Strategies to Prevent Viral Escape

A major problem for the long-term inhibition of viruses is the emergence of escape mutants. This limitation, which is well-known for conventional antiviral therapy with low molecular weight drugs, is applicable to RNAi approaches as well. Virus escape as a consequence of the accumulation of point mutations in or close to the siRNA target site has been observed for various types of viruses, including poliovirus (Gitlin *et al*., 2005) and HIV (von Eije *et al*., 2008). Three counter-strategies have been followed to counter the problem of viral escape: (1) targeting of conserved regions of the virus genome; (2) combination of efficient antiviral siRNAs; and (3) silencing of host factors that are essential for the viral life cycle. Mutations in highly conserved regions of the virus genome are likely to cause a loss of virulence. Comparison of silencing by siRNAs against different genomic regions of CVB3 revealed that targeting of non-structural protein coding regions is superior to selecting structural protein coding regions, since enzymes often lose activity when mutations occur (Merl and Wessely, 2007). Several groups have initially directed siRNA against the 5' untranslated region (5'UTR) of the CVB3 genome, which harbors the internal ribosome entry site (IRES). Interestingly, none of the tested siRNAs exerted significant antiviral activity (Merl and Wessely, 2007; Kim *et al*., 2007). A possible explanation for this unexpected finding is that tight structures such as the IRES are detrimental to siRNA-mediated gene silencing (Schubert *et al*., 2005). Only after laborious screening for accessible sites of complementary oligonucleotides in the 5' UTR region could ensure siRNAs to target efficiently the IRES and inhibit CVB3 (Dutkiewicz *et al*., 2008).

The antiviral activity of the siRNA was improved further by its partial modification with locked nucleic acids (LNA), which have a high affinity towards complementary RNAs. Another highly conserved region of the CVB3 genome is the *cis*-acting replication element (CRE) located in the 2C protein coding region. A siRNA directed against this region conferred sustained protection against CVB3 and prevented the emergence of viable escape mutants (Lee *et al*., 2007). Since the CRE sequence is identical in other enteroviruses such as echoviruses 6 and 7, and A-type as well as other B-type coxsackieviruses, the siRNA has a universal and persistent anti-enteroviral activity.

A widely employed strategy to minimize viral escape in conventional virus therapy is to combine various agents with antiviral activity. For HIV, this approach is known as highly active anti-retroviral therapy (HAART) or combined anti-retroviral therapy. The adaptation of this idea to RNAi is the combination of two or more active siRNAs or shRNAs. Combination of two shRNA expression cassettes in one vector was found to maintain silencing activity

against mutated target RNAs of CVB3 in a reporter system, since the second shRNA can compensate for the loss of silencing activity of the shRNA directed against the mutated target site (Schubert *et al*., 2005). A systematic investigation of viral escape in cell culture revealed that cocktails of three siRNAs targeting distinct sites of the virus genome could maintain therapeutic efficacy, while virus inhibition with dual- or single-molecule-based RNAi was abrogated by viral escape (Merl and Wessely, 2007). In the long run, however, resistant mutants are likely to develop even against combinations of three or more site-specific siRNAs. Therefore, using a pool of siRNAs covering 3.5 kb of CVB3 genomic sequence was developed (Nygardas *et al*., 2009). The pool was generated by synthesizing a long double-stranded RNA covering the region encoding most of the non-structural proteins of CVB3, which was subsequently cleaved into siRNAs by a recombinant Dicer. The pool of siRNAs was found to be significantly more effective than single-site siRNAs. Although this strategy can be expected to prevent viral escape, its therapeutic application is questionable since the antiviral agent consists of a heterogenous mixture of hundreds of different siRNA molecules. It is currently unclear whether the pool of siRNAs will induce more severe off-target effects than single siRNAs, since it consists of numerous sequences, each of them being able to potentially regulate non-target RNAs. The third strategy for sustained inhibition of viral spread is silencing of genes of the host cells that are required by the virus to enter cells and replicate. The advantage of this approach is that viruses have a limited capacity to adapt to host cell changes. CVB3 initially attaches to the decay-accelerating factor (DAF), which serves as a co-receptor, prior to the virus entering the host cells via CAR. Whereas, CAR is essential for cardiac CVB3 infection (Shi *et al*., 2009), most CVB2, CVB4, CVB6, as well as some strains of CVB1, CVB3, and CVB5 do not bind DAF (Freimuth *et al*., 2008). Silencing of CAR was found to prevent infection of the treated cells by CVB3 (Werk *et al*., 2005; Fechner *et al*., 2007). CAR is located in the tight junctions of epithelial cells (Freimuth *et al*., 2008; Coyne and Bergelson, 2005) and its constitutive gene knockout was found to result in an embryonic lethal condition associated with cardiac defects (Asher *et al*., 2005). Animals with a conditional knockout of CAR at a later time point of embryonic development (E11) survived to adulthood and did not have evident cardiac abnormalities (Chen *et al*., 2006). Very detailed investigations of heart function, however, revealed a block of atrio-ventricular conduction developed in the absence of CAR in the adult mouse heart, which may lead to arrhythmia (Lisewski *et al*., 2008; Lim *et al*., 2008). Conditional knockout mice exhibited a complete atrio-ventricular block and various phenotypes in other organs as well (Pazirandeh *et al*., 2011). It should, however, be noted that no direct conclusions can be drawn from knockout experiments for RNAi applications, since knockout animals completely lack CAR, while RNAi only results in a partial knockdown of target gene expression, possibly leaving enough for preventing these defects from occurring. Since CAR might have essential cellular functions, other host factors should be considered as targets for inhibitors that block CVB3 indirectly (Coyne *et al*., 2011). Conserved regions from VP1 of the coxsackievirus genome was identified and used as targets for RNAi (Petrov *et al*., 2011). dsRNAs specific for conserved part of VP1 of coxsackievirus B1 (CVB1) and CVB3 reduced virus titer from 4 to 3 Lg $CCID_{50}$ *in vitro* in HEp-2 cells (Petrov and Galabov, 2012a). siRNAs from the same gene region reduced CVB1 and CVB3 virus titer from 5 to 3 *in vitro* in HEp-2 cells (Petrov and Galabov, 2012b).

Application of PTGS against CVB3 in Animal Models

A siRNA targeting the 2A protease encoding genomic region was found to lead to significant reduction of viral tissue titers, attenuate tissue damage, and prolong survival in highly susceptible type I interferon receptor-knockout mice (Merl *et al*., 2005). The siRNAs were applied by hydrodynamic transfection. This method involves high pressure injection of the nucleic acid into the tail vein, which does not lend itself to a therapeutic setting for humans. It was therefore necessary to develop alternative application routes for efficient RNAi-mediated inhibition of CVB3. Since delivery of chemically synthesized siRNAs across the endothelial barrier to cardiomyocytes is inefficient, even in the presence of transfection agents, transfer of shRNA expression cassettes by viral vectors appears to be the method of choice. Kim *et al*. used a lentiviral vector to deliver the expression cassette for the above mentioned shRNA against the highly conserved CRE region (Kim *et al*., 2008). Mice were injected intraperitoneally with the lentiviral vector and were subsequently challenged with CVB3. Treated animals had significant reductions in viral titers, viral myocarditis, and pro-inflammatory cytokines, and,

most importantly, the survival rate was improved from 20% to 50% at Day 10 after infection (Fechner *et al*., 2008). The *in vitro* studies demonstrate that RNAi is an efficient approach to inhibit CVB3 and subsequent *in vivo* studies confirmed viral vectors to be suitable vehicles for the delivery of shRNA expression cassettes to the heart. Thus, the technology has the potential to develop into a therapeutic option to treat humans with virus-induced myocarditis (Schonhofer-Merl and Wessely, 2010).

Conclusion

The implications of PTGS, once viewed as an impediment to genetic engineering of plants, may instead be of fundamental importance both for controlling gene expression of pathogenic viral genes, (thus, inhibiting virus replication) and for use as an instrument for functional genomics. RNAi can target specific genes and control their expression. The problem with escape virus mutants has an alternative solution via a method, based on the replication complex of the bacteriophage phi 6. The technology produces a pool of siRNAs targeted to a specific gene region, which overlaps and silences the target (from any eukaryotic organism) whether a mutation or recombination appear.

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