Drugs of the Future 2006, 31(6): 513-525

CCC: 0377-8282/2006
DOI: 10.1358/dof.2006.031.06.995893

Review Article

Potential applications of small interfering RNAs in the cardiovascular field

F. Agostini[1], B. Dapas[1], R. Farra[1], M. Grassi[2*], G. Racchi[3], K. Klingel[3], R. Kandolf[3], O. Heidenreich[4], A. Mercatanti[5], G. Rainaldi[5], N. Altamura[1], G. Guarnieri[1] and G. Grassi[1, 3]

[1]Department of Internal Medicine, University Hospital of Trieste, Cattinara, Trieste, Italy; [2]Department of Chemical Engineering, University of Trieste, Piazzale Europa 1, Trieste, I-34127, Italy; [3]Department of Molecular Pathology, Eberhard Karls University Hospital of Tuebingen, Germany; [4]Interfaculty Institute for Cell Biology, Department of Molecular Biology, Eberhard Karls University of Tuebingen, Germany; [5]C.N.R. - Area della Ricerca di Pisa, Istituto di Fisiologia Clinica, Pisa, Italy.
**Correspondence: e-mail: ggrassi@units.it*

CONTENTS

Abstract

Since the demonstration of the ability of small interfering RNAs (siRNAs) to downregulate gene expression in mammalian cells, many potential therapeutic applications have been explored. Here we present an update on the studies performed in the cardiovascular field, which at the moment are rather limited compared to the applications studied for other pathological conditions. So far, in the cardiovascular field, three main research topics can be discerned: 1) studies devoted to ameliorating and understanding the mechanisms regulating cardiomyocyte function; 2) studies aimed at preventing viral myocarditis; and 3) studies dealing with vascular pathologies. Although the potential applications of siRNAs in cardiovascular pathologies are limited compared to other human diseases, the reported studies suggest that siRNAs may help to develop novel effective therapeutic approaches in the cardiovascular field.

Introduction

The possibility of downregulating the expression of abnormally expressed genes causing disease appears to be of use in the development of novel therapeutic approaches. In this regard, RNA interference (RNAi) represents an attractive strategy. RNAi, first discovered in nematodes (1), derives from an evolutionarily conserved mechanism involved in the defense against biological phenomena based on the formation of double-stranded RNA intermediates such as those occurring during RNA virus invasion. More recently, RNAi has also been shown to be responsible for regulating the expression of several cellular genes by destroying mRNA, affecting translation (2-4) and influencing promoter activity inducing DNA methylation (for further information see Ref. 5). RNAi is triggered by both single hairpin and double-stranded RNA (dsRNA) precursors, which are processed to small RNA duplexes of 21 nucleotides able to guide the destruction or the translational repression of complementary single-stranded RNA (ssRNA). Since it is possible to specifically downregulate the expression of the desired genes by designing specific short RNA duplexes, RNAi not only represents a novel attractive therapeutic strategy, but may also facilitate functional genomic research. For these reasons, several pharmaceutical companies have considered RNAi as an attractive opportunity.

The therapeutic potential of RNAi was initially demonstrated in 2001 (6) and was then further developed. For example, in cultured cancer cells, the possibility of specifically reducing the intracellular level of a mutant form of *p53* led to a novel potential approach to the treatment of cancer (7). Promising results have also been observed in the downregulation of hepatitis C virus (HCV) infection and spread (8). Even more striking is the potential use of RNAi to treat leukemia and HIV infection (9). Among

other potential applications of RNAi, in this review we focus on its use in the cardiovascular field. A detailed description and discussion of siRNA selection and delivery via viral vectors will be not addressed here. For this topic, we recommend previously published reviews (10-13).

Mechanism of RNA interference

To date, the two most common types of small RNAs mediating the process of RNAi are represented by microRNA (miRNA) (4) and short interfering RNA (siRNA) (3). miRNAs are formed by an initial long precursor processed by specific RNase type III endonucleases called Drosha and Dicer (Fig. 1) (3). Drosha, which is active in the nucleus, promotes the formation of double-stranded fragments with two nucleotides overhanging at the 3'-end and a phosphate at the 5'-end. Subsequently, exportin 5 mediates the export of dsRNA fragments to the cytoplasm, allowing further processing by the second RNAse type III endonuclease, *i.e.,* Dicer. Dicer can also process dsRNA originated by RNA viruses and artificially introduced dsRNAs. Dicer produces dsRNA similar to but shorter than that produced by Drosha, 21 nucleotides long and paired in the 19 central nucleotides with 2 unpaired nucleotides at each end (14).

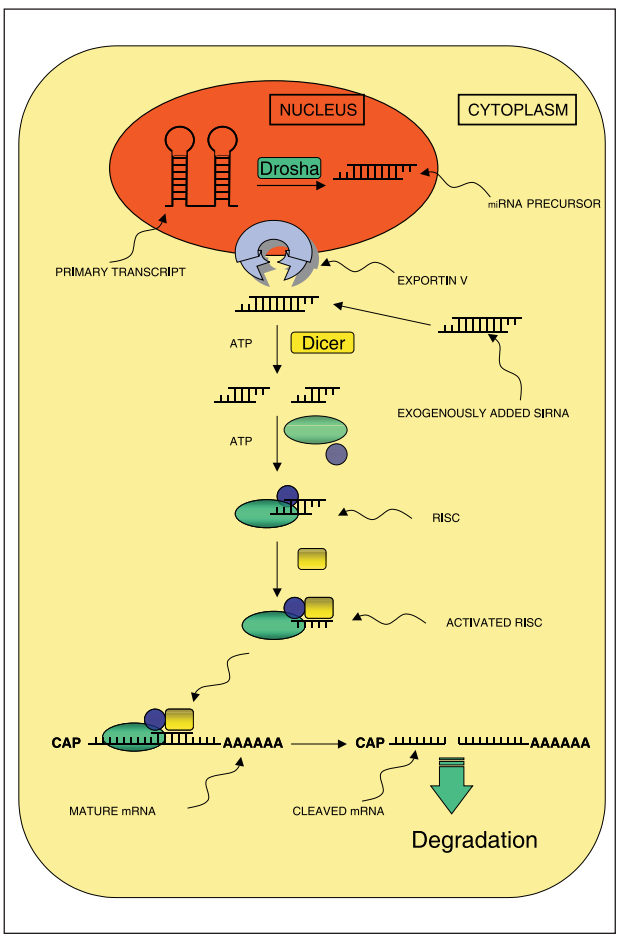

Fig. 1. Sequence of the events occurring during RNA interference. Following Dicer action, the siRNA is incorporated into the RISC complex, where the antisense strand guides the protein complex to the complementary region on the target mRNA; following recognition, the target RNA is cut and eventually degraded.

At this step of the pathway, dsRNA, now called siRNA can be considered fully synthesized. To exert their biological role, siRNAs need to be embedded into a protein complex called RISC (RNA-induced silencing complex) where the siRNA strand complementary to the target RNA is retained (15). The RISC complex, bound to a specific short ssRNA, can now perform the final step of the RNAi process, *i.e.,* the catalytic cleavage of the target RNA (16, 17). The specific cleavage of the target RNA leads to the formation of 5'-phosphate- and 3'-hydroxyl-ending fragments and to subsequent rapid degradation of the cleavage fragments. The RNA destined for degradation may be encoded by genes of an invading viral genome, by transgenes, parasitic transposons or repetitive elements contained in the host genome itself. Alternatively, the target RNA may be related to a gene of scientific or therapeutic interest, which can be downregulated by the administration of exogenous siRNA specifically synthesized for this purpose.

siRNA delivery

Despite the great variety of possible therapeutic applications of siRNAs, their *in vivo* delivery is still a problem (18, 19). Indeed, while *in vitro* the major requirement is to achieve efficient crossing of the cellular membrane, *in vivo* siRNA must first access the target tissues/cells, and ideally, should reside at the required location for a sufficient length of time. Additionally, it needs to be protected from degradation mediated by extracellular nucleases.

Basically, two main categories of siRNA delivery systems exist – viral and nonviral (19). Viral constructs have the advantage of being very efficient, but they may induce serious adverse effects *in vivo* (20). Nonviral approaches are considered to have far fewer drawbacks, although the delivery efficiency needs improvement (21). To overcome this limitation, a number of innovative approaches are being intensively pursued.

Nonviral approaches consist of the delivery of naked nucleic acid by physical methods (electroporation, for example), by hydrodynamic injection and by the use of synthetic carriers. Hydrodynamic tail vein injection in mice (22) consists of the rapid injection of a large volume into the tail vein such that a transient increase in venous pressure and extravasation of the nucleic acid occur. Nucleic acid molecules are rapidly internalized when presented to parenchymal cells using this technique. This strategy is effective in the liver, but in other organs such as the kidney, pancreas, spleen, lung and heart the efficiency is reduced (23) to approximately one-tenth of that achieved in the liver. Interestingly, this approach appears to be more efficient for the delivery of siRNAs than for plasmid DNA, possibly due to the smaller size of siRNAs and the

low intracellular siRNA concentrations required for gene knockdown. Additionally, siRNAs, acting in the cytoplasm, do not require nuclear import, which is a significant barrier to the delivery of biologically active plasmid DNA (24). Finally, an important advantage of hydrodynamic tail vein injection lies in its simplicity, with no specialized equipment being required. However, while applicable in murine models, hydrodynamic delivery is of very limited clinical value. Calculating from a 20-g mouse to a 70-kg man, this type of siRNA delivery would require an intravenous injection of 3-7 l of siRNA solution within 5 seconds.

A second nonviral approach for *in vivo* siRNA delivery is based on synthetic vehicles that contain cationic polymers and/or lipids (19). In order to be effective and reliable, this strategy requires that the delivery vehicle must not only overcome cellular barriers, but also form stable complexes with siRNAs during membrane crossing. Positively charged agents (cationic nature is essential for complex formation with anionic siRNAs) are often toxic after systemic administration (25). To eliminate this drawback, the use of uncharged, hydrophilic polymers has been proposed. These polymers, inhibiting nonspecific hydrophobic and charge-charge interactions, increase circulation time *in vivo* and thus facilitate enrichment in the target organ (19), with decreased toxicity. Examples of these polymers include polyethylene glycol (PEG), polyhydroxypropylmethacrylic acid and cyclodextrins (26).

Another way to reduce the toxicity of positively charged compounds is to add polyanion to cationic complexes in order to form layered structures (27). Heyes *et al.* (28) employed a homologous series of cationic lipids with incremental degrees of saturation to encapsulate and deliver siRNA. Notably, they demonstrated that the degree of saturation of cationic lipids affects lipid pKa, fusogenicity, cellular uptake and gene silencing ability. Bartlett *et al.* (29) considered a cyclodextrin-based polycation vector stabilized by surface PEGylation intended for systemic administration. Schiffelers *et al.* (30) focused their attention on strategies based on the development of self-assembling nanoscale carriers for the targeted delivery of siRNA to the vasculature, with the aim of inhibiting tumor or ocular angiogenesis. In particular, nanoparticle delivery systems have been developed with a ligand-polymer-polymer conjugate. Kim *et al.* (31) used folate-modified cationic polyethylenimine (PEI) for siRNA delivery. Kushibiki *et al*. (32) considered cationized gelatin delivery systems of plasmid DNA expressing siRNA for the TGF-β type II receptor to prevent interstitial renal fibrosis. Moriguchi *et al.* (33) designed the multifunctional envelope-type nano device (MEND) modified with stearylated octa-arginine (STR-R8) (Fig. 2). MEND consists of a polycation-condensed plasmid DNA core and a lipid envelope. The lipid envelope is modified with the membrane-penetrating peptide STR-R8 to improve cellular uptake and intracellular trafficking. Woodle and Lu (34) proposed nanoparticles allowing both nucleic acid binding into a core that can release siRNAs into the cytosol and protection from nonspecific interactions and tissue targeting. As no single material possesses all of these required properties, they considered modular conjugates of three materials to obtain the multiple properties required. Kakizawa *et al.* (35) studied the possibility of using organic-inorganic hybrid nanoparticles based on the self-associating properties of a block copolymer such as poly(ethylene glycol)-block-poly(aspartic acid) (PEG-PAA) with calcium phosphate.

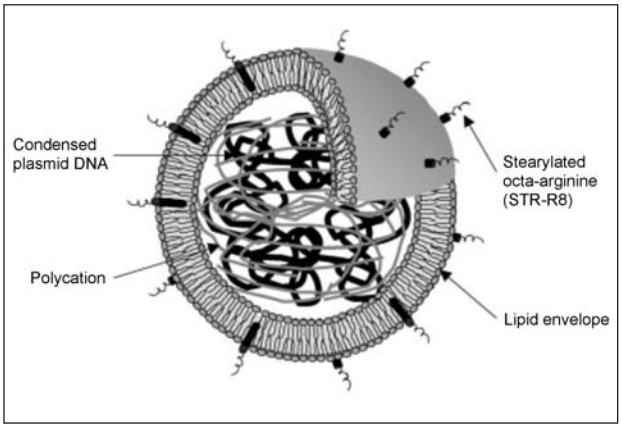

Fig. 2. A multifunctional envelope-type nano device (MEND) modified with stearylated octa-arginine (STR-R8) is depicted. MEND consists of a polycation-condensed plasmid DNA core and a lipid envelope structure. The lipid envelope is modified with the membrane-penetrating peptide STR-R8 to improve cellular uptake and intracellular trafficking. Reprinted from Int J Pharm 301, Moriguchi, R., Kogure, K., Akita, H. et al. *A multifunctional envelope-type nano device for novel gene delivery of siRNA plasmids*, 277-85. Copyright 2005, with permission from Elsevier.

Our group (36, 37) proposed a polymeric blend matrix (consisting of alginates and pluronics) containing siRNA-liposome complexes in the context of restenosis prevention. Our approach aimed to confer siRNA protection against degradation and to minimize blood wash, thereby substantially increasing the intravascular residence time of siRNAs.

Finally, an emerging approach consists of conjugation of siRNA to lipid molecules, a strategy with the potential to improve siRNA stability *in vivo* and to facilitate membrane association because of the lipophilic conjugate. Soutschek *et al.* (38) conjugated a cholesterol molecule to the 3'-end of an siRNA by means of a pyrrolidine linker. The activity of this novel composite molecule was then tested *in vivo* in a rat by i.v. injection. The authors showed improved pharmacokinetic properties for the cholesterol-siRNA molecule as compared to unconjugated siRNAs. Additionally, it was possible to show that the cholesterol conjugate does not substantially impair siRNA activity. The cholesterol-siRNA molecule showed broad tissue distribution 24 h after injection, with significant levels detected in heart, liver, kidney, adipose and lung tissues. This study, together with another (39), indicated a novel strategy with the potential to efficiently deliver siRNA *in vivo*.

Cardiovascular applications

Whereas the use of siRNA in the cardiovascular field for both therapy and as a tool for basic research purposes is still in its infancy, many promising investigations have indicated the great potential of this type of molecule. To date, three main research areas can be identified: 1) studies devoted to ameliorating and understanding the mechanisms regulating cardiomyocyte function; 2) studies aimed at preventing viral infection of the heart; and 3) studies dealing with the treatment of vascular pathologies.

Cardiomyocyte function

Despite current treatments, heart failure still represents an important problem in clinical practice. Thus, novel therapeutic approaches are now being developed, including those based on the use of siRNAs. In this regard, Watanabe *et al.* (40) explored an innovative approach with potential utility for future therapeutic application, based on inhibition of phospholamban (PLB) expression in cardiomyocytes. PLB forms complexes with and inhibits sarcoplasmic reticulum Ca^{2+}-ATPase 2 (SERCA2) (41) (Fig. 3). SERCA2 mediates Ca^{2+} uptake into the sarcoplasmic reticulum and is thus a major determinant in regulating cardiac contraction (42). In addition to PLB binding, SERCA2 activity depends on several other variables, including its intracellular levels. Low SERCA2 levels underlie, at least in part, impaired cardiac function in heart failure (43).

Watanabe *et al.* (40) counteracted the reduced activity of SERCA2 in heart failure cardiomyocytes by reducing levels of the inhibitor PLB. For this purpose, siRNAs directed against PLB were delivered to cultured rat cardiac myocytes using the hemagglutinating virus of Japan (HVJ) envelope vector. After demonstrating concentration-dependent activity of the siRNAs directed against

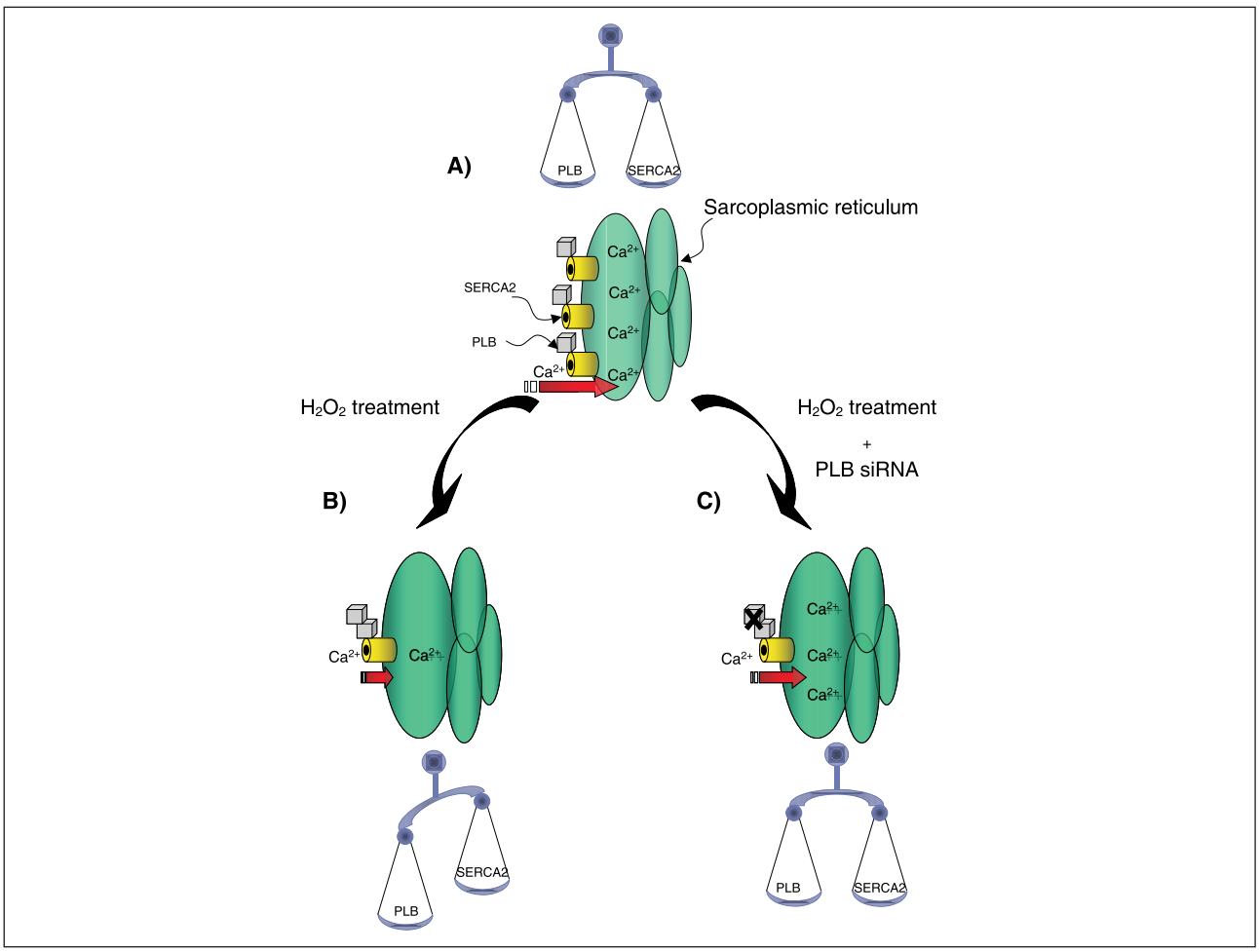

Fig. 3. PLB regulation of SERCA2-mediated Ca^{2+} intake in the sarcoplasmic reticulum. The physiological balance between PLB and SERCA2 (**A**) is disrupted by the addition of H_2O_2 to cultured cardiomyocytes (**B**). The increase in H_2O_2 levels, a condition which partially simulates the *in vivo* situation of failing cardiomyocytes, determines a significant and parallel reduction in SERCA2 and PLB levels, which leads to a reduction in Ca^{2+} uptake by the sarcoplasmic reticulum (**B**). The reduction in PLB levels by specific siRNAs determines an increased affinity of SERCA2 for Ca^{2+} (**C**).

PLB mRNA, the authors studied the effect of PLB ablation on the affinity of SERCA2 for Ca^{2+}. They showed that downregulation of PLB by siRNA increases the affinity of SERCA2 for Ca^{2+}, thereby enhancing the sarcoplasmic reticulum Ca^{2+} uptake rate. To better mimic conditions found *in vivo* in the failing heart, they tested the efficacy of the siRNA in cardiac myocytes treated with H_2O_2, which is typically produced in the failing heart. Exposure of rat cardiomyocytes to H_2O_2 led to a significant and parallel reduction in SERCA2 and PLB levels and a concomitant reduction in both sarcoplasmic reticulum Ca^{2+} uptake affinity and maximum velocity. Under these conditions, which partially simulate the *in vivo* conditions of failing cardiomyocytes, the authors demonstrated that siRNA-treated cardiomyocytes displayed an increased affinity of SERCA2 for Ca^{2+}, most likely due to a decrease in the levels of PLB. Thus, the authors provided proof of concept that it is feasible to improve Ca^{2+} uptake by acting on the SERCA2-PLB complex. It should be noted, however, that even if sarcoplasmic reticulum Ca^{2+} uptake is an initial and critical step to confer normal contractility to cardiomyocytes, other factors are also involved in this process and should be considered for future therapeutic applications.

siRNAs not only have the potential to correct the pathological cardiomyocyte contraction typical of heart failure, but they can also be used to dissect the role of genes regulating contraction. In this regard, an interesting study was carried out by Seth *et al*. (44), who investigated the consequences of SERCA2 downregulation by siRNA. Cultured neonatal rat and embryonic chicken cardiomyocytes were transduced using an adenoviral vector expressing an siRNA directed against position 173 of SERCA2 mRNA. As a result of SERCA2 downregulation, it was possible to demonstrate a compensatory mechanism with regard to Ca^{2+} homeostasis. The intracellular store deficiency of Ca^{2+} due to SERCA2 downregulation was compensated by upregulation of the expression of the transient receptor potential channel proteins TRPC4 and TRPC5 and of Na^+/Ca^{2+} exchanger (NCX) genes, which are also involved in Ca^{2+} export/import. Thus, variation in Ca^{2+} homeostasis leads to a transcriptional crosstalk that results in remodeling of Ca^{2+} signaling pathways. These findings have clear relevance not only for the better understanding of Ca^{2+}-associated cardiac pathologies such as heart failure and cardiac hypertrophy (45), but also for the identification of novel therapeutic strategies.

Another example of how siRNA techniques can be used to understand the role of genes involved in Ca^{2+} regulation and cardiomyocyte contractility emerged from the work of Hurtado *et al.* (46). In these experiments, the authors examined the effects of depletion of the exchanger protein NCX1. This protein is involved in the excitation-contraction coupling via Ca^{2+} efflux regulation in the heart. Rat cultured cardiomyocytes were transduced with an adenoviral vector expressing an siRNA directed against region 2040-2069 of the NCX mRNA. In contrast to the lethal phenotype of NCX1 knockout mice, Hurtado *et al.* could show grossly normal cardiomyocyte contractility in NCX1 knockout cultured cells. However, even if spontaneous beating was normal, a reduction in Ca^{2+} transient amplitude, a depression in the rate of Ca^{2+} rise and decline, elevated diastolic Ca^{2+} concentrations and shorter action potentials were observed. The authors concluded that these data indicate an important but nonessential role for NCX1 in the regulation of cardiac contractility. The relationship between NCX1 and SERCA2, however, still remains unclear. Whereas Seth *et al.* (44) showed that the downregulation of SERCA2 is followed by a compensatory increase in NCX1 expression, Hurtado *et al*. (46) reported that suppression of NCX1 expression is followed by downregulation of SERCA2 expression, instead of the expected upregulation. Further studies are required to understand the mechanism regulating Ca^{2+} homeostasis.

In addition to research aimed at improving and understanding cardiomyocyte contractility, studies have also been conducted to explore the mechanisms regulating myocardial damage following hypoxia-ischemia. Natarajan *et al*. (47) studied hypoxia inducible factor-1 (HIF-1), a key regulator of genes involved in oxygen homeostasis in response to reduced oxygen tension (Fig. 4). Under physiological conditions of normoxia, HIF-1 levels are negatively regulated by HIF-1α prolyl 4-hydroxylase-2 (PHD-2) which targets HIF for ubiquitination and proteasomal degradation. In hypoxia-ischemia, PHD-2 is less active and HIF levels therefore increase and drive the expression of several genes, such as iNOS, which protect the myocardium against hypoxia-ischemia-related damage. The authors hypothesized that the elevation in HIF levels immediately before ischemia-hypoxia might reduce myocardial damage. For this purpose, an siRNA directed against PHD-2 was prepared and tested both *in vitro* and *in vivo. In vitro* tests performed under conditions of normoxia in the murine endothelial cell line SVEC4-10 in the presence of 100 nM siRNA transfected with the liposome RNAiFect, showed a clear downregulation of PHD-2. This effect was paralleled by an increase in HIF-1 and *iNOS*, a gene regulated by HIF-1 that has a protective effect against ischemia-hypoxia. These data were confirmed *in vivo* in a mouse model of ischemia-reperfusion, where it was also possible to show a reduction in the size of cardiac damage, paralleled by a significant improvement in hemodymamic performance. Although these interesting findings may open the way to novel approaches to the treatment of ischemic-hypoxic cardiac damage, further studies are required to examine the effects of this treatment in long postischemic periods.

Connected to the above-mentioned study are reports from Yin *et al*. (48) and Davidson *et al.* (49), who used siRNA to explore the role of HSF1 (heat shock factor 1) and FLIP (FLICE-inhibitory protein), respectively, in cardiac ischemia-reperfusion models. Yin *et al.* (48) used siRNA (i.p. injection) to downregulate the expression of HSF1, a transcription factor known to be associated with pathophysiological stresses such as whole-body hyperthermia (WBH) and ischemia-reperfusion, in mice. The authors demonstrated that HSF1 plays an essential role in WBH preconditioning against ischemia-reperfusion

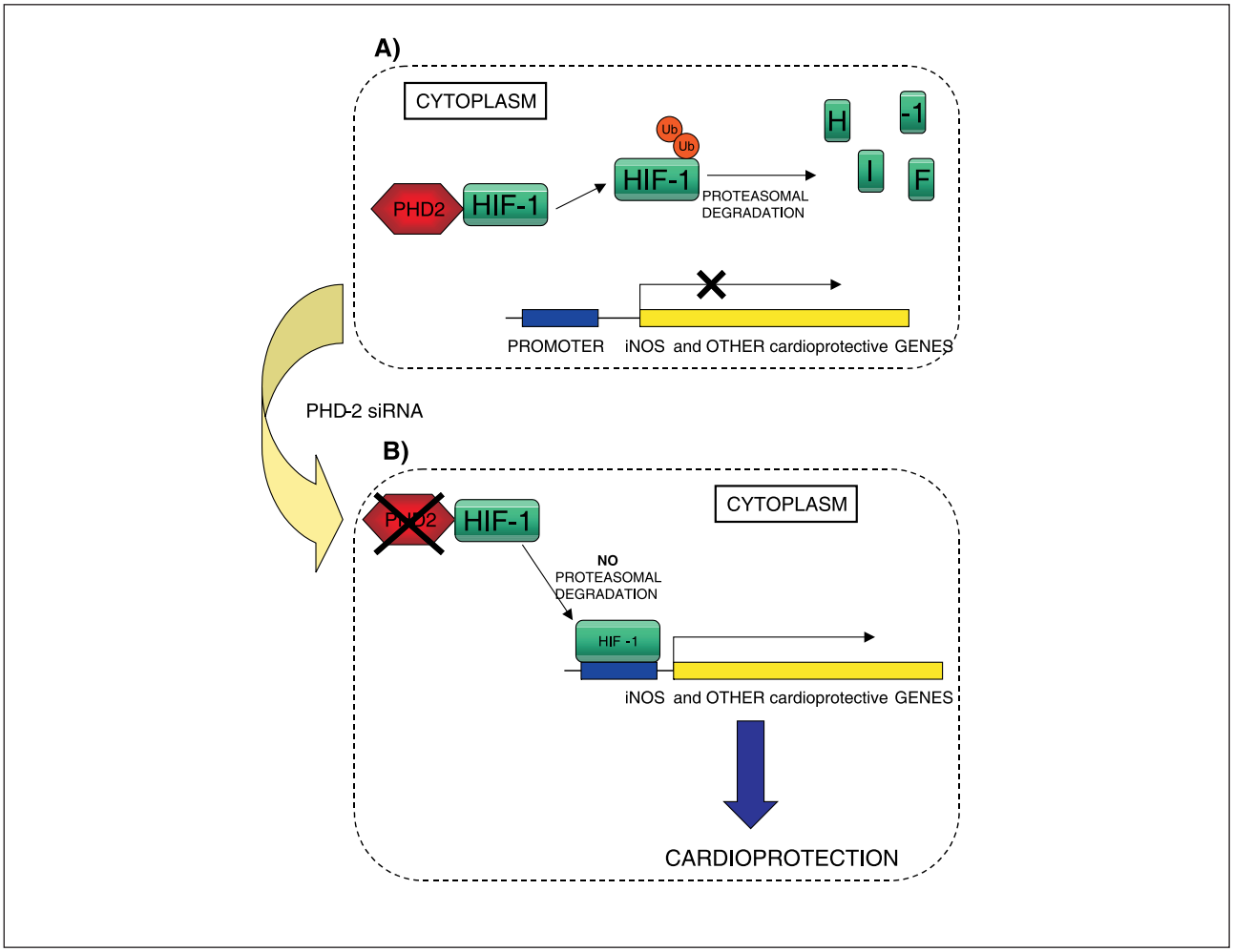

Fig. 4. HIF regulation by PHD-2. HIF-1α prolyl 4-hydroxylase-2 (PHD-2) targets HIF-1 (hypoxia inducible factor-1) for ubiquitin-dependent proteasomal degradation under conditions of normoxia; this prevents the expression of cardioprotective genes regulated by HIF itself (A). The downregulation of PHD-2 by a specific siRNA increases the levels of HIF, allowing the HIF-dependent activation of cardioprotective genes (B).

injury. Davidson *et al.* (49) directed siRNAs against FLIP, which regulates death receptor-mediated apoptosis in a wide variety of cell types. In cultured cardiomyocytes, the authors demonstrated that FLIP confers protection from ischemia-reperfusion, possibly through more than one pathway.

In conclusion, although potential siRNA-based therapeutic applications in the cardiovascular field are presently limited, basic research studies have had a broader diffusion. We believe that this is due, at least in part, to the lack of an ideal *in vivo* delivery system which can guarantee safe, specific and controlled release of siRNA to cardiovascular cells. Despite this limitation, we believe siRNA-based applications have the potential to be used to ameliorate cardiac dysfunction.

Viral myocarditis

Despite current therapeutic approaches, myocarditis is a major cause of morbidity and mortality worldwide (50) and contributes significantly to the social burden of heart failure. Affected individuals may require long-term medical therapy, and in many cases heart transplantation. Significant evidence from animal models and clinical studies (51) indicates that viruses represent major etiological causes of myocarditis. In particular, group B coxsackieviruses (CVB) are the most frequent cause of symptomatic viral myocarditis. CVB3 is a nonenveloped single-stranded RNA virus of positive polarity, which, upon entry into host cells, induces the synthesis of viral capsid proteins and nonstructural proteins using the host translational apparatus (Fig. 5).

Prominent cytopathic alterations in CVB3-infected cardiac cells, as well as CVB persistence in the myocardium of affected individuals, have been well documented (52-55). Heart tissue damage depends not only on a direct viral cytolytic effect, but also on the immune response elicited by the virus. In this regard, the contribution of the immune system to tissue injury relies on a complex balance between protective and detrimental

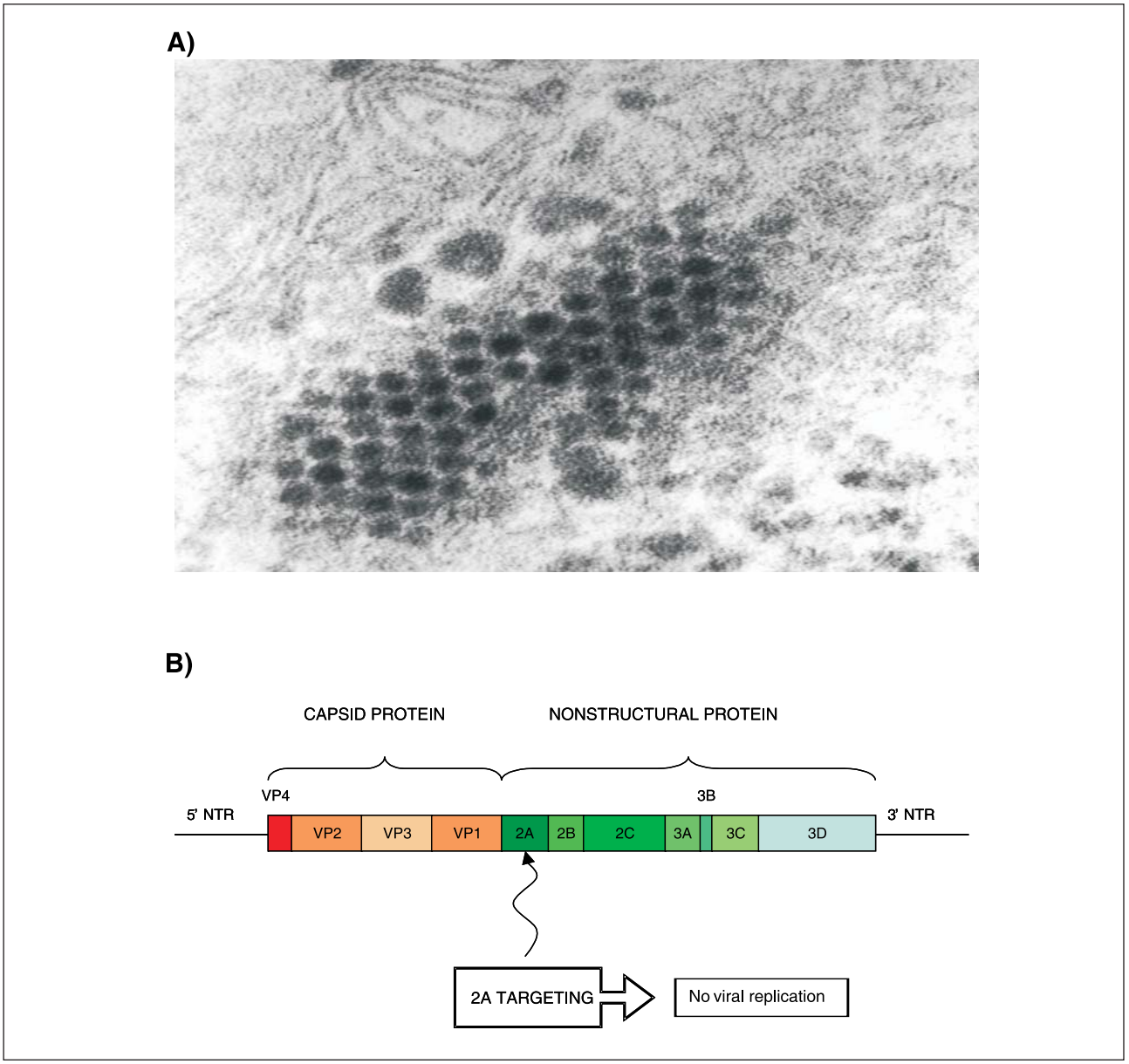

Fig. 5. Electron microscopic image of CVB3 (**A**) and genome structure (**B**). Within the viral genome, the portion encoding for the protease 2A appears to be the most indicated for siRNA-mediated destruction of the CVB3 genome.

immunological pathways (56). This implies that any interference with the immune response may have unpredictable, if not deleterious, effects on the course of the disease. Additionally, the observation that immunosuppressive therapy did not lead to an improvement in cardiac ejection fraction when compared with conventional therapy (57) further suggests that targeting the immune system is not the optimal strategy for the treatment of CVB myocarditis. Moreover, we demonstrated in a murine model of CVB3 myocarditis that early limitation of viral load is an important factor in the prevention of chronic myocarditis (58). Thus, novel strategies focused mainly on the selective destruction of the CVB3 genome have been developed.

Yuan *et al*. (59) designed siRNAs directed against the UTR viral region, the capsid protein VP1, the viral RNA polymerase 3D, the AUG start codon region and the viral protease 2A. siRNAs (300 nM) were transfected with Oligofectamine into HeLa cells and the HL-1 cardiac muscle cell line. Subsequently, the cells were infected with CVB3 at a multiplicity of infection (MOI) of 10. The most active siRNA, *i.e*., that directed against viral protease 2A, achieved 90% inhibition of CVB3 replication. Since the siRNA should be able to effectively inhibit an ongoing viral infection in the clinical setting, the authors also tested the efficacy of the most active siRNA in downmodulating a pre-existing viral infection. For this purpose, HeLa cells were infected with an MOI of 0.01 for 1 h and

subsequently transfected with the protease 2A siRNA. They found effective inhibition of ongoing viral replication, strengthening the potential therapeutic value of this siRNA. Finally, the reported enhanced antiviral effect of multiple siRNAs directed against HIV-1 prompted the authors to test whether combined administration of different siRNAs would also improve anti-CVB3 efficacy. Disappointingly, neither an additive nor a synergistic effect on CVB3 replication could be observed, as recently reported by others, including our group (60, 61). Whether this depends on the siRNAs tested or on the biology of CVB3 remains to be clarified. However, despite the lack of enhancement, this approach remains potentially relevant for long-term treatment. Indeed, mutated virus variants may be produced to escape from siRNA destruction, whereas the presence of multiple siRNAs may render escape less likely, thus maintaining the efficacy of the siRNA-based approach.

Consistent with the above-mentioned work, Merl *et al.* (62) confirmed the efficacy of siRNA-mediated targeting of CVB3 *in vitro*, expanding this observation to the *in vivo* situation. An siRNA directed against a part of the CVB3 genome coding for protease 2A, different from that used by Yuan (59), was evaluated. Protease 2A RNA was chosen as a target for several reasons. The protease is of fundamental importance for virus replication (63), as it processes the viral polyprotein into individual structural and nonstructural proteins; it cleaves the eukaryotic translation initiation factor 4G (eIF4G) (64), resulting in host cap-dependent translation inhibition; and it cleaves (65) the poly(A)-binding protein (PABP), an RNA-binding protein essential for protein translation. Additionally, in both cultured cardiomyocytes and murine hearts infected with CVB3 (66), protease 2A proteolytically cleaves dystrophin and dystrophin-associated glycoproteins, subverting cardiac tissue architecture and thereby contributing to the pathogenesis of dilated cardiomyopathy, a late sequela of CVB3 infection (67, 68). Finally, reduced dystrophin levels in cardiac tissue was associated with higher viral replication rates (68), suggesting a negative regulation function of dystrophin on viral replication. Thus, reducing the levels of protease 2A should attenuate virion production, host protein synthesis shut off and modification of cardiac tissue architecture. In HeLa cells transfected with 300 nM of siRNA in the presence of Oligofectamine and subsequently infected with CVB3 at 5 MOI, the authors observed a protective effect up to 48 h postinfection, with a peak in the reduction of viral titers > 90% of controls. *In vivo*, the authors administered the protease 2A siRNA by hydrodynamic transfection to lethally infected mice. Compared to controls, mice receiving the protease siRNA after viral infection showed a significantly improved life span, with 1 of 8 animals surviving infection. In addition, it was shown that CVB3 titers in various organs, such as the heart, liver, lung and kidney, were markedly reduced in treated animals compared to controls. Despite these encouraging results, most of the siRNA-treated animals died, most likely because of the persistence of low amounts of CVB3, which may have triggered an immune-mediated remodeling process in the infected organs.

The clearance of CVB3 and thus the attenuation of CVB3-mediated tissue damage might be achieved by improving the efficacy and specificity of siRNA delivery systems. So far, however, the lack of ideal delivery systems has forced the use of increased amounts of anti-CVB3 siRNA and/or to repeated siRNA administration. As this solution can have important negative consequences in terms of systemic toxicity, alternative strategies are required. In this regard, attention has been focused on inhibition of the expression of the coxackievirus and adenovirus receptor (CAR) required for CVB3 internalization. In HeLa cells transfected with a CAR-directed siRNA (100 nM), Werk *et al.* (69) showed a specific reduction in CAR protein and a reduction in viral titer of about 60% compared to controls. A more pronounced inhibition of CVB3 titers was not achieved. This is probably due to the presence of the decay accelerating factor (DAF), which functions as a co-receptor for CVB3 internalization. Thus, it would be interesting to evaluate whether dual inhibition of the expression of CAR and DAF could enhance the protective effect against CVB3 infection. Moreover, it may be appropriate to explore the efficacy of a combined approach based on the targeting of CVB3 as well as the CAR and DAF receptors.

Our group is in the process of developing novel strategies based on the downmodulation of CVB3 infection by targeting cellular gene(s) implicated both in the CVB3 life cycle and in the development of cardiac tissue remodeling. We have studied permissive (ABY/SnJ) and resistant (C57BL/6) mice infected with 10^5 plaque forming units of CVB3 Nancy strain. The animals were sacrificed 4, 8 and 28 h postinfection, the hearts were perfused and explanted, and total RNA was isolated and subjected to microchip analysis (Affymetrix; whole mouse genome). Based on the results, we have selected a pool of candidate genes related to the CVB3 life cycle and myocardial remodeling. We now plan to explore the effect of downregulation of the selected genes alone or in combination with a previously selected siRNA directed against protease 2A (61). For our approach, as well as any strategy based on targeting cellular genes, caution should be observed in terms of the biological consequences of downmodulating genes which, in addition to the regulation of the CVB3 life cycle, also have other biological functions.

Vascular-related studies

Complications of atherosclerosis are the most common cause of death in Western societies. The pharmacological lowering of blood LDL cholesterol has been shown to be protective with regard to atherosclerosis-associated morbidity and mortality. Nevertheless, alternative/complementary treatments targeting different aspects of the atherogenic process may significantly improve the outcome of patients. Blaschke *et al.* (70) reported that C-reactive protein (CRP) induces apoptosis in cultured human vascular smooth muscle cells (VSMCs). As the

presence of excessive apoptotic cells within the plaque renders the plaque itself more prone to rupture (71), it is evident that understanding the mechanisms regulating this process may contribute to the discovery of novel therapeutic approaches. In this regard, Blaschke *et al*. demonstrated that the proapoptotic effects of CRP are mediated through the protein growth arrest- and DNA damage-inducible gene 153 (*GADD153*). This finding was also substantiated by the use of an siRNA directed against *GADD153*. In cultured VSMCs transfected with 100 nM siRNA complexed with siPORT Lipid, the researchers demonstrated that, upon reduction of *GADD153* expression, the proapoptotic effect of CRP was reduced about 2-fold in comparison to controls. Besides providing a novel mechanism through which plaque rupture may occur, this work suggests the possibility that downregulation of CRP and/or *GADD153* expression may have therapeutic value in the stabilization of atherosclerotic plaques.

In addition to treatments aimed at downmodulating the atherosclerotic process, novel strategies are also required to treat the sequelae of atherosclerosis. A typical case is represented by the need to restore blood flow in coronary vessels occluded by atherosclerotic plaque. A possible approach is represented by coronary artery bypass grafting (CABG) using autologous saphenous veins. Unfortunately, this technique is plagued by 20% and 50% graft occlusion, respectively, 1 month and 10 years after intervention. This leads to vein graft occlusion and thus a new risk for myocardial damage. Intimal thickening of the graft, comparable to early lesions seen in atherosclerotic vessels, depends on different factors, among which endothelial cell dysfunction seems to play a pivotal role. In particular, it has been proposed that endothelial cells increase the expression of adhesion molecules, such as the intercellular adhesion molecules (ICAMs). ICAMs, in turn, induce leukocyte adherence and penetration, events which trigger intimal thickening and eventually graft occlusion. Walker *et al.* (72) designed an siRNA directed against ICAM-1 and tested it in human vein endothelial cells (HVECs) stimulated by TNF-α. At an siRNA concentration of 100 nM and using Cellfectin as the transfection agent, a decrease in ICAM expression to 13% of controls was observed, indicating the potential of the proposed approach, especially as the vein graft can be treated *ex vivo* after harvesting and before implantation, eliminating the problem of the *in vivo* delivery of siRNAs. Further animal experiments are needed to confirm the *in vitro* data and to demonstrate a reduction in intimal thickening and graft occlusion.

Another strategy followed to circumvent the problem of artery occlusion by atherosclerotic plaques involves the mechanical elimination of the obstruction by means of a balloon catheter introduced in the artery (percutaneous transluminal angioplasty, or PCTA) (73). Subsequently, a rigid scaffold, or stent, is placed at the site of intervention (Fig. 6). The stent can efficiently prevent the early arterial elastic recoil and late remodeling (restenosis) associated with PCTA (74). Stents, however, do not completely solve the problem of artery re-occlusion. Indeed, they induce particularly pronounced intimal hyperplasia (75) characterized by proliferation of VSMCs. Recently, the use of stents releasing antiproliferative drugs has substantially reduced VSMC proliferation, and consequently

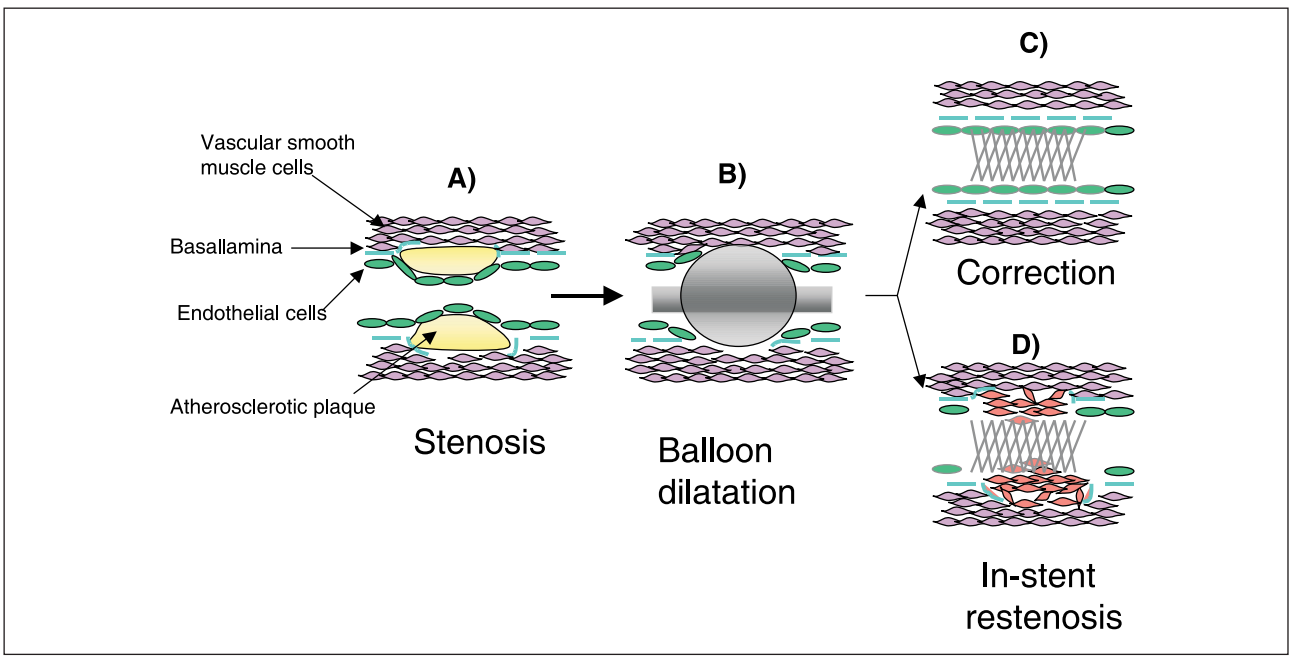

Fig. 6. In-stent restenosis. Stenotic arteries (**A**) are re-vascularized by balloon dilatation (**B**) and implantation of a rigid scaffold, or stent (**C**). Especially in diabetic patients, this procedure may be followed by re-occlusion of the vessel (in-stent restenosis) (**D**), mainly due to the hyperproliferation of vascular smooth muscle cells.

restenosis rates in single primary lesions in native coronary arteries (76, 77). Unfortunately, however, when drug-releasing stents have been applied to more challenging coronary lesions, such as lesions present in diabetic patients, success rates have been considerably lower (78, 79).

As an alternative to commonly used drugs, we are in the process of developing siRNAs directed against relevant cell cycle-promoting genes. In particular, we are focusing on the selection of siRNA directed against cyclin E1, cyclin E2 and the transcription factors E2F1 and serum response factor (SRF). Preliminary data obtained using siRNAs and ribozymes (80-83) suggest that these are amenable targets to downmodulate VSMC growth *in vitro.* Further studies will examine efficacy *in vivo* and explore the biological consequences of their downregulation, in addition to downmodulation of cell proliferation.

Recently, we prepared an *in vitro* model to test the ability of active siRNAs to counteract the activation of porcine coronary smooth muscle cells (84) or to identify factors that promote activation (85).

The possibility of constructing vectors for the expression of siRNAs targeted against more than one gene would be a further challenge in RNAi applications. All the isoforms of the same gene or a cluster of genes involved in the same pathway could be knocked out at once, with a conceivable increase in efficiency. We demonstrated the feasibility of the multicopy siRNA cassette delivery approach and its efficacy in achieving optimal gene knockout efficiency at different levels of target mRNA gene expression (86).

Conclusions

Since the demonstration of the ability of siRNAs to downregulate gene expression in mammalian cells, many potential therapeutic applications have been explored. Among these, cardiovascular applications represent an emerging field still in its infancy. The limited number of siRNA-based studies in this area may be attributed to different factors, including the lack of reliable *in vitro* models of the pathological conditions and the lack of ideal delivery systems. In the latter case, the problem may be overcome by linking the sRNAs to appropriate carriers able to efficiently direct the siRNA to the target cell. Despite these problems, studies reported indicate the potential relevance of siRNA both for the correction of cardiovascular pathologies and for elucidating the mechanisms involved in such pathologies. In our opinion, the siRNA approach may be of particular interest in the treatment of coxsackievirus myocarditis. The data reported so far clearly indicate the potency of protease 2A-directed siRNAs in inhibiting CVB3 replication. Efforts are now required to completely eliminate the virus from the infected organs either by prolonging the action of the siRNAs or by finding additional targets for further downregulating the CVB3 life cycle. The application of siRNAs to improve the outcome of CABG and PCTA/stent implantation used to re-vascularize occluded vessels is also attractive. In both cases, the efficacy of the selected siRNAs and the possibility of local delivery may substantially simplify siRNA-based applications.

In conclusion, whereas the potential applications of siRNA in cardiovascular pathologies are presently limited compared to other human diseases, studies suggest that siRNAs may contribute to the development of novel therapeutic approaches in the cardiovascular field.

Acknowledgements

This work was supported in part by the Dr. Karl-Kuhn-Award and *f*ortuene program (project no. 1271), University of Tuebingen (SFB/TR19-04), by the "Fondazione Cassa di Risparmio of Trieste", the "Fondazione per il Sostegno delle Strutture Cardiovascolari, Mirano", the "Fondazione Casali of Trieste" and the Fondo Trieste 2006. G. Grassi is supported by the program "Rientro cervelli" art. 1 DM n.13, MIUR (Ministero dell'Istruzione, dell'Università e della Ricerca Scientifica").

References

1. Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C. *Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans*. Nature 1998, 391: 806-11.

2. Hannon, G.J., Rossi, J.J. *Unlocking the potential of the human genome with RNA interference*. Nature 2004, 431: 371-8.

3. Meister, G., Tuschl, T. *Mechanisms of gene silencing by double-stranded RNA*. Nature 2004, 431: 343-9.

4. Ambros, V. *The functions of animal microRNAs*. Nature 2004, 431: 350-5.

5. Matzke, M.A., Birchler, J.A. *RNAi-mediated pathways in the nucleus*. Nat Rev Genet 2005, 6: 24-35.

6. Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T. *Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells*. Nature 2001, 411: 494-8.

7. Martinez, L.A., Naguibneva, I., Lehrmann, H. et al. *Synthetic small inhibiting RNAs: Efficient tools to inactivate oncogenic mutations and restore p53 pathways*. Proc Natl Acad Sci USA 2002, 99: 14849-54.

8. Smolic, R., Volarevic, M., Wu, C.H., Wu, G.Y. *Potential applications of siRNA in hepatitis C virus therapy*. Curr Opin Invest Drugs 2006, 7: 142-6.

9. Sledz, C.A., Williams, B.R. *RNA interference in biology and disease*. Blood 2005, 106: 787-94.

10. Uprichard, S.L. *The therapeutic potential of RNA interference*. FEBS Lett 2005, 579: 5996-6007.

11. Zentilin, L., Giacca, M. *In vivo transfer and expression of genes coding for short interfering RNAs*. Curr Pharm Biotechnol 2004, 5: 341-7.

12. Poliseno, L., Evangelista, M., Mercatanti, A., Mariani, L., Citti, L., Rainaldi, G. *The energy profiling of short interfering RNAs is*

highly predictive of their activity. Oligonucleotides 2004, 14: 227-32.

13. Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W.S., Khvorova, A. *Rational siRNA design for RNA interference*. Nat Biotechnol 2004, 22: 326-30.

14. Hammond, S.M. *Dicing and slicing: The core machinery of the RNA interference pathway*. FEBS Lett 2005, 579: 5822-9.

15. Novina, C.D., Sharp, P.A. *The RNAi revolution*. Nature 2004, 430: 161-4.

16. Sharp, P.A. *RNA interference – 2001*. Genes Dev 2001, 15: 485-90.

17. Elbashir, S.M., Lendeckel, W., Tuschl, T. *RNA interference is mediated by 21- and 22-nucleotide RNAs*. Genes Dev 2001, 15: 188-200.

18. Lee, S.H., Sinko, P.J. *siRNA – Getting the message out*. Eur J Pharm Sci 2006, 27: 401-10.

19. Rozema, D., Lewis, D. *siRNA delivery technologiesa for mammalian systems*. Targets 2003, 2: 253-60.

20. Chen, D., Murphy, B., Sung, R., Bromberg, J.S. *Adaptive and innate immune responses to gene transfer vectors: Role of cytokines and chemokines in vector function*. Gene Ther 2003, 10: 991-8.

21. Luo, D., Saltzman, W.M. *Synthetic DNA delivery systems*. Nat Biotechnol 2000, 18: 33-7.

22. McCaffrey, A.P., Meuse, L., Pham, T.T., Conklin, D.S., Hannon, G.J., Kay, M.A. *RNA interference in adult mice*. Nature 2002, 418: 38-9.

23. Liu, F., Song, Y., Liu, D. *Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA*. Gene Ther 1999, 6: 1258-66.

24. Ludtke, J.J., Zhang, G., Sebestyen, M.G., Wolff, J.A. *A nuclear localization signal can enhance both the nuclear transport and expression of 1 kb DNA*. J Cell Sci 1999, 112(Pt. 12): 2033-41.

25. Chollet, P., Favrot, M.C., Hurbin, A., Coll, J.L. *Side-effects of a systemic injection of linear polyethylenimine-DNA complexes*. J Gene Med 2002, 4: 84-91.

26. Pun, S.H., Davis, M.E. *Development of a nonviral gene delivery vehicle for systemic application*. Bioconjug Chem 2002, 13: 630-9.

27. Decher, G. *Fuzzy nanoassemblies: Toward layered polymeric multicomposites*. Science 1997, 277: 1232-7.

28. Heyes, J., Palmer, L., Bremner, K., MacLachlan, I. *Cationic lipid saturation influences intracellular delivery of encapsulated nucleic acids*. J Control Release 2005, 107: 276-87.

29. Heidel, J.D., Hu-Lieskovan, S., Bartlett, D.W., Triche, T.J., Davis, M.E. *Systemic administration of siRNA against EWS-FLI1 using a targeted, non viral formulation inhibits growth in a disseminated murine model of Ewing's sarcoma*. Mol Ther 2005, 11: S28.

30. Schiffelers, R.M., Mixson, A.J., Ansari, A.M. et al. *Transporting silence: Design of carriers for siRNA to angiogenic endothelium*. J Control Release 2005, 109: 5-14.

31. Kim, S.H., Jeong, J.H., Cho, K.C., Kim, S.W., Park, T.G. *Target-specific gene silencng by siRNA plasmid DNA complexed with folate-modified poly(ethylenimine)*. J Control Release 2005, 104: 223-32.

32. Kushibiki, T., Nagata-Nakajima, N., Sugai, M., Shimizu, A., Tabata, Y. *Delivery of plasmid DNA expressing small interference RNA for TGF-beta type II receptor by cationized gelatin to prevent interstitial renal fibrosis*. J Control Release 2005, 105: 318-31.

33. Moriguchi, R., Kogure, K., Akita, H. et al. *A multifunctional envelope-type nano device for novel gene delivery of siRNA plasmids*. Int J Pharm 2005, 301: 277-85.

34. Woodle, M.C., Lu, P.Y. *Nanoparticles deliver RNAi therapy*. Nanotoday 2005, 97: 345-56.

35. Kakizawa, Y., Furukawa, S., Kataoka, K. *Block copolymer-coated calcium phosphate nanoparticles sensing intracellular environment for oligodeoxynucleotide and siRNA delivery*. J Control Release 2004, 97: 345-56.

36. Farra, R., Voinivic, D., Rubessa, F. et al. *Characterization of liposomes for the delivery of siRNAs in the treatment of coronary restenosis*. Eur J Pharm Sci 2005, 25(Suppl. 1): S99.

37. Grassi, G., Crevatin, A., Farra, R. et al. *Rheological properties of aqueous Pluronic-Alginate systems containing liposomes*. J Colloid Interface Sci 2006, In press.

38. Soutschek, J., Akinc, A., Bramlage, B. et al. *Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs*. Nature 2004, 432: 173-8.

39. Lorenz, C., Hadwiger, P., John, M., Vornlocher, H.P., Unverzagt, C. *Steroid and lipid conjugates of siRNAs to enhance cellular uptake and gene silencing in liver cells*. Bioorg Med Chem Lett 2004, 14: 4975-7.

40. Watanabe, A., Arai, M., Yamazaki, M., Koitabashi, N., Wuytack, F., Kurabayashi, M. *Phospholamban ablation by RNA interference increases Ca^{2+} uptake into rat cardiac myocyte sarcoplasmic reticulum*. J Mol Cell Cardiol 2004, 37: 691-8.

41. MacLennan, D.H., Kranias, E.G. *Phospholamban: A crucial regulator of cardiac contractility*. Nat Rev Mol Cell Biol 2003, 4: 566-77.

42. Vangheluwe, P., Raeymaekers, L., Dode, L., Wuytack, F. *Modulating sarco(endo)plasmic reticulum Ca^{2+} ATPase 2 (SERCA2) activity: Cell biological implications*. Cell Calcium 2005, 38: 291-302.

43. Meyer, M., Schillinger, W., Pieske, B. et al. *Alterations of sarcoplasmic reticulum proteins in failing human dilated cardiomyopathy*. Circulation 1995, 92: 778-84.

44. Seth, M., Sumbilla, C., Mullen, S.P. et al. *Sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) gene silencing and remodeling of the Ca^{2+} signaling mechanism in cardiac myocytes*. Proc Natl Acad Sci USA 2004, 101: 16683-8.

45. Hunton, D.L., Lucchesi, P.A., Pang, Y., Cheng, X., Dell'Italia, L.J., Marchase, R.B. *Capacitative calcium entry contributes to nuclear factor of activated T-cells nuclear translocation and hypertrophy in cardiomyocytes*. J Biol Chem 2002, 277: 14266-73.

46. Hurtado, C., Ander, B.P., Maddaford, T.G., Lukas, A., Hryshko, L.V., Pierce, G.N. *Adenovirally delivered shRNA strongly inhibits Na^{+}-Ca^{2+} exchanger expression but does not prevent contraction of neonatal cardiomyocytes*. J Mol Cell Cardiol 2005, 38: 647-54.

47. Natarajan, R., Salloum, F.N., Fisher, B.J., Kukreja, R.C., Fowler, A.A., III. *Hypoxia inducible factor-1 activation by prolyl 4-hydroxylase-2 gene silencing attenuates myocardial ischemia reperfusion injury*. Circ Res 2006, 98: 133-40.

48. Yin, C., Xi, L., Wang, X., Eapen, M., Kukreja, R.C. *Silencing heat shock factor 1 by small interfering RNA abrogates heat shock-induced cardioprotection against ischemia-reperfusion injury in mice*. J Mol Cell Cardiol 2005, 39: 681-9.

49. Davidson, S.M., Stephanou, A., Latchman, D.S. *FLIP protects cardiomyocytes from apoptosis induced by simulated ischemia/reoxygenation, as demonstrated by short hairpin-induced (shRNA) silencing of FLIP mRNA*. J Mol Cell Cardiol 2003, 35: 1359-64.

50. Dec, G.W., Fuster, V. *Idiopathic dilated cardiomyopathy*. New Engl J Med 1994, 331: 1564-75.

51. Sole, M.J., Liu, P. *Viral myocarditis: A paradigm for understanding the pathogenesis and treatment of dilated cardiomyopathy*. J Am Coll Cardiol 1993, 22: 99A-105A.

52. Kandolf, R., Ameis, D., Kirschner, P., Canu, A., Hofschneider, P.H. *In situ detection of enteroviral genomes in myocardial cells by nucleic acid hybridization: An approach to the diagnosis of viral heart disease*. Proc Natl Acad Sci USA 1987, 84: 6272-6.

53. Kandolf, R., Klingel, K., Mertsching, H. et al. *Molecular studies on enteroviral heart disease: Patterns of acute and persistent infections*. Eur Heart J 1991, 12(Suppl. D): 49-55.

54. Klingel, K., Rieger, P., Mall, G., Selinka, H.C., Huber, M., Kandolf, R. *Visualization of enteroviral replication in myocardial tissue by ultrastructural in situ hybridization: Identification of target cells and cytopathic effects*. Lab Invest 1998, 78: 1227-37.

55. Klingel, K., Hohenadl, C., Canu, A. et al. *Ongoing enterovirus-induced myocarditis is associated with persistent heart muscle infection: Quantitative analysis of virus replication, tissue damage, and inflammation*. Proc Natl Acad Sci USA 1992, 89: 314-8.

56. Ayach, B., Fuse, K., Martino, T., Liu, P. *Dissecting mechanisms of innate and acquired immunity in myocarditis*. Curr Opin Cardiol 2003, 18: 175-81.

57. Mason, J.W., O'Connell, J.B., Herskowitz, A. et al. *A clinical trial of immunosuppressive therapy for myocarditis. The Myocarditis Treatment Trial Investigators*. New Engl J Med 1995, 333: 269-75.

58. Klingel, K., Schnorr, J.J., Sauter, M., Szalay, G., Kandolf, R. *Beta2-microglobulin-associated regulation of interferon-gamma and virus-specific immunoglobulin G confer resistance against the development of chronic coxsackievirus myocarditis*. Am J Pathol 2003, 162: 1709-20.

59. Yuan, J., Cheung, P.K., Zhang, H.M., Chau, D., Yang, D. *Inhibition of coxsackievirus B3 replication by small interfering RNAs requires perfect sequence match in the central region of the viral positive strand*. J Virol 2005, 79: 2151-9.

60. Ahn, J., Jun, E.S., Lee, H.S. et al. *A small interfering RNA targeting coxsackievirus B3 protects permissive HeLa cells from viral challenge*. J Virol 2005, 79: 8620-4.

61. Racchi, G., Klingel, K., Kandolf, R., Rainaldi, G., Mercatanti, A., Grassi, G. *siRNAs targeted against coxsackieviruses B3 genome down regulate virus proliferation thus showing their potential use in the prevention of viral myocarditis*. 13th Annu Cong Eur Soc Gene Ther (Oct 29-Nov 1, Prague) 2005.

62. Merl, S., Michaelis, C., Jaschke, B., Vorpahl, M., Seidl, S., Wessely, R. *Targeting 2A protease by RNA interference attenuates coxsackieviral cytopathogenicity and promotes survival in highly susceptible mice*. Circulation 2005, 111: 1583-92.

63. Rueckert, R.R. *The enteroviruses.* In: Fundamental Virology. Fields, B.N., Knipe, D.M., Howley, P.M. (Eds.). New York, Raven Press, 1996, 477-522.

64. Krausslich, H.G., Nicklin, M.J., Toyoda, H., Etchison, D., Wimmer, E. *Poliovirus proteinase 2A induces cleavage of eukaryotic initiation factor 4F polypeptide p220*. J Virol 1987, 61: 2711-8.

65. Kerekatte, V., Keiper, B.D., Badorff, C., Cai, A., Knowlton, K.U., Rhoads, R.E. *Cleavage of poly(A)-binding protein by coxsackievirus 2A protease in vitro and in vivo: Another mechanism for host protein synthesis shutoff?* J Virol 1999, 73: 709-17.

66. Badorff, C., Lee, G.H., Lamphear, B.J. et al. *Enteroviral protease 2A cleaves dystrophin: Evidence of cytoskeletal disruption in an acquired cardiomyopathy*. Nat Med 1999, 5: 320-6.

67. Liu, P.P., Mason, J.W. *Advances in the understanding of myocarditis*. Circulation 2001, 104: 1076-82.

68. Xiong, D., Lee, G.H., Badorff, C. et al. *Dystrophin deficiency markedly increases enterovirus-induced cardiomyopathy: A genetic predisposition to viral heart disease*. Nat Med 2002, 8: 872-7.

69. Werk, D., Schubert, S., Lindig, V. et al. *Developing an effective RNA interference strategy against a plus-strand RNA virus: Silencing of coxsackievirus B3 and its cognate coxsackievirus-adenovirus receptor*. Biol Chem 2005, 386: 857-63.

70. Blaschke, F., Bruemmer, D., Yin, F. et al. *C-reactive protein induces apoptosis in human coronary vascular smooth muscle cells*. Circulation 2004, 110: 579-87.

71. Bennett, M.R. *Apoptosis of vascular smooth muscle cells in vascular remodelling and atherosclerotic plaque rupture*. Cardiovasc Res 1999, 41: 361-8.

72. Walker, T., Wendel, H.P., Tetzloff, L., Heidenreich, O., Ziemer, G. *Suppression of ICAM-1 in human venous endothelial cells by small interfering RNAs*. Eur J Cardiothorac Surg 2005, 28: 816-20.

73. Califf, R.M. *Restenosis: The cost to society*. Am Heart J 1995, 130: 680-4.

74. Labinaz, M., Pels, K., Hoffert, C., Aggarwal, S., O'Brien, E.R. *Time course and importance of neoadventitial formation in arterial remodeling following balloon angioplasty of porcine coronary arteries*. Cardiovasc Res 1999, 41: 255-66.

75. Kearney, M., Pieczek, A., Haley, L. et al. *Histopathology of in-stent restenosis in patients with peripheral artery disease*. Circulation 1997, 95: 1998-2002.

76. Morice, M.C., Serruys, P.W., Sousa, J.E. et al. *A randomized comparison of a sirolimus-eluting stent with a standard stent for coronary revascularization*. New Engl J Med 2002, 346: 1773-80.

77. Colombo, A., Drzewiecki, J., Banning, A. et al. *Randomized study to assess the effectiveness of slow- and moderate-release polymer-based paclitaxel-eluting stents for coronary artery lesions*. Circulation 2003, 108: 788-94.

78. Colombo, A., Moses, J.W., Morice, M.C. et al. *Randomized study to evaluate sirolimus-eluting stents implanted at coronary bifurcation lesions*. Circulation 2004, 109: 1244-9.

79. Holmes, D.R., Jr., Leon, M.B., Moses, J.W. et al. *Analysis of 1-year clinical outcomes in the SIRIUS trial: A randomized trial of a sirolimus-eluting stent versus a standard stent in patients at high risk for coronary restenosis*. Circulation 2004, 109: 634-40.

80. Grassi, G., Dapas, R., Farra, R. et al. *siRNAs targeted against cyclin E1 and E2F1 down regulate coronary smooth muscle cells proliferation thus showing their potential use in the prevention of in-stent restenosis*. 13th Annu Cong Eur Soc Gene Ther (Oct 29-Nov 1, Prague) 2005, Abst p004.

81. Werth, D., Heidenreich, O., Nordheim, A., Herdeg, C., Grassi, G. *siRNAs targeting serum response factor as potential tools to interfere with in-stent restenosis*. 12th Annu Cong Eur Soc Gene Ther (Nov 4-7, Tampere) 2004, Abst P 163.

82. Grassi, G., Schneider, A., Engel, S., Racchi, G., Kandolf, R., Kuhn, A. *Hammerhead ribozymes targeted against cyclin E and E2F1 co-operate to down regulate coronary smooth muscle cells proliferation*. J Gene Med 2005, 7: 1223-4.

83. Grassi, G., Dawson, P., Guarnieri, G., Kandolf, R., Grassi M. *Therapeutic potential of hammerhead ribozymes in the treatment of hyper-proliferative diseases*. Curr Pharm Biotechnol 2004, 5: 369-86.

84. Poliseno, L., Evangelista, M., Ricci, F., Bonotti, A., Nannipieri, M., Rainaldi, G. *Identification of active siRNAs against IGF-IR of porcine coronary smooth muscle cells in a heterologous cell line*. Int J Mol Med 2005, 15: 713-8.

85. Poliseno, L., Cecchettini, A., Mariani, L. et al. *Resting smooth muscle cells as a model for studying vascular cell activation*. Tissue Cell 2006, 38: 111-20.

86. Poliseno, L., Evangelista, M., Giacca, M., Rainaldi, G. *The analysis of dose response curve cimes is useful for the assembly of multi-siRNAs expressing cassettes*. Gene Ther Mol Biol 2006, 10: 101-8.