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REVIEW

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Nucleic acid-based modulation of cardiac gene expression for the treatment of cardiac diseases

Approaches and perspectives

Nucleinsäure-basierte Modulation der kardialen Genexpression zur Behandlung kardialer Erkrankungen

Zusammenfassung In den letzten Jahren wurden wichtige konzeptionelle und technische Fortschritte erzielt auf dem Weg zur therapeutischen Modulation der kardialen Genexpression bei kardialen Erkrankungen. Zu diesen Fortschritten zählt 1. Die Identifikation neuer therapeutischer Ziele bei der Herzinsuffizienz, oft anhand genetischer Tiermodelle. 2. Ein besseres Verständnis der molekularen und zellulären Determinanten kardialen Gentransfers *in vivo*, in Tiermodellen und in ersten klinischen Studien. 3. Die Entwicklung regulierbarer und langzeitstabiler Vektorsysteme. Diese Übersicht fokussiert sich auf die Nukleinsäure-basierte Modulation der kardialen Calcium-Homöostase als ein Paradigma für diese gentherapeutischen Ansätze, da jüngere bahnbrechende Arbeiten annehmen lassen, dass die Calcium-Homöostase ein Therapieziel von zentraler Bedeutung bei der Herzinsuffizienz darstellt. So konnte die Entwicklung einer schweren Herzinsuffizienz im genetischen MLP$^{-/-}$ Tiermodell vollständig blockiert werden durch die gezielte genetische Ausschaltung des Phospholambans (PL), eines zentralen Regulators der kardialen Calcium-Homöostase. Dieser eindrucksvolle Effekt einer permanenten PL-Ausschaltung liefert – in Verbindung mit wichtigen früheren Arbeiten über Störungen der Calcium-Homöostase im insuffizienten menschlichen Herzen – eine rationale Basis für den therapeutischen Ansatz einer *ad hoc*-Suppression von PL mit Hilfe von *antisense*-Strategien (*antisense*-RNAs, Ribozyme, RNA-*Interferenz*) oder PL-Varianten. Wegen des sehr breiten Spektrums an Methoden, die zur Charakterisierung dieser Strategie eingesetzt worden sind, können PL-gerichtete Strategien als Paradigma für zukünftige genetische Therapien kardialer Erkrankungen angesehen werden, trotz bekannter komplexer Unterschiede zwischen Tiermodellen und dem Menschen. Hohe Sicherheit jeglicher solchen Therapie ist eine Voraussetzung für ihre mögliche klinische Anwendung und neue Kontrollmethoden werden daher entwickelt: 1. Regulation des Vektors durch biochemische Anomalien, die mit der Zielkrankheit selbst assoziiert sind („*Induction-by-Disease*“ Gentherapie). 2. Externe Kontrolle der Vektoraktivität durch den Einsatz pharmakon-sensitiver Promotoren. Darüber hinaus konnte das wichtige Ziel kardialer Langzeit-Stabilität der therapeutischen Vektoren in jüngster Zeit tierexperimentell mit Vektoren auf der Basis von Adeno-assoziierten Viren erreicht werden.

Schlüsselwörter Antisense-RNA – kardiale Calcium-Homöostase – Gentherapie – Herzinsuffizienz – Phospholamban – RNA-Interferenz – Ribozyme

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Summary During the past few years major conceptual and technical advances have been made towards the therapeutic modulation of cardiac gene expression for the treatment of cardiac diseases. Among these are 1) the identification of new molecular therapy targets in cardiac disorders, often derived from genetic animal models. 2) A better understanding of the molecular and cellular determinants of cardiac gene transfer *in vivo*, in animal models and in first clinical trials. 3) The development of novel regulatable and long-term stable vector systems. This review is focused on nucleic acid-based modulation of cardiac calcium homeostasis as a paradigm for the new gene therapeutic approaches, since recent landmark papers have suggested this to be a molecular target of key importance in heart failure. In particular, the development of severe heart failure in the genetic $MLP^{-/-}$ animal model could be completely abolished by the targeted ablation of phospholamban (PL), a key regulator of cardiac calcium homeostasis. This impressive effect of permanent germline PL ablation provides – in conjunction with former important work on disturbed calcium handling in the failing human heart – a rationale for the gene therapeutic approach of *ad hoc* suppression of PL by *antisense* strategies (*antisense* RNAs, ribozymes, RNA *interference*) or PL variants. Based on the broad spectrum of methods employed to characterize this general strategy, PL-targeted approaches may be considered as a paradigm of future genetic treatments of cardiac disorders, although the differences between animal models and humans must be kept in mind. High safety of any such therapy will be a prerequisite for any possible clinical application and therefore novel methods to improve control are being devised: 1) The regulation of gene therapy vectors by biochemical abnormalities associated with the target disease itself (*"Induction-by-Disease"* gene therapy). 2) External control of vector activity by the employment of drug-sensitive promotors. In addition, the important goal of cardiac long-term stability of the therapeutic vectors has recently been achieved in animal models using vectors derived from adeno-associated viruses (AAVs).

Key words Antisense RNA – cardiac calcium homeostasis – gene therapy – heart failure – phospholamban – RNA interference – ribozymes

Clinical background and problems

During the past few years major conceptual and technical advances have been made towards the therapeutic modulation of cardiac gene expression for the treatment of cardiac diseases. Among these are 1) the identification of new molecular therapy targets in cardiac disorders. 2) A better understanding of the molecular and cellular determinants of cardiac gene transfer *in vivo*. 3) The development of novel regulatable and long-term stable vector systems. As a paradigm for these new therapeutic approaches this review focuses on nucleic acid-based modulation of cardiac calcium homeostasis, since recent landmark papers have suggested this to be a molecular target of key importance in heart failure.

In an effort to improve upon the current standard therapy of heart failure novel strategies are being developed. Since even under optimal standard therapy, heart failure has an adverse prognosis [1, 2], the need for additional treatment options is obvious. The most severly ill heart failure patients could possibly benefit most from any new treatment option and therefore highly invasive approaches have already been employed in this subgroup, *e.g.*, the REMATCH study evaluating the efficacy of a cardiac assist devices in patients with chronic end stage heart failure [3]. Current heart failure treatment takes advantage of additive or synergistic effects between individual drugs [4–6] and other options such as multisite pacing [7] or left ventricular assist devices [3]. Stem cell therapy may become an option in the future [8–18]. Current combined approaches are to some degree effective even in the most severely ill. Any novel future option will at most become *one part* of the general scheme, not a substitute for any of the current options, or it will be able to address

Abbreviations

AAV	adeno-associated virus
AdV	replication-deficient adenovirus-based vector
CAR	Coxsackie virus-adenovirus-receptor
DCM	dilated cardiomyopathy
Dox	doxycycline
*ds*RNA	*double-stranded* RNA
ET-1	endothelin-1
GFP	green fluorescent protein
HCM	hypertrophic cardiomyopathy
MLP	muscle LIM protein
ON	oligonucleotide
PL	phospholamban
PL*as*	phospholamban *antisense* RNA
PTCA	percutaneous transluminal coronary angioplasty
RISC	RNA-induced silencing complex
RNA*i*	RNA *interference*
rtTA	reverse transcriptional transactivator
SERCA	sarcoplasmic reticulum Ca^{2+} ATPase
si RNA	*small interfering* RNA

specific important subgroups. For the treatment of diastolic heart failure [19, 20], for instance, no large controlled clinical trials comparable to those of systolic heart failure are currently available. Blockade of the renin-angiotensin system, NO donors, and drugs enhancing NO release have been employed in smaller clinical trials [21–23]. Novel approaches, however, based on specific diastolic pathophysiology may significantly improve the treatment options for the large number [24, 25] of patients suffering from predominantly diastolic heart failure.

Current gene therapy protocols are technically still very demanding, but due to recent progress in several field of gene therapy research it appears more likely now that genetic therapies may play a role in the treatment of cardiac diseases in the foreseeable future. Besides the identification of new molecular targets in heart failure, often derived from genetic animal models in conjunction with clinical studies, a more detailed understanding of the molecular and cellular determinants [26–29] of vector-based cardiac therapeutic gene transfer *in vivo* has been achieved, both in animal models [30–38] (Fig. 1) and in first clinical trials [39–42] (Fig. 1). Furthermore, improved vector systems for cardiac gene transfer are now available [43]. This review is focused on the genetic modulation of cardiac calcium homeostasis, since recent landmark papers have suggested this to be a molecular target of key importance in heart failure [30, 44]. In particular, the development of severe heart failure in the genetic $MLP^{-/-}$ animal model of heart failure [45] could be completely abolished by the targeted ablation of phospholamban (PL) [44], a key regulator of cardiac calcium homeostasis. This impressive effect of permanent germline PL ablation lends strong support to the closely related gene therapeutic approaches of *ad hoc* suppression of PL by using *antisense* strategies (*antisense*-oligoncleotoides and -RNA, ribozymes and DNA enzymes, RNA *interference*) [33, 43] or dominant negative PL mutants [30, 46]. Due to the unusually broad spectrum of methods employed to characterize this general strategy, PL-targeted approaches may be considered as a paradigm of future genetic treatments of severe heart failure. Tight control over such therapies will be of paramount importance before clinical applications may be considered, and novel methods to improve control have recently been devised: the regulation of gene therapeutic vectors by exploiting biochemical abnormalities associated with heart failure itself ("*Induction-by-Disease*" gene therapy [43], see Fig. 5 C, D), or pharmacological control of vector activity by the employment of drug-sensitive promotors [47, 48] (see Fig. 5 A, B). Another approach to transgene regulation by the disease process itself uses hypoxia-inducible promotors [49–52]. Cardiac long-term stability of the therapeutic vectors is also under intense investigation and may be achieved by vector systems derived from adeno-associated viruses (AAVs) [30, 51, 53–57], by novel hybrid-vectors combining advantageous properties of different viral species [58], or by multiply attenuated, self-inactivating lentiviral vectors [59, 60]. The basic biology of AAV [54, 55] makes recombinant AAV-derived vectors endowed with additional advantageous properties [61, 62] particularly suitable for long-term stability in eucaryotic tissues, in particular second generation AAV vector systems [62 a].

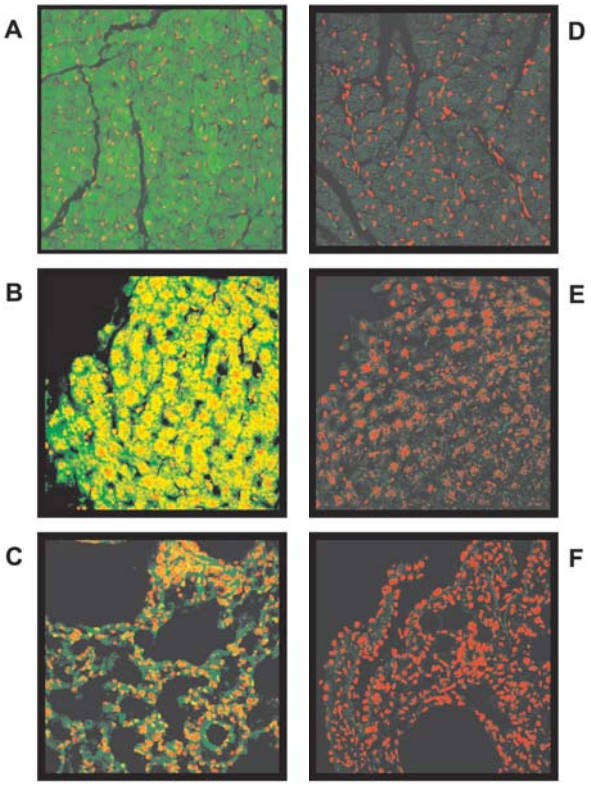


Fig. 1 *Targeting of recombinant adenovectors to the myocardium*. **A** After aortic root injection of an adenovector expressing the marker *green fluorescent protein* (GFP), the rat heart expressed high levels of GFP evenly distributed throughout the myocardium, as shown by confocal laser scanning microscopy of cardiac section. **B** shows a heart after treatment with a luciferase control vector, displaying only red propidium iodide staining of cardiomyocytes and very low background green fluorescence. Whereas cardiac gene transfer was successfully enhanced by several orders of magnitude by aortic root as compared to intravenous injection, gene transfer was not confined to the heart. Instead, **B** and **C** still show significant GFP expression in liver and heart, respectively, as compared to controls (**E** and **F**) (for further details see reference 26) (Reprinted with permission from *Gene Therapy*)

New molecular targets for heart failure treatment

Several new molecular targets for heart failure therapy have been identified during the past few years (for review see ref. [2]). Among those targets several components of the cardiac calcium homeostasis have gained particular interest due to landmark observations in genetic animal models [44, 45]. These observations have been made possible by the synthesis of two lines of research. First, a genetic mouse model of severe dilated cardiomyopathy (DCM) was generated by targeted ablation of a particular Z-band protein, the muscle LIM protein (MLP) [45]. These homozygous $MLP^{-/-}$ *knock out* animals developed heart failure due to severe DCM and were the first genetic animal model presenting with a phenotype closely resembling DCM in humans. Recently, a cellular function of the MLP protein as part of the cardiac mechanical stretch sensor machinery was proposed which provides a possible molecular mechanism for the development of the severe cardiac dysfunction observed in $MLP^{-/-}$ *knock out* mice [63]. The recent detection of human MLP gene mutations in a subset of patients presenting with the clinical phenotype of DCM may be of major clinical importance, since in these individuals a novel molecular pathomechanism appears to contribute to the development of DCM [63]. Interestingly, a genetic founder effect was observed for the heterozygous carriers (all of them of Central European origin) of the human W4R mutation, a missense point mutation resulting in a severe charge change at amino acid position 4 of the MLP protein [63]. In summary, these recent studies show that the MLP protein may play an important role in the pathogenesis of heart failure in animals, but also in man. Second, genetic ablation of the gene encoding phospholamban (PL), a key regulator of the cardiac calcium cycle, resulted in mice with enhanced cardiac contractility [64]. When $PL^{-/-}$ animals were crossed with $MLP^{-/-}$ mice, a complete rescue of the DCM phenotype of $MLP^{-/-}$ mice was observed [44]. This finding is of outstanding importance, since it suggests a central role of PL in the treatment of DCM. PL also appears to play a role in the development of DCM in humans [65]. These data provide a rationale for attempts to modulate PL expression and function not only in order to improve cardiac function in DCM, but also to delay or halt the progression of the disease. Disturbed cardiac calcium homeostasis had already been descibed several years ago in human failing hearts [66–69] which in conjunction with the above data from the genetic animal models lends further support to the concept of treating heart failure by calcium cycle modulation [70, 70 a].

The classical approach to achieve PL modulation would be pharmacological in nature, *e.g.*, using compounds blocking PL interaction with its physiological target, the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA), or by modulation of its phosphorylation state through interference with any step of the β-adrenergic signal transduction system. The major problem of this therapeutic approach is that the β-adrenergic signal transduction system has several other protein targets such as troponin I, myosin-binding protein C and the L-type calcium channel which each have their own specific roles in excitation-contraction coupling. However, due to the clear molecular definition of PL as a target of outstanding importance, the highly specific approaches of gene therapy also appear to be suitable to achieve this therapeutic goal, since clear definition of a single target gene or mRNA is a prerequisite for any genetic therapy. For this methodological reason and because of the high clinical relevance of the disease to be treated, gene therapeutic modulation of the cardiac calcium cycle has been attempted by several groups during the past few years [30, 33, 43, 44, 71–74], based on extensive previous basic work using genetically engineered models with alterations in cardiac calcium-handling proteins [64]. Another calcium-related therapeutic approach in heart failure is the modulation of excitation-contraction coupling, *e.g.*, by FKBP-mediated stabilization of the cardiac calcium release channel (ryanodine receptor) [75–77]. In the following two sections the different strategies used, with their respective advantages and limitations, shall be discussed.

Recent developments in cardiac 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 primarily manifested in the myocardium could theoretically be cured by this approach. Among them are the various long-QT syndromes [78], arrhythmogenic right ventricular dysplasia, Brugada syndrome [79], hypertrophic cardiomyopathies caused by a host of mutations in various sarcomeric proteins [80, 81], and dilated cardiomyopathies [82, 83]. A second concept of gene therapy is the *enhancement* of gene functions to cells, tissues, and organs by overexpression of primarily endogenous genes using gene transfer vectors. 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 truely novel gene functions to the target organ by vector-based expression of foreign genes [84]. In the field of heart failure gene therapeutics, one transgene of interest is the SERCA gene whose expression level influences the efficacy of diastolic calcium reuptake from the cytosol to the SR and may be *enhanced* by gene transfer. SERCA "gene therapy" has first been performed in cardiomyocyte cultures *in vitro* for *proof-of-principle*, and later on also *in vivo* using viral vector systems. Other approaches to achieve an improvement of cardiac function using the *enhancement* concept have used components of the β-adrenergic signal transduction system [31, 35–38, 85] and of cardiomyocyte cytoskeletal [86] or membrane proteins [87]. These transgenic gene therapeutic strategies are often first evaluated in *proof-of-principle* studies using cultured cardiomyocytes *in vitro* under carefully controlled conditions. Further investigation of the principle *in vivo* may then provide an important further step towards possible therapeutic applications, in particular if animal disease models closely resembling human disorders are employed. Although the concepts of gene *substitution*, *enhancement*, or *addition* therapy are rather simple, several technical issues need to be resolved to make them efficient *in vivo*.

The first issue is the mere physical steering (*targeting*) of the vector to the target tissue, *e.g.*, the myocardium, needs to be achieved which is not a simple task. Various approaches [30, 31, 35, 37, 85, 87] including intracoronary application [34, 36] have been employed to achieve this goal, also already in clinical trials [39–42]. A better understanding of the molecular and cellular determinants of *vector targeting* in the cardiovascular system has evolved during the past few years based on studies of the expression patterns of *vector receptors* (*i.e.*, the receptors primarily mediating the cellular uptake of the virus from which the vector is derived) in animals [26, 27] and humans [28, 29, 88], and on the discovery of *anatomical barriers* such as the vascular endothelium or intracellular matrix [26] inhibiting or preventing vector transfer to particular parts of the heart and other organs and tumors. Interestingly, the expression of a particularly important vector receptor, the Coxsackie virus-adenovirus-receptor (CAR), was found to be highly variable in human hearts (generalized induction in dilated cardiomyopathy) [29, 89] (Fig. 2) and in animals (local induction after myocardial infarction) [27] (Fig. 3). Receptor-directed strategies [90] for the improvement of myocardial vector targeting are currently being developed and CAR may be of particular interest as a cardiac vector receptor in those disorders (DCM, myocardial infarction) where it is induced in the diseased regions of the heart [26, 27, 29] (Fig. 3). If ideal physical vector targeting and highly selective homing of the transgene to the target tissue cannot be achieved, additional *transcriptional confinement* of the transgene may be achieved by using cardiac-specific promotors [91–93]. The recognition of the strong receptor-dependency of vector targeting has prompted investigation dealing with the possible therapeutic potential of *tropism modifications* by altering the receptor-interaction domains of the viruses used as vectors. A number of studies on the vector susceptibility of different primary cell types for *tropism modification* have been conducted during the past few years, both for adenoviral [94] and AAV-based vectors [95–100]. Basically, these approaches employ alterations of viral surface structures mediating cel-

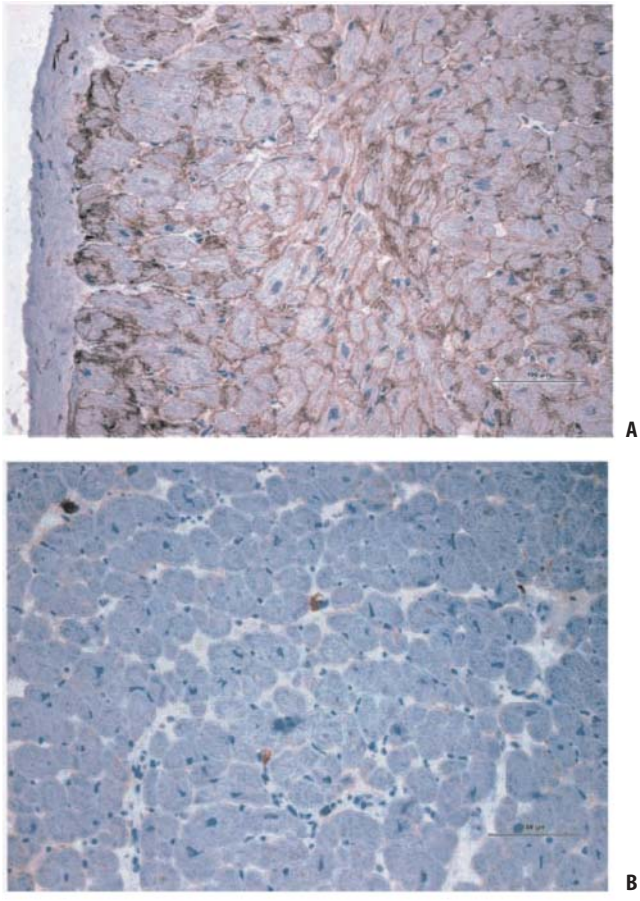


Fig. 2 *Variable expression patterns of the adenovector receptor CAR in normal and diseased human hearts*. **A** Normal human donor hearts showed low immunoreactivity for the cellular vector receptor CAR on cardiomyocytes, as assessed by immunoperoxidase staining. A few interstitial cells and the subendothelial wall layers od cardiac vessels stained positive for CAR (reddish stain). **B** In contrast, DCM hearts showed *generalized* induction of CAR on cardiomyocytes, either due to de *novo* synthesis of CAR or as a consequence of the exposure of immunoreactive sites of CAR (29). In contrast to **B**, which shows *generalized* CAR induction, Fig. 3 depicts *localized* induction of CAR after myocardial infarction (Reprinted with permission from *Zeitschrift für Kardiologie*)

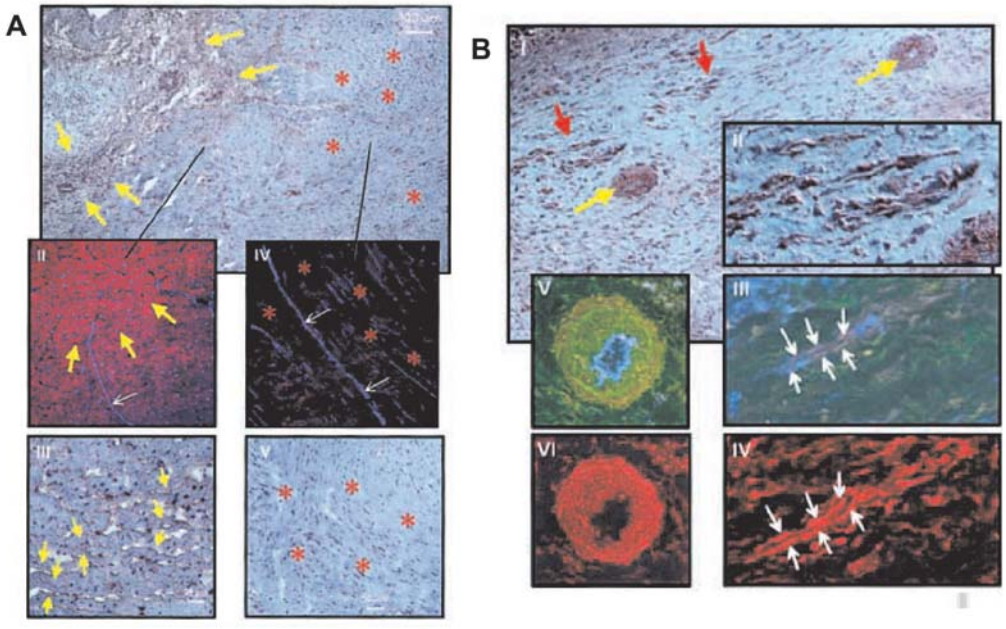


Fig. 3 *Localized induction of the adenovector receptor CAR after myocardial infarction in the rat*. **A** Peroxidase immunohistochemistry (parts I, III, V) and confocal laser scanning microscopy (parts II, IV) for CAR (Cy3-red) and CD31 (Cy5-blue) in infarcted rat hearts. Part I gives an overview with reddish immunohistochemistry staining for CAR. Parts II and III are from the infarct border zone with yellow arrows indicating sites of high Cy3-red CAR immunorectivity. Parts IV and V are from remote uninfarcted areas show low CAR signals. **B** Peroxidase immunohistochemistry (parts I, II) and confocal laser scanning microscopy (parts III, IV) of arterioles (parts V, VI – arrows showing Cy3-red-CAR) and CD31-positive capillary-like structures (parts III, IV). Whereas in arterioles (parts V, VI) and venules (not shown) Cy5-blue-CD31 was confined to the endothelium and Cy3-red-CAR to the subendothelial wall layers, the capillary-like structures (parts III, IV) showed co-localization of Cy5-blue-CD31 and Cy3-red CAR, resulting in magenta colouring of the structures on confocal analysis (Reprinted with permission from *Circulation*)

lular receptor binding and internalization of the vector. This has been achieved by genetic engineering of vector genes encoding vector surface components [94, 101], by bi-functional antibodies binding both to a vector surface epitope and a cellular structure specific for the desired target cells [93], or by bi-specific targeting proteins conjugated to the vector surface *via* avidin-biotin complexes [97]. An interesting new concept along the line of *tropism modifications* is the use of drugs to "enhance" virus uptake pathways, *e.g.*, 5α-dihydrotestosterone strongly increased liver-directed transfer of AAV vectors [102].

It should be emphasized that in order for the above *tropism modifications* to become useful *in vivo* the vectors first need to obtain direct access to the desired target cells which may be prevented, however, by *anatomical barriers* (vascular endothelium, basal membrane, extracellular matrix). In addition to studies addressing the receptor issue a number of approaches to overcome the *anatomical barriers* have been evaluated [103–109], but none of these methods is simple or as yet appropriate for application in the clinical setting. In myocardial gene therapy the final target cells – beyond the *anatomical barriers* – are particularly difficult targets as compared to other cell types and at the current state of the art, viral vectors are the only option to achieve transfer rates sufficient for therapeutic efficacy. So far no report on successful *in vivo* treatment of cardiac diseases using non-viral systems (including nanoparticles) has been published. Given the obstacles against efficient and targeted vector delivery, the recent development of imaging techniques for the *in vivo* detection of transgene expression may greatly facilitate the further development, monitoring, and assessment of cardiac gene therapeutic procedures [110–112].

The second issue is the stability of transgene function. If genetic therapy for heart failure is not only meant to serve as a *bridge-to-transplant* or *bridge-to-recovery*, then long-term stability of the therapeutic vector plus transgene is required. Among the virus-based vector systems [60] (Table 1) used for myocardial gene transfer, the currently most promising systems to achieve long-term stability are derived from adeno-associated viruses (AAVs). AAV vectors – although still difficult to produce at the high titers needed for *in vivo* applications – have shown stability for than one year in fully immunocompetent hemophilic dogs treated intramuscularly with a coagulation factor IX-producing AAV vector [54, 113]. Based on the experimental

Table 1 Viral vectors for cardiac gene therapy

Parent virus	Vector genome modificaitons	Nucleic acids expressed	Stability of expression	Genomic integration	Immmunogenicity and cellular effects	Natural tropism and modifications
Adenovirus (AdVs) Types 2, 5 efficient transduction of non dividing cells	Safety and capacity modifications: E1, E3 deletions in standard helper-independent AdV vectors Large-scale deletions in helper-independent, high-capacity AdV vector [188–194] Expression cassette: Cardiac-specific promotors (MLC-2, ANF, BNP) [47, 48, 91] Tetrcycline-inducible promotor [47, 48, 120] Promotors (ANF, HIF) for *"induction-by-disease"* strategies [43, 50, 51] Polymerase III (H1, U6) or polymerase II promotors for *si*RNA transcription [167]	cDNAs: [31–38, 71, 72, 74, 84, 86, 87, 181–184] *antisense*-RNAs: [43, 47] *si*RNAs: [167, 171, 172]	Vector stability: Limited to a few weeks in immunocompetent hosts due to immune reactions against vector-transduced cells and/or transgene products	Wild-type virus: Normally no genomic integration with AdV types 2 and 5 AdV vectors AdV may be changed in integrating vectors [58]	Humoral and cellular immune responses: Against both vector/transgene products limit expression to several weeks in immunocompetent hosts Cellular effects: Dose-dependent induction of inflammatory cytokines: RANTES, inferferon-inducible protein 10, interleukin-8, MIP-1β, MIP-2 [126, 195] Induction of cellular "stress response" with augmented expression from cellular cytokine and chemokine gene [126, 195]	Cellular receptor: CAR [26–29, 88] Cellular co-receptors: $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins [88] Alternative pathways: [196] Vector re-targeting strategies: Viral survae protein modifications [94, 95, 101] Bi-functional antibodies mediating interactions between viral surface and cellular target protein [93]
Adeno-associate viruses (AAVs types 1 to 6)	Safety and capacity modificaitons: Large parts of wil-type AAV genome are removed from AAV vectors, thus vector production is possible only by using viral or plasmid helper systems providing all missing functions "Duples" AAV vectors synthesizing dsAAV genomes after cell entry, leading to a far more rapid starting of transgene expression Expression cassette: Tetracycline-inducible promotors [121]	cDNAs: [30, 53–57, 114, 197]	Vector stability: Very high as compared to AdV (several months in myocardium), in part due to genomic integration events, in part due to minimal immune responses	Wild-type virus: Wild-type AAV integrates into a specific site on chromosome 19 AdV vectors: AAV vectors have multiple integration sites on the same chromosome, but at different sites [115–117] AAV vectors for preferential integration into AAVS1 have been developed [61] Chromosomal effects of AAV vector integration constitute a possible safety risk [155]	Humoral and cellular immune responses: Very low as compared to adeno-vectors [195, 198] Cellular effects: Very low as compared to AdV [195, 198]	Cellular receptor: Heparan sulfate proteoglycan (HSPG) [199, 200] Cellular co-receptors: $\alpha v\beta 5$ integrin [201] Vector re-targeting strategies: Packaging of identical vector genomes into different virus envelopes [96] AAV-3 [202] AAV-5 [203] AAv-6 [204] Bi-specific targeting proteins [95, 97] RGD incorporation into the VP3 [98] Hormonal virus uptake pathway modifications [102] Search strategy: AAV display [99]
Retrovirus Including lentiviridae which efficiently tansduce non-dividing cells, in contrast to the other retroviruses [205–207]	Safety and capacity modificaitons: Multiply attenuated self-inactivating vectors [59, 60]	cDNAs: [60, 208–215] *si*RNAs: [173, 174, 216, 217]	Vector stability: Very high as compared to AdV (possibly life-long), in part due to regular genomic integration			Vector re-targeting strategies: Pseudotyping for the broadening of vector tropism [218–221] Cardiomyocyte transduction capacity [60, 214, 215]

Numbers in brackets refer to references
AAV adeno-associated virus; *AdV* adenovirus; *CAR* coxsackievirus-adenovirus-receptor; *siRNA* small interfering RNA

data a factor IX AAV vector has been evaluated in a clinical phase I trial [114]. The high stability appears to be a consequence of inherent fundamental biological properties of the AAV genome [55]. Although it is not always clear whether transgene expression occurs from integrated or episomal vector genomes, sequencing of vector-genome junctions has demonstrated the presence of integrated AAV genomes in cultured human cells and in mice after treatment with AAV vectors [115–117]. AAV vectors have only recently been employed for the cardiac genetic treatment of heart failure [30], for the systemic treatment of Fabry disease [56], and for substitution gene therapy of an inherited cardiomyopathy due to a δ-sarcoglycan gene defect [53], with transgene expression over several months in all cases. Recently, transgene expression in cardiomyocytes for extended periods of time has also been reported for multiply attenuated, self-inactivating lentiviral vectors which fall into the group of retroviral vectors [59]. The relative merits of retroviral *versus* AAV vectors need to be studied further.

A third issue – of paramount importance before any clinical application may be considered – is the safety of the gene transfer protocols. One safety feature under intense investigation are approaches towards regulation of the transgene after injection into the host. This may not be required for any application but is necessary in situations when adaptation of transgene activity to the physiologically required level is desired. For instance, recent data suggest that PL suppression by *antisense* approaches should not be attempted using non-regulatable vectors, since certain defects of the PL gene in humans apparently resulting in permanent suppression of PL function were associated with the development of heart failure [65, 118]. An option to shut down the transgene completely at any time in the case of serious adverse effects is highly desirable for safety considerations [119]. Vectors systems that can be shut off by withdrawal of an inducer drug have been described, most of them using doxycycline as an inducer [48, 120, 121].

Safety issues inherent to specific vector types are, *e.g.*, the inflammatory responses against adenoviral vectors which were transient, however, and appeared to be of no clinical significance in two recent cardiological clinical trials in 2002 and 2003 employing intracoronary injection of adenoviral vectors expressing VEGF [41, 42]. In a previous trial in 1999 [122], a liver-targeted gene therapy protocol aiming at the treatment of ornithin transcarboxylase deficiency had used vector doses three orders of magnitude higher than those in the recent cardiological trials. The highest dose used therein was 3.3×10^{10} vector particles [41], whereas in the former liver-directed study multiorgan failure and death occurred in one patient who had received the highest dose of 3.8×10^{13} particles [122]. That tragic event prompted thorough additional investigations [123, 124] into the risks of adenovector-mediated gene therapy including serious adverse events even if they may occur only very rarely, in predisposed individuals, or at very high vector doses. A survey of clinical trials in 100 cancer patients employing intravascular adenoviral vectors reports that doses up to 2.5×10^{13} had an acceptable safety profile [125]. Even very rare serious side effects will be considered unacceptable, however, for non-malignant diseases.

With respect to possible risks specific to the AAV vectors their immunogenicity is low as compared to adenovectors [126]. Another possible risk has been deduced from the capacity of wild-type AAV and AAV-based vectors for chromosomal integration [116, 117]. A recent study has searched for possible chromosomal effects of AAV vector integration [115] which deserve particular attention after a recent report on the late and unexpected occurrence of leukemia in children treated with retroviral vectors for severe combined immmunodeficiency (SCID), after successful correction of the primary genetic defect [127]. Whereas retroviral vectors such as used in the SCID study are integrating into the human genome at random sites, AAV vectors show a preference for chromosome 19 [115, 117]. The possible consequences of genomic integration of AAV vectors deserves close attention over years, since the retrovector-associated leukemia occurred only years after successful *ex vivo* gene therapy for SCID by retrovirally mediated transfer of the γ c gene into $CD34^+$ cells [127–130].

Nucleic acid-based modulation of cardiac gene expression

Whereas the above transgenic concepts to gene therapy seek *substitution*, *enhancement*, or *addition* of gene functions, recent years have brought the emergence of complementary concepts attempting the *inhibition* of gene expression and protein functions for therapeutic purposes. This concept may be realized by using RNA-based technologies: A) *antisense* and *decoy* oligonucleotides [131–133], B) *antisense* RNAs [43], C) ribozymes and DNA enzymes [134–138], D) RNA *interference* (RNA*i*) using *small interfering* RNAs (*si* RNAs) [139–143]. Another approach to realize therapeutic inhibition of gene function employs dominant negative or other suppressive mutants of the target protein [30, 46]. Among all these approaches the only recently discovered biological

principle of RNA *interference* appears to carry a particularly high therapeutic potential.

Antisense and decoy oligonucleotides

Among the RNA-based technologies for inhibition of gene function the first approach was the use of short antisense oligonucleotides (ONs). These ONs bind to "appropriate sequences" of the target gene mRNA transcript and induce its degradation by RNase H or block translation. In 1998, the first antisense drug was approved for the treatment of cytomegalovirus-induced retinitis in patients with AIDS [144] and a number of antisense ONs are currently being tested in clinical trials [145]. For cardiovascular research this approach has, for example, been used to reduce neointimal formation in the porcine stent restenosis model and the ON targeting c-myc is now being tested in a clinical trial [146]. ONs have also been successfully used for the inhibition of stenotic lesions in coronary bypass grafts [147] or restenosis after PTCA [148]. In recent years valuable progress has been made through the development of novel chemically modified nucleotides with improved properties like enhanced serum stability, higher target affinity and low toxicity [131]. In addition, new delivery systems have been developed to facilitate the cellular uptake of ONs [149]. Intracoronary infusion of a phosphorothioate-modified c-myc ON has been evaluated with respect to its capacity to inhibit restenosis after PTCA in a randomized trial [150], but did not significantly reduce the restenosis rate at the dose and application route employed.

Antisense RNA

The use of longer antisense-RNAs may result in inhibition of gene function by annealing of the *antisense* sequence with its complementary "physiologic" mRNA transcribed from the target gene to be inhibited. Several groups have successfully employed *antisense* RNAs to inhibit the expression of specific cardiovascular genes, but reliable model-based prediction of appropriate *antisense* sequences capable of efficient inhibition is not yet possible and therefore a trial-and-error procedure still needs to be followed in many cases. Essentially, the same is true for *antisense* ONs. In contrast to the *antisense* ONs, however, which may efficiently enter the target cells under certain conditions, the delivery of longer *antisense* RNAs requires their recombinant expression from a vector capable of efficiently entering the cells [43] (Fig. 4). This constitutes a close link between the *antisense* RNA and the vector-based gene transfer technology.

Ribozymes and DNA enzymes

In contrast to *antisense* ONs and RNAs, which inhibit gene expression *via* formation of duplexes with their complementary sequence on the target mRNA, the so-called ribozymes do not only anneal to a complementary RNA by virtue of their substrate binding arms but also cut the target mRNA. The enzyme-like catalytic activity of ribozymes was initially described by Cech et al. [151–153] and Altman and coworkers [154]. Due to the cleavage activity much lower concentrations of a ribozyme as compared to *antisense* ONs or RNAs should be needed to inactivate a target mRNA. For therapeutic purposes, ribozymes can be delivered in two ways: as presynthesized ribozymes (exogenous delivery) or as ribozyme gene for their intracellular expression. For the former method, stabilization of the ribozymes by the introduction of chemically modified nucleotides and the means of transfection are important factor, whereas the choice of promoter (pol II, pol III, tissue-specific) and efficient delivery systems (adenovirus-based or AAV-derived vectors) are the most important factors for the latter approach [138] (for review see reference 155]. Inhibition of restenosis after PTCA has been achieved with different ribozymes in animal models [137, 138, 156], anti-MMP-2 ribozyme treatment has been used to attenuate cardiac allograft vasculopathy [157], and several ribozymes have been applied in clinical trials to treat viral infections and cancer [134, 158].

A major disadvantage of ribozymes is the need to use unmodified ribonucleotides in certain positions of the ON, which are highly susceptible to nucleolytic degradation. Catalytically active nucleic acids, which are entirely composed of DNA have been obtained by *in vitro* selection using a combinatorial library [159]. These DNA enzymes are easier to prepare, less sensitive to enzymatic degradation and in some cases more active than conventional ribozymes [160]. DNA enzymes against the *early growth response factor-1* and *c-jun* have been employed *in vivo* to inhibit neointima formation after arterial injury [161, 162]. In addition, a DNA enzyme targeting TNF-β improved hemodynamic performance in rats with postinfarction failure [163].

RNA Interference

The most recent RNA-based technology capable of specific inhibition of gene function is RNA interference (RNA*i*). This phenomenon was first recognized in the nematode *Caenorhabditis elegans* as a biological response to exogenous *double-stranded* RNA (*ds* RNA) [164]. The apparently widespread nature of

RNA*i* in eukaryotes, ranging from trypanosomes to mice, has elicited great interest from both applied and fundamental standpoints. RNA*i* is initiated by the Dicer enzyme, which processes long double-stranded RNA into 21–23 nucleotide *small interfering* RNAs (*si* RNA). These *si* RNAs are incorporated into the RNA-induced silencing complex (RISC), a multicomponent ribonucleoparticle. Recent reports suggest that RISC must be activated from a latent form, containing double-stranded *si* RNA to an active form, RISC*, by unwinding of *si* RNAs. The unwound *si* RNA is then used by RISC* as a guide to cleave the target mRNA [140] (see Fig. 6).

The application of RNA*i* in mammals was hampered by the fact that double-stranded RNA molecules longer than 30 nucleotides induce a strong interferon response. This limitation was overcome by the discovery that presynthesized 21-nucleotide-long *si* RNA molecules with 3′-overhangs can also be used to suppress target gene expression in a sequence-specific manner in mammalian cells [165]. Subsequently, several strategies have been developed to transcribe *si* RNAs or *short hairpin* RNAs (*sh* RNAs) that can be used to investigate effects of long-term gene silencing or to generate stably transfected cells or transgenic animals (for review see ref. [141, 166]). *si* RNAs may be transcribed under the control of RNA polymerase III promotors that normally regulate transcription of the small nuclear RNA U6 or the H1 RNA component of RNase P, but have also been transcribed from a CMV promotor using an adenoviral vector [167].

Early on the potential of RNA*i* for the investigation of gene functions in mammalian cells, but also for the development of novel gene-specific therapeutics, has been recognized [165]. *si* RNAs have been sucessfully employed for the silencing of specific genes in mammalian cells [168–171]. For the first studies a large volume of physiological solution containing the *si* RNA or a plasmid coding for *si* RNA was rapidly injected into the mouse tail vein to suppress the expression of reporter genes that were either encoded on cotransfected plasmids or in transgenic strains [142, 143]. Although *si* RNAs can be delivered directly into cells *in vitro* [165] and mice *in vivo*, their efficacy for the therapeutic suppression of gene functions is significantly enhanced when specialized *in vivo* delivery systems are employed. *si* RNA delivery to target cells has been achieved with various viral vector including recombinant adenoviruses [167, 172] as well as lentiviruses [173] and other retroviruses [170]. The carcinogenic *K-ras* allele was downregulated by *si* RNA expressed from a retroviral vector [170, 173]. An adenovector has been successfully used to deliver *si* RNA to diminish target gene expression in brain and liver tissue *in vivo* [172]. A dose-dependent silencing of the enhanced green fluorescent protein expressed in stable cell lines was achieved for at least 25 days after infection with a lentiviral vector expressing *si* RNA [174]. Lentiviruses can be used to transfect a broad range of non-cycling and post-mitotic cells and they can be used to generate transgenic animals through infection of embryonic stem cells or embryos. Therefore, a lentiviral system was developed for the delivery of *si* RNA into cycling and non-cycling mammalian cells, stem cells, zygotes and their differentiated progeny [174]. Delivery of *si* RNA by the lentiviral vector was also used to generate transgenic animals, in which a target gene expression was specifically silenced. Some clinically most interesting applications are foreseeable in the field of infectious diseases: inhibition of hepatitis C virus by RNA *interference* has been reported [175] and silencing of the endogenous *Fas* gene protected mice from fulminant hepatitis [176]. The latter study showed that after injection of agonistic *Fas*-specific antibodies all control animals died within three days, whereas more than 80% of the *si* RNA treated animals survived the observation period of ten days.

In addition to refinements of the structure of the *si* RNA transcription cassette, positional effects of *si* RNA targets [177] as well as mutations and chemical modifications of *si* RNA are under intense investigation [178]. In has already become clear that it is not trivial to achieve high inhibitory efficacy and target specificity of a *si* RNA at the same time. Nevertheless, the fact that RNA*i* does not rely on equimolar interactions with a target mRNA (as *antisense* RNA) may make it a highly efficient tool for therapeutic gene silencing *in vivo* in the near future. Independent of these therapy-directed studies RNA*i* has also been used for a systematic analysis of gene functions in *C. elegans* [179, 180] and that genome-wide RNA*i* library approach is expected to be extended to mammalian cells soon. Due to similarities between conventional *antisense* and the newly developed RNA*i* strategies, researchers will benefit from lessons learned over the past decade and RNA *interference* may finally fulfill the promises made in the *antisense* field 20 years ago.

Experimental gene therapies for heart failure

Both enhancement and inhibition of gene functions have been used to alleviate cardiac dysfunction and/or attentuate the progression of heart failure in animal models. Interestingly, the majority of these studies attempts to modulate cardiac calcium homeostasis. The following two sections deal with enhancement strategies and with inhibitory approaches, respectively.

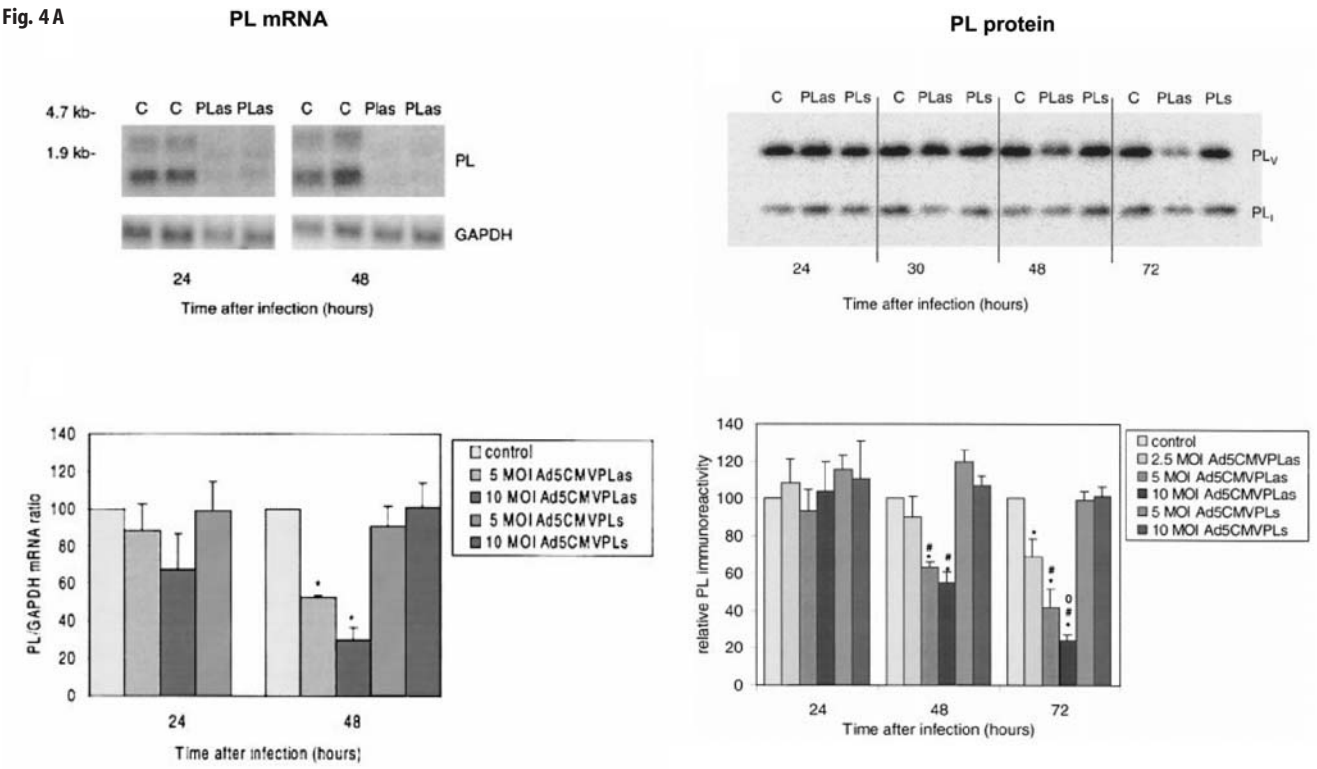


Fig. 4 *Antisense-RNA-mediated suppression of phospholamban (PL) in cardiomyocytes.* **A** Reduction of PL mRNA (upper left: Northern blots, lower left: quantification) and PL protein (upper right: Western blots, lower right: quantification) after treatment of the cardiomyocytes with the CMV promotor vector Ad5*CMV*PL*as*. **B** Modulation of the cardiomyocyte action potential by treatment with Ad5CMVPLas. Ca^{2+} transients, represented by time-dependent changes in F_{380}/F_{360} of fura 2-loaded cardiomyocytes stimulated at 1 Hz, of uninfected cardiomyocytes (top) and cardiomyocytes infected with Ad5*CMV*PL*as* (middle) and Ad5*CMV*PL*s* (bottom). Times to 50% recovery of Ca^{2+} transients are 719 ms (top), 398 ms (middle), and 615 ms (bottom), respectively. F_{380}/F_{360} is an inverse measure of intracellular free Ca^{2+} concentration (for further details see reference 43)

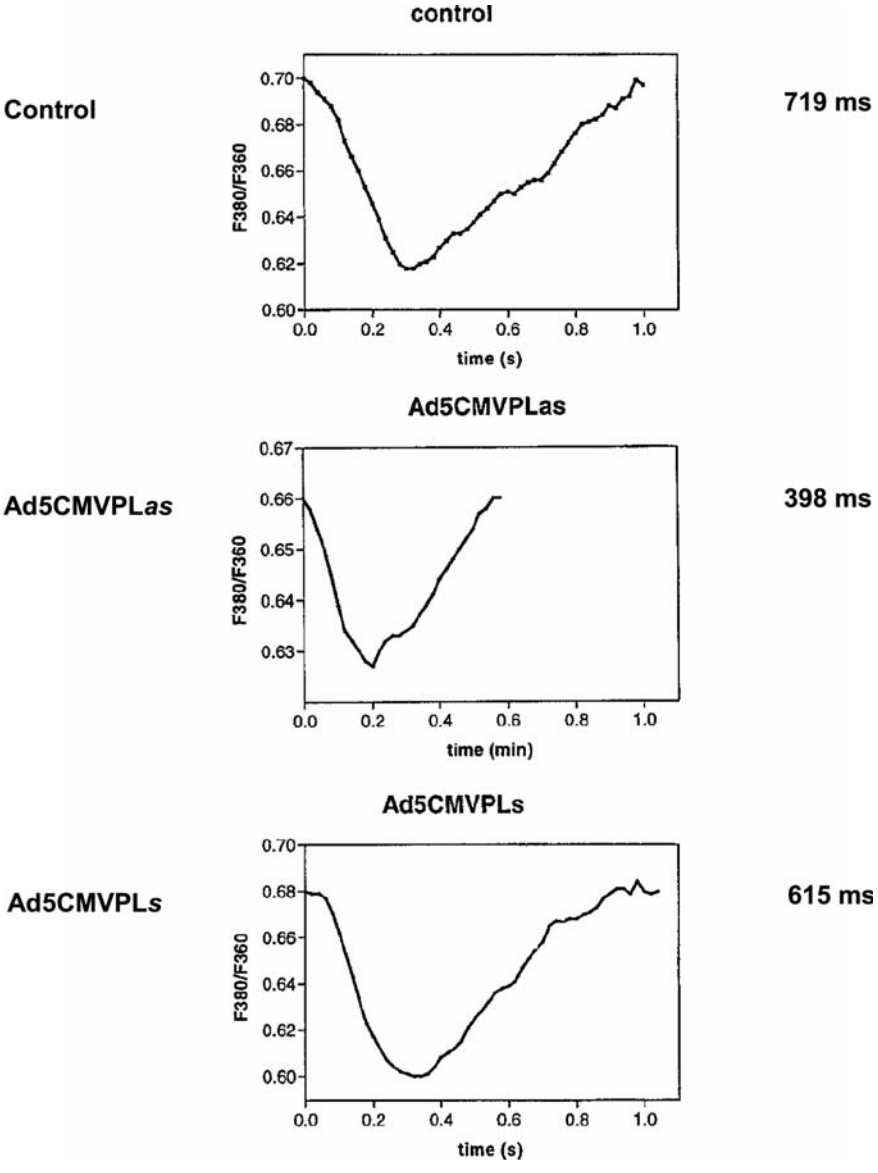
Substitution or enhancement of cardiac gene functions

The influence of altered expression of the sarcoplasmic reticulum ATPase (SERCA) upon the cardiac calcium cycle has been documented by several transgenic animal models, by SERCA gene transfer in cardiomyocyte cultures, and finally also by SERCA gene transfer *in vivo*. Nakayama et al. [74] have generated transgenic mice overexpressing a high calcium affinity SERCA2a mutant (K397/400E) lacking a functional association with PL. The transgenics showed increased contraction and relaxation and upon induction of pressure overload by transverse aortic constriction they developed less cardiac hypertrophy than controls suggesting SERCA2a activity as a new therapeutic target for the prevention and treatment of cardiac hypertrophy. Del Monte et al. showed improved survival and cardiac metabolism after SERCA gene transfer in a rat model of heart failure [181], restoration of diastolic function in senescent rat hearts [182], and functional improvement in aortic-banded rats in transition to heart failure [183] by the SERCA transgene. Other transgenes unrelated to cardiac calcium homeostasis genes have also been employed for experimental gene therapy of heart failure: components of the β-adrenergic signal transduction system [31, 35–38, 85, 184], cardiomyocyte cytoskeletal [86] or membrane proteins [87], and V2 vasopressin receptors [185]. Recently, successful treatment of an inherited cardiomyopathy by AAV-vector-based δ-sarcoglycan substitution gene therapy has been achieved [53].

Suppression of cardiac gene functions

In the field of heart failure the currently most important papers on the concept of therapeutic *inhibition* of gene function attempt inhibition of phospholamban expression to mimic the beneficial effect upon cardiac function and disease progression observed in the $PL^{-/-} \times MLP^{-/-}$ germline *double knock out* animal model [44]. In contrast with germline *knockout* animal models, *ad hoc* ablation of gene function in adults will have no effects related to or-

Fig. 4 B



gan development, but the effects are solely due to changes in the physiological functions of the gene in the adult organism. Both RNA-based technology and dominant negative protein mutants have been used to achieve this goal [33, 43, 186]. Both the transcription of PL-*antisense*-RNA [33, 43, 186] and of mutant PL proteins [30, 33, 46] have been successfully used to inibit PL expression in cardiomyocytes.

Several investigators have taken advantage of mutant PL proteins. A study by Hoshijima et al. [30] used a recombinant AAV-based vector (rAAV/S16EPLN) delivered by the transcoronary route to express a pseudophosphorylated PL mutant in a cardiomyopathic animal model. This treatment enhanced myocardial sarcoplasmic reticulum Ca^{2+} re-uptake and suppressed the progressive impairment of left ventricular (LV) systolic function and contractility characteristic of this heart failure model. Furthermore, the deterioration of diastolic LV function were also largely prevented by the rAAV/S16EPLN treatment which appears to be a potential therapy for progressive dilated cardiomyopathy and the associated heart failure. Zhai et al. [46] have employed the cardiac-specific overexpression of a superinhibitory pentameric phospholamban mutant and achieved inhibition of cardiac function *in vivo* [46].

We have recently use a PL-*antisense*-RNA to suppress PL-RNA expression and thereby the *de novo* synthesis of PL protein in cardiomyocytes. Using either a constitutive promotor or an ANF-promotor

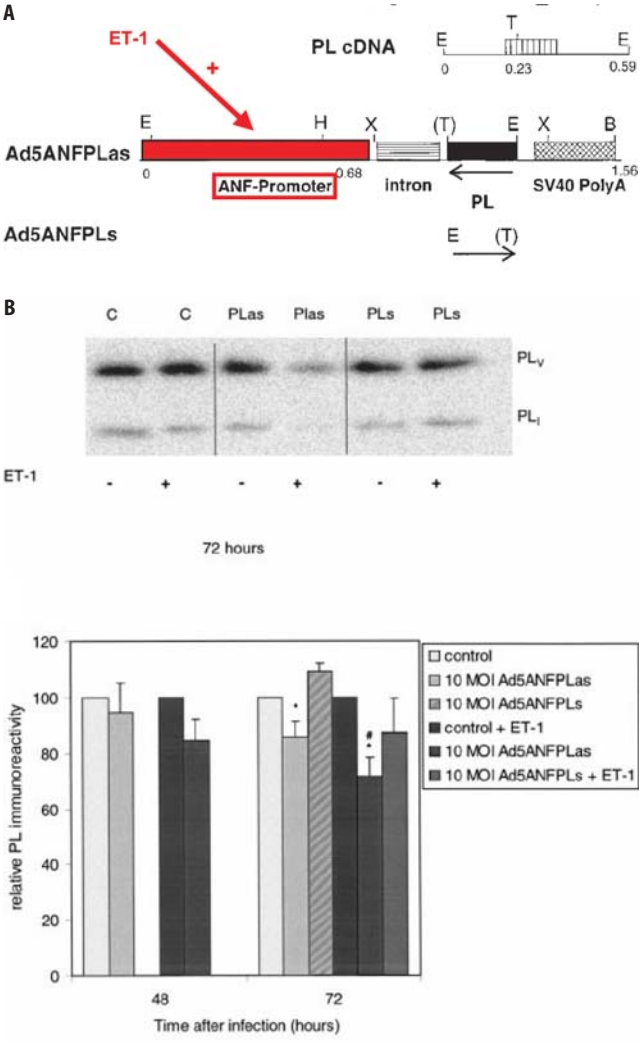


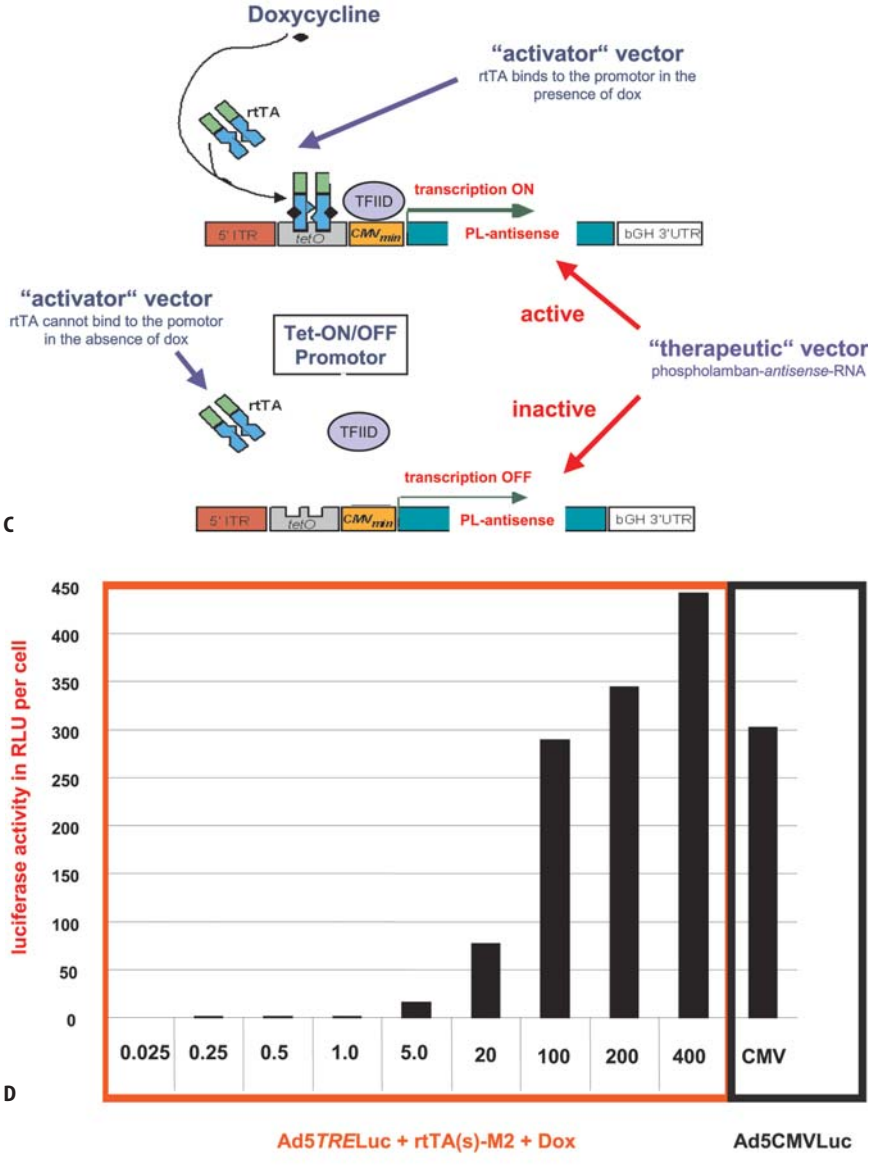
Fig. 5 *"Induction-by-Disease" gene therapy and pharmacological control of therapeutic vectors.* **A** The structure of vectors expressing a PL-*antisense*-RNA under the control of an endothelin-1-inducible ANF promotor. **B** Endothelin-1-dependent activation of the ANF promotor vector, resulting in significant reduction of PL protein in cardiomyocytes (top: Western blots showing pentameric and monomeric PL, bottom: quantification) in the presence of 10 nM ET-1 (for further details see reference (43)). **C** The principle of a novel, doxycyclin-(Dox) regulatable two-vector system employing a second-generation Dox-on/off-promotor (48). A first, "therapeutic" vector containing a Dox-inducible expression cassette (depicted for PL-*antisense*-RNA) is regulated by a transactivator protein (rtTA) produced by a second, "activator" vector. rtTA binds to and activates the Dox-on/off-promotor in the presence of Dox only, whereas in its absence rtTA dissociates and the expression cassette becomes inactive. **D** Application of this new system (Ad5TRELuc + rtTA + doxycyclin) for Dox-regulated expression of a marker luciferase transgene in cardiomyocytes. Background activity of the vector in the absence of Dox is very low, whereas at increasing ambient Dox concentrations (red box, the numbers below the columns give the Dox concentration in ng/ml) transgene expression increases up to levels similar as achieved by using the vector Ad5-*CMV*Luc driven by a very strong, constantly active viral CMV promotor

inducible by endothelin-1 (ET-1), this approach resulted in improved SR calcium re-uptake and parameters of diastolic function. When the strong constitutive viral CMV promotor was used to drive PL-*antisense*-RNA transcription, endogenous PL mRNA and protein expression were reduced to 30 ± 7% and 24 ± 3% of baseline (Fig. 4 A) and to modulation of the cardiomyocyte action potential (Fig. 4 B). When the ANF promotor vector (Fig. 5 A) was used instead, significant suppression of the endogenous PL was induced only in the presence of endothelin-1 (Fig. 5 B). Since the ANF promoter used here is upregulated in hypertrophied and failing heart, thereby employing an inherent abnormality of the target disease for the steering of the gene therapeutic vector, we have used the term *"Induction-by-Disease"* gene therapy to describe this general principle [43]. A conceptually similar approach has been developed for other diseases, by using hypoxia-inducible vectors [49–52]. Another approach suitable for exogenous control of the expression level of a therapeutic nucleic acid is the use of drug-inducible promotor systems such as outlined in Fig. 5 C and D (for further details see [47, 48, 120]). A system analogous to that in Fig. 5 C has been used for tight control of factor IX plasma levels over two orders of magnitude [120] and may become useful also for drug-regulatable transgene expression in the myocardium.

Clinical perspective and unanswered questions

During the past few years the molecular and structural foundations and the problems associated with myocardial gene therapy have been defined in considerable detail. It has become obvious that efficient and selective transport of the gene transport vehicle (*"targeting"*) to the myocardium is a first key issue. Future cardiac gene therapy of the myocardium will most probably have to take advantage of direct intracoronary infusion of the vector [34] to achieve a first level of target selectivity, which should be enhanced by a second level of transcriptional confinement of vector expression by the use cardiac-specific promotors. Intracoronary infusion of an adenoviral vector has already been employed in first clinical trial [39–42]. Even after intracoronary infusion there occur two limitations to vector targeting. First, the existence of naturally occurring *anatomical barriers* which impede or may even abolish vector transit from the coronary circulation to the myocardium [26]. These barriers (vascular endothelium, basal membrane, extracellular matrix) are revelant for all virus-based vectors and significantly change in certain diseases. Second, specific attachment and entry

Fig. 5 C and D



structures on the target cells, *i.e.*, vector receptors do determine if and at which rate vectors can be taken up into the cardiomyocytes. It is now known that the CAR receptor mediating cellular entry of standard adenovectors is highly variably expressed both in experimental diseases (rat MI) and in human cardiac disorders (dilated cardiomyopathy) [27–29]. Favorable conditions at both the barrier and receptor level need to be achieved for efficient gene transfer. Unless the disease process itself creates such conditions (breakdown of endothelial barrier and receptor induction) pharmacological measures might be taken into account.

A second key issue of cardiac gene therapy is the durability of vector expression in the myocardium. Certain therapeutic tasks such as the enhancement of angioneogenesis or favourable modulation of remodeling after myocardial infarction may be achieved by vector expression for only a few days or weeks. Currently available adenovectors may already be capable of achieving this "*short-term*" goal [41, 42]. On the other hand, definitive treatment of heart failure and all genetic cardiac disorders would require very long-term stability of the vector, unless only a limited *bridge-to-recovery* or *bridge-to-transplant* strategy is followed. Very long-term expression appears to become possible by the introduction of AAV-based vectors which differ grossly from adenovectors by several peculiar biological properties. Whereas adenovectors have already entered the stage

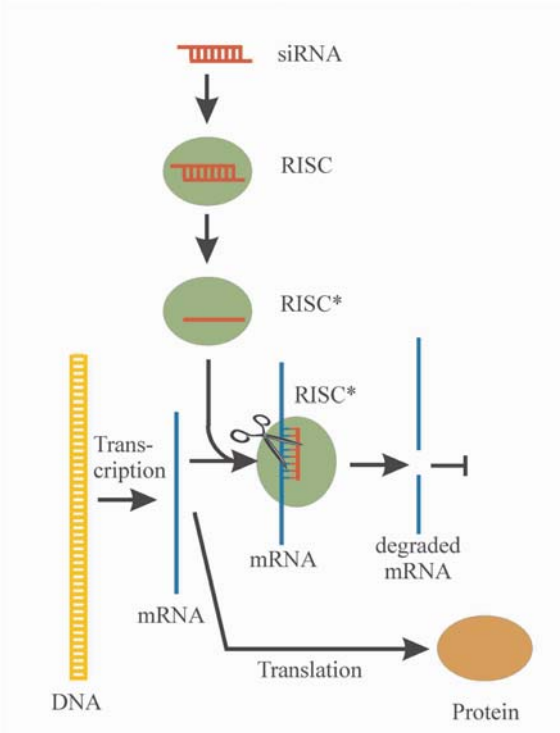


Fig. 6 *The principle of RNA Interference.* RNA interference is initiated by the *Dicer* enzyme, which processes long double-stranded RNA into 21–23 nucleotide *small interfering* RNAs (*si* RNA). The *si* RNAs are incorporated into the *RNA-induced silencing complex* (RISC), a multicomponent ribonucleoparticle. Recent reports suggest that RISC must be activated from a latent form, containing double-stranded *si* RNA to an active form, RISC*, by unwinding of *si* RNAs. The unwound *si* RNA is then used by RISC* as a guide to cleave the target mRNA (for further details see text and reference 140)

of first cardiovascular clinical trials [39–42], AAV vector application has so far been confined to experimental models in the cardiovascular field. In these models, however, impressive long-term stability and function and morphological improvements could be achieved [30, 51, 53–57]. In non-cardiovascular diseases, however, AAV vectors have already been applied in a clinical setting [114, 114a].

When these two issues will have been solved at a high safety level, the effort of a gene therapeutic approach may be justified if the specific therapeutic target is not adequately accessible by other means (*e.g.*, by pharmacotherapy) and if the disease to be treated is sufficiently severe to justify the efforts and possible risks of a still experimental therapeutic approach. Many current applications of nucleic acid-based modulation of cardiac gene expression still aim at the understanding of molecular mechanisms rather than treatment. In the field of heart failure, the cardiac calcium homeostasis provides paradigmatic targets for gene therapy research, although the complex differences between animal models [30, 44, 187] and humans [65, 118] and the requirement of adequate transgene regulation must be kept in mind. The PL-directed approaches developed during the past years reflect the continuing development of molecular tools – based upon but reaching far beyond gene transfer technology – for selective, efficient, and long-lasting modulation of cardiac gene functions. These tools provide now a solid basis for highly innovative therapeutic approaches, irrespective of the specific targets which will ultimately prove to be successful in the clinical setting. Two most recent and promising tools available are AAV-based vectors and the principle of RNA *interference*. The potential of a synthesis of current vector and *antisense* technologies addressing key cardiac pathomechanisms will be further evaluated experimentally in the near future and may in the long term provide novel tools for clinical therapy.

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