Targeting Viral Heart Disease by RNA Interference

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Abstract Viral heart disease (VHD) is an important clinical disease entity both in pediatric as well as adult cardiology. Coxsackieviruses (CVBs) are considered an important cause for VHD in both populations. VHD may lead to dilated cardiomyopathy and heart failure which can ultimately require heart transplantation. However, no specific treatment modality is so far available. We and others have shown that coxsackieviral replication and cytotoxicity can be successfully targeted by RNA interference, thus leading to increased cell viability and even prolongation of survival in vivo. However, considerable limitations have to be solved before this novel therapeutic approach may enter the clinical trials arena.

1 Introduction

Picornaviridae comprise one of the largest families of human infectious pathogens of major clinical significance. Among the different genera, particularly rhinoviruses and enteroviruses such as coxsackievirus and poliovirus are of particular medical and economical interest. Coxsackievirus B3 (CVB3) has been identified as the most causative agent for the pathogenesis of viral heart disease both in adult and

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V.A. Erdmann et al. (eds.) *RNA Technologies in Cardiovascular Medicine and Research*,
doi: 10.1007/978-3-540-78709-9, © Springer-Verlag Berlin Heidelberg 2008

pediatric patients (Woodruff 1980). Coxsackieviral heart disease encompasses acute as well as chronic forms of myocarditis (Feldman and McNamara 2000) and may lead to arrhythmias, heart failure, and sudden death. In addition, the coxsackieviral genome can establish a persistent infection of the myocardium without clinical signs of myocarditis and thus may induce viral cardiomyopathy (Kandolf et al. 1993; Wessely et al. 1998b). Viral cardiomyopathy may evolve to a life-threatening progressive disease, characterized by enlargement of the heart chambers, myocardial and ventricular dysfunction and finally heart failure that may ultimately lead to heart transplantation. Besides its relevance for the pathogenesis of viral heart disease, extracardiac diseases including hepatitis (Wessely et al. 2001), pancreatitis (Zaragoza et al. 1999), aseptic meningitis (Feuer et al. 2003) as well as encephalomyelitis (Bauer et al. 2002), have been attributed to CVB3. At present, treatment with ß-interferon is clinically under investigation to attenuate clinical symptoms and improve cardiac function. However, a specific antiviral drug therapy for coxsackievirus-mediated disease is currently not available.

RNA interference (RNAi) is an evolutionarily highly conserved endogenous mechanism for the regulation of gene expression by sequence-specific RNA degradation. First described by Fire et al. (1998) as an unexpected anomaly during antisense inhibition of gene expression in *Caenorhabditis elegans*, the process of RNA interference proved to be conserved also in many higher organisms (Billy et al. 2001; Elbashir et al. 2001a,b). The post-transcriptional gene silencing is triggered by long, double-stranded RNA molecules, which are cleaved by a cytoplasmatic dsRNA specific endonuclease known as Dicer into 21–23 nucleotides long, double-stranded short interfering RNA (siRNA) molecules (Bernstein et al. 2001; Elbashir et al. 2001a; Hammond et al. 2000). The siRNA molecules associate with helicase and nuclease molecules to form the targeting complex, known as RNA-induced silencing complex (RISC). Within RISC, the double-stranded siRNA molecule is unwound by helicase activity and the sense strand released. The antisense strand of the siRNA directs the complex to homologous complementary target mRNA sequences and hybridizes by Watson-Crick base pairing. Finally, the endonuclease Argonaute 2, also a component of the multiprotein complex RISC, promotes the precise and highly sequence-specific degradation of target RNA. Since RNA interference has probably evolved besides its role in basic cellular processes determining cell fate and differentiation (Abrahante et al. 2003; Brennecke et al. 2003) as an endogenous defence mechanism for protection from invading genetic elements like transposons and particularly viruses (Downward 2004), this ubiquitous pathway has opened exciting possibilities for exploiting it experimentally for functional genomics and in particular therapeutically in the fight against infections.

Initially, the attempt to provoke a specific knockdown of an individual gene by treating mammalian cells with long double-stranded RNAs failed. In mammalian cells, duplex RNA molecules longer than 30 nucleotides induce an interferon response, which triggers nonspecific degradation of RNA and inhibition of protein synthesis (Stark et al. 1998), leading ultimately to cell death. The crucial finding of 21–23 bp long siRNA molecules mediating the sequence-specific degradation of homologous RNA without affecting cellular functions (Elbashir et al. 2001a) alleviated

this problem. By entering the RNA interference pathway equally to Dicer products downstream of the interferon pathway, these molecules mostly circumvent the stimulation of a stress response. In this process, gene silencing may either be achieved by exogenous delivery of chemically synthesized siRNAs (Elbashir et al. 2001a) or by vector-derived expression of either siRNAs or double-stranded short hairpin RNAs (shRNA), which are subsequently processed by Dicer in functional siRNAs (Brummelkamp et al. 2002; Miyagishi and Taira 2002; Paul et al. 2002).

The technique of siRNA-mediated silencing rapidly turned out to be an excellent experimental tool to analyze the function of individual genes, resulting in several studies demonstrating the impact of siRNAs to interfere with cellular processes such as apoptosis (Kartasheva et al. 2002; Lassus et al. 2002), or cell cycle regulation (Chen et al. 2002). Notably, the finding of synthetic siRNA mediating highly sequence specific RNA degradation provided a novel therapeutic tool to target distinct RNAs involved in the pathogenesis of human disease, such as cancer, autoimmune disease, dominant genetic disorders and in particular viral diseases. In this context, potential targets for RNAi-based antiviral therapies constitute of viral genes that are essential for virus replication or, on the other hand, cellular genes that are essential for virus entry or involved in the virus life cycle. For RNA viruses, theoretically any region of the viral genome, even a non-coding sequence, may serve as a potential target site for nucleic-acid based silencing. Presently, RNAi targeted pathogens of clinical importance encompass the human immunodeficiency virus (Boden et al. 2003; Coburn and Cullen 2002; Das et al. 2004; Hu et al. 2002; Jacque et al. 2002; Ji et al. 2003; Lee et al. 2002, 2003; Novina et al. 2002; Park et al. 2003), influenza virus (Ge et al. 2003), herpes simplex virus (Palliser et al. 2006), respiratory syncytial virus (Bitko and Barik 2001), hepatitis B virus (Hamasaki et al. 2003; McCaffrey et al. 2003; Shlomai and Shaul 2003; Ying et al. 2003) and poliovirus (Gitlin et al. 2002). In vivo approaches demonstrating RNAi mediated intracellular immunity encompass hepatitis B (Giladi et al. 2003; McCaffrey et al. 2003; Morrissey et al. 2005b), hepatitis C (McCaffrey et al. 2002; Wang et al. 2005), influenza (Ge et al. 2004; Tompkins et al. 2004), and respiratory syncytial virus (RSV) (Bitko et al. 2005; Zhang et al. 2005). Ongoing clinical trials utilize RNAi-based immunity to protect cells from infection with viruses such as respiratory syncytial virus infection (Alnylam Pharmaceuticals, Cambridge, Mass., USA) and trials protecting against infection with the human immunodeficiency virus (Benitec, Melbourne, Vic, Australia).

Our laboratory has focused on the investigation of coxsackievirus induced pathophysiologic processes in the context of viral heart diseases, particularly in consideration of potential therapeutic implications. Coxsackievirus B3 belongs to the picornaviridae virus family and is a close relative of poliovirus. Its genome is a messenger-like positive strand RNA molecule with a single open reading frame encoding a monocistronic polyprotein that is processed post-translational. Upon CVB3 infection, cardiac tissue injury occurs either by a direct viral cytopathic effect (Badorff et al. 1999; Wessely et al. 1998a) and/or by immunomodulatory mechanisms precipitated by viral infection (Knowlton and Badorff 1999). Therefore, a therapeutic drug concept promoting virus elimination can be considered as a key therapeutic approach to cure or attenuate CVB3-related disease. The

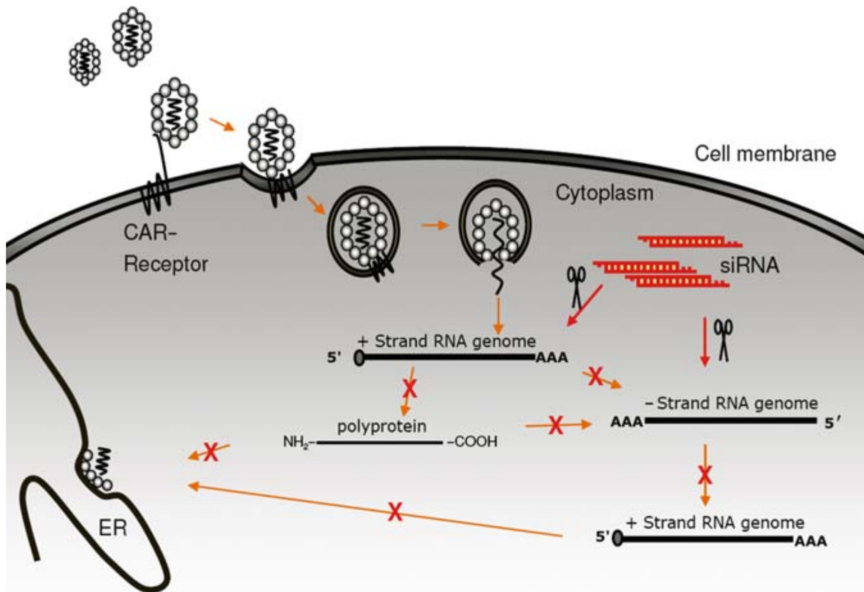

Fig. 1 Targeting coxsackieviral infection cycle by RNA interference. The coxsackieviral life cycle begins with the entry of the host cells, which express the coxsackievirus-adenovirus receptor (*CAR*). Upon cell entry, coxsackievirus releases its messenger like plus-stranded RNA genome. Subsequently, the viral genome mediates its own translation into a monocistronic polyprotein, which is processed post-translationally by viral proteases. The viral RNA dependent RNA polymerase is essential for virus replication via a negative-stranded intermediate. Ultimately the viral RNA assembles with viral proteins at the endoplasmatic reticulum (*ER*) to form infectious virions that are released from the host cell. Therapeutic antiviral RNA interference should result in the degradation of positive-stranded viral RNA as well as negative-stranded intermediates, resulting in the complete abrogation of the subsequent viral maturation process

pivotal observation that siRNAs may facilitate viral clearance and intracellular immunity without apparent side effects during infections with pathogenic human viruses such as the human immunodeficiency virus (HIV) (Rossi 2006) has raised exciting possibilities. The use of highly specific siRNAs targeting distinct regions of the viral genome (Fig. 1) as well as host genes that are relevant for virus entry and maturation represents a novel therapeutic strategy to cure or attenuate in particular coxsackievirus-mediated diseases.

2 Preclinical Results of Therapeutic Antiviral RNA Interference: Focus on CVB3-Associated Disease

In recent years, the evidence of siRNA conferring genomic immunity via the endogenous RNA interference pathway generated vigorous enthusiasm to exploit the RNAi mechanism therapeutically in the fight against a broad range of intractable

diseases, including neurodegenerative diseases (Rodriguez-Lebron and Gonzalez-Alegre 2006) such as Alzheimer, Huntington and Parkinson's disease, as well as macular degeneration and cancer (Alisky and Davidson 2004; Barik 2005; Dorsett and Tuschl 2004; Shankar et al. 2005). The RNAi-mediated silencing of various oncogenes and cancer-associated genes proved to be efficient for the attenuation of tumor growth, tumor survival, cellular invasion and metastasis both in vitro and in vivo (Friedrich et al. 2004; Gartel and Kandel 2006). Given its primordial antiviral function, the RNA interference pathway soon became important for antiviral drug development (Table 1).

The interest to identify RNAi-based therapeutic strategies has been particularly strong for the reverse-transcribing human immunodeficiency virus (HIV). Several laboratories obtained significant inhibition of the HIV-1 replication applying both synthetic and vector-derived siRNAs/shRNAs directed against the viral genome and HIV-encoded RNAs, such as the TAR element, tat, rev, gag, env, vif, nef and reverse transcriptase (Boden et al. 2003; Coburn and Cullen 2002; Das et al. 2004; Hu et al. 2002; Jacque et al. 2002; Lee et al. 2002, 2003; Novina et al. 2002; Park et al. 2003). Novina et al. (2002) used RNAi to substantially suppress the viral *gag* gene, which encodes a precursor protein being a key component for uncoating as well as maturation processes during the HIV infection cycle. Due to its enormous genetic diversity as well as long-term siRNA treatment evolving mutational variants presenting nucleotide substitutions and deletions in their siRNA target sequences (Boden et al. 2003; Das et al. 2004), HIV can escape the antiviral RNAi. Indeed, the antiviral effectiveness was retained by simultaneously targeting different regions of the viral RNA (Boden et al. 2003; Gitlin et al. 2002). As an alternative strategy, cellular cofactors essential for the virus entry have also been successfully silenced, resulting in a profound inhibition of HIV entry and subsequent replication, encompassing the viral receptor CD4 (Novina et al. 2002) and the co-receptors CCR5 (Lee et al. 2003; Qin et al. 2003) as well as CXCR4 (Ji et al. 2003). In a previous study, the therapeutic effectiveness was significantly enhanced by the lentiviral-based delivery of a combination of shRNAs targeting the HIV tat and rev RNAs, a ribozyme directed against the host co-receptor CCR5, and a nucleolar-localizing TAR RNA decoy, each blocking HIV by a distinct mechanism (Li et al. 2005). At present, a clinical trial using simultaneous application of multiple siRNAs is in progress (Benitec, Melbourne, Vic, Australia).

The first evidence for the in vivo antiviral effectiveness of RNAi was provided by targeting hepatitis B virus (Giladi et al. 2003; McCaffrey et al. 2003; Morrissey et al. 2005a,b). The expression of anti-HBV shRNAs efficiently suppressed HBV expression plasmids in the liver upon hydrodynamic co-transfection via the tail vein (McCaffrey et al. 2002), resulting in a substantial reduction of HBV RNA and replicated HBV genomes as well as a significant decrease of secreted hepatitis B surface antigen levels (McCaffrey et al. 2003; Morrissey et al. 2005a).

Additional proof for the antiviral potential of RNAi has come from studies with poliovirus. Poliovirus is an enterovirus such as CVB3 and belongs to the family of Picornaviridae, utilizing a messenger-like positive-sense single-stranded RNA genome. The transfection of short interfering RNAs directed against genomic

Table 1 RNAi in vitro and in vivo approaches targeting viral diseases

Genome		Virus family	Virus	RNAi target	In vitro/ in vivo	Reference
DNA	dsDNA	Herpesviridae	Herpes simplex virus	Helicase/primase associated protein UL5	In vitro	Palliser et al. (2006)
				Glycoprotein B (UL27)	In vitro/ in vivo	Palliser et al. (2006)
				Single-stranded DNA binding protein UL29	In vitro/ in vivo	Palliser et al. (2006)
				Glycoprotein E (US8)	In vitro	Bhuyan et al. (2004)
	dsDNA reverse transcribing	Hepadnaviridae	Hepatitis B virus	Pre-genomic RNA	In vitro/ in vivo	Hamasaki et al. (2003), McCaffrey et al. (2003), Morrissey et al. (2005a)
				Core protein HBcAG	In vitro/ in vivo	Hamasaki et al. (2003), McCaffrey et al. (2003), Shlomai and Shaul (2003), Ying et al. (2003)
				Non-structural transactivator protein HBx	In vitro	Shlomai and Shaul (2003)
RNA	Plus-stranded	Flaviviridae	Hepatitis C virus	5′ untranslated region (UTR)	In vitro	Krönke et al. (2004), Wang et al. (2005), Yokota et al. (2003)
				Core protein	In vitro	Randall and Rice (2004)
				Helicase/protease NS3	In vitro	Kapadia et al. (2003)
				Non-structural protein NS4B	In vitro	Randall and Rice (2004)
				Polymerase NS5B	In vitro	Kapadia et al. (2003), Wilson et al. (2003)
				Polymerase NS5B	In vivo	McCaffrey et al. (2002)
			West Nile virus	Capsid protein	In vitro	McCown et al. (2003)
				Polymerase NS5	In vitro	McCown et al. (2003)
		Picornaviridae	Coxsackievirus B3	Capsid protein VP1	In vitro/ in vivo	Ahn et al. (2005), Kim et al. (2007), Merl and Wessely (2007), Yuan et al. (2005)
				Capsid protein VP2	In vitro	Merl and Wessely (2007)
				Protease 2A	In vitro/ in vivo	Merl et al. (2005), Yuan et al. (2005)

			Protease 3C	In vitro	Merl and Wessely (2007)
			Polymerase 3D	In vitro/ in vivo	Ahn et al. (2005), Kim et al. (2007), Merl and Wessely (2007), Schubert et al. (2005), Werk et al. (2005), Yuan et al. (2005)
		Poliovirus	Capsid protein	In vitro	Gitlin et al. (2002)
			Polymerase 3D	In vitro	Gitlin et al. (2002)
Minus-stranded	Paramyxoviridae	Respiratory syncytial virus	Fusion protein F	In vitro	Bitko and Barik (2001)
			Phosphoprotein P	In vitro	Bitko and Barik (2001)
			Phosphoprotein P	In vivo	Bitko et al. (2005)
			Non-structural protein NS1	In vivo	Zhang et al. (2005)
	Orthomyxoviridae	Influenza virus	Nucleocapsid protein NP	In vitro/ in vivo	Ge et al. (2003, 2004), Tompkins et al. (2004)
			RNA transcriptase PA	In vitro/ in vivo	Ge et al. (2003, 2004), Tompkins et al. (2004)
Reverse transcribing	Retroviridae	Human immune deficiency virus	Group-specific antigen (gag)	In vitro	Hu et al. (2002), Novina et al. (2002)
			Cellular receptor CD4	In vitro	Novina et al. (2002)
			Cellular co-receptor CCR5	In vitro	Lee et al. (2003), Qin et al. (2003)
			Cellular co-receptor CXCR4	In vitro	Ji et al. (2003)
			Transactivator of transcription (tat)	In vitro	Boden et al. (2003), Coburn and Cullen (2002), Lee et al. (2003)
			Regulator of virion (rev)	In vitro	Coburn and Cullen (2002), Lee et al. (2002)
			Integrase	In vitro	Hu et al. (2002)
			Long terminal repeat (LTR)	In vitro	Jacque et al. (2002)
			Viral infectivity factor (vif)	In vitro	Jacque et al. (2002)
			Negative regulatory factor (nef)	In vitro	Das et al. (2004), Jacque et al. (2002)
			Envelope protein (env)	In vitro	Park et al. (2003)

regions encoding either a viral capsid-protein or the RNA dependent RNA polymerase effectively protected mammalian cells against polioviral infection, promoting viral clearance from most infected cells (Gitlin et al. 2002, 2005). In this context it was also observed that poliovirus can escape siRNA challenge due to emerging mutational variants harboring unique point mutations within their siRNA binding motif.

The development of an RNAi-based therapeutic strategy to combat coxsackievirus-induced heart diseases is due to its medical as well as economic impact and the lack of a specific anti-coxsackieviral drug therapy under focus of intense research. A number of independent laboratories, including ourselves, showed as a proof of concept that intracellular immunity against coxsackieviral infection could be successfully conferred by RNAi in vitro and in vivo.

Several groups achieved significant attenuation of viral replication and cytopathogenicity in cell culture by directly targeting the coxsackieviral genome. The process of target site validation revealed numerous effective siRNA binding motifs in genomic regions coding for the viral capsid protein VP1 (Ahn et al. 2005; Merl and Wessely 2007; Yuan et al. 2005), the capsid protein VP2 (Merl and Wessely 2007), the viral protease 2A (Merl et al. 2005; Yuan et al. 2005), the viral protease 3C (Merl and Wessely 2007) and the RNA dependent RNA polymerase 3D (Ahn et al. 2005; Kim et al. 2007; Merl and Wessely 2007; Schubert et al. 2005; Werk et al. 2005; Yuan et al. 2005). Of note, siRNAs targeting the 5′ as well as 3′ UTR had no substantial effect on viral cytopathogenicity and replication (Kim et al. 2007; Merl and Wessely 2007; Werk et al. 2005). The silencing was achieved either by treatment with synthetic siRNAs or by vector derived expression of shRNAs.

Yuan and co-workers reported a 92% inhibition of CVB3 replication in human HeLa cells and murine immortalized cardiomyocytes pre-treated with siRNA directed against the viral protease 2A (Yuan et al. 2005). In this study, the administration of siRNA post-infection proved to be effective in limiting even ongoing coxsackieviral infections, a fact that is of major importance for the clinical applicability of antiviral RNAi. Using fusion constructs composed of a reporter gene and subgenomic fragments of viral RNA, Werk et al. identified effective target sites in the genomic 3D region, which exhibited therapeutic potential to inhibit the virus propagation up to 80–90% (Werk et al. 2005). In our previous work, the application of the most effective siRNA directed against the RNA dependent RNA polymerase 3D resulted in an approximately fourfold prolonged survival of coxsackievirus-infected cells and an inhibition of viral replication by more than 10^5-fold compared to control siRNAs (Merl and Wessely 2007). Indeed, it has been demonstrated that therapeutic anti-coxsackieviral RNAi effectiveness can be hampered by the emergence of viral escape mutants harboring single point mutations in the central part of their siRNA target sites. Yet, the appearance of mutated coxsackievirus progeny can be sufficiently suppressed by the simultaneous application of at least three siRNAs targeting distinct genomic regions, thereby improving therapeutic outcome in terms of prolongation of cell survival by a factor of more than 6 compared to infected, control-siRNA treated cells (Merl and Wessely 2007). Similarly, Schubert et al. used a vector simultaneously expressing two different short hairpin RNAs to preserve therapeutic effectiveness

when targeting viral RNA harboring subtle mutations in its target sites (Schubert et al. 2005). Upon administration of a dual shRNA cocktail directed against the genomic 3D region, the virus propagation in HeLa cells was efficiently inhibited resulting in an 80–90% decrease of the virus titer. As an alternative strategy, the coxsackievirus–adenovirus receptor (CAR), which is a host gene essential for the cell entry of group B coxsackieviruses and various adenovirus subtypes (Bergelson et al. 1997), has also been successfully silenced by RNAi. The suppression of CAR led to an attenuation of virus propagation by approximately 60% in HeLa cells (Werk et al. 2005). Similar results were obtained by Fechner et al. reporting shRNA-mediated silencing of CAR resulting in the inhibition of CVB3 infection for up to 97% in human immortalized cardiomyocytes (Fechner et al. 2007).

First evidence for the in vivo anti-coxsackieviral effectiveness of RNAi was provided in Type I IFNR–knockout mice (Muller et al. 1994), a knockout mice strain highly susceptible to CVB3 infection with increased mortality even when infected with low viral titers (Wessely et al. 2001). The application of siRNA directed against the viral protease 2A was carried out via hydrodynamic tail vein injection. siRNA-2A had a profound therapeutic impact promoting significant inhibition of viral replication, attenuated organ damage and consequently prolonged survival (Merl et al. 2005). Repeated siRNA administration further improved survival in this animal model compared to single application. Kim et al. examined the antiviral effectiveness of siRNA directed against the viral capsid protein VP1 as well as the RNA dependent RNA polymerase following hydrodynamic transfection of vector-derived shRNAs in Balb/c mice (Kim et al. 2007). Irrespective of the time point of the shRNA application prior or subsequent to viral infection, this therapeutic strategy led to a reduction of viral replication and tissue damage.

3 Factors That Limit Early Efficacy of Antiviral RNA Interference

Considering that enteroviruses undergo high-level replication predominantly at initial stages of infection, early therapeutic effectiveness is considered of critical importance for the antiviral impact of RNAi. The early antiviral efficacy of RNAi depends on various important factors that include optimized siRNA design as well as target site accessibility and off-target effects. Though numerous determinants for optimal silencing potency still remain to be elucidated, many features conferring enhanced siRNA functionality have been unraveled. siRNA design criteria being crucial for the success of the RNAi approach encompass structural features such as moderate to low G/C content (30–50%) and a lack of siRNA intrinsic secondary structures as well as internal repeats. Internal secondary structures may potentially hamper the association of the siRNA molecules with the components of the targeting complex. Another decisive feature determining enhanced effectiveness is the low thermodynamic stability at the 5′ terminus of the antisense strand. The thermodynamic 5′ end stability of the antisense strand versus the sense strand determines the

rate of asymmetric antisense strand incorporation in the targeting complex (Khvorova et al. 2003; Schwarz et al. 2003). In addition, position specific determinants, such as base preferences at certain positions of the siRNAs, are of major importance to ensure high silencing activity of the siRNA. To impede any off-target effect, which is the unintended silencing of distinct genes, bioinformatics database queries for sequence homologies between the potential siRNA and the transcriptome of the targeted organism such as a Blast search should be conducted. Yet, several guidelines on siRNA design have been published including recommendations of fundamental importance by Reynolds et al. (2004). In addition, several academic and commercial entities provide online algorithms, to design siRNAs with enhanced target specificity according to thermodynamic, structural as well as position specific criteria (http://www.dharmacon.com; http://www.ambion.com/techlib/misc/siRNA_finder.html; http://www1.qiagen.com/Products/GeneSilencing/CustomSiRna/SiRnaDesigner.aspx).

The development of algorithms for rational siRNA design in consideration of thermodynamic and structural determinants led to a widespread use of siRNA-mediated gene silencing in research and development in life sciences. Yet, occasionally, siRNAs designed in conformity with theses algorithms exhibit no silencing activity, suggesting that additional factors impact on silencing activity. Several studies demonstrated that siRNA efficacy is at least in part influenced by structural properties of the targeted RNA, such as local intramolecular folding (Bohula et al. 2003; Heale et al. 2005; Vickers et al. 2003). In a previous study, Luo and Chang (2004) attributed this positional effect of different siRNAs mostly to the local secondary structure of the mRNA at the target site. They showed that the target site accessibility can be characterized by a single parameter, the "hydrogen bond (H-b) index." The gene-silencing effect inversely depends on the (H-b) index, which is the average number of hydrogen bonds formed between nucleotides in the target region and the rest of the mRNA. Ding et al. (2004) predicted the target site accessibility using a statistical probability profile of single-stranded regions generated for the entire target RNA. In this context, Yoshinari et al. could demonstrate that the silencing effectiveness of distinct siRNAs directed against the HIV-1 transactivation response element (TAR) was significantly influenced by the tight stem–loop structure of TAR (Yoshinari et al. 2004). Brown et al. supported the findings of the inaccessibility of the highly structured TAR, showing that silencing activity can be recovered upon enhancing the target site accessibility by disrupting the secondary structure of TAR using 2′-*O*-methyl oligonucleotides complementary to regions 5′ or 3′ of the siRNA binding motif (Brown et al. 2005).

Albeit the messenger-like positive stranded RNA genome of coxsackievirus suggests RNAi susceptibility for the complete genomic sequence, it has been shown frequently that genomic target selection has a major impact on therapeutic outcome. In general, siRNAs directed against the coding regions of CVB3 conferred considerable antiviral immunity. However, genomic targets located in the 5′ and 3′ untranslated, noncoding regions were less effective compared to targets in the protein coding region, despite designing siRNAs under careful consideration of all recommended criteria (Kim et al. 2007; Merl and Wessely 2007; Werk et al. 2005;

Yuan et al. 2005). The loss of antiviral effectiveness might be at least in part be due to steric hindrance by secondary and tertiary structures of the viral target RNA, as well as to proteins binding to regulatory genomic regions rendering the siRNA binding sites not fully accessible for the RNAi targeting complex. Notably, the highly folded structure of the 5′ UTR region of the coxsackieviral genome with its pronounced stem–loop motifs may hinder siRNA binding significantly. Even though previous studies reported efficient suppression of hepatitis C virus replication by siRNAs targeting single-stranded regions inbetween two stem–loop motifs of the viral 5′ UTR (Yokota et al. 2003) or even double-stranded regions in a stem–loop motif (Kanda et al. 2007; Prabhu et al. 2006), the overall findings implicate that RNAi efficacy can be optimized by selection of sequences located in viral protein coding regions, at least in the context of an enterovirus.

4 Factors That Limit Subsequent Efficacy of Antiviral RNA Interference

Several organisms such as plants (Palauqui and Vaucheret 1998; Voinnet et al. 1998) and nematodes (Grishok and Mello 2002) have developed mechanisms to amplify the dsRNA-induced RNA degradation signal resulting in a robust and long-lasting silencing even through cell division (Fire et al. 1998; Kennerdell and Carthew 1998). The local triggered gene silencing can spread to untreated cells throughout the organism and is often inherited by the next generation when spreading into germ line cells (Grishok and Mello 2002; Hammond et al. 2001). However, mammalian cells lack an RNA-dependent RNA polymerase to generate new siRNAs (Chi et al. 2003; Stein et al. 2003). As a result, gene silencing and thus antiviral efficacy is temporarily restricted to progressive degradation of siRNA by cellular nucleases and dilution to non-efficient levels due to cell division. Indeed, the silencing effect may last in terminally differentiated and cell cycle-arrested cells, such as neurons, for 3 weeks or more (Omi et al. 2004). However, in proliferating cells, RNAi activity induced by synthetic siRNA duplexes persists in dependence of the cell proliferation status about 3–7 days (Holen et al. 2002; Yang et al. 2001).

Since the coxsackieviral genome can persist chronically in the myocardium, the long-term efficacy is of significant importance for the antiviral RNAi approach. As a result, a number of groups have focused either on the use of chemical modifications to improve the siRNA intrinsic pharmacokinetic properties (deFougerolles et al. 2005) or the use of vector derived siRNA/shRNA. Chemical modifications are conducted to increase siRNA stability and to protect against degradation by endogenous nucleases while retaining full silencing activity. These modifications include RNA phosphate backbone modifications, replacing some phosphates of the siRNA duplex by RNase-protecting phosphorothioate (Braasch et al. 2003; Harborth et al. 2003), introduction of 2′-fluoro (2′-F) pyrimidines into the siRNA duplex (Layzer et al. 2004), or the modification of the ribose (Braasch et al. 2003), such as the introduction of 2′-*O*-methyl, 2′-deoxy-2′-fluorouridine. The incorporation of a

number of synthetic RNA-like high affinity nucleotide analogues (LNA) substantially improved the siRNA molecule bio-stability in serum significantly. Furthermore, therapeutic application of siRNA targeting the genomic RNA of the pathogen SARS corona virus provided evidence that LNA is compatible with the intracellular targeting machinery and actually showed enhanced silencing effectiveness (Elmen et al. 2005). Similarly, Allerson et al. demonstrated that the introduction of 2′-*O*-methyl and 2′-fluoro nucleotides conferred greatly enhanced plasma stability and additionally a more than 500-fold increase of in vitro potency (Allerson et al. 2005). A previous work reported that a combination of diverse chemical modifications can dramatically augment siRNA serum stability while maintaining high silencing activity. In this context, the siRNA duplex consisted of a sense strand with all pyrimidines substituted by 2′-fluoro pyrimidines, all purines substituted by deoxyriboses, as well as inverted abasic caps at the 5′ and 3′ termini. Within the antisense strand, all pyrimidines were replaced by 2′-fluoro pyrimidines and all purines by 2′-*O*-methyl purines. The 3′ end of the antisense strand exhibited a phosphorothioate linkage (Morrissey et al. 2005a).

An alternative approach to achieve long-term silencing effects suggests the use of vector-based systems for endogenous siRNA expression in target cells (Tuschl and Borkhardt 2002; Yu et al. 2002). The expression cassettes contain RNA polymerase III promoters which either express separately sense and antisense strands that subsequently constitute the active siRNA duplex, or express short hairpin RNAs as stem–loop structures that are processed by Dicer into functional siRNAs. Upon transfection, the plasmid expresses large amounts of shRNAs resulting in a profound and long lasting suppression (McCaffrey et al. 2002) in contrast to chemically synthesized siRNA. Kim et al. could demonstrate the anti-coxsackieviral impact of plasmid-derived short hairpin RNAs in Cos-7 cells and in mice (Kim et al. 2007). Potential adverse side effects of the long-term silencing still remain unknown; however, inducible expression systems with the transcription being under tight control of a specific inducer or repressor are available.

Indeed, the use of shRNA-expressing plasmids is often limited due to the low plasmid transfection efficiencies of the majority of mammalian cells, in particular primary cells (Dykxhoorn et al. 2003). Therefore, efficient delivery as well as highly stable expression of shRNA can currently only be achieved in a broad range of proliferating and non-proliferating mammalian cells, stem cells and transgenic mice using retroviral (Brummelkamp et al. 2002), lentiviral (Rubinson et al. 2003), as well as adenoviral (Arts et al. 2003) expression systems. Viral gene transfer provides high transduction efficiencies, yet, albeit significant progress has been achieved, important safety issues remain (Thomas et al. 2003). Besides safety concerns, involving immunogenic as well as inflammatory host responses and the possibility of emerging replication competent viruses, insertional mutagenesis due to non-specific retroviral integration into the host genome potentially triggering oncogenic transformation is an important risk during viral gene therapy. By contrast, synthetic, chemically modified siRNA molecules facilitate indeed a timely restricted, though robust, silencing effect avoiding the risks of insertional mutagenesis, immunogenicity or the potential toxic effects of long-term RNAi expression.

Another critical factor that strongly influences the efficacy of RNAi-based antiviral strategies at subsequent stages in a clinical setting is the highly error-prone nature of the virally encoded RNA-dependent RNA polymerase. Incomplete silencing may enable the RNA-dependent RNA polymerase to generate several mutational variants, including viruses harboring subtle mutations within the respective siRNA target sequences. These so-called viral escape mutants can evade the siRNA recognition and augment the viral replication, thereby neutralizing the therapeutic siRNA treatment. The ability to counteract therapeutic RNAi by emergence of escape mutants has been observed in a number of viruses (Boden et al. 2003; Das et al. 2004; Gitlin et al. 2002, 2005). During HIV-1 inhibition, the appearance of a siRNA resistant, mutated virus progeny was observed after several weeks of culture. These RNAi-resistant viruses presented nucleotide substitutions or deletions in the siRNA binding motif (Boden et al. 2003; Das et al. 2004). Emergence of viral escape mutants from siRNA challenge has also been described for members of the enteroviruses family. During the therapeutic application of siRNA targeting the polioviral genome, a siRNA resistant virus progeny emerged after infection at a high multiplicity of infection (Gitlin et al. 2002, 2005). These escape mutants harbored single nucleotide alterations in their siRNA target sequences. Equally, the anti-coxsackieviral efficacy of RNAi proved to be highly dependent on the emergence of a siRNA resistant virus progeny. The resistant viruses exhibited single point mutations in the central part of their respective siRNA target site that allowed for a rapid escape from siRNA-mediated viral genome degradation, thus resulting in the complete loss of therapeutic efficacy (Merl and Wessely 2007). Mutational diversity within the virus progeny was mainly restricted to silent transversions on wobble positions as well as transversions leading to conservative amino acid changes retaining the physicochemical properties thereby ensuring the propagation of viable mutants.

The emergence of escape mutants might be hampered partly by targeting highly conserved genomic regions, since nucleotide substitutions or deletions occurring in these regions often affect viral viability. However, these regions are often not fully accessible to the RISC complex due to proteins binding to regulatory genomic regions as well as steric hindrance by highly ordered secondary and tertiary structures. For example, the pronounced stem–loop motifs render the 5′ UTR of the coxsackieviral genome less susceptible to siRNA-induced degradation as noted below (Kim et al. 2007; Merl and Wessely 2007; Werk et al. 2005; Yuan et al. 2005). In addition, under selective pressure of RNAi, the emergence of a siRNA-resistant virus progeny harboring subtle point mutations in their respective siRNA target sequences was observed despite targeting the conserved genomic regions encoding the viral RNA dependent RNA polymerase 3D and the viral protease 3C (Merl and Wessely 2007).

An additional option to overcome the hindrance of viral escape is to target simultaneously different genomic regions thereby reducing the probability of emerging escape mutants limited by the mutation rate of the viral polymerases. An enhanced antiviral effectiveness was achieved by the application of siRNA pools simultaneously targeting multiple regions of the viral RNA during HIV-1 as well as

polioviral infection (Boden et al. 2003; Gitlin et al. 2002; Ji et al. 2003). Similarly, viral escape was minimized during coxsackieviral infections upon simultaneous administration of siRNA directed against distinct genomic regions (Merl and Wessely 2007). The occurrence of mutated viruses was reduced by more than half following simultaneous treatment with two independent antiviral siRNAs compared to single siRNA treatment, from more than 90% to 40%. However, only the combined application of three different siRNAs could achieve an almost complete suppression of mutated virus progeny, suggesting the combined treatment with at least three different siRNA molecules to be sufficient to suppress significantly viral escape. Schubert et al. used a vector simultaneously expressing two different short hairpin RNAs to retain high therapeutic effectiveness actually when targeting viral RNA harboring subtle mutations in its target sites (Schubert et al. 2005). In summary, exogenous or endogenous delivery of cocktails of siRNAs that target multiple viral sequences may be the best option to prevent viral escape that represents the major reason for the subsequent limitation of antiviral RNAi efficacy.

5 Conclusions and Future Prospects

In recent years, the discovery of the endogenous RNA interference pathway conferring genomic immunity has opened exciting possibilities for experimental exploitation for functional genomics and in particular therapeutically in the fight against a broad range of yet intractable diseases. In this perspective, RNAi-based antiviral therapy has great potential to cure or attenuate in particular viral diseases that are not efficiently treatable to date.

In the context of viral heart disease, several independent laboratories, including ourselves, were recently able to demonstrate that intracellular immunity can be successfully conferred by siRNA-mediated targeting of the coxsackieviral genome or the coxsackievirus-adenovirus receptor (CAR), thus leading to attenuated coxsackieviral replication and cytopathogenicity indicating a potential therapeutic role for RNAi against CVB3-related diseases. The in vivo application of siRNA directed against the viral genome had profound therapeutic impact promoting significant inhibition of viral replication, attenuated organ damage and consequently prolonged survival in highly susceptible mice. Despite these encouraging findings, major obstacles must still be overcome to enhance therapeutic efficacy before the value of therapeutic antiviral RNAi can be investigated clinically. These obstacles include the complete suppression of viral escape, the prevention of unwanted off-target effects, security aspects, tissue-specific delivery, siRNA stability, in vivo delivery systems and prolongation of therapeutic effectiveness. Yet, the development of non-viral delivery methods in terms of liposomal siRNA encapsulation as well as conjugation with specific ligands, antibodies, and aptamers have been first promising steps towards the clinical applicability of antiviral RNAi that may be available in the not too distant future to provide a novel therapeutic strategy to attenuate or cure life-threatening viral heart diseases.

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