24

Gene-Silencing for Treatment of Cardiovascular Diseases

A. Nolte, M. Schneider, T. Walker and H. P. Wendel
Clinical Research Laboratory, Department of Congenital and Paediatric Cardiac Surgery, University Children´s Hospital Tuebingen, Tuebingen Germany

1. Introduction

In the last decade, great advances in RNA biology have been achieved. Micro- and short-interfering RNAs were once thought to be degradation products of larger RNA molecules. With the knowledge of today we know that they represent independent classes of small noncoding (~ 20-30 nt) RNAs regulating various cellular processes across the eukaryotes. The first miRNA lin4 was discovered in the Nematode *C. elegans* in the year 1993, when Lee et al. [1] demonstrated that this transcript works as an endogenous regulator of genes that control developmental timing. Five years later Fire et al. [2] showed the ability of exogenous double stranded RNA to targeted posttranscriptional gene silencing, which recognizes each target by Watson-Crick base pairing. This scientific breakthrough was awarded with the novel price 2007.

The RNA interference (RNAi) is a naturally happening catalytic process, offering the possibility to silence every pathological interesting gene which is defective expressed in a given disease. Thus it will give new approaches in the development and applications of siRNA-based therapeutics.

Since cardiovascular diseases (CVDs) are the main cause of mortality and morbidity in the Western world this chapter will discuss possible applications of si- and miRNA-based therapeutics and delivery systems with special regard to atherosclerosis, ischemic heart-disease and hypertension.

2. Molecular mechanism of RNAi

2.1 siRNA pathway

SiRNAs are ~ 20-25 nt regulatory molecules that play a primitive role in the defence against any foreign nucleic acid molecule derived from viruses or tranposons to preserve genome integrity. This hypothesis is supported by a study that demonstrates the passive uptake of siRNAs trough a membrane receptor protein called Systemic RNA Interference – Defective 1 (SID-1) [3].

RNAi is induced by linear, long perfectly complementary dsRNA which is directly introduced in the cytoplasm or is taken up by the environment [4]. First the dsRNA is processed by the RNase III-type endonuclease Dicer in 21-23 bp long siRNA duplexes that have 3´overhangs and 5`phosphates. In general Dicer posseses six domains including DExH

Helicase, DUF283, PAZ, RNAse IIIa, RNAse IIIb and RNA Binding Domain (RBD). The PAZ domain (Piwi, Argonaute, Zwille) binds to the 3'-nt overhangs of the cleaved RNA substrate while RBD recognizes duplex structures of RNA [5]. The next step is the incorporation of the duplex siRNAs into the nuclease-containing multiprotein complex RISC (RNA-induced silencing complex). The duplex is then unwound by the helicase activity (PAZ-domain) of the nuclease Ago2 which activates RISC. The unwinding process starts from the end of the siRNA with lower thermal energy. One strand called guide strand remains within the RISC, while the passenger strand is degraded by exonucleases [6]. The 5`end of the guide strand contains the seed region between the ribonucleotides at position 2-7 and are responsible for governing the RISC to its binding to target sequences [7]. Then the guide strand activates the RNase activity (PIWI domain) of Ago 2 which cleaves the complementary target mRNA. As result of these two unprotected mRNA ends are built, that are fast degraded by intracellular nucleases. This gene silencing by mRNA cleavage is an effective and catalytic process due to the fact that the activated RISC is freed after one round and is ready for further destruction of target mRNAs resulting in target repression [8] (See also Figure 1).

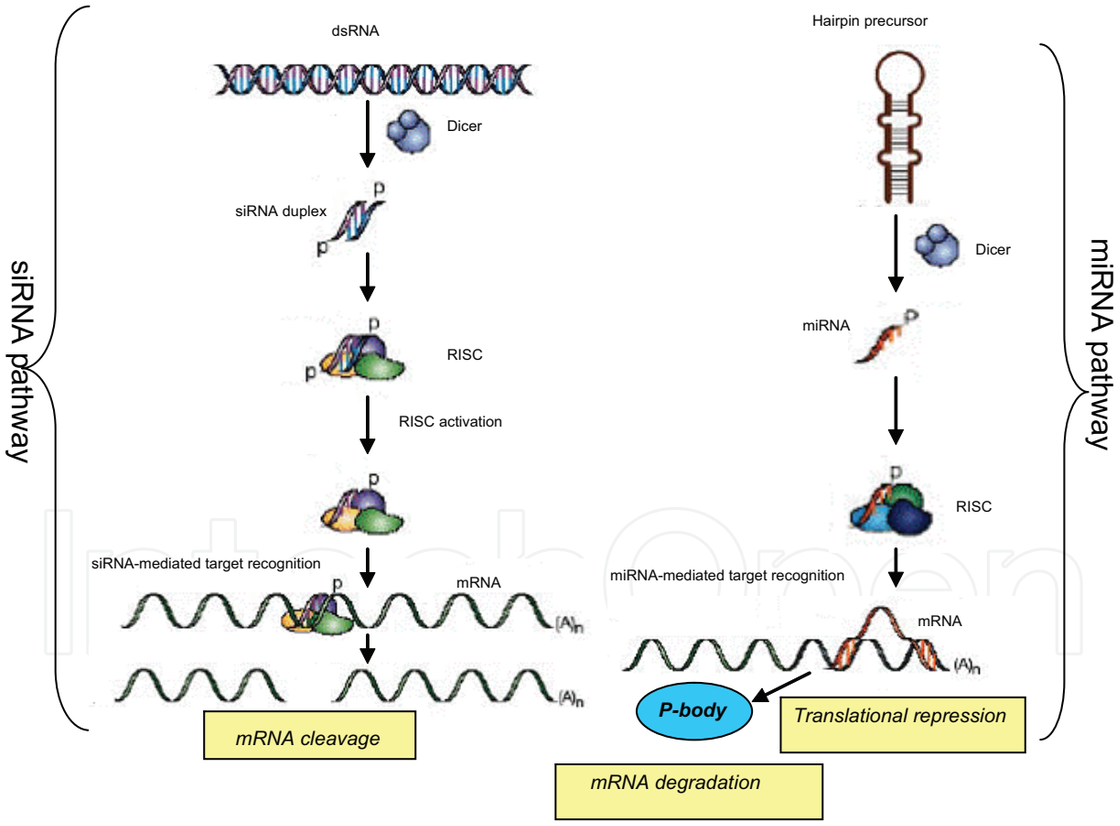

Fig. 1. Mechanism of RNA interference (RNAi) in mammalian systems

2.2 miRNA pathway

MiRNAs are ~22 nt long single-stranded RNAs with endogenous distinct that serve as regulators for proteins that repress gene expression [9]. Initially their precursors (pri-

miRNAs) are transcribed by the RNA polymerase II as single-stranded transcript that contain a local hairpin structure and also possess 5`cap and poly-A tail [10]. Secondly the pri-miRNAs are processed by the Drosha/Pasha complex (RNAse III) and they are excised as stem-loops (pre-miRNAs). Some pri-miRNAs are originated from introns and are called mitrons. They are generated by the pre-miRNA splicing machinery rather than by Drosha [11]. The pre-miRNA are then transported out of the nucleus in the cytoplasm and this transport is mediated by Exportin 5 and Ran-GTPase [12]. Subsequently the pre-miRNA is processed and shortened by Dicer to generate imperfectly-matched ds miRNA. Then the duplex miRNA is loaded on the Ago2-RISC complex and in case of imperfect sequence complementary the passenger strand is unwound by Ago2 producing a mature miRNA that is bound to active RISC.
The repression of the mature miRNA to genetic processes is made on two different levels: it recognizes and binds the 3`UTR of target mRNAs blocking translation or this binding results in mRNA degradation in special processing bodies, termed p-bodies [13, 14] (See also Figure 1).

3. Delivery systems and designs of siRNA- and miRNA-based therapeutics

According to its large molecular weight (~13 kDa) and high content of anionic charge (~40 negative phosphate charge), unmodified naked siRNA does not freely enter cell membranes. Therefore delivery systems are needed to give an access for the nucleic acids to its intracellular sites of actions since all other contributing parts of the RNAi based machinery are provided by the target cell / target tissue [39, 75]. Another critical hurdle to overcome is the low biological stability of nucleic acids under physiological conditions, which is the topic of the following passage.

3.1 Chemical modifications of siRNA: improving biological stability

Both single-stranded nucleic acids and double-stranded nucleic acids are relatively fast degraded trough nuclease attack if they are applied in unmodified and naked form. These finding indicates that they have short half-lives in blood and serum *in vivo* because of the activity of endo-and exonucleases [15]. To avoid the rapidly degradation there have been made efforts in the chemical modifications of siRNAs including strategies that improve the cellular uptake due to the conjugation with cholesterol, peptides, aptamers or antibodies.
These chemical modifications mainly focus on the backbone, base or sugar of the RNA. One strategy is to modify the 2`-position of the ribose enhancing duplex stability (Tm) and nuclease resistance of the phosphodiester bond between the nucleotides against hydrolysis. The 2`O-Methyl-modification (2`OMe) is a naturally found RNA which is untoxic but can implement the potency of the siRNA. To undergo this effect an alternation of 2`OMe with RNA or other 2`modifications can be used so that siRNA function and nuclease stability is preserved [16,17]. Another well studied 2`modification is the 2`fluoro (2`-F) modification at pyrimidine positions which show compliance with the siRNA potency and stabilisation of the duplex against nuclease degradation in vitro [18] and in vivo [19]. Morrissey et al [20] demonstrated that a combination of 2`OMe purines with 2`-F pyrimidines can generate RNA duplexes that are highly stable in serum and have good in vivo performance. The modification of internucleotide phosphate linkage through the replacement of the non-bridging oxygen with sulfur is also an efficient approach to

improve nuclease stability without reducing the potency of siRNA. This was revealed through several studies [21-23].
Furthermore the covalent conjugation of siRNA with various chemical groups can direct cell uptake and alter biodistribution. The attachment of hydrophobic ligands like chosterol is a prominent example to extend serum lifetime. Soutschek et al. demonstrated in a mice model that i.v. administration of anti-ApoB siRNA that contained two 2`OMe RNA residues, phosphorthioate 3`-end modification and a cholesterol group resulted in silencing of the apolipoprotein B gene and reduced total cholesterol and plasma levels of apoB protein [24]. These data suggest that chemical modifications are a useful tool to improve stability and cellular uptake of siRNAs in general.

3.2 Off-targets: activation of the innate immune system

In the design and selection of a siRNA sequence their effect on innate immune system has also to be considered. It have been shown that the introduction of too long ds RNA molecules (>30 nt) can initiate an antiviral IFN-response of the cell or can activate a cellular pathway that involves the serin/threonine protein kinase PKR [25, 26]. A suppression of these effects could be the use of smaller (21-23 nt) siRNAs and additional in vitro tests or BLAST search of desired siRNA sequences to avoid further immune responses. SiRNAs have also the potential to interfere with the innate immune system due to the activation of Toll-like receptors (TLRs). Especially TLR 3, 7 and 8 are expressed in endosomal compartments and exposure to these receptors is affected by strength of the entry of synthetic RNA enters the cell. TLR 3 recognizes motifs in ssRNA and TLR 7 and 8 notice motifs in dsRNA. TLR 7 is binding favourable to GU-rich sequences and selecting of sequences that are not recognized by this receptor will help to solve this problem [13]. Moreover it has been demonstrated that the use of chemical modification like 2`OMe can prevent the detection of siRNAs by the immune system. The study of Judge et al. showed that incorporation of two or three 2`OMe residues in a siRNA duplex can be enough to evade immune detection [27]. Another category of off-targets are the cross-reactions between the si- and mi-RNA-pathway. If one of the siRNA strands have partly complementary regions with the 3`UTR of not targeted mRNA it can result in imperfect base-pairing of guide strand and "target" strand. This will end in translational repression or exonucleolytic degradation in a way similar like miRNA silencing [28]. This mismatch effect would be avoided if the seed-region of the guide strand between the 2 and 8 nucleotid position (5`end) is fully complementary with the target mRNA sequence. Thus homology screenings of seed regions of siRNA in the 3`UTR of all interfering and target genes would be useful [29].

3.3 In vivo delivery systems

Beyond the importance of effective design of siRNA to achieve specific and potent target gene silencing, the therapeutic use of siRNA need reliable delivery into tissues and target cells. Due to the fact that small RNA molecules like si- and miRNA have a strong anionic charge of their backbone consisting of phosphodiesters and they have a considerable molecular weight it is not possible that they can simply enter the cell membrane by passive diffusion. This lead to the development of two main methods of delivery including viral and nonviral strategies, which have been already successfully applied *in vitro* and *in vivo*. Although viral transfection in patients is regarded very critically, the next paragraph should give a small overview of this technique.

Viral delivery sytems	Nonviral delivery systems
• Viral vectors: • Adenovirus, adenovirus-associated virus, retrovirus (e.g. lentivirus)	• Systemic delivery *in vivo*: cationic delivery systems (e.g. PEI, Acetocollagen, Chitosan) cationic liposomal delivery systems (e.g. *in vitro*: Lipofectamin, e.g. *in vivo*: SNALPs) • Local delivery strategies *in vivo*

Table 1. Overview of following discussed delivery systems

3.3.1 Viral delivery systems

Viral vectors commonly consists of nucleic acids (shRNAs or miRNA mimics) that are incorporated in the backbone of the virus genome and are induced through a Pol II or Pol III promoter. They are surrounded by a viral capsid of proteins that usually interact with ECM-molecules to enter the cell by membrane fusion or receptor binding [31]. The gene transfer with viral vectors is a highly effective method because only one application is able to silence the target gene for a long time *in vivo* [32]. But despite the great potency of viral vectors for delivery, one has to consider that threats like host immune responses, potential oncogen and mutagen properties can occur. The common used viral vectors are adenovirus, adenovirus-associated virus (AAV) and retrovirus [31]. Especially lentiviral vectors, a class of retroviruses show great potential of infecting a wide variety of non-dividing and dividing cells, stable integration in host genome, and results in long term expression of the transgene [32]. This kind of vector are of great interest for the injection in non-dividing cells like neurons and are suitable in the therapy of brain specific diseases or expression of shRNAs targeting disease associated genes [33, 34]. A possible target of shsiRNA could be in the case of a neurodegenerative disease like alzheimer, the γ-secretase BACE 1 which generates the toxic amyloid ß-peptid. This was demonstrated in a Alzheimer mouse model with reduction of endogenous levels of BACE1 by the use of a lentiviral vector expressing shRNA [35]. Adenoviral vectors have also been used for delivery of a shRNA in the brain directed against the mRNA expressing the polyQ-harbouring SCA-1 encoding transcript of spinocerebellar ataxia type 1 [36].

Another field of treatment would be chronic viral infections like chronic hepapitis b that need long-term treatment. Kim et al. demonstrated the *in vitro* lentivirus-mediated delivery of shRNA against HBx can effectively suppress the replication of HBV and reduce HBV covalently closed circular DNA [37]. Nevertheless, the safety issue remains a parameter in the decision in the use of a viral vector.

3.3.2 Nonviral delivery systems

There have been made many investigations for the use of nonviral delivery systems, including formulations like cationic polymers, cationic lipids, antibodies, conjugates or naked siRNA. The first consideration of the choice of a delivery strategy must be if a local or systemic administration is needed for the intended disease target.

Systemic delivery strategies in vivo

Cationic delivery systems

All cationic vectors share the ability to form complexes with the polyanionic nucleic acids through electrostatic interactions. Another possibility is the formation of nanoparticles in which the nucleic acid is entrapped within the particle through ionic interactions. The advantage of these formulations is that the siRNAs are not reachable for nucleases until it reaches its destination through vesicular transport (endocytosis and/ or macropinocytosis). Nanoparticles and complexes must have a limited size of 100 nm to be taken up by cells and to suppress renal excretion [38].

Cationic polymer delivery systems of siRNA

Cationic polymers that have been applied include both natural (e.g., acetocollagen, chitosan) and synthetic (e.g, polyethylenimine). Polyetylenimine (PEI) is one of the best investigated polymers for nucleic acid delivery [39].

The polymer is linear or branched and has high cationic charge density.

The PEI-siRNA complexes are endocytosed in the cell through electrostatic interactions where it escapes the endosome through the "proton-sponge-effect".

PEI has the ability to accept protons which results in a buffering-effect in the vesicular system and a subsequent influx of Cl- and water leading to a osmotic release of the complexes in the cytoplasm [40]. PEI is available in different weights, but low-molecular PEI is favourable because high molecular weight is highly toxic [41]. The linear 22kDa jetPEI was successfully used in a antiviral therapy of guinea pigs against a lethal Ebola virus [42]. PEI-siRNA complexes have also been effective applied as a antiviral agent in a murine model of influenza infection [43]. PEI-siRNA complexes have also been efficient targeted against many proteins like HER2 [44], VEGF [45] and many more [39].

Acetocollagen is a natural polymer which is produced through pepsin treatment of type I collagen of calf dermis [46]. Acetocollagen/siRNA-mediated targeting have been demonstrated in the treatment of prostata and pancreatic cancer as well as virus replication [47, 48, 49]. Another well-tolerated natural polymer is chitosan that builds cationic complexes with siRNAs. Howard et al. showed effective RNAi delivery in epithelial cells in the lung of mice through the intrasal application of transgenic enhanced green fluorescent protein (EGFP) [50].

Cationic liposomal delivery systems

Cationic liposomes and lipoplexes have been successfully applied for in vitro and in vivo delivery of siRNA. Liposomes are vesicels that can improve drug delivery through their fusion with cell membranes and they consist of a soluable compartment enclosed in a bilayer of phosholipids in which polar drugs can be entrapped. Lipoplexes are particles that are built through a complex formation of nucleic acids and lipids. The advantage of lipid formulations is the increase of siRNA stability in serum and the protection to overcome renal excretion, but unfortunately cationic liposomes can interfere with proteins like lipoproteins or serum proteins which can result in off effects as aggregation. For in vitro use there are many commercial available formulations like Oligofectamine, Lipofectamine (Invitrogen), RNAifect (Qiagen). An example of liposomes is SNALP (stable nucleic acid lipid particles) that have been efficient used in the *in vivo* silencing of the apoB gene in mice and primates [51]. The silencing effect of only one intravenous dose of 2.5 mg per kg SNALP-formulated siRNA lasted for 11 days and showed a reduction of 90% of the apoB mRNA.

Local delivery strategies in vivo

Especially the local application of siRNA at site-specific delivery targets offers the possibility to reduce the doses of use as well as preventing the threat of systemic off targets.

Intraocular delivery

Up to now it is possible to treat wet-age related macular degeneration (AMD) with si-RNA based therapeutics. This disease affects blood vessels behind the retina that show a overgrowth which can cause a loss of vision. A human clinical study with complete status show the potency of VEGF-targeted siRNA Bevasiranib (Acuity Pharmaceuticals) to reduce neovascularisation and is also thought for the treatment of diabetic macular oedema (clinical status: complete). A mouse model in which Bevasiranib (Cand 5) was used also showed downregulation of VEGF after ocular injection [52]. The Drug Ranibizumap (Sirna-027/AGN211745) which was developed by the Merck-Sirna Therapeutics is also already under complete status in the treatment of AMD. Another siRNA called RTP-801i from Silence Therapeutics is also under clinical investigation for AMD and renal injury use.

Intratumoral delivery

Calando Pharmaceuticals has induced a now active (not recruiting) status of a clinical trial in which the siRNA CALLAA-01 is encapsulated in the polymer cyclodextrin to built particles that are linked with transferrin. This siRNA is directed against a subunit of the ribonucleotide reductase to stop the synthesis of DNA required for growth within solid tumors. This is the first clinical attempt of connecting a siRNA particle with a receptor that is highly expressed on cancer cells, like transferrin to facilitate the uptake. Another example is the Atu-027 siRNA (Silence Therapeutics) which is thought to be effective in the treatment of gastrointestinal cancer that has achieved the status recruiting.

Intranasal delivery

Intranasal application of formulated siRNA is resulting in effective gene silencing in the lung. This has great benefit in the treatment of pulmonary diseases.

Alnylam Pharmaceuticals have developed the first antiviral siRNA ALN-RSV01 which is directed against the respiratory syntical virus through the silencing of the nucleocapsid N-gene of the virus [75].

Other clinical trials are summed up in the following table

siRNA/ miRNA	Disease	Clinical Status
TD101	Pachyonychia congenita	Completed
PRO-040201	Hypercholesterolemia	Recruiting
Anti-miRNA SPC3649	Chronic Hepatitis C	Recruiting

Table 2. Clinical trials (a selection)

4. Therapeutical applications of si- and miRNAs with focus on CVDs

The treatment of CVDs with si- and mi-RNA based therapeutics is a very novel strategy with high potential for clinical applications.

4.1 Possible targets of atherosclerosis therapy

Atherosclerosis is a chronic, multifactorial, smoldering inflammatory disease of medium sized and large conductive arteries characterized by lipid-fuelled lesions. The major players

in the development of the disease are endothelial cells, monocytes, leukocytes and intimal smooth muscle cells [53]. Atherosclerotic lesions start to develop under an intact but activated, dysfunctional and partly leaky endothelium. Through the sites of defective endothelium especially lipoprotein particles can accumulate in the vessel wall where they can undergo modifications like oxidations. OxLDL is highly proatherogenic and is generated by myeloperoxidase, 15-lipoxygenase (or 12/15-LO), and/ or nitric oxide synthase (NOS) [53]. Especially the 12-15-LO is associated with cellular growth, migration, adhesion, and inflammatory gene expression in monocytes/macrophages, endothelial cells and vascular smooth muscle cells (VSMC). Li et al. demonstrated that shRNA was able to knockdown 12/15-LO in mouse macrophages and also in rat and mouse VSMCs. The knockdown of 12/15-LO had also functional effects which was showed through reduced monocyte-chemoattractant protein-1 (MCP-1) expression in a differentiated mouse monocyte line as well as reduced cellular adhesion and fibronectin expression in VSMCs [54]. Making 12/15-LO an interesting target of atherosclerotic RNAi based prevention. Endothelium is activated through atherogenic stimuli like oxLDL and cytokines, that induces expression of adhesion molecules like VCAM-1, ICAM-1 and selectins like P- and E-selectin which have proven to be important in atherosclerotic lesion development [55, 56, 57]. These adhesion molecules can contribute to the recruitment of monocytes as well as leukocytes and VSMCS. During the development of atherosclerosis and neointimal hyperplasia a transformation of VSMCs in the media from a contractile into a synthetic phenotype is ongoing and after the arrival of VSMCs in the intima of the arterial wall they begin to generate extracellular matrix, resulting in the formation of intimal lesions. Petersen et al. isolated VSMCs from the aorta of C57BL/6 mice and transfected them with siRNA targeting VCAM-1. In a migration assay they revealed that with the treatment of VCAM-1 siRNA the number of migrated VSMCs was significant reduced [58]. These findings indicate that VCAM-1 is necessary for the migration of VSMCs in the intima and VCAM-1 siRNA expression is an eventually potent approach to prevent and treat atherosclerosis and restenosis. Another possible target of atherosclerotic therapy is the inhibition of leukocyte entry to lesion areas to stop inflammatory progression. Pluvinet et al demonstrated that efficient blockage of the CD40-CD154 signaling by RNAi-mediated silencing of human CD40 expression on vascular endothelial cells leads to inhibition of VCAM-1, ICAM-1 and E-selectin expression and to a concomitant reduction of leukocyte adherence on these cells [59]. This signalling way is very interesting because the CD40-CD154 interaction also triggers matrix metalloproteinase (MMP) expression and these enzymes destabilize lipid-rich core of plaques which can cause thrombosis. SMCs are also responsible for the fibrin cap production which contributes also to neointimal thickening after arterial injury which can lead to restenosis. The discoidin domain receptor 2 (DDR2) plays potential roles in the regulation of collagen turnover mediated by VSMCs in atherosclerosis. DDR 1 and DDR 2 are nonintegrin receptors of collagen [60]. Shyu et al applied in a balloon injured rat model the DDR2 siRNA for attenuating the neointimal formation and decreasing the MMP2 protein labeling *in vitro* and *in vivo* [61]. Another study also showed successful *in vivo* transplantation of coated stents with a cationized pullulan-based hydrogel loaded with MMP2 siRNA. San Juan et al demonstrated in this study an uptake of siRNA into the arterial wall and a decrease of pro-MMP2 activity [62]. This stent coating technology could be a auspicious therapeutic approach for prevention of restenosis.

4.2 Possible targets of hypertension therapy

Hypertension also represents a severe riskfactor for many chronic CVDs. The use of cationic liposomal gene delivery vectors like DOTAP could be one possible strategy to reduce blood pressure. This finding was recently confirmed by Arnold et al., who showed that a single dose of 1mg/kg i.v. of siRNA-DOTAP (N-[1-(2,3-dioleoyloxy)]-N-N-N trimethyl ammonium propane) lipoplexes lead to the selective inhibition of β1-adrenoreceptor expression and reduced blood pressure lasting for 12 days [63]. The reduced blood pressure was also not greater if the β1-adrenoreceptor were blocked by β-blockers.

Sun et al. also used RNAi to inhibit α1D-adrenerig receptor gene in rat VSMCs and the therapeutic implications of this treatment are of great interest, for the reason that pharmacologic blockage of α1-ARs is a commonly used treatment for hypertension [64]. A other area of gene targeting in hypertension are the blockage of receptors that are affected by potent vasoconstrictors like Angiotensin II (Ang II). Ang II exerts its physiological effects by activating multiple subtypes of its receptor such as AT1a-, AT1-b and AT2-receptors. It regulates diverse functions of the cardiovascular system as increases in blood pressure, extracellular fluid volume, hormone secretion, stimulation of sympathetic nerve activity, damping of baroreflexes and vascular and cardiac remodelling [65, 66]. Mostly the effects are mediated through AT1R. Vázquez et al. used AT1147siRNA to silence the AT1a receptor for up to 7 days, with decrease of Ang II binding to cells that were transfected [67]. Thus this target will be an interesting field in CVD therapy.

4.3 Possible targets of ischemic heart-disease (IHD) therapy

The therapeutic opportunities for the treatment of IHD range from medical therapy achieving to decrease myocardial oxygen consumption and increase coronary flow to revascularization. Revascularization is done through coronary artery bypass grafting (CABG) or percutaneous coronary angioplasty (PCI) in most cases with stent positioning [68].

Through hypoxia the hypoxia inducible factor-1 transcriptional factor (HIF1-TF) can activate several angiogenic genes. HIF1-TF is naturally degraded by prolyl hydroxylase-2 (PHD2). Huang et al. imaged the biological role of shRNA therapy for improving cardiac function. Inhibition of PH2D by shRNA led to significant improvement in angiogenesis and contractility by in vivo and in vitro experiments and this process can be followed by molecular imaging [69]. Markkanen et al. reviews also that combined use of growth-factors or of factors that are capable of up-regulating other factors (e.g. HIF-1) will improve collateral vessel growth in case of IHD [70]. The potential use of miRNAs could also be a strategy for IHD treatment. MiRNAs contribute in the regulation of angiogenesis. Especially miR-126 can regulate angiogenesis and vascular integrity due to two recent studies [71, 72]. MiR-126 was demonstrated to be one of the most expressed miRNAs in cardiovascular tissue. Wang et al. generated miR-126 null mice that showed decreased sprouting potential of endothelial cells in vitro and also defective response to angiogenic factors in vivo [72]. These mice also showed decreased vascularization of infarcted myocardium, stronger fibrosis and loss of functional myocardium and were more prone to cardiac rupture. The search of mir-126 targets identified Spred-1, which is an negative regulator of the MAP kinase pathway that signals the pathway of angiogenic regulators like VEGF [68].

Thus an upregulation of miR-126 could be effective in the repression of antiangiogenesis. Another study by Fasanaro et al. also revealed a hypoxia induced upregulation of miR-210

in endothelial cells [73]. They used endothelial cells that were cultured under oxygen deprivation, which were derived from human, umbilical veins. It was shown that the overexpression of miR-210 stimulated angiogenesis in mormoxic endothelial cells, while miR-210 blockage inhibited it [73, 68]. A target of miR-210 is Ephrin-A3 which is a regulator of angiogenesis and VEGF signaling.

5. Conclusions and future perspectives

Since CVDs are the leading cause of morbidity and mortality in the world, siRNA and miRNA-based therapeutics represent a new approach to treat CVDs. Due to the fact, that RNAi technology has shown a fast development from the research level to human clinical trials as an effective gene-silencing method since its discovery in 1998, it shows great advantages for use in routine clinical practice as an adjuvance of existing therapies. The challenging hurdle remains the delivery of nucleic acids *in vivo* including the improvement of biological properties such as delivery efficacy, cellular uptake and well pharmacokinetics to achieve drug release to the wished target cell type. But there have been already made great efforts in the establishment of siRNA delivery methods like cationic polymer-mediated siRNA delivery, targeted siRNA delivery and conjugation or chemical modifications of siRNAs. Thus on going research and pratical use will improve the safety issue in general making RNAi-based therapeutics a new class of drugs with high potential, especially in the treatment of a wide field of diseases.

6. References

[1] Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity lin-14. *Cell*. 1993; 75(5): 843-54.

[2] Fire A, Xu S, Montgomery M, et al. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature*. 1998; 391(6669): 806-11.

[3] Feinberg EH, Hunter CP. Transport of dsRNA into cells by the transmembran protein SID-1. *Science*. 2003; 301(5639): 1545-7.

[4] Mello CC, Conte D Jr, Revealing the world of RNA interference. *Nature* 2004; 431: 338-42.

[5] Yan KS, Yan S, Farooq A, et al. Structure and conserved RNA binding of the PAZ domain. *Nature*. 2003; 426(6965): 468-74.

[6] Matranga C, Tomari Y, Shin C, Bartel DP, Zamore PD. Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell*. 2005; 123(4): 607-20.

[7] Lewis B, Burge C, Bartel D. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*. 2005; 120(1): 15-20.

[8] Hutvanger G, Zamore P.D. A micro RNA in a multiple-turnover RNAi enzyme complex. *Science*. 2002; 297: 2056-2060.

[9] Matranga C, Zamore PD. Small silencing RNAs. *Current Biology*. 2007; 17:R789-93.

[10] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004; 116(2): 281-97.

[11] Barringhaus KG, Zamore PD. miRNAs: Regulating a change of heart. *Circulation.* 2009; 119(16): 2217-24.

[12] Lund E, Guttinger S, Calado A, Dahlberg JE, Kutay U. Nuclear export of microRNA precursors. *Science*. 2004; 303(5654): 95-8.
[13] De Fougerolles A, Vornlocher HP, Maraganore J, Liebermann J. Interfering with disease: a progress report on siRNA-based therapeutics. *Nature Reviews Drug Disvovery*. 2007; 6:443-52.
[14] Lui J et al. MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nature Cell Biol*. 2005; 7:719-23.
[15] Dowler T, Bergeron D, Tedeschi AL, Paquet L, Ferrari N, Damha MJ. Improvements in siRNA properties mediated by 2'-deoxy-2'-fluoro-beta-D-arabinonucleic acid (FANA). *Nucleic Acids Res*. 2006; 34: 1669-75.
[16] Czauderba F, Fechtner M, Dames S, Aygun H, Klippel A., Pronk GJ , Giese K, Kaufmann J. Structural variations and stabilising modifi cations ofsynthetic siRNAs in mammalian cells. *Nucleic Acids Res.* 2003; 31:2705–16.
[17] Choung S , Kim YJ, Kim S, Park HO, Choi, YC.Chemical modification of siRNAs to improve serum stability withoutloss of efficacy. *Biochem. Biophys. Res. Commun*. 2006; 342:919–27.
[18] Kraynack BA, Baker BF. (2006). Small interfering RNAs containing full 2′-O-methylribonucleotide-modified sense strands display Argonaute2/eIF2C2-dependent activity. *RNA*. 2006; 12:163–76.
[19] Layzer JM, MCCaffrey AP, Tanner AK, Huang Z, Kay MA, Sullenger BA. In vivo activity of nuclease-resistant siRNAs. *RNA*. 2004; 10:766–71.
[20] Morrissey DV et al. Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. *Nat. Biotechnol.* 2005; 23:1002–07.
[21] Amarzguioui M, Holen T, Babaie E, Prydz H. Tolerance for mutations and chemical modifi cations in a siRNA. *Nucleic Acids Res*. 2003; 31:589–95.
[22] Braasch DA, Jensen S, Liu Y, Kaur K, Arar K, White MA, Corey DR. RNA interference in mammalian cells by chemically-modified RNA. *Biochemistry*. 2003; 42:7967–75.
[23] Li ZY, Mao H, Kallick DA, Gorenstein DG. The effects of thiophosphate substitutions on native siRNA gene silencing. Biochem. *Biophys. Res. Commun*. 2005; 329:1026–30.
[24] Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M, et al. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature*. 2004; 432:173-8.
[25] Sledz CA, Holko M, de Veer MJ, Silverman RH, Williams BR. Activation of the interferon system by short-interfering RNAs. *Nat Cell Biol* 2003; 5: 834-9.
[26] Schlee M et al. SiRNA and isRNA: two edges of one sword. *Mol. Ther*. 2006; 14: 463-70
[27] Judge AD, Bola G, Lee AC, Machlachlan I.Design of noninflammatory synthetic siRNA mediating potent gene silencing in vivo. *Mol. Ther*. 2006; 13:494–505.
[28] Carthew RW, Sontheimer EJ. Origins and mechanisms of miRNAs and siRNAs. *Cell.* 2009; 136(4): 642-655.
[29] Anderson EM , Birmingham A, Baskerville S, Reynold A , Maksimova E, Laeke D, Federov Y, Karpilow J, Hvorova A. Experimental validation of the importance of seed complement frequency to siRNA specificity. *RNA.* 2008; 14: 853–86.
[30] Koostra NA, Verma IM. Gene therapy with viral vectors. *Ann. Rev. Pharmacol. Toxicol*. 2003; 43: 413-39.
[31] Loser P et al. Advances in the development of nonhuman viral DNA-vectors for gene delivery. *Curr. Gene Ther*. 2002; 2(2): 161-71;

[32] Singer O, Verma IM. Application of lentiviral vectors for shRNA delivery and transgenesis. *Curr. Gene Ther*. 2008; 8(6): 483-88.
[33] Naldini L, Blomer U, Gallay P et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science*. 1996; 272: 263-67.
[34] Marr RA, Rockenstein E, Mukherjee A, et al. Neprilysin gene transfer reduces human amyloid pathology in transgenic mice. *J Neurosci.* 2003; 23: 1992-96.
[35] Singer O, Marr RA, Rockenstein E, et al. Targeting BACE1 with siRNA ameliorates Alzheimer disease neuropathology in a transgenic model. *Nat Neurosci*. 2005; 8: 1343-49.
[36] Xia H, et al. RNAi supress polyglutamine-induced neurodegeneration in a model of spinocerebellar ataxia. *Nature Med*. 2004; 10: 816-820.
[37] Kim JW, et al. Inhibition of in vitro hepatitis B virus replication by lentivirus-mediated short-hairpin RNA against HBx. *Korean J Hepatol*. 2009; 15(1): 15-24.
[38] Li W, Szoka FC Jr. Lipid-based nanoparticles for nucleic acid delivery. *Pharm. Res*. 2007; 24: 438-49.
[39] Aigner A. Cellular delivery in vivo of siRNA-based therapeutics. *Current Pharmaceutical Design*. 2008; 14:1-17.
[40] Boussif O, et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc. Natl Acad. Sci. USA*. 1995; 92:7297–7301.
[41] Kichler, A. Gene transfer with modified polyethylenimine. *J.Gene Med*. 2004; 6:3-10.
[42] Geisbert, T. W. et al. Postexposure protection of guinea pigs against lethal ebola virus challenge is conferred by RNA interference. *J. Infect. Dis.* 2006;193:1650–1657.
[43] Ge Q, et al. Inhibition of Influenza virus production in virus-infected mice by RNA interference. *Proc. Natl Acad. Sci. USA*. 2004; 101: 8676-81.
[44] Urban-Klein B, Werth S, Abuharbeid S, Czubayko F, Aigner A.RNAi-mediated gene-targeting through systemic application ofpolyethylenimine (PEI)-complexed siRNA in vivo. *Gene Ther*. 2005; 12: 461-6.
[45] Kim SH, Jeong JH, Lee SH, Kim SW, Park TG. Local and systemic delivery of VEGF siRNA using polyelectrolyte complex micelles for effective treatment of cancer. *J Control Release* 2008.
[46] Ochiya T, Nagahara S, Sano A, Itoh H, Terada M. Biomaterials for gene delivery: atelocollagen-mediated controlled release of molecularmedicines. *Curr Gene Ther* 2001; 1: 31-52.
[47] Takei Y, Kadomatsu K, Yuzawa Y, Matsuo S, Muramatsu T. A small interfering RNA targeting vascular endothelial growth factor as cancer therapeutics. *Cancer Res* 2004; 64: 3365-70.
[48] Iwaki K, Shibata K, Ohta M, Endo Y, Uchida H, Tominaga M, et al. A small interfering RNA targeting proteinase-activated receptor-2 is effective in suppression of tumor growth in a Panc1 xenograft model. *Int J Cancer* 2007.
[49] Matoba T, Orba Y, Suzuki T, Makino Y, Shichinohe H, Kuroda S, et al. An siRNA against JC virus (JCV) agnoprotein inhibits JCV infection in JCV-producing cells inoculated in nude mice. *Neuropathology*. 2008; 28: 286-94.
[50] Howard, K. A. et al. RNA interference in vitro and in vivo using a novel chitosan/siRNA nanoparticlesystem. *Mol. Ther*. 2006; 14: 476–84.
[51] Zimmermann TS et al. RNAi-mediated gene silencing in non-human primates. *Nature*. 2006; 441:111-14.

[52] Shen J, et al. Suppression of ocular neovascularisation with siRNA targeting VEGF receptor 1. *Gene Ther*. 2006; 13: 225-234.
[53] Falk E. Pathogenesis of atheroclerosis. *J. Am. Coll. Cardiol*. 2006; 47: C7-C12.
[54] Li S et al. Effects of silencing leucocyte-type 12/15-lipoxygenase using short interfering RNAs. *Journal of Lipid Research*. 2005; 46: 220-29.
[55] Dong ZM, et al. The combined role of P- and E-selectins in atherosclerosis. *J Clin Invest*. 1998; 102: 145-52.
[56] Shih PT, Brennan ML, Vora DK, Territo MC, Strahl D, Elices MJ, et al. Blocking very late antigen-4 integrin decreases leukocyte entry and fatty streak formation in mice fed an atherogenic diet. *Circ Res*. 1999;84: 345–51.
[57] Collins RG, Velji R, Guevara NV, Hicks MJ, Chan L, Beaudet AL. P-Selectin or intercellular adhesion molecule (ICAM)-1 deficiency substantially protects against atherosclerosis in apolipoprotein E deficient mice. *J Exp Med*. 2000; 191: 189–94.
[58] Petersen EJ, et al. siRNA silencing reveals role of vascular adhesion molecule-1 in vascular smooth muscle cell migration. *Atherosclerosis*. 2008; 198 (2): 301-306.
[59] Pluvinet R, et al. RNAi-mediated silencing of CD40 prevents leukocyte adhesion on CD154-activated endothelial cells. *Blood*. 104(12): 3642-46.
[60] Vogel WF, et al. Sensing extracellular matrix: an update on discoidin domain receptor function. *Cellular Signaling*. 2006; 18:1108-16.
[61] Shyu KG, et al. RNA interference for discoidin domain receptor 2 attenuates neointimal formation in balloon injured rat carotid artery. *Ateriosler Thromb Vasc Biol*. 2008; 28: 1447-53.
[62] San Juan A, et al. Development of a functionlized polymer for stent coating in aterial delivery of small interfering RNA. *Biomacromolecules*. 2009.
[63] Arnold AS, et al. Specific beta1-adrenergic receptor silencing with small interfering RNA lowers high blood pressure and improves cardiac function in myocardial ischemia. *J. Hypertens*. 2007; 25: 197-205.
[64] Sun B, et al. Inhibition of α1-adrenergic receptor gene by RNAi in rat vascular smooth muscle cells and its effects on other adrenergic receptors. *Vascul Pharmacol*. 2007; 46(5): 367-72.
[65] Saavedra JM. Brain and pituitary angiotensin. *Endocr Rev*. 1992; 13: 329-380.
[66] Veerasingham SJ, et al. Brain renin-angiotensin system dysfunction in hypertension: recent advances and perpectives. *Br J Pharmacol*. 2003; 139: 191-202.
[67] Vázquez J et al. Selective silencing angiotensin receptor subtype 1a (AT1aR) by RNA interference. *Hypertension*. 2005; 45: 115-19.
[68] Silvestri P et al. MicroRNAs and ischemic heart disease: towards a better comprehension of pathogenesis, new diagnostic tools and new therapeutic targets. Recent Patents of Cardiovascular *Drug Discovery*. 2009; 4: 109-118.
[69] Huang M, et al. Short hairpin RNA interference therapy for ischemic heart disease. *Circulation*. 2008; 118: 226-33.
[70] Markkanen JE, et al. growth factor-induced therapeutic angiogenesis and ateriogenesis in the heart-gene therapy. *Cardiovascular Research*. 2005; 65:656-64.
[71] Fish JE, et al. miR-126 regulates the angiogenic signaling and vascular integrity. *Dev Cell*. 2008; 15: 272-284.
[72] Wang S, et al. The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. *Dev Cell*. 2008; 15:261-71.

[73] Fasanaro P, et al. MicroRNA-219 modulates endothelial cell response of hypoxia and inhibits the receptor tyrosine-kinase ligand Ephrin-A3. *J Biol Chem*. 2008; 283: 15878-83.

[74] Akhtar S, Benter IF. Nonviral delivery of synthetic siRNAs in vivo. *The Journal of Clinical investigations*. 2007; 117(12): 3623-32.

[75] Castanotto D, Rossi JJ. The promises and pitfalls of RNA-interference-based therapeutics. 2009; *Nature*. 457(7228): 426-33.

Regenerative Medicine and Tissue Engineering - Cells and Biomaterials

Edited by Prof. Daniel Eberli

ISBN 978-953-307-663-8
Hard cover, 588 pages
Publisher InTech
Published online 29, August, 2011
Published in print edition August, 2011

Tissue Engineering may offer new treatment alternatives for organ replacement or repair deteriorated organs. Among the clinical applications of Tissue Engineering are the production of artificial skin for burn patients, tissue engineered trachea, cartilage for knee-replacement procedures, urinary bladder replacement, urethra substitutes and cellular therapies for the treatment of urinary incontinence. The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues reconstructed from readily available biopsy material induce only minimal or no immunogenicity when reimplanted in the patient. This book is aimed at anyone interested in the application of Tissue Engineering in different organ systems. It offers insights into a wide variety of strategies applying the principles of Tissue Engineering to tissue and organ regeneration.

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

IntechOpen