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REVIEW

Cardiac-targeted delivery of regulatory RNA molecules and genes for the treatment of heart failure

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Ribonucleic acid (RNA) in its many facets of structure and function is becoming more fully understood, and, therefore, it is possible to design and use RNAs as valuable tools in molecular biology and medicine. Understanding of the role of RNAs within the cell has changed dramatically during the past few years. Therapeutic strategies based on non-coding regulatory RNAs include RNA interference (RNAi) for the silencing of specific genes, and microRNA (miRNA) modulations to alter complex gene expression patterns. Recent progress has allowed the targeting of therapeutic RNAi to the heart for the treatment of heart failure, and we discuss current strategies in this field. Owing to the peculiar biochemical properties of small RNA molecules, the actual therapeutic translation of findings *in vitro* or in cell cultures is more demanding than with small molecule drugs or proteins. The critical requirement for animal studies after pre-testing of RNAi tools *in vitro* likewise applies for miRNA modulations, which also have complex consequences for the recipient that are dependent on stability and distribution of the RNA tools. Problems in the field that are not yet fully solved are the prediction of targets and specificity of the RNA tools as well as their tissue-specific and regulatable expression. We discuss analogies and differences between regulatory RNA therapy and classical gene therapy, since recent breakthroughs in vector technology are of importance for both. Recent years have witnessed parallel progress in the fields of gene-based and regulatory RNA-based therapies that are likely to significantly expand the cardiovascular therapeutic repertoire within the next decade.

Keywords Regulatory RNA molecules • RNA interference • MicroRNAs • Gene therapy • Heart failure • Vector technology

1. Introduction

The need to develop novel strategies for the treatment of heart failure (HF) arises from the limited efficacy of current therapeutic approaches[1] for this serious disorder, which has a high and still rising prevalence.[2] HF may result from multiple cardiac diseases including myocardial infarction and genetic or exogenous cardiomyopathies. Drug-based and surgical approaches including heart transplantation (HTX)[3] and assist devices [left ventricular assist device (LVAD)][4] have significantly improved the quality of life and prognosis of HF patients. However, LVAD and HTX are available only for a very small fraction of HF patients worldwide, and drug therapy has limited efficacy in advanced stages. Current novel approaches to HF treatment include cell transfer-based therapies[5] and recombinant protein-based approaches.[6–9] We focus here on treatment strategies based on regulatory ribonucleic acid (RNA) or gene transfer to the heart. For several therapeutic targets of major interest, inhibitory or enhancing small molecule drug development has failed, and recombinant protein substitution was not feasible due to intracellular or membrane localization of the target. One recent prominent example is the important regulator of cardiac contractility, phospholamban (PLB), for which no inhibitory drug or antibody has been developed, despite considerable efforts. Specifically, for 'hard' targets of this type, which are not accessible by conventional pharmaceutical agents, regulatory RNA- or gene-based therapy should be considered as an alternative. It may be anticipated that therapeutically successful target protein enhancement by gene transfer, or target protein suppression by RNAi, will subsequently trigger research in conventional

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drug or recombinant protein development to modulate the newly established therapeutic target.

2. Regulatory RNA- vs. gene-based therapies

2.1 RNA world

The structure and function of RNA is becoming better understood, and it is possible to design and use RNA molecules as valuable tools in molecular biology and medicine. An understanding of the role of RNAs within the cell has fundamentally changed during recent years[10] (*Figure 1*). Its status expanded with reports on catalytic RNAs (ribozymes) 25 years ago, of endogenous RNAi 15 years later, and other non-coding RNA very recently. Today, it is obvious that RNAs are not merely the intermediary molecules between DNA and proteins, but that they can also be functional end products. Large stretches of genomic DNA do not contain protein-coding sequences and have, therefore, been considered as 'junk'. However, a significant fraction of this non-coding DNA has actually been found to hold the information for some of these functional non-coding RNAs. Diverse eukaryotic organisms harbour a class of non-coding small RNAs which are thought to function as regulators of gene expression. Thus, RNAs can be the transmitters [messenger RNAs (mRNAs)] of genetic information to the ribosome for proteins to be synthesized, and also the regulators in protein synthesis. The conclusion to be drawn is that RNA is much more than solely an mRNA, and, therefore, molecules of this class are truly renaissance molecules.

RNA has become a focus of investigations into novel therapeutic schemes. Ribozymes, antisense RNAs (asRNAs), RNA decoys, aptamers and spiegelmers,[11,12] microRNAs (miRNAs), and small interfering (siRNAs) have been used to down-regulate undesired gene expression (*Figure 2*). Multiple challenges, such as optimization of selectivity, stability, delivery, and long-term safety, have to be addressed in order for 'RNA drugs' to become successful therapeutic agents. The recognition of the biological roles of small molecular weight RNAs have been one of the most significant discoveries in molecular biology. These RNA molecules influence the translation of mRNAs in a post-transcriptional manner that makes the regulation of RNAs even more complex. The use of RNA interference (RNAi) mediated by siRNAs or short hairpin RNAs (shRNAs) for the silencing of specific genes has provided a powerful tool for loss-of-function studies. RNAi-mediated gene silencing by degradation of target mRNAs has been widely used in gene function characterizations. Compared with the very laborious, time-consuming, and costly gene knockout models, RNAi may provide an efficient, specific, and cheap solution to inhibit target genes. However, efficient RNAi delivery is essential for the success of specific gene silencing and difficult to achieve *in vivo*, in particular if silencing is desired only in one organ.

In several recent studies, microarray and other analyses were performed to determine whether another class of small regulatory RNA molecules designated as miRNAs are deregulated in cardiac diseases diseases.[13–18] A major challenge for the future will be to identify the targets of miRNAs that participate in the respective pathomechanisms and to understand their mechanistic contribution in detail. This may be the most important threshold to pass before regulatory agencies allow these RNA tools into the clinic. Finally, the recent application of advanced vector technologies developed initially in the classical gene therapy field has had an enormous impact on the efficacy by which RNAi and miRNAs can be employed for therapeutic purposes *in vivo*. These most recent developments have brought clinical translation of certain RNA-based therapies within reach.

2.2 RNA interference

The discovery of co-suppression in Petunia plants about 18 years ago turned out to be a foundation of the dynamically expanding

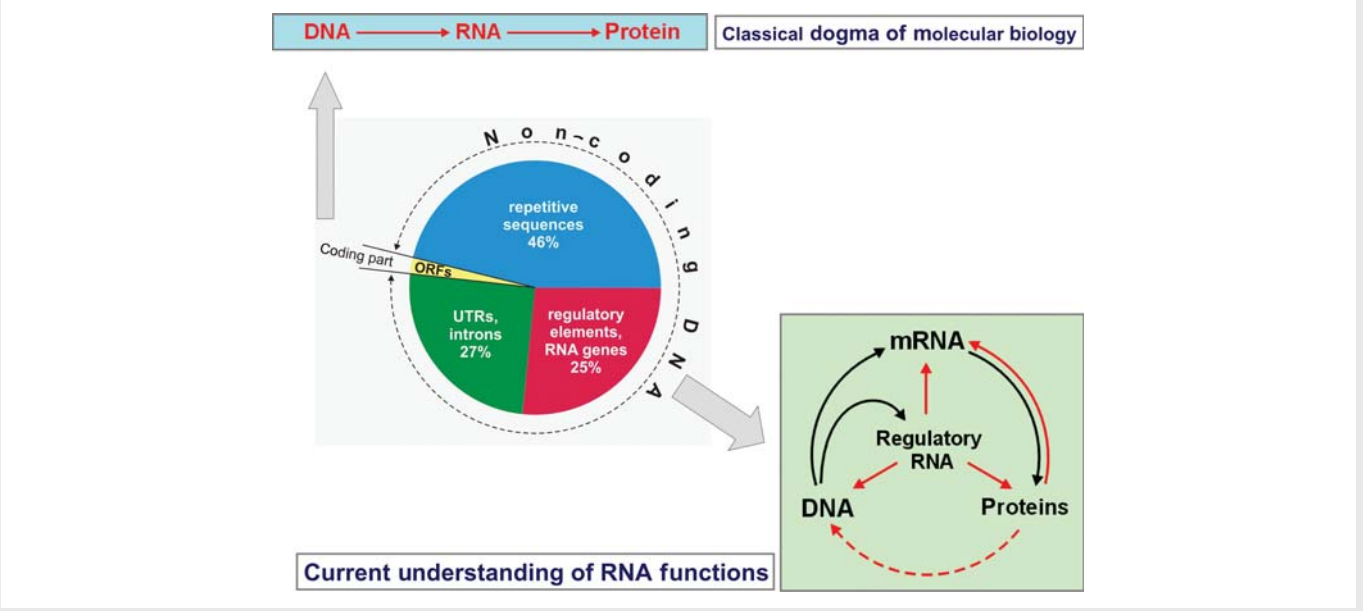

Figure 1 Traditional and current concept for coding vs. non-coding DNA functions. Only a small fraction of the RNA species found in eukaryotic cells has protein-coding function, the traditional role for RNA. During the past decade, a multitude of RNAs arising from the huge non-coding part of the genome was discovered to exert regulatory functions of fundamental importance to maintain normal cell function.

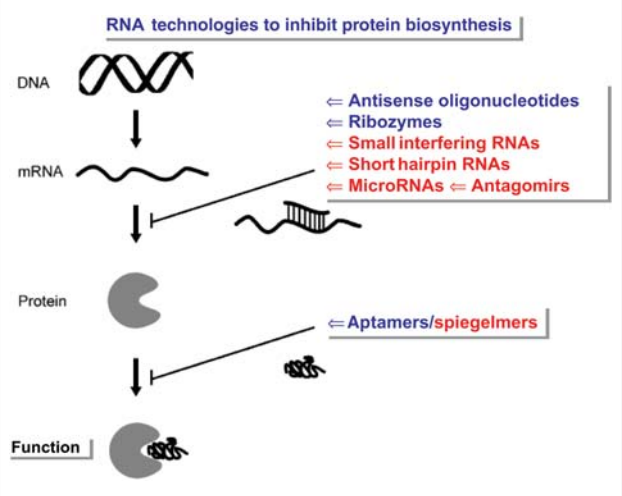

Figure 2 RNA-based technologies for gene and protein silencing. A broad spectrum of 'tools' made from RNA molecules has been developed that are capable of either suppressing specific gene/protein functions or of altering complex expression patterns (miRNAs). By these capacities, RNA tools significantly extend the therapeutic repertoire beyond that of classical pharmacology.

technology of RNAi, with scientific and medical impact comparable to the discovery of polymerase chain reaction or mouse knockout technology.[10] That original discovery revealed that introduction of double-stranded RNA (dsRNA) into a cell can trigger a target sequence-specific gene-silencing mechanism.[19] Within the cell, the dsRNA is processed in short ~22 nt-long fragments by an evolutionary ancient, endogenous machinery later designated as RNAi. It was later discovered that the silencing effect of dsRNA may be mimicked by chemically synthesized siRNA.[20] The challenge to produce siRNAs *in vivo* was met by designing vectors expressing shRNAs from RNA polymerase III promoters (U6, H1, and 7SK), which were in cells processed into active siRNAs. In parallel with the study of RNAi, progress in understanding the cellular processing and function of miRNAs was achieved. RNAi and miRNA systems share most components of the silencing pathway (*Figure 3*). Theoretical predictors for efficient and target-specific silencing RNA sequences have been incorporated in numerous dedicated software, but still always need to be verified first in the relevant target cells and finally in respective animal models *in vivo*. Ideally, an RNAi tool will silence one single gene without any 'off-target' effects.

2.3 MicroRNAs

In computer science, 'macro' describes a single command programmed to replace multiple separate commands performing a series of actions, thus simplifying, expediting, and minimizing error. By analogy, living cells may employ a single miRNA to modulate the expression pattern of a whole group of genes that coordinate a particular cell function. Regulation of multiple genes by a single miRNA obviates the need for transcriptional regulation of individual genes and facilitates synchronization of the regulatory effects and promptness of the response. miRNA are targets of significant therapeutic interest. In advance of therapeutic targeting of an miRNA, however, detailed knowledge of its gene targets, functions, and tissue distribution are required. Since mammalian miRNA research is still in an early phase, informations in that regard are as yet incomplete. Nevertheless, certain miRNA targets with major therapeutic relevance for cardiovascular diseases have already been well characterized and investigated in animal models.[13–15,17,18,21,22]

Therapeutic miRNA modulations may be classified into inhibition of a specific miRNA or overexpression of an miRNA in the target cells (*Figure 3*). In contrast to RNAi which is intended to serve ideally as a single-target silencing tool, miRNA modulations are a priori intended to address multiple targets. Commonly but not always,[23] an miRNA silences gene expression by binding to complementary mRNA sequences in the 3′-untranslated regions (UTRs) of their target genes. Up-regulation of protein expression by miRNA binding to the 5′-UTR has also been described, so that miRNA overexpression does not necessarily results only in silencing of the target genes. Therefore, both inhibition and overexpression of a single miRNA are likely to have complex ramifications for the function of the treated cells. As outlined in the left of *Figure 3*, miRNA inhibition has been achieved by 'antagomirs',[24] anti-microRNA oligodeoxyribonucleotides,[25] miRNA 'target masking' that blocks miRNA binding,[26] and 'sponges'[27] or 'erasers'[28] both of which act by scavenging miRNAs. Targeting of these RNA tools is commonly dependent on expression from a vector with appropriate tropism, whereas the antagomir approach instead attempts to achieve this goal by direct coupling of the RNA tool itself to a targeting ligand. The first antagomirs employed an miRNA-specific, anti-sense, cholesteryl-conjugated, 2′-*O*-methyl oligoribonucleotide which is cell permeable and inhibits the miRNA by inducing its elimination. Interestingly, it offers a relatively long-lived effect and for certain applications, circumvents the need for a specific delivery vehicle such as an organotropic viral vector. Pioneering work on antagomir-based ablation of the HF-associated miRNA-21 overexpression in cardiofibroblasts has shown impressive therapeutic potential, although this miRNA is expressed in multiple tissues and intravenously injected antagomir enters most organs except the brain. Since no side effects in non-cardiac organs were observed, it is obviously not mandatory to achieve targeting of an 'RNA drug' exclusively to the diseased organ.[29] It will be interesting to see which other diseases may be efficiently treated by this 'untargeted' and elegant approach without significant side effects.

The replenishment of depleted miRNAs or their deliberate overexpression with therapeutic intention, on the other hand, requires the delivery of the premature miRNA (pre-miRNA) in its native form (*Figure 3*) in order to allow proper processing and incorporation into the RISC complex. For this group of strategies, other delivery methods similar to those employed in classical gene therapy are needed, but important additional restriction has to be met if small RNA molecules are to be generated.[30] Pioneering studies in the cancer field have described impressive therapeutic effects by overexpression of miRNA-206[31] and miRNA-205,[32] whereas to our knowledge, analogous therapeutic miRNA overexpression has not yet been employed for cardiovascular diseases.

Although major progress is being made in deciphering the molecular foundations of RNAi, e.g. by solving the structure of an argonaute silencing complex,[33] reliable prediction of RNA sequences with high and specific silencing capacity for single genes (by RNAi) or miRNA (e.g. by antagomirs) by calculation alone is not yet possible and sequences and constructs have always to be validated in the relevant experimental models *in vivo*.

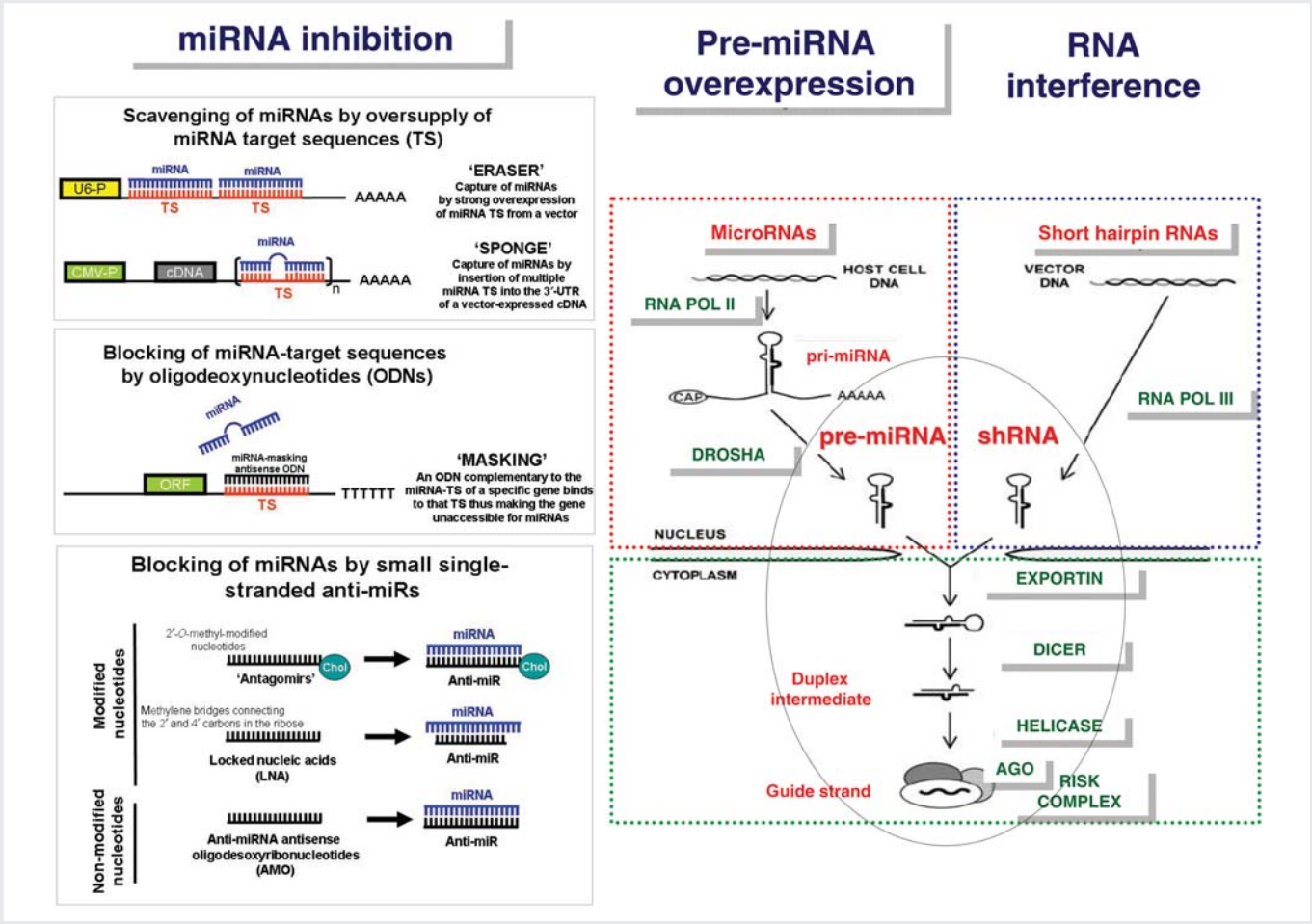

Figure 3 Current strategies for regulatory RNA modulation. The cartoon outlines the principle of RNAi alongside that of miRNA enhancement, both of which share multiple cellular components (boxes on the right), as well as strategies towards miRNA inhibition (left).

2.4 Common principles of regulatory RNA and gene therapy

The classical concept of gene therapy which has been developed for monogenic disorders caused by deficiency of a single gene is gene substitution therapy. In the field of cardiology, several monogenic disorders manifested in the myocardium could theoretically be cured by this approach. Among them are long-QT syndromes, arrhythmogenic right ventricular dysplasia, Brugada syndromes, hypertrophic cardiomyopathies caused by a host of mutations in sarcomeric proteins, and dilated cardiomyopathies. A second concept is the enhancement of gene functions by overexpression of primarily endogenous genes using gene transfer. Under appropriate conditions, a therapeutic effect may be achieved not only in genetically determined but also in acquired diseases. A third concept is the addition of truly novel gene functions to the target organ by vector-based expression of foreign genes. Novel concepts of far more recent origin involve the use of non-coding, small regulatory RNA molecules for therapeutic purposes. They include the concept of shRNA transcription for the suppression of gene functions in the diseased organ by the mechanism of RNAi, and the concept of pre-miRNA generation to achieve modulation of cell functions on the level of the cellular miRNA systems. Naturally, all key issues of classical gene therapy apply similarly to the RNA-based therapies. *Figure 4* illustrates some fundamental differences between gene and regulatory RNA therapies, but at the same time points to significant methodological overlaps between these distinct strategies. Common to both is the need to deliver the therapeutic tool (cDNA or regulatory RNA) to the right place, at appropriate concentration for a sufficient period of time, and with adequate safety features. This allows researchers to employ advanced vector technologies, initially developed in the gene therapy field, also for several types of RNA therapy.

2.5 Technical challenges common to regulatory RNA and gene therapy

2.5.1 Targeting of the tools

A key issue of vector technology is the physical steering (targeting) of the vector (expressing a protein-coding cDNA, shRNA, or pre-miRNA) to the target tissue. Cardiac targeting proved to be an extremely difficult task that could not be solved in a simple and efficient way using earlier gene transfer systems. A breakthrough in the field has been achieved with the introduction of adeno-associated virus (AAV) vector systems,[34–38] in particular the discovery of highly cardiotropic AAV serotypes[39–41] and the reengineering of AAV with therapeutically desirable properties.[35,38] This has not only led to successful gene therapy[42–45] and recently also regulatory RNA therapy[30] *in vivo* by direct intravenous injection in animal models, but is already in the

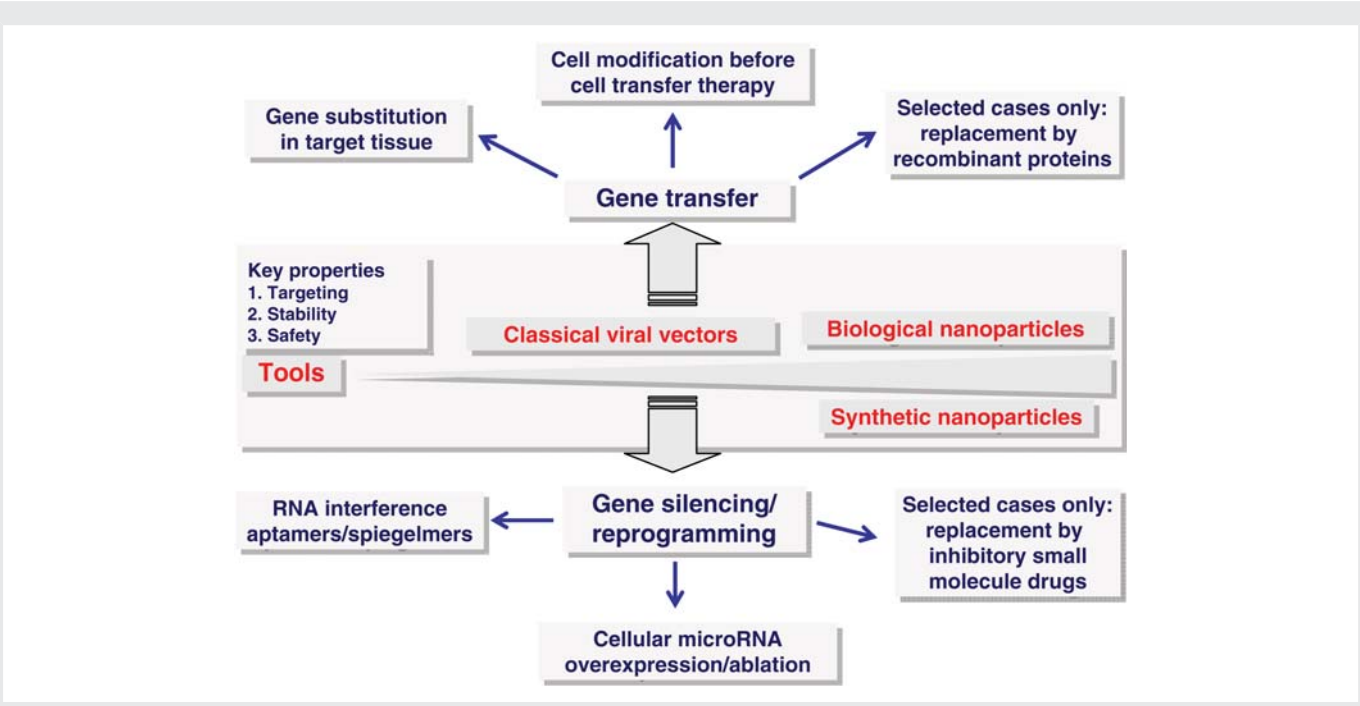

Figure 4 RNA and gene therapy—relation to other therapeutic concepts. Regulatory RNA- and gene-based strategies have recently been successfully used for cardiac therapies *in vivo*. Recent developments in vector technology laid the foundation for efficient, cardiac-targeted, and stable RNA and gene delivery. Parallel evolution of RNA technology enabled stable expression of RNAi-mediating small RNAs over months. A fully synthetic class of RNA molecules, spiegelmers, displays high stability *in vivo* and constitutes a novel class of RNA drugs with significant therapeutic potential. Only in selected cases, these fundamentally new approaches may be replaced by conventional drugs or proteins.

status of clinical translational trials.[46] If perfect targeting cannot be achieved (transductional targeting), further transcriptional confinement of the transgene may be achieved by using cardiac-specific promotors. It should be emphasized that the novel generation of cardiotropic AAV vectors is distinct from previous systems not only by cellular receptor affinity (resulting from virion surface properties), but even more importantly by their ability to overcome key anatomical barriers on their way to the heart (endothelium, basal membrane, and extracellular matrix) by some as yet unidentified mechanism (e.g. transmigration through the endothelium). Their practical application is therefore far more simple than former approaches employing sophisticated catheter delivery and endothelium-permeabilizing drugs.[47–49]

2.5.2 Stability and regulation of the tools

A second key issue of vector technology is the stability of therapeutic tool (cDNA, shRNA, and pre-miRNA). Small regulatory RNAs are inherently unstable, and viral vectors are currently the only tools for non-topical *in vivo* therapy using these molecules. On the other hand, multiple proteins are also inherently unstable and/or intracellular, which likewise suggest the use of long-term stable vectors for long-term therapy. If RNA- or gene-based therapy for HF is not only meant to serve as bridge-to-transplant or bridge-to-recovery, then long-term persistence and functionality of the therapeutic vector is required. The high stability appears to be a consequence of inherent fundamental biological properties of the AAV genome. AAV vectors have developed into the vectors of choice for the treatment of HF,[30,48,50] cardiac storage as in Fabry disease,[51] and gene substitution as in δ-sarcoglycan deficiency,[52] with transgene expression over several months in all cases.

In contrast to therapy of viral infections where complete inhibition of viral gene expression is desirable, for many cardiac disorders, successful therapy would require the regulation of gene expression levels within a narrow therapeutic window to avoid side effects. RNAi-mediated ablation of PLB which plays a key role in the regulation of cardiac Ca^{2+}-homeostasis showed high therapeutic potential in HF animal models. However, humans lacking functional PLB due to genomic mutations developed lethal cardiomyopathy[53] so that unregulated long-term inhibition of PLB by RNAi may therefore be of potential risk in humans, despite successful application in rodents. RNAi may also exert serious side effects *in vivo* under adverse circumstances.[54,55] A mode to switch on/off shRNA expression in therapeutic settings is therefore highly desirable before application in man. The basis for regulatable RNAi to date is a panel of drug-inducible gene expression systems where a drug-dependent transactivator and transrepressor protein activates and suppresses a responsive promoter and transcription, respectively. Regulatable 'on' system seems to be more suitable than 'off' systems for therapy since here transgene expression is induced by drug application and simply switched off by withdrawal of the drug (further details in Poller *et al.*[56]).

2.5.3 Safety issues

A third key issue of vector technology, of paramount importance before any clinical application, is the safety of the therapeutic tools which is determined by the properties of the regulatory RNA only, if it is applied as a synthetic molecule, or in addition by those of the delivery vehicle in the case of vector-based RNA therapies. As depicted in *Figure 3*, toxicity of an shRNA may arise from competition

with the normal cellular miRNA processing system. Serious liver toxicity arising from this mechanism has been described,[54] but could be overcome by selecting alternative non-toxic shRNA sequences and appropriate dose.[55,57] For other target diseases approached by the use of cardiotropic RNAi vectors with low affinity to the liver,[30] no hepatic or other toxicity was observed, but certainly always needs to be taken into consideration. Off-target effects of regulatory RNAs are another possible source of toxicity and difficult to assess. Microarray studies may analyse genomic expression pattern changes induced by an siRNA or shRNA, but even for these single-target tools, multiple changes will arise secondary to the silencing of the single intended gene. Essentially useless is this approach if unwanted miRNA effects are to be identified, since miRNA modulation will a priori result in complex expression pattern changes. As a consequence, side effects at the cellular level and *in vivo* need to be investigated to assess possible toxicity of the RNA drugs.

Their delivery system is another issue of concern. We focus here on AAV vectors and derived biological nanoparticles (BNPs) since their safety profile is superior to the formerly used adenoviral, retroviral, and lentiviral vectors. Beyond regulation of the transgene to adapt expression to the actual needs of the patient, an option to shut down the transgene at any time in the case of serious adverse effects is highly desirable under safety aspects. Adenoviral vectors are associated with several safety issues likely to prevent their clinical application for non-malignant diseases, given the availability of naturally occurring or engineered AAVs with significantly more favourable safety profiles. Their immunogenicity is very low compared with adenovectors, although not absent.[58–60] Another possible risk has been deduced from the capacity of wild-type AAV for chromosomal integration which is fortunately lost, however, in recombinant AAV vectors. Chromosomal effects of integration deserve attention after a report on late occurrence of leukaemia in children treated with retroviral vectors for severe combined immunodeficiency, after successful correction of the primary genetic defect.[61] The development of non-invasive methods for vector tracking *in vivo* is also desirable as an additional safety and control feature, but has not yet been achieved at the required sensitivity.

3. Regulatory RNA and gene therapies in cardiology

3.1 Two classes of therapeutic targets

HF remains a leading cause of mortality in the developed world. During the past few years, a several molecular targets have been identified and investigated in animal models (and in one case in humans) to improve the deteriorated function of the failing heart. Several therapeutic targets are relatively independent of the specific origin of HF, and therefore deserve particular clinical interest due to the large number of patients which may in principle profit from modulations of those targets. The cartoon of *Figure 5* distinguishes groups of targets that fall into two distinct classes, one of which ('Class A') needs to be enhanced, whereas the other one ('Class B') needs to be suppressed for therapeutic purposes. Groups 1–4 are well documented to be altered in HF of diverse aetiologies: (1) myocardial Ca^{2+} and Na^{+} homeostasis[30,42,62–64] (for details, see paragraph below), (2) β-adrenergic signal transduction,[37,45,65] (3) extracellular matrix remodelling,[66,67] and (4) innate immune signalling.[68,69] Recent investigations suggest that targets associated with immune cell trafficking and cardiac immune cell infiltration (Group 5) are also altered across a spectrum of cardiac disorders beyond the classical viral/autoimmune pathology.[70–78] In contrast, narrowly specific therapeutic targets are found in monogenic (Fabry disease,[51] δ-sarcoglycan deficiency,[44,52] and others[49,72] (Group 6) and virus-induced cardiomyopathies[79–81] (Group 7).

Excellent recent reviews have described several aspects of gene therapy for cardiac diseases. We therefore focus here on the fundamentally novel approach of therapies employing regulatory RNA molecules for that purpose, and on the most recent developments for the cardiac delivery of both regulatory RNA molecules and genes. The

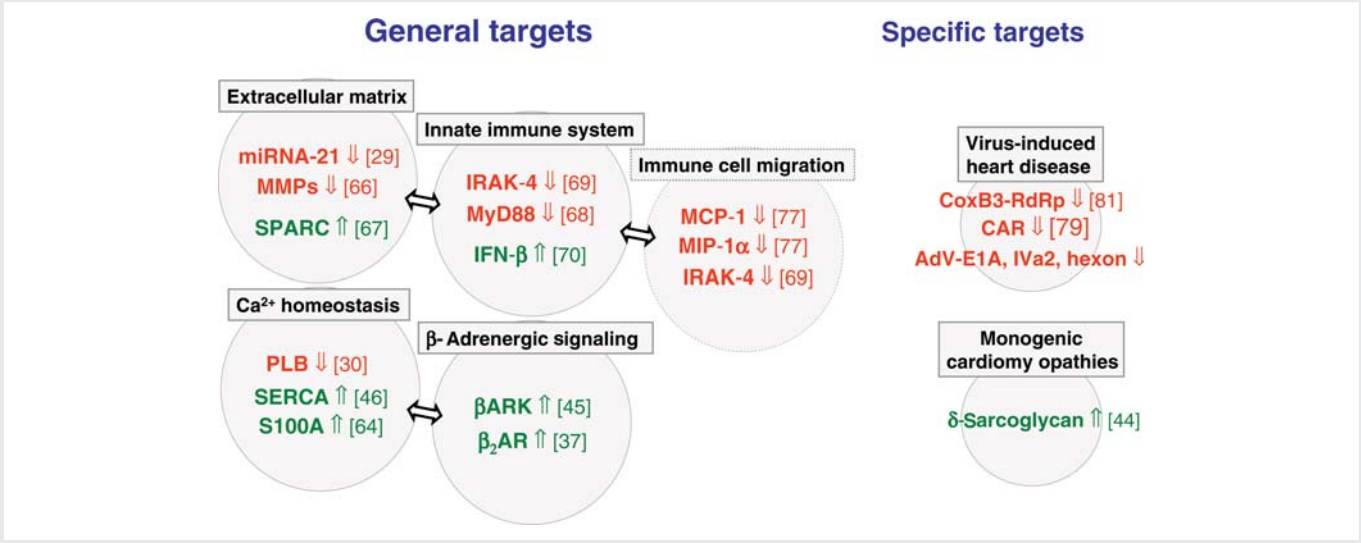

Figure 5 Spectrum of targets for heart failure therapy. The cartoon distinguishes two different classes of targets that need to be approached by using fundamentally distinct methods. 'Class A' targets (green) need to be enhanced for therapeutic purposes, whereas 'Class B' targets (red) require suppression. Only a fraction of the currently known targets is listed for illustration. "General" denotes therapeutic targets relevant to HF of any origin, whereas "specific" targets are relevant for specific etiologies only.

above discussed challenges of targeting, stability and regulation, and safety of the therapeutic tools apply to all the targets summarized in *Figure 5*. Until the discovery of induced RNAi for gene silencing and consecutive protein ablation, Class B targets were only accessible by drugs or antibodies. As a first successful example of this new RNAi approach for a Class B target, we describe below PLB silencing to treat HF. As evident from *Figure 5*, however, a multitude of other Class B targets has already been identified and may be approached by RNAi silencing the specific gene only that expresses the Class B target protein. Other RNA therapeutic technologies involving miRNA ablation and silencing of multiple targets by a single tool are summarized in *Figure 3* and have already been discussed in above paragraph 'miRNAs'. Clinical translation of RNAi is a demanding challenge, but is likely to fundamentally expand the therapeutic repertoire for cardiovascular diseases during the next decades.[82]

3.2 RNAi: a novel approach towards Class B targets

For long-term treatment of HF, novel approaches are currently being explored, including gene and cell therapies[5] and recombinant protein-based approaches[6–9] and combinations thereof,[83] whereas the use of RNAi to modulate cardiac functions had not yet been evaluated. As first successful example of this new RNAi approach for a Class B target, we describe PLB silencing to treat HF. Whereas gene therapy rests on recombinant protein expression as its basic principle, RNAi therapy instead uses small regulatory RNAs to achieve its effect. Targeting, kinetics, and toxicity of these RNAs *in vivo* are grossly different from those of recombinant proteins.[10,55,82,84,85] HF may result from multiple causes, but defective cardiac Ca^{2+} homeostasis is an import final common pathway. We recently demonstrated treatment of HF by the use of a cardiac-targeted RNAi strategy addressing PLB as a key regulator of cardiac Ca^{2+} homeostasis. Cardiac malfunction is in part due to dysfunction of the PLB-controlled sarcoplasmic reticulum Ca^{2+}ATPase pump (SERCA2a), as a consequence of reduced SERCA2a expression or PLB phosphorylation. Unphosphorylated PLB keeps the Ca^{2+} affinity of SERCA2a low, resulting in decreased sarcoplasmic reticulum (SR) Ca^{2+} uptake, slowed relaxation, and decreased SR Ca^{2+} load. In contrast, PLB phosphorylation in response to β-adrenergic stimulation relieves this inhibition.[62] Germline ablation of the PLB gene, gene transfer for dominant-negative PLB mutants,[48,86] asRNAs,[87] and inhibitory antibodies[88] have been employed to enhance SERCA2a activity and rescue experimental HF.[89] RNAi by synthetic siRNAs in cardiomyocytes displayed very low efficacy and stability even *in vitro*,[90] and pharmacological approaches to PLB modulation have failed so far. Inherent limitations of synthetic siRNAs are their very rapid degradation in plasma and target cells, and the unsolved problem of *in vivo* delivery and tissue targeting. Viral vectors have the potential to overcome these limitations, and efficient PLB ablation was achieved in cultured cardiomyocytes [neonatal rat cardiomyocyte (NRCMs)] by the use of an adenoviral RNAi vector.[91] No change in the expression of other cardiac proteins, including Ca^{2+} handling proteins, occurred indicating high target specificity. On the basis of this *in vitro* work, we evaluated the principle of RNAi against PLB for short- and long-term treatment of HF *in vivo*. Functional characterization of a series of vectors and the determinants of their efficacy was required, before investigation of both an optimized recombinant adeno-associated virus pseudotype 9 vector (rAAV9) alongside a traditional adenoviral vector in an animal model of HF became possible.

Building upon landmark articles describing highly cardiotropic vector systems,[39,40] we were able to achieve *in vivo* restoration of cardiac function and reduction of pathological hypertrophy and dilation in an HF animal model by RNAi. Comparison of the two vector systems used suggested that for intermediate timescales, adenoviral vectors may suffice and even provide advantages over long-term stable rAAVs,[38–40,92] because RNAi may be desirable only temporarily in acute and potentially reversible HF. In fact, the significant improvement of diastolic and systolic function and LV morphology 1 month after adenovirus (AdV)-shPLB treatment was evidence of at least a functional therapeutic benefit from the adenoviral system. What has been shown previously for classic gene transfer therapy[42] may obviously work for RNAi-based strategies, too, but strict additional constraints exist for RNAi vector structure to avoid loss of therapeutic efficacy and disturbance of miRNA pathways.[54,93] The experiments further suggest that rAAV-based RNAi may be suitable for the long-term treatment of chronic HF by RNAi strategies. In classic gene therapy studies, rAAV vectors have supported stable transgenic protein expression for more than 1 year, which was never achieved with adenoviral systems in immunocompetent hosts. Although shRNA production from rAAVs is different in several aspects from classic gene transfer, the data from the rAAV arm of the present study provided the first evidence that cardiac rAAV9-based shRNA production remains stable for a period of time sufficient for long-term improvement of cardiac function and possibly also survival in HF. The classical method for gene delivery by recombinant AdV uses cross-clamping of the aorta and the pulmonary arteries in rat hearts and yields homogeneous transgene expression.[47] In our study, employing an advanced AAV vector developed from the highly cardiotropic AAV9 serotype, fluorescent imaging and immunohistochemistry showed that a single intravenous injection of an rAAV9-GFP marker vector induces strong and grossly homogeneous cardiac GFP expression 1 month after treatment (*Figure 6*).

The important observation that co-expression of GFP together with shPLB extinguishes its silencing capacity currently prevents direct demonstration of spatial and temporal uniformity of cardiac shRNA synthesis *in vivo* by standard GFP co-expression. Uniformity was deduced, however, by inference from analogous *in vivo* imaging of GFP expression from adenoviral and rAAV9 vectors. With rAAV9-GFP, the cardiac transduction rate increased over time and reached 90% after 1 month. The improvement of HF by both RNAi therapeutic protocols was mediated via ablation of PLB protein in cultured NRCMs[91] and in rat hearts *in vivo* (*Figure 6*).

After RNAi therapy, cardiac SERCA2a expression was increased compared with HF groups, consistent with the facts that RNAi therapy normalized LV function, and that SERCA2a expression is a marker of the degree of HF.[94] Both RNAi therapies induced a decrease in cardiomyocyte size. Notably, AdV-based therapy over 1 month did not influence the fibrosis induced in failing hearts, whereas long-term rAAV9-based treatment resulted in significantly reduced fibrosis after 3 months.[30]

Irrespective of such subtle differences, however, the effect of the RNAi vectors *in vivo* on PLB ablation, haemodynamics, and morphology[30] confirmed that the doses chosen for both systems are within the therapeutic range. The AAV9 vector used in this study fulfils one important requirement for application in human HF, because it is cardiotropic in primates.[39] In addition to its long-term

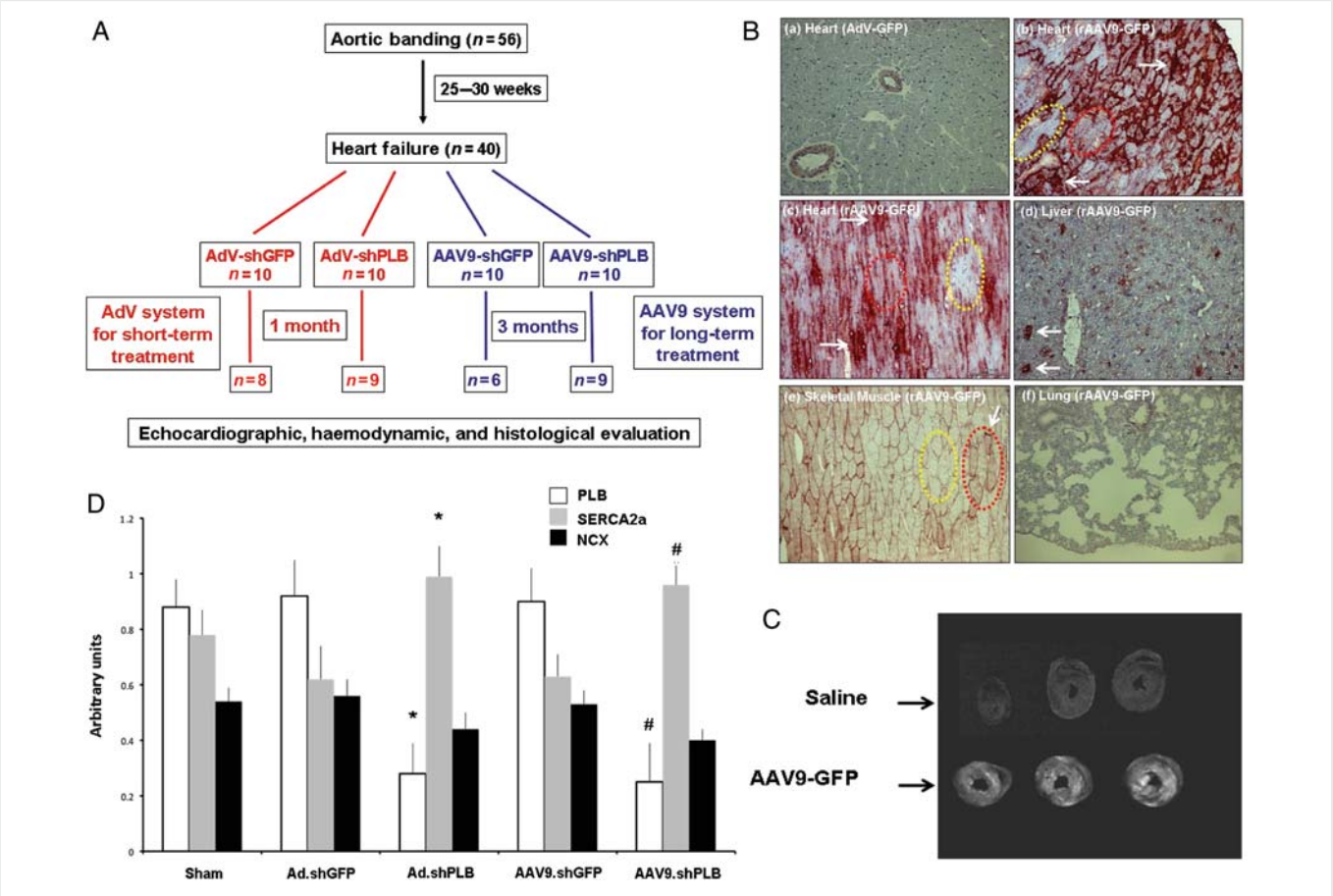

Figure 6 Protocol for RNA interference therapy of heart failure (reproduced by permission from Suckau *et al.*).[30] (*A*) Animals for RNAi therapy were divided into two groups: one of 56 animals with aortic banding and a second of 12 sham-operated animals. In aortic-banded animals, we waited for them to develop LV dilatation and a decrease in fractional shortening by 25% before cardiac RNAi vector transfer. Of 56 aortic-banded animals, 40 survived and were divided into groups receiving AdV-shGFP, AdV-shPLB, rAAV9-shGFP, or rAAV9-shPLB. In the aortic root, 3×10^{10} pfu of each AdV was injected. For experiments with rAAV9, tail-vein injection was done with 5×10^{11} genomes of either vector. Outcome evaluation by echocardiography, tip catheter, morphometry, and histology was performed after 1 month in the adenoviral and after 3 months in the rAAV9 groups (see ref.[30]). (*B*) Immunohistochemical staining of GFP in different organs 1 month after intravenous injection of rAAV9-GFP. Whereas after intravenous injection of an adenoviral vector (AdV-GFP), no GFP was detected in the heart (*a*), rAAV9-GFP treatment resulted in strong GFP expression (*b* and *c*) which was grossly homogeneous. An average of 70% of cardiomyocytes were positive by immunohistochemistry. (*e*) Skeletal muscle with faint staining of a fraction of cells, whereas the liver shows prominent signal of individual cells only (*d*). No signal was visible in the lungs (*f*). Few areas were completely devoid of GFP immunoreactivity (encircled yellow areas); others showed homogeneous cytoplasmic staining (red circles). Staining was particularly dense at sites where high expression over 1 month had obviously resulted in the formation of precipitates (white arrows) of GFP. (*C*) Rats were injected intravenously with an rAAV9-GFP vector expressing GFP or with saline. One month later, GFP imaging showed a grossly homogeneous cardiac GFP signal in the rAAV9-GFP group (bottom) and no signal in the saline group (top). (*D*) Representative western blots show a significant decrease in cardiac PLB protein after 1 month of AdV-shPLB and 3 months of rAAV9-shPLB therapy, compared with shGFP control groups. The sodium–calcium exchanger and GAPDH protein remained unchanged. SERCA2a was decreased in the shGFP groups that were in HF compared with sham, whereas SERCA2a was increased significantly in both shPLB groups. $^{*}P < 0.05$ compared with AdV-shGFP; $^{\#}P < 0.05$ compared with rAAV9-shGFP.

stability, rAAV9 offers further advantages of clinical interest through cardiac targeting after intravenous injection (*Figure 6*) and low immunogenicity. In contrast to rodents, regulatable PLB modulation will likely be required in humans, because permanent PLB deficiency or PLB dysfunction due to genomic mutations has been linked to cardiomyopathies.[95,96] Drug-regulatable RNAi appears possible on both vector platforms used in our study. However, transcriptional control of shRNA expression is technically significantly more demanding than the use of the tissue-specific promoters employed for traditional gene therapy, as these promoters are unable to support proper formation and cleavage of shRNA. New promoters compatible with shRNA biosynthesis are still under development and may add an additional safety feature to organ-targeted RNAi therapies.

3.3 Pathogenetic analyses employing advanced RNA technology

Independent of its therapeutic potential, organ-targeted RNAi may be of use to identify novel gene functions in that organ by functional ablation, analogous to classic tissue-specific inducible knockout

models. The extent of ablation observed in the present study may suffice to recognize unknown gene functions, and the efficacy of this novel analytical approach is likely to increase with the advent of more sophisticated RNAi delivery systems. Importantly, gene ablation by RNAi may be induced at any desired age or disease state and would be more rapid and inexpensive than the traditional models.

3.4 Consideration of side effects of RNAi

The cellular machinery of RNAi evolved over millions of years and is the most efficient and versatile mechanism known for specific gene silencing. shRNAs exploit this machinery to mediate therapeutic effects by mimicking the endogenous process and achieve silencing at far lower concentrations than asRNAs, but they may disturb cellular miRNA pathways[54,55] and thereby cause hepatotoxicity. When using a cardiotropic rAAV9 serotype with low affinity for the liver, we observed no histological evidence of acute or chronic liver damage. We also studied the cardiac-expressed miRNAs 1 and 133 during RNAi treatment. Because malignant arrhythmias are important complications in HF, deregulation of an arrhythmia-related miRNA such as miRNA-1,[15] by a novel treatment, should be considered as a possibly serious adverse effect. None of the RNAi vectors changed miRNA-1 levels in NRCMs, but interestingly, shPLB treatment was instead associated with rescue of the miRNA-1 depression induced by phenylephrine in these cells, and rat hearts that underwent shPLB therapy had higher miRNA levels than controls. In conjunction with the trend towards improved survival in the AAV-shPLN treatment group, no evidence was thus found for arrhythmogenic side effects. Although improved contractile function during shPLB therapy results immediately from its influence on excitation–contraction coupling via the cytoplasmic Ca^{2+} transients, the marked reduction of LV hypertrophy and dilation is not deduced as easily, because it involves major reprogramming of the cardiac transcriptome. The RNAi-induced changes of Ca^{2+} homeostasis may affect not only the cytoplasmic Ca^{2+} transients but also the separate and insulated Ca^{2+} signals generated in the perinuclear space[97,98] that influence transcription. With respect to hypertrophy, it is of interest that shPLB treatment was associated with rescue of the phenylephrine-induced miRNA-133 depression in NRCMs and higher levels in rat hearts on shPLB treatment. The rescue of miRNA-133, which plays a critical role in the control of cardiomyocyte size,[21,99] appears to be linked to RNAi at the cell level, because it also occurs *in vitro*, where haemodynamic stress, neurohumoral, or cytokine activation, as in HF *in vivo*, cannot play any role. Although our rationale to measure miRNA expression was to test whether shPLB may 'poison' the RNAi machinery, differential miRNA induction can also be viewed as 'master switches' that control reinduction of foetal genes during HF.[14] A clear distinction between side effect and master-switch aspects of miRNA regulation is very difficult and certainly beyond the scope of the present report. In addition, clarification of the mechanism by which shPLB-RNAi is linked to the changes in miRNA-1 and miRNA-133 (passive association or component of the therapeutic process) requires future studies.

The investigation outlined above[30] provides a first example for high efficacy of a locally targeted RNAi therapeutic strategy in a cardiac disease. The paper emphasizes the critical need for careful vector adaptation to the specific requirements of RNAi, and for extensive characterization of candidate regulatory RNA sequences with respect to high intrinsic activity and target specificity. Under these preconditions, RNAi therapy may achieve long-term cardiac benefit without apparent toxicity. When functionally optimized RNAi vectors were used, and aortic root vector injection or cardiotropic rAAV9 capsids were used to target RNAi to the heart, no evidence was found of side effects. Short-term PLB silencing led to improved cardiac function 1 month after aortic root injection of an adenoviral RNAi vector. Long-term RNAi after simple intravenous injection of an optimized rAAV9 vector resulted in restored cardiac function and reduction of cardiac dilation, hypertrophy, and fibrosis after a period of 3 months. The rAAV9 approach uses a vector known to target the heart in primates,[39] thus offering potential for clinical translation. Specifically, for targets such as PLB in which pharmacological approaches have failed thus far, the RNAi approach may enhance the therapeutic repertoire for cardiac diseases. Multiple other Class B targets (*Figure 5*) are likely to be investigated in the near future.

4. Translation issues and clinical trials

With respect to the clinical translation of RNA therapeutic strategies, it may be anticipated that some of them may ultimately be substituted by more traditional 'pharmacological' approaches than the pioneering studies which mostly employ highly sophisticated methods. One important recent example is the use of an intravenously injected, untargeted antagomir to ablate the HF-associated miRNA-21. Whereas the landmark papers that have elucidated the biological and pathogenic roles of this miRNA have used transgenic and knockout animals and viral vectors, the antagomir approach has shown impressive therapeutic potential, although its target is expressed in multiple organs.[29] It will be interesting to see which other diseases may be efficiently treated by this 'untargeted' and elegant approach without significant side effects in non-target organ due to miRNA depletion at these sites.

In contrast to this 'pharmacological' approach at miRNA ablation, there was so far no successful *in vivo* therapeutic application of RNAi based on synthetic siRNAs or chemical derivatives. The reason for this is their still very short half-lifes in plasma and target cells and the lack of proven concepts for organ targeting. In the field of RNAi therapy, the introduction of AAV vectors with favourable safety profiles has nevertheless allowed to use the powerful approach of RNAi for successful therapy of both systemic[54] and local diseases[30,81] *in vivo*. Such proof-of-concept studies have triggered major interest in the pharmacological industry to investigate to what extent these therapies may be mimicked by modified synthetic siRNAs. Obviously, pharmacological approaches face the challenge of delivering extremely unstable small RNAs, even if in some cases targeting may not be required. Both stability and targeting may be achieved by the use of advanced vector technologies[35,38] for both gene therapy and regulatory RNA delivery. A first clinical trial has recently demonstrated the feasibility of a vector-based approach for HF therapy by cardiac SERCA2a gene transfer in humans[46] and an analogous RNAi therapeutic strategy has been successfully applied in a rodent model.[30] Gene therapy has also been employed to induce 'cardiac regeneration', initially attempted by cell transfer strategies only.[5,100] Important recent work has demonstrated that promotion of cardiac repair is also possible by delivery of proteins such as periostin,[7] thymosin-β4,[9,101] or cyclin A2.[102] Gene transfer

has also improved the efficacy of cells to be used for adoptive cell therapy[83] and may foster this strategy in general.

Independent of such translational applications, regulatory RNA and gene transfer are indispensable for research. They have helped to define the hierarchy of progenitor cells suitable for cardiac therapy,[103,104] and to discover new important roles for miRNAs in cellular differentiation.[105] As outlined in *Figure 4*, the technologies for RNA- and gene-based therapies are rapidly evolving. New types of viral vectors and BNPs already have multiple properties required for *in vivo* applications and clinical translation. Whereas traditional nanotechnology has so far been unsuccessful for RNA and gene delivery to the heart,[106,107] 'biomimetic' nanoparticles designed to mimic key physicochemical properties of virion shells with known cardiotropism may in the future develop into an alternative to the current biological technologies. Recent progress in the field suggests that RNA and gene transfer technology will have a major impact on clinical practice in the near future, by fundamentally expanding our therapeutic repertoire beyond classical drugs and recombinant proteins.

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