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REVIEW

Gene therapy for cardiovascular disease: advances in vector development, targeting, and delivery for clinical translation

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Abstract

Gene therapy is a promising modality for the treatment of inherited and acquired cardiovascular diseases. The identification of the molecular pathways involved in the pathophysiology of heart failure and other associated cardiac diseases led to encouraging preclinical gene therapy studies in small and large animal models. However, the initial clinical results yielded only modest or no improvement in clinical endpoints. The presence of neutralizing antibodies and cellular immune responses directed against the viral vector and/or the gene-modified cells, the insufficient gene expression levels, and the limited gene transduction efficiencies accounted for the overall limited clinical improvements. Nevertheless, further improvements of the gene delivery technology and a better understanding of the underlying biology fostered renewed interest in gene therapy for heart failure. In particular, improved vectors based on emerging cardiotropic serotypes of the adeno-associated viral vector (AAV) are particularly well suited to coax expression of therapeutic genes in the heart. This led to new clinical trials based on the delivery of the sarcoplasmic reticulum Ca^{2+}-ATPase protein (SERCA2a). Though the first clinical results were encouraging, a recent Phase IIb trial did not confirm the beneficial clinical outcomes that were initially reported. New approaches based on S100A1 and adenylate cyclase 6 are also being considered for clinical applications. Emerging paradigms based on the use of miRNA regulation or CRISPR/Cas9-based genome engineering open new therapeutic perspectives for treating cardiovascular diseases by gene therapy. Nevertheless, the continuous improvement of cardiac gene delivery is needed to allow the use of safer and more effective vector doses, ultimately bringing gene therapy for heart failure one step closer to reality.

Keywords Gene therapy • Cardiovascular disease • Heart failure • Clinical trials • Adeno-associated viral vector • SERCA2a • S100A1 • Adenylate cyclase • miRNA • CRISPR

1. Introduction

Cardiovascular disease (CVD) remains a major public health problem with increasing prevalence, poor clinical outcomes, and large health care costs. Approximately 1–2% of the adult population in developed countries suffer from heart failure (HF), with prevalence rising to ≥10% among individuals aged 70 years or older.[1] While pharmacological[2] and invasive therapies for CVD achieve symptom reduction and slow disease progression, there is still an urgent need for alternative therapeutic approaches to effectively treat or even cure CVD and HF. The molecular pathways and causative genes involved in the induction and progression of CVD have been elucidated through advances in molecular cardiology.[3] These emerging insights pave the way towards the use of gene therapy as a novel treatment modality for CVD and HF,[3] fueled by the emerging clinical successes in the field at large.[4]

Depending on the underlying CVD, short-term or sustained expression of a given therapeutic gene may be required that in turn determines the type of gene delivery vector needed to achieve the desired therapeutic effect[5] (*Table 1*). For instance, short-term expression may suffice to induce vasculogenenesis for the treatment of myocardial infarction (MI), whereas more sustained expression may be required to achieve left ventricular remodelling in HF. The obvious target cells for CVD gene therapy include cardiomyocytes and endothelial cells/smooth muscle cells. However, distal cell types can also be considered (i.e. skeletal muscle, liver) that could be used for the systemic release of paracrine factors.[6–8]

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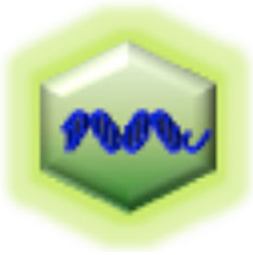
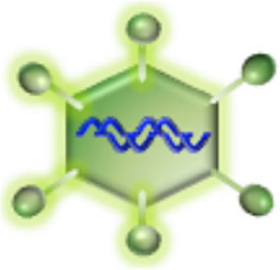
Table 1 Plasmids and viral vectors for CVD

Delivery method	Plasmid	AAV	Lentivirus	Ad
Schematic diagram				
Diameter (nM)	N/A	20	80–100	70–90
Genome/Size (Kb)	DNA/N/A	(ds)ssDNA/± 4,8	RNA/± 10	dsDNA/± 36
Cardiac gene transfer	Low cardiac transfection	Cardiotropic AAV serotypes	Low cardiac transduction	High cardiac transduction
Duration of expression	Expression up to 2 months	Long-term cardiac expression	Long-term cardiac expression	Expression up to 2 weeks
Major disadvantage	Low transfection efficiency	Risk of neutralizing antibodies and T-cell responses	Risk of insertional mutagenesis	High antibody and inflammatory response
Used in clinical trials for CVD	+	+	−	+

In this review, we will focus on gene therapy approaches that have been explored in clinical trials or are approaching clinical translation. Since the use of miRNA for modulating heart function has been extensively reviewed elsewhere,[9] this falls out of the scope of this review.

2. Gene therapy and CVDs

2.1 Non-viral vectors for CVD

Despite the initial promising results based on non-viral vectors (mainly plasmid transfections) and the hype that surrounded the initial trials, most recent studies have shown that this approach is generally not very efficient to treat CVD by gene therapy.[10,11] Different strategies have been developed to improve its overall efficiency such as the use of liposome-DNA complexes that increase plasmid stability, though the plasmid is rapidly cleared from the systemic circulation.[12] Polymer-based DNA complexes based on poly-L-lysine (PLL) and polyethyleneimine (PEI) products protect plasmids from nuclease digestion and facilitate cellular uptake;[10] however, when used *in vivo*, these complexes tend to aggregate and accumulate in different tissues. Electroporation and the use of micro-bubbles as echo contrast agents have also been explored but with variable results.[13] The ultrasound-targeted microbubbles (UTM) strategy has gained interest as an alternative delivery strategy for CVD due to its intrinsic low levels of toxicity and immunogenicity, which is compounded by the potential for re-administration and organ-specific delivery of the genes of interest.[14] Recent reports have shown benefits in MI and HF animal models, using UTM for targeted delivery of DNA or microRNA. In particular, lipid micro-bubbles carrying VEGF and stem cell factor (SCF) genes significantly improved myocardial perfusion and ventricular function after coronary artery ligation in rodent models.[15] Similarly, repeated UTM-based delivery of SCF and stromal cell-derived factor (SDF)-1α genes in a rat model of MI resulted in an increased vascular density, improved myocardial function, and reduced infarct size.[16] UTM was also well suited to enhance delivery of microRNAs to cardiomyocytes without discernable toxicity. In particular, UTM-mediated delivery of miR-133 in cardiomyocytes *in vitro* led to a reversal of hypertrophy.[17] One of the challenges consists of translating these findings to large animal models and ultimately to the clinic, which is compounded by the relative low efficiency and/or short-term gene expression.

2.2 Viral vectors for CVD

Viral vectors consist of genetic material surrounded by a protein-based capsid or a lipidic envelope that interacts with specific cell surface receptors to aid binding, internalization, and delivery of the therapeutic gene into the target cell.[18] The capsid or envelope protein directs trafficking of the therapeutic gene towards the nucleus and protects it from degradation in the lysosomes.[4] In general, viral vectors are more efficient than non-viral vectors and have the potential for long-term gene expression (*Table 1*). Viral vectors resemble the parental viruses from which they have been derived except for the lack of viral genes in the vector backbone. With the exception of early-generation adenoviral vectors (see below), that retain some residual viral genes in their backbone, all other vector types are devoid of viral genes. Consequently, vector manufacturing is more challenging than when non-viral vectors are employed and requires packaging cells that complement the missing viral genes *in trans*. The clinical use of viral vectors also raises important regulatory challenges.[19] Depending on the type of viral vector used, the immune response to the vector and/or the gene-modified cells may be a limiting factor.[20] Vector-specific immune responses can preclude gene transfer after vector re-administration and/or limit the duration of gene expression or result in immune clearance of gene-modified cells.

2.2.1 Adenoviral vectors

Adenoviral (Ad) vectors are non-enveloped, non-integrating double-stranded (ds)DNA vectors that enter the cells, predominantly via clathrin-mediated endocytosis upon binding with coxsackie-adenovirus

receptor (CAR). The dsDNA is subsequently transported to the nucleus allowing efficient transduction of a wide range of dividing and non-dividing cells, including cardiomyocytes, skeletal muscle, or smooth muscle cells.[21–23] In the heart, transgene expression after Ad vectors transduction is robust but transient (1–2 weeks),[24,25] which limits its applications in CVD for HF. Nevertheless, it is a useful system for short-term pro-angiogenic therapies in ischaemic heart disease,[26] peripheral arterial occlusive disease, and limb ischaemia.[27] One major disadvantage of Ad vectors relates to their ability to induce inflammation, which compromise their efficacy and safety in clinical trials.[28,29] In particular, the early-generation Ad vectors contain residual adenoviral genes in the vector backbone that can be induced *in vivo* and trigger T-cell-mediated immune responses that eliminate the gene-modified cells. The latest generation Ad vectors exhibit decreased T-cell immune responses by eliminating all of the residual viral genes (i.e. gutless or helper-dependent Ad vectors) expanding the cargo capacity to 30 kb.[30] Nevertheless, both early- and late-generation Ad vector particles can rapidly activate the innate immune system contributing to significant dose-limiting toxicity.[31] Though catheter-mediated localized delivery in the myocardium may minimize this risk,[32] the intrinsic risks associated with immune system activation remain. This risk is compounded by the broad tropism of Ad vectors resulting in ectopic transduction of non-target cells (e.g. hepatocytes, antigen-presenting cells).[33] Consequently, the utility of Ad vectors in cardiovascular gene therapy trials in humans must be carefully evaluated.

Recombinant vectors derived from the serotype 5 adenovirus (Ad5) have been predominantly used in preclinical and clinical trials in gene therapy for CVD.[34] The CAR is the primary cell surface receptor for Ad5, though other cellular co-receptors are also implicated in vector entry (i.e. integrins). CAR is highly expressed on cardiomyocytes, whereas its expression is reduced in vascular smooth muscle and endothelial cells. This impacts on the transduction efficiency in these different cell types after systemic administration.[35] Although Ad vectors cannot easily cross the endothelial barrier after systemic administration, it has been reported that Ad vectors can selectively transduce endothelial cells after local administration.[36] Additionally, Ad vectors also achieve high levels of myocardial transduction after local delivery, either by intracoronary infusion or by direct intramyocardial injection.[37] The transduction efficiency varies depending on the Ad serotype. In particular, Ad serotype 49 (Ad49) showed increased transduction of endothelial cells and smooth muscle cells *in vitro* and in vascular graft *ex vivo*.[38]

2.2.2 Adeno-associated viral vectors

Adeno-associated viral vectors (AAV) are single-stranded (ss)DNA vectors with a favourable safety profile and capable of achieving persistent transgene expression in a wide range of target tissues, including the heart.[39] Since they provoke much less inflammation compared with Ad vectors, they have garnered a lot of interest for cardiac gene therapy applications. Long-term expression is predominantly mediated by episomally retained, non-integrated AAV genomes, mostly organized as high molecular weight concatemers.[40] More than 100 serotypes of the wild-type AAV have been reported.[41] Many exhibit a distinct tissue tropism that is determined by the capsid protein structure.[3] AAV1, AAV6, AAV8, and AAV9 have been identified as the most cardiotropic serotypes after systemic delivery.[42] These distinct serotypes predominantly transduce cardiomyocytes. However, the extent of AAV transduction in other cardiac cells (e.g. fibroblasts) has not been carefully examined. We and others demonstrated that AAV9 was the most efficient serotype for cardiac gene delivery, at least in mice.[40,43,44] Recent studies using mouse models have reported a >200-fold increase in myocardial transduction efficiency after only a single intravenous dose of AAV9 compared with AAV1, confirming the superiority of AAV9 for cardiac gene transfer.[44] The ability of certain AAV serotypes to efficiently transduce cardiomyocytes following intravenous vector administration suggests that these AAV vectors are capable of transcytosis, depending on the serotype.[45] Though direct intramyocardial AAV injection may also result in transduction of cardiomyocytes, its effect is typically more localized and restricted to the injection site itself.[46] However, the superior cardiotropic properties of AAV9 in mouse models may not necessarily translate to larger animal models and ultimately to human subjects and may depend—at least in part—on the delivery method (e.g. anteriograde vs. retrograde coronary artery delivery) warranting further preclinical studies (*Table 2*).[47,48] Overall, transduction of smooth muscle cells and endothelial cells in blood vessels using AAV has been relatively modest, even after using retargeted, peptide-modified AAV.[49–51]

Despite their increased cardiotropism, AAV9 transduction is not restricted to the myocardium, since other tissues, including liver and skeletal muscle can also be transduced.[40,52] To improve the cardiac specificity of AAV, AAV2 variants were selected that exhibit different receptor-binding properties, with improved transduction profiles and the ability to at least partially evade neutralizing antibodies[53] (*Figure 1*). One particular approach consisted of constructing a random viral display peptide AAV library allowing subsequent *in vivo* selection of cardiotropic AAV variants.[54] Alternatively, using an AAV *cap* gene library produced by DNA shuffling of different AAV serotype capsid genes, Yang *et al.* obtained a myocardium-tropic AAV strain, AAVM41, through direct evolution strategies and DNA shuffling. This variant exhibited enhanced transduction to cardiac muscle and diminished tropism to the liver after systemic administration.[55] Finally, Samulski *et al.* replaced a hexapeptide in a previously identified heparan sulfate receptor footprint sequence from an AAV2 vector with corresponding residues from other AAV strains. Consequently, such an AAV2/AAV8 chimera designated AAV2i8 selectively transduced cardiac and whole-body skeletal muscle tissues, while exhibiting significantly reduced hepatic tropism.[56] Such liver-detargeted AAV vectors could also be obtained by random mutagenesis of residues within a surface-exposed region of the major AAV9 capsid protein.[57] Using a combination of sequence analysis, structural models, and *in vivo* screening, several functionally diverse AAV9 variants were identified, notably, variants AAV9.45 and AAV9.61 that displayed a 10- to 25-fold lower gene transfer efficiency in liver, while transducing the myocardium as efficiently as AAV9. In conclusion, these emerging strategies can be used to enhance tissue tropism and can also be utilized to produce AAV that are able to evade neutralizing antibodies, at least partially.

Cardiac-specific promoters could be employed to restrict expression to the heart. Typically these promoters are quite large, consequently restricting the size of the therapeutic gene that can readily be accommodated in an AAV vector.[58] Moreover, gene expression levels are often reduced compared with when more robust (viral) promoters were used, such as the CMV promoter.[44,58] The alpha myosin heavy chain (αMHC) promoter has been among the most commonly used myocardial-specific promoters. It drives transgene expression throughout the entire heart, including the ventricles and atria.[59,60] The myosin light-chain (MLC) 2v promoter has also been used in various cardiac gene therapy applications, by virtue of its cardiac-specific

Table 2 Gene therapy delivery strategies for CVD targeting the heart

Delivery method	Procedures	Indications	Advantages	Disadvantages
Catheter-based delivery methods				
Anterograde arterial infusion	a. Intracoronary perfusion b. Intracoronary perfusion + Balloon occlusion c. Intracoronary perfusion + Balloon occlusion + Venous occlusion	Patients with unstable and advanced heart failure	– Simple and minimally invasive with cardiac selectivity – Can achieve homogeneous distribution	– Not possible in patients with advance atherosclerosis or significative coronary artery disease – Without occlusion risk of non-cardiac transduction
Retrograde intravenous	a. Intravenous perfusion + Venous occlusion	Patients with impaired coronary artery circulation and limited potential for revascularization	– Can achieve higher levels of transduction in cardiomyocytes	– Risk of ischaemic events for blockage of arterial circulation
Direct intramyocardial injection	a. Percutaneous for endocardial delivery b. Surgically invasive intramyocardial administration c. NOGA system	Therapeutic angiogenesis and focal arrhythmia therapy when restricted area is needed	– Decrease risk of immune response and ectopic expression of the transgen – Highly successful for plasmid delivery	– Restricted area of vector delivery can be insufficient. – Physical damage with then needle on healthy myocardium
Pericardial delivery	a. Percutaneous approach	Patients not suitable for previous methods or with high level of circulatory neutralizing antibodies	– Long time of vector exposure can increase transduction levels – Safe and minimally invasive approach	– Transgene expression limited to superficial epicardium – Minimal risk of pericardial effusion and pneumothorax

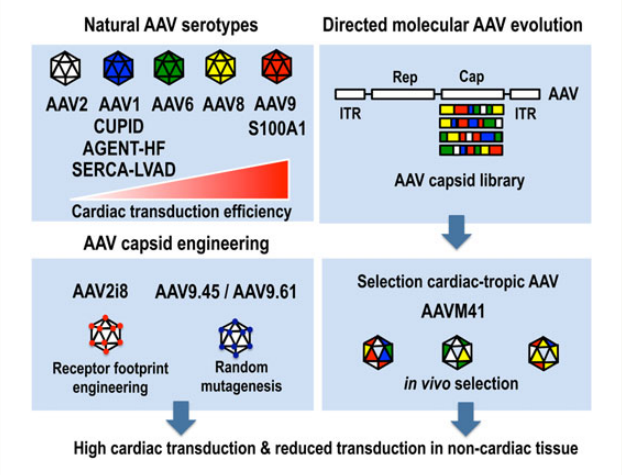


Figure 1 Strategies to increase cardiac-specific gene transfer using AAV vectors using naturally occurring AAV serotypes, AAV capsid engineering, or directed molecular evolution and *in vivo* selection of cardiotropic AAV variants (see text for details). The relative cardiac transduction efficiency of the naturally occurring AAV serotypes (AAV2, AAV1, AAV6, AAV8, and AAV9) based on preclinical murine studies is shown schematically.

expression pattern.[61–65] Comparative analysis after intra-vascular gene delivery in newborn mice using AAV vectors revealed that the αMHC promoter is among the most cardiac-specific promoters, whereas the desmin promoter resulted in robust expression in both heart and skeletal muscle.[44] Nevertheless, both promoters were not as potent compared with the CMV promoter. We have recently explored novel computational approaches, to identify potent, yet small evolutionary conserved cardiac-specific *cis*-regulatory modules (CRMs) that boost the performance of cardiac-specific promoters, as in the case of the αMHC promoter.[66] We have also validated this *in silico* approach to identify and validate tissue-specific CRMs that improve gene expression in other tissues, such as liver.[48,67] This underscores the validity of computational vector design as a novel strategy to improve vector performance in gene therapy. An alternative approach to achieve cardiac-specific expression consists of incorporating a miRNA-regulated cassette that selectively represses gene expression in non-cardiac tissues.[68,69]

One major drawback of AAV is the limited packaging capacity of the vector particles (i.e. 4.7 kb), which constrains the size of the transgene expression cassette that can be used.[70] Dual vector strategies have been developed to overcome the packaging constraints that rely on the concatemerization of AAV genomes to generate functional expression cassettes *in situ* in the transduced cells.[21] In particular, the use of *trans*-splicing AAV enables the *in situ* reconstitution a functional expression cassette of up to 10 kb.[71] Such *trans*-splicing AAV9 vectors were able to transduce the hearts of neonatal and adult dystrophic mdx mice at the same efficiency as conventional non-split ssAAV9.[72]

Though the risk of inflammatory immune responses following AAV transduction is significantly reduced compared with when Ad vectors are employed, there are still some important immune issues to address. In particular, the high prevalence of pre-existing antibodies to wild-type AAV in the population can result in rapid neutralization of AAV precluding gene transfer.[73] Similarly, the induction of neutralizing antibodies after AAV-based gene therapy will prevent gene transfer after AAV vector re-administration.[39,74] However, the use of

alternative AAV serotypes or capsid variants, AAV capsid decoys, pharmacological immune modulation, and/or plasmapheresis treatment may allow AAV transduction in the face of pre-existing antibodies.[39]

In addition, T-cell immune responses against the AAV capsid antigens that are presented in association with MHC class I (MHC-I) antigens on the surface of transduced target cells may curtail long-term gene expression after immune rejection of the gene-modified target cells.[20] The use of transient immune suppression and/or AAV capsid variants that results in reduced MHC-I presentation of AAV capsid-derived antigenic peptides may reduce this risk.[75–77]

2.2.3 Lentiviral vectors

The quintessential lentiviral vectors (LV) are derived from the HIV type 1 (HIV-1).[78] LV are enveloped single-stranded (ss)RNA vectors that have the ability to stably integrate their genome as cDNA into the chromosomes of both dividing and non-dividing target cells. They are therefore well suited to achieve long-term expression of the therapeutic gene.[79] LV have been used successfully for the treatment of monogenetic haematopoietic disorders, resulting in long-term therapeutic effects.[80,81] However, their use in gene therapy applications for CVD/HF is more limited given their relatively poor transduction of myocardium after *in vivo* LV gene delivery. This may depend, at least on part, on the species, age, and/or mode of LV delivery.[82] Indeed, recently it was shown that rat myocardium could be transduced by LV.[83,84] These results are very promising in that the LV platform could be tailored towards an effective treatment option for CVD that require long-term expression of the therapeutic gene, without the adverse effects of adenoviral vectors obviating the need for repeated viral vector administration.

Since LV could also transduce endothelial cells or endothelial progenitors, these properties have potential implications for the treatment of peripheral vascular diseases by LV gene therapy or as a means to induce therapeutic angiogenesis/vasculogenesis.[85,86] The endothelial tropism of the LV can be enhanced either by using single-chain Fv retargeting moieties or by pseudotyping the LV with naturally occurring endotheliotropic envelopes (e.g. Nipah virus).[87,88] It would be important to now validate the therapeutic potential of such endothelial-specific LV in an experimental model of CVD. Despite their promise, it is important to also consider some of the safety issues of using LV. Since they can integrate randomly into the target cell genome, with a preference for genes, their use carries an intrinsic risk of triggering insertional oncogenesis.[89] However, this risk can be reduced by optimizing the vector design and depends on the target cell type. Consequently, the risk of insertional oncogenesis may be significantly lower in post-mitotic terminally differentiated cardiomyocyte than in lentivirally transduced gene-modified haematopoietic stem cells (HSC) in the context of myeloablative haematopoietic reconstitution.

2.3 Enhancing uptake of viral vectors

Several strategies have been developed to improve the delivery of therapeutic genes and increase the vector uptake by cardiomyocytes. Initially, improvements in vector delivery techniques were evaluated, from intracoronary delivery of the viral particles by percutaneous administration to direct intramyocardial injections and image-guided injection strategies based on NOGA® electromechanical mapping of the heart.[11] Though these methods have increased the overall vector uptake, they still yielded relatively low transduction efficiencies in cardiomyocytes. Consequently, this requires high doses of vector particles, increasing the risk of immune responses against the vector and/or the gene-modified cells. Moreover, the need for higher vector doses also resulted in an increased risk of ectopic transduction in undesired tissues. Therefore, the necessity to achieve higher cardiac transduction efficiencies by using minimally invasive techniques required the development of adjuvants that enhanced vector uptake into the myocardium. Sasano *et al.*[90] reported an increase of up to 80% in cardiac transduction efficiencies in a porcine model by using a combination of VEGF, adenosine, calcium, and nitroglycerin (NTG) infusion prior to the viral vector administration. However, this adversely impacted on the haemodynamic parameters, which impedes the possible clinical translation of this particular strategy. Prior to the CUPID trial, Hajjar and co-workers[91] reported a modest yet significant increase in mRNA and protein concentration of SERCA2a in the left ventricle of treated pigs compared with the control groups by simultaneous intravenous infusion of NTG and the vector of interest (i.e. AAV1/SERCA2a). This strategy was posteriorly implemented in the CUPID trial (see below).

3. Therapeutic genes for gene therapy for CVD

To ultimately achieve the greatest therapeutic impact by gene therapy, it is necessary to identify the appropriate therapeutic gene. Promising results have been obtained with gene delivery systems that induce angiogenesis/vasculogenesis or that target proteins involved in the handling of cardiomyocytic calcium (Ca^{2+}) (e.g. sarcoplasmic reticulum Ca^{2+}-ATPase, S100A1, and phospholamban) and the β-adrenergic system (the β1- and β2-adrenergic receptors, or the G-protein-coupled receptor (GPCR) kinase-2 (GRK2) (*Table 3*).

3.1 Angiogenic factors for CVD: ischaemic heart disease

Angiogenic factors induce the formation of new vascular networks, which make them suitable therapeutic options for treating acute coronary syndromes and peripheral vascular diseases. It is beyond the scope of this review to provide an exhaustive overview and discussion of the underlying molecular biological aspects of angiogenesis and vasculogenesis. Instead, only the most salient features and controversies relevant to the subsequent clinical trials (see below) will be highlighted.

Several types of angiogenic factors that exhibit different properties have been explored in gene therapy for CVD. These include the different subtypes of VEGF, such as VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF).[110] Some of these factors also yield distinct isoforms (e.g. VEGF-A^{165}). The intracellular signals of these VEGF subtypes are mediated mainly by three different tyrosine kinase receptors: VEGFR1, VEGFR2, and VEGFR3. Specific interaction between these VEGF subtypes and their cognate cellular receptors evokes a differential cellular response in endothelial cells and cardiomyocytes. In particular, an isoform of VEGF-A (VEGF-A^{165}) reportedly has high angiogenic activity and plays a significant role in ischaemic diseases.[111] VEGF-A^{165} interacts principally with VEGFR1 and VEGFR2. Interaction of VEGF-A^{165} with VEGFR1 on endothelial cells contributes to vascular stability of newly formed vessels (*Figure 2*). Its interaction with VEGFR2 on endothelial cells induces angiogenesis, vasculogenesis, and arteriogenesis, vasodilation, cell survival, and increase of cell permeability (*Figure 2*). Simultaneously, activation of VEGFR2 in newly formed cardiomyocytes increased expression of anti-apoptotic proteins and

Table 3 Preclinical gene therapy studies for heart failure and other cardiovascular diseases

Molecular target	Model	Vector	Findings	Refs
VEGF-A^{165}	Coronary artery occlusion model in adult sheep	Plasmid DNA CMVenh/p-VEGF-A^{165} (3.8 mg)	– Reduction of 30% in infarct size – Improvement in myocardial perfusion and LV wall motion	92
	Occlusion of the LAD coronary artery in dogs	Adeno-associated virus AAV6-CMVp- VEGF-A^{165} (5×10^{12} vp)	– LV and arterial pressure, and ejection fraction were not significantly different between the groups – Improvement in tissue viability and cardiac function	93
	Occlusion of the LAD coronary artery in pigs	Adeno-associated virus AAV1-MLCp- VEGF-A^{165} (1×10^{12} vp)	– Co-expression VEGF and Ang1 induced angiogenesis, stimulated cardiomyocyte proliferation, and reduced apoptosis	94
FGF4	Pig model of chronic myocardial ischaemia	Adenovirus Ad5-CMVp-PR39 (3×10^{9} vp)	– Increase in myocardial blood flow at the peak of dobutamine-induced stress – Improved regional ventricular function	95
	Stress-induced myocardial ischaemia in pigs	Adenovirus Ad5-CMVp-FGF4 (up to 1.6×10^{12} vp)	– Improve regional and myocardial function and perfusion after 12 weeks post-injection	34
Sarcoplasmic reticulum Ca^{2+}-ATPase	Human ventricular myocytes from patients with end-stage heart failure	Adenovirus Ad5-CMVp-SERCA2a	– Increased pump activity and contraction velocity – Improved Ca^{2+} concentration in systole and diastole	96
	Ascending aortic constriction in rats	Adenovirus Ad5-CMVp-SERCA2a	– Improved left ventricular systolic pressure and contractility parameters	97
	Heart failure post-MI model in sheep	Adeno-associated virus AAV6-CMVp-SERCA2a (5×10^{12} vp)	– Improved left ventricular remodelling – Decreased caspase 3 levels (anti-apoptotic effect)	98
	Rats with ligation of the left anterior descending artery	Adenovirus Ad-CMVp-SERCA2a (2×10^{11} vp)	– Improvements in regional-wall motion and anterior-wall thickening – Reduction of ventricular arrhythmias during Ischaemia/ Reperfusion	99
	Pressure-overload model of heart failure in guinea pigs	Adeno-associated virus AAV1-CMVp-SERCA2a	– Increased left ventricle fractional shortening – Reduced susceptibility to inducible ventricular arrhythmias	100
S100A1	Rat model of heart failure	Adeno-associated virus AAV6-actin-S100A1 (2.5×10^{11} vp)	– Improved contractility – Improvement of left ventricular dysfunction and remodelling	101
	Heart failure due to balloon occlusion of the left circumflex coronary artery in domestic pigs	Adeno-associated virus AAV9-CMVenh/ MLCp-S100A1 (1.5×10^{13} vp)	– Restoration of S100A levels with improved Ca^{2+} handling and energy homeostasis – Improved contractility	102
β-Adrenergic receptor	Catheterization of coronary artery in rabbits	Adenovirus Ad-CMVp-β2-AR (5×10^{11} vp)	– Improved left ventricular systolic function	103
	Rat model of hypertrophied heart failure	Plasmid DNA	– Improved contractility response to isoproterenol	104
	Rat model of post-MI heart failure	Adenovirus Ad-β2-AR	– Improved basal and isoproterenol-stimulated cardiac contractility	105
Adenylyl-cyclase 6	Wild-type C57/B6 mice	Adenovirus Ad.AC6 (2.5×10^{10} vp)	– Increased myocardial contractility – Improved LV function	106
	Transgenic mice overexpressing adenylyl cyclase (AC) type 6	n/a	– Increased LV ejection fraction – Improvements in both systolic and diastolic LV function	107
	Transgenic mice overexpressing adenylyl cyclase (AC) type 6	n/a	– Poor response to chronic pressure overload and increased deterioration in contractility parameters	108
	Porcine model of heart failure	Adenovirus Ad-CMVp-AC6 (1.4×10^{12} vp)	– Improved left ventricular remodelling parameters – Improved LV function (increased fractional shortening and velocity of circumferential fibre shortening) – Increased cAMP levels	109

reduced expression of pro-apoptotic proteins,[111] suggesting a direct effect on cardiomyocytes. Additionally, VEGFR2 activation induces recruitment of local cardiac stem cells in ischaemic areas.[112] Importantly, the angiogenic response to VEGF is depending on the blood flow. In normal flow conditions, VEGF response is characterized by dilatation of the existing capillaries. In contrast, in an ischaemic situation, a more robust angiogenic response is observed. PlGF also binds on VEGFR1 and synergizes with VEGF.[113] PlGF stimulates angiogenesis and collateral growth in ischaemic heart and limb with at least a comparable efficiency to VEGF through its action on different cell types (i.e.

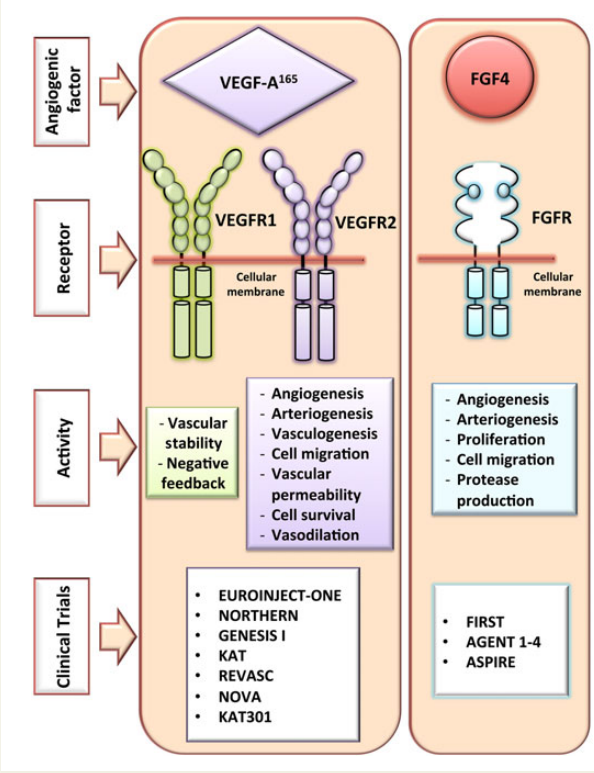


Figure 2 Angiogenic factors in gene therapy for CVD. Description of the membrane receptor involved in the angiogenic factor pathways and the physiological effects. VEGF-A[165], vascular endothelial growth factor; FGF-4, fibroblast growth factor 4; VEGFR, vascular endothelial growth factor receptor; FGFR, fibroblast growth factor receptor.

endothelial, smooth muscle, and inflammatory cells and their precursors) that play a cardinal role in blood vessel formation. However, PlGF did not cause side effects associated with VEGF, such as oedema or hypotension.

Studies in small and large animal ischaemic heart disease models have reported that myocardial overexpression of VEGF following naked DNA transfection, Ad vectors, or AAV transduction induce angiogenesis, improving myocardial blood flow and overall left ventricular function.[114] Preclinical animal studies demonstrated that VEGF-A[165] gene therapy resulted in the induction of angiogenesis, arteriogenesis, and vasculogenesis,[92,93] recovery of ventricular function during ischaemia, reduction in apoptosis of the infarcted area.[94] VEGF also plays a role in the recruitment and homing of cardiac and endothelial progenitor cells[115] and maintenance of physiological homeostasis in the heart.[116] Translational studies in large animal models confirmed some of the beneficial effects of VEGF, either by itself or in combination with other angiogeneic factors (e.g. Ang 1). For instance, direct intramyocardial injection of AAV expressing VEGF and Ang1 resulted in activation of pro-survival pathways and reduction of cell apoptosis, consistent with improvement in the perfusion and function of the heart in a porcine MI model.[94]

There are 22 identified members of the fibroblast growth factor (FGF) family in humans and they interact with four high-affinity specific co-receptor systems consisting of tyrosine kinase FGF receptors (FGFRs) and heparin-like GAGs (HLGAG).[117] The FGFR2 is mainly present in cardiovascular cells and interacts with FGF1, 2, 4, and 5. FGF induces cell proliferation, migration, and production of proteases in endothelial cells and cardiomyocytes. Additionally, interaction of FGF5 with cardiomyocytes has been reported to stimulate angiogenesis, enhance collateral blood flow, and relieve stress-induced ischaemia.[118] Previous studies reported that FGF2 can act on most cardiac cells, including cardiomyocytes, endothelial cells, smooth muscle cells, and fibroblasts inducing a cardio-protective effect in ischaemic events by inducing angiogenesis and increasing vascular remodelling.[119] In pig models of MI, the injection of a plasmid encoding FGF2 resulted in increased vascular perfusion and cardiac contractility compared with control pigs.[95] Lastly, FGF4 has additional paracrine effects compared with FGF1 and FGF2. Intracoronary injection of Ad vectors encoding human FGF4 in a pig model of stress-induced MI resulted in improved myocardial perfusion and regional myocardial function (*Figure 2*).[34,120–122] Other angiogenic factors, including VEGF-B, PlGF, and hypoxia-inducible factor-1α (HIF1α), were also shown to increase angiogenesis and improve the therapeutic outcome in small and large animal models following treatment by either recombinant proteins and/or gene therapy (*Table 3*).

These preclinical studies justified the use of angiogenic gene therapy in clinical trials (see below). Nevertheless, despite their promise, angiogenic gene therapies suffered several limitations: (i) an increase of vascular permeability was often apparently raising important safety concerns (i.e. vascular leak syndrome);[123] (ii) a single angiogenic factor was often insufficient to obtain fully functional, stable, and mature blood vessels, requiring the combination of multiple growth factors instead; (iii) unnatural large capillary structures were often formed[124] requiring approaches to also recruit smooth muscle cells to obtain a more effective revascularization; and (iv) finally, expression of angiogenic factors may contribute to pathological angiogenesis and increase the risk of haemangioma formation[125] and tumour progression and metastasis. Nevertheless, careful selection of the type of angiogenic factor used may mitigate some of these risks (i.e. PlGF).[113]

3.1.1 Clinical trials using angiogenic factors for CVD

The initial gene therapy trials for CVD focus on the administration of genes encoding angiogenic growth factors, such as VEGF-A[165], angiopoietins, FGF,[126] and HIF-1α[26] (*Table 4*). The main objective of these trials is to promote the development of collateral blood vessels in ischaemia-related conditions, such as chronic critical limb ischaemia, myocardial ischaemia, angina, or peripheral arterial occlusive disease.[11,115,127–129] EUROINJECT-ONE, a multicentre, double-blind, randomized trial, included 80 patients with severe stable ischaemic heart disease. VEGF-A[165] cDNA plasmid (0.5 mg) was administered by intramyocardial catheter injection. The study reported functional and symptomatic improvements in patients, but without any significant difference in perfusion compared with placebo.[11] In the NORTHERN trial, the dose of VEGF-A[165] cDNA plasmid was increased to 2 mg. The plasmid was administered through an endocardial route using an electro-anatomical guidance catheter in patients with Class 3 or 4 angina. After 6 months of follow-up, no evidence of improvement was observed in myocardial perfusion assessed by single-photon emission tomography (SPECT).[130]

In an effort to improve the gene delivery efficiency of the angiogenic factors, viral vectors were used instead. The Phase II REVASC trial[131] was therefore conducted to evaluate the efficacy of an adenoviral vector-containing VEGF (Ad. VEGF-A[121]) in patients with severely symptomatic coronary artery disease who are not candidates for conventional revascularization. Though one of the primary endpoints of

Table 4 Gene therapy clinical trials for therapeutic angiogenesis

Trial	Gene	Country	Vector	Delivery method	Dose	Clinical condition (no. of patients)	Findings
FIRST (**F**GF **I**nitiating **R**eva**S**cularization Trial) 2002	FGF2	USA	Plasmid DNA	Intracoronary infusion (single dose)	Dose escalation (0.3, 3, and 30 μg/kg)	Coronary artery disease (337)	– No improvement in exercise tolerance – No improvement in myocardial perfusion – Trend towards symptomatic improvements
AGENT-I **A**ngiogenic **GEN**e **T**herapy (2002)	FGF4	USA	Adeno-virus	Intracoronary infusion (single dose)	Five doses: (3.3×10^8, 1.0×10^9, 3.3×10^9, 1.0×10^{10}, 3.3×10^{10} viral particles)	Chronic stable angina (79)	– No major adverse events – Favourable anti-ischaemic effects
AGENT-Ii **A**ngiogenic **GEN**e **T**herapy (2003)	FGF4	USA	Adeno-virus	Intracoronary infusion (single dose)	10^{10} adenoviral particles	Chronic stable angina (52)	– Trend for improved myocardial perfusion without statistical difference with placebo
KAT **K**uopio **A**ngiogenesis **T**rial (2003)	VEGF-A^{165}	Finland	Adeno-virus/ plasmid liposome	Intracoronary infusion (single dose)	Two groups: VEGF-Adv, 2×10^{10}pfu VEGF-P/L; 2000 μg of DNA with 2000 μL of DOTMA:DOPE	Coronary artery disease (103)	– No major adverse events – No differences in clinical restenosis rate or minimal lumen diameter
VIVA (**V**ascular endothelial growth factor in **Ischaemia** for **V**ascular **A**ngiogenesis) (2003)	VEGF-A^{165}	USA	Plasmid DNA	Intracoronary infusion (day 0) plus intravenous infusions (Days 3, 6, and 9)	Low dose: 17 ng kg^{-1} min^{-1} High dose: 50 ng kg^{-1} min^{-1}	Stable exertional angina (178)	– No safety concerns – No evidence of myocardial perfusion improvement – Trend towards improvements in angina class and frequency
EUROINJECT—One (2005)	VEGF-A^{165}	Denmark, Poland, Sweden, and Austria	Plasmid DNA	Direct intramyocardial injection via NOGA-Myostar©	0.5 mg of phVEGF-A_{165}	Severe stable ischaemic heart disease (80)	– No difference with placebo in clinical, perfusion, and wall motion characteristics – Improved regional wall motion
REVASC-II (2006)	VEGF-A^{121}	Canada	Adeno-virus	Direct intramyocardial injections	4×10^{10} viral particles AdVEGF121	Severe coronary artery disease (67)	– No improvement in myocardial perfusion by SPECT nuclear imaging
AGENT-III/IV **A**ngiogenic **GEN**e **T**herapy (2008)	FGF4	USA, Europe	Adeno-virus	Intracoronary infusion (single dose)	Low dose of 4, 1×10^9 viral particles (vp), and a high dose of 1×10^{10} vp	Recurrent stable angina (AGENT-III: 416; AGENT-IV: 116)	– No differences between placebo and treatment for any primary or secondary endpoints – No significant safety concerns – Difference in placebo response between men and women

Continued

Table 4 *Continued*

Trial	Gene	Country	Vector	Delivery method	Dose	Clinical condition (no. of patients)	Findings
Phase I intracoronary administration of Ad-Hhgf (2009)	hHGF	China	Adeno-virus	Intracoronary infusion (single dose)	Three doses: 5.0×10^9, 1.0×10^{10}, 2.0×10^{10} pfu	Severe and diffuse triple vessel coronary disease (18)	– No adverse events related to the vector – Improved activity tolerance
NORTHERN (**NO**GA angiogenesis **R**evascularization **THE**rapy: assessment by **R**adio**N**uclide imaging) (2009)	VEGF-A^{165}	Canada	Plasmid DNA	Endocardial route using an electro-anatomical NOGA guidance catheter	Total dose, 2 mg	Refractory Canadian Cardiovascular Society (CCS) Class 3 or 4 angina symptoms (93)	– No evidence of improved myocardial perfusion assessed by single-photon emission tomography (SPECT)
Multicentre Phase I and Safety Study (2010)	HIF1α	Germany, UK	Adeno-virus	Intramyocardial injections during CABG	Three doses: 1.0×10^{10}, 3.0×10^{10}, and 1.0×10^{11} viral particles	Hypo-perfused area of viable ventricular muscle (13)	– No safety concerns related to vector administration
VIF-CAD (2011)	Bicistronic [VEGF/FGF] plasmid	Poland	Plasmid DNA	Percutaneous intramyocardial injection using NOGA guidance catheter	Total dose, 0.5 mg	Refractory coronary artery disease (52)	– No demonstrated improvement in cardiac perfusion assessed by SPECT – Functional class improved – Improved exercise tolerance
GENESIS I (2012)	VEGF-A^{165}	Argentina	Plasmid DNA	Intramyocardial injections	Total dose, 3.8 mg	Severe CAD not amenable for revascularization (10)	– Safe at 2 years follow-up – Trend towards improved myocardial perfusion assessed by SPECT
ASPIRE (2013)	FGF4	Russia	Adeno-virus	intracoronary infusion during induced transient ischaemia	6×10^9 viral particles Ad5FGF4	Stable angina pectoris (100)	– In recruitment phase – Assessments for safety and efficacy after 1 year of follow-up
KAT301 (2013)	VEGF-D	Finland	Adeno-virus	Endocardial injection system (NOGATM)	Escalating dose of 1×10^9, 1×10^{10}, and 1×10^{11} vpu injected into 10 sites of the myocardium	Severe coronary artery disease (30)	– In recruitment phase – Assessments for safety and efficacy after 1 year of follow-up

the trial, exercise treadmill evaluation was significantly improved after 6 months of follow-up, there was no evidence of improvement in myocardial perfusion, evaluated by SPECT. Different limitations inherent to the study such as the surgical vector delivery technique employed, lack of blindness for the treatment groups, and the advances in catheter systems for percutaneous intramyocardial delivery prompted new clinical studies. The KAT301 trial was based on the use of an adenoviral vector to administer VEGF-D cDNA. Using a catheter-mediated *trans*-endocardial injection strategy, patients with coronary heart disease (CHD) with no other therapeutic options will be recruited in an escalating dose protocol. Doses of 1×10^9, 1×10^{10}, and 1×10^{11} vp of Ad.VEGF-D will be injected into 10 myocardial sites. The main goals of the study are to evaluate safety and efficacy (http://clinicaltrials.gov/show/NCT01002430). These trials constitute the major advances in VEGF-based gene therapy.

The AGENT trial (Angiogenic GENe Therapy)[118] evaluated the safety and anti-ischaemic effects of five ascending doses of adenoviral vectors carrying the FGF4 cDNA (Ad5-FGF4). Selected patients with chronic stable angina exhibited symptomatic improvement in exercise time at 4 weeks after injection, and the overall safety profile of the viral vector system was ascertained. The same strategy was followed in the AGENT-2 trial.[132] Intracoronary administration of Ad5-FGF4 was

evaluated in patients with coronary artery disease. After 8 weeks of follow-up, a trend towards improvement in stress-induced myocardial perfusion was observed compared with baseline. Nonetheless, there was no significant difference compared with the placebo group. In the AGENT-3 and AGENT-4 trials, low and high doses of Ad5-FGF4 for chronic angina were evaluated. The results demonstrated a sex-specific beneficial effect on the exercise treadmill test; however, this effect was mainly due to a poor placebo response among women.[133] Moreover, both studies were stopped after an interim analysis of the AGENT-3 trial indicated that there were no significant differences regarding the primary endpoint in the treatment arm compared with placebo.

Several factors could have influenced the negative results obtained in the clinical trials using angiogenic factors compared with the promising preclinical data or earlier clinical trials.[134,135] The clinical data were only conclusive when the later studies were randomized and blinded compared with the initial ones. This suggested a strong placebo effect, in part due to the strict selection of patients, the delivery method used (intramuscular injection can increase the production of growth factors), or a possible bias by lack of a blinded protocol. The lack of efficacy could also be explained by the fact that the patients selected for clinical trials were in end stages of the disease, with the more severe presentation, despite previous pharmacological interventions. It cannot be excluded that these angiogenic gene therapies may be more effective in the less severe patients, which constitutes the higher percentage of patients in the population. Finally, especially for naked plasmid strategies, the poor DNA uptake of cardiac and muscle cells is low, the short transient expression limited up to 2 weeks can be insufficient for achieve an effective angiogenic stimulus with substantial quantifiable changes in the heart parameter in comparison to the results obtained in preclinical models. Given the intrinsic complexity of angiogenesis, it is unlikely that administration of a single gene would suffice to obtain sustainable effects in patients with CVD. Indeed, preclinical studies have shown that additional factors are needed to lead to optimal endothelial and smooth cell proliferation and integration into sustainable and functional blood vessel development.[136,137]

3.2 Myocyte Ca^{2+} handling/contractility target genes: HF

In healthy cardiomyocytes, the norepinephrine (NE) released by the sympathetic system stimulates the β-adrenergic receptor, thereby inducing the entry of Ca^{2+} through the L-channels into the cells. The increase of calcium concentration activates the ryanodine receptor (RyR) to release stored Ca^{2+} from the sarcoplasmic reticulum into the cytoplasm, which in turn leads to contraction of the myofilaments. The NE stimulus also activates adenylyl-cyclase 6 (AC6), which induces the conversion of ATP in cyclic-AMP (cAMP), which in turn causes the phosphorylation (P) of phospholamban (PLN). This phosphorylation step, which is mediated by phosphokinase-A (PKA), releases PLN's inhibitory effect on the sarcoplasmic reticulum Ca^{2+}−ATPase pump (SERCA2a). Next, SERCA2a binds Ca^{2+} ions from the cytoplasm and pumps them back into the sarcoplasmic reticulum, reducing the cytoplasmic concentration of calcium and permitting the relaxation of the myofilaments. The alteration of Ca^{2+}-handling proteins during the process of excitation−contraction coupling plays an important role in the development and evolution of HF. In failing cardiomyocytes, a reduced Ca^{2+} cycling activity by a reduced expression of SERCA2a, an imbalance in phosphorylation of the RyR and abnormal expression of regulatory proteins such as S100A1 and AC6 have been demonstrated, suggesting these proteins as possible therapeutic targets for gene therapy (*Table 3*).

3.2.1 Sarcoplasmic reticulum Ca^{2+}ATPase up-regulation improves cardiac function in HF

Initial studies demonstrated that HF is partly caused by decreased sarcoplasmic/endoplasmic reticulum Ca^{2+}ATPase2a (SERCA2a) levels or activity, independent of the aetiology of the HF.[138] The calcium pump SERCA2a causes muscle relaxation by lowering the cytosolic calcium and restores the calcium reserves in the sarcoplasmic reticulum, which are necessary for muscle contraction.[139] Subsequent studies demonstrated an association between decreased SERCA2a mRNA level and low SERCA2a protein concentration and Ca^{2+}-ATPase activity, especially during the transition from compensated hypertrophy to decompensated HF, leading to faster and more severe HF.[140] Overexpression of SERCA2a in human ventricular cardiomyocytes obtained from patients with ischaemic and dilated cardiomyopathy resulted in increased SERCA2a pump activity consistent with improved contraction and relaxation velocity.[96] In a rat model of pressure-overload hypertrophy HF, Ad vector-mediated overexpression of SERCA2a raised left ventricular, systolic, and diastolic function to levels similar to those observed in control mice.[97] Using a percutaneous delivery system for AAV6 encoding SERCA2a, Beeri *et al.*[98] demonstrated long-term overexpression of SERCA2a in a sheep model of mitral regurgitation and MI, with improved contractility and inhibition of the remodelling process.

Most importantly, SERCA2a gene transfer improves contractile function survival rates and the energy potential in failing hearts without increasing mortality or worsening metabolism.[99] In addition, SERCA2a overexpression in a rat model of ischaemia/reperfusion injury significantly decreased ventricular arrhythmias and (more importantly) led to reduced infarct size and improved wall thickening in the anterior wall.[99] It is also encouraging that long-term *in vivo* overexpression of SERCA2a after AAV1-mediated gene transfer in cardiomyocytes in a preclinical volume-overload model of HF can preserve cardiac function by increasing eNOS expression in coronary arteries.[100] Furthermore, Ad SERCA2a transduction in an animal model of pressure-overload hypertrophy with transition to HF resulted in decreased pro-apoptotic (e.g. Bax) and apoptotic and inflammatory (e.g. TNF-α) cytokines, suggesting a SERCA2a regulatory role in controlling inflammation and apoptotic events during HF and MI.

Before clinical translation, the effects of SERCA2a were evaluated in a Yorkshire-Landrace swine model of volume-overload HF. An AAV1 vector was used to deliver human SERCA2a cDNA. The CMV immediate-early promoter/enhancer was incorporated into the transgene cassette. Two months after HF induction, pigs received AAV1-SERCA2a vectors, resulting in normalization of SERCA2a expression levels consistent with improvements in left ventricular ionotropic activity and remodelling.[141] Nevertheless, the results obtained in these preclinical models must be carefully and critically evaluated. In particular, it was shown that human cardiomyocytes rely on SERCA2a pump function (around 71%) to a lesser extent than rat and mouse cardiomyocytes (around 90%).[142] Ultimately, only more clinical trials will provide the necessary data to corroborate the therapeutic potential of SERCA2a gene therapy in patients with HF.

Apart from Ad vectors and AAV vectors, LV have also been used to deliver SERCA2 to the myocardium. LV containing the SERCA2 gene were delivered by a hypothermic intracoronary delivery method in rat myocardium, 2 weeks after MI, Significantly, this LV-SERCA2 gene

therapy resulted in long-term improvement of systolic and diastolic function, prevention of left ventricular remodelling even up to 6 months after gene therapy, consistent with significant improvement of the survival rate.[84] Though these findings offer a potentially translatable therapy, the results would still need to be confirmed in a preclinical large animal model.

3.2.2 S100A1 is a key protein in cycling Ca^{2+} capacity of myofilaments

S100A1 is preferentially expressed in myocardial tissue though low levels have also been reported in other tissues.[143] It exerts profound ionotropic actions through the modulation of cardiomyocyte Ca^{2+} homeostasis and myofilament function independent of β-adrenergic stimulation.[144] S100A1 interacts in a Ca^{2+}-dependent manner with the RyR and stabilizes the SERCA2a-PLN complex.[145] S100A1 diminishes the diastolic leak of Ca^{2+} and influences cardiac titin and mitochondrial F1-ATPase. Cardiomyocytes that overexpress S100A1 present a higher ATP content than control cells, suggesting a role for S100A1 in energy metabolism.[146] Considering the potential therapeutic effects of S100A1 protein, gene therapy studies have evaluated its performance in HF models. Pleger *et al.* evaluated the response of an AAV6-S100A1 vector in a rat model of HF. The results demonstrated long-term improvement in cardiac dysfunction and attenuated left ventricular remodelling. Even non-transduced cardiomyocytes showed a trend towards functional improvement, suggesting that S100A1-overexpressing cardiomyocytes have an indirect bystander effect on neighbouring cardiomyocytes. The therapeutic effects of S100A1 were preserved during β-adrenergic antagonist treatment with metoprolol, improving its cardiac reverse remodelling, ionotropic action, and anti-arrhythmic effects.[101] Intracoronary adenovirus-mediated S100A1 gene delivery in a rat model of post-MI improved myocardial contractility and Ca^{2+} handling.[147] Additionally, S100A1 gene transfer improved force generation in engineered cardiac grafts without interfering with the mechanical structure of the engrafted heart.[148] In a large animal model of HF, Pleger *et al.*[102] evaluated the long-term effectiveness of an AAV9 vector encoding the S100A1 gene. Retrograde coronary venous delivery strategy was used in post-ischaemic pig model of HF after 2 weeks of occlusion of the left circumflex coronary artery. The follow-up resulted in long-term benefits in cardiac performance by improving mitochondrial function in failing cardiomyocytes and reverse remodelling by improving systolic and diastolic left ventricular performance at 12 weeks post intervention. The use of S100A1 gene therapy for the treatment of HF was further supported by Brinks *et al.*[149] using an *in vitro* evaluation of human failing ventricular cardiomyocytes strategy. An Ad vector encoding S100A1 was used to transduce cardiomyocytes isolated from the heart of patients undergoing transplant surgery. S100A1 levels returned to normal in transduced cardiomyocytes, with clear improvement in contractibility performance and restoring of sarcoplasmic reticulum functions. These preclinical data support the use of S100A1 as a gene therapy modality in patients with HF.

3.2.3 Adenylyl-cyclase type 6 is a central regulator of calcium cycling in cardiomyocytes and SERCA2a activity.

Adenylyl-cyclase type 6 (AC6) catalyzes the conversion of ATP to 3′,5′-cyclic-AMP (cAMP) and pyrophosphate. It improves the affinity of SERCA2a for calcium by activating a cAMP-dependent protein kinase of PLN. Upon phosphorylation, PLN loses its inhibitory effect on SERCA2a. This AC6 pathway also results in the transcription factor-3-dependent suppression of PLN promoter activity. The overall effect of PLN activation is a decrease in contractility and the rate of muscle relaxation, thereby decreasing stroke volume and heart rate.[150] Conversely, inhibiting PLN expression of function has the reverse effect and is therapeutically beneficial. Indeed, studies in transgenic mice overexpressing AC6 demonstrated increased cAMP generation in cardiomyocytes, which in turn triggered an improvement in cardiac function, particularly left ventricular contractile function.[106] A decrease in ventricular hypertrophy and increased survival were reported in cardiomyopathy after AC6 activation.[151] This finding is consistent with improved left ventricle function, reduced ventricular dilation, and reduced mortality rates in transgenic mice overexpressing AC6 compared with control mice.[107] In contrast to conventional sympathomimetic interventions, increasing AC6 expression in a post-MI mouse model did not increase susceptibility to ventricular arrhythmias. Nevertheless, AC6 overexpression in a long-term murine model of pressure-overload HF was correlated with a worse outcome, possibly because of increased systolic ventricular wall stress. These results suggest a different molecular imbalance depending on the cause of the HF.[108] Intracoronary delivery of an Ad vector encoding AC6 into a large-animal model of HF improved left ventricle function and attenuated deleterious left ventricular remodelling. These results were associated with increased cAMP-generating capacity.[109] In summary, the pivotal effects of AC6 on the β-AR signalling pathway and calcium handling, consistent with its impact on improving left ventricular function and survival rates, reaffirm the use of AC6 as a rational approach to HF treatment. Moreover, intracoronary delivery of AC6 in small and large preclinical models confirms its favourable safety profile.

3.2.4 Alterations of the β-adrenergic system modulate cardiomyocyte contractile function in HF.

It was demonstrated >20 years ago that the β-adrenergic receptor (β-AR) system is centrally involved in HF.[152] Activation of the sympathetic system in patients with HF correlated with morbidity and mortality levels. Reduction and de-sensitization of the β-AR receptors caused by the up-regulation of β-AR kinases and increased function of the inhibitory guanine nucleotide-binding protein (Gi) during HF have led to the employment of this system as a treatment option for HF.[153] Results from transgenic mice revealed differential roles for different components of the β-AR system. Up-regulation of β1-AR is related to cardiomyocyte hypertrophy, followed by fibrosis and HF induced by activation of the β1-AR-Gs pathway.[154] In contrast, moderate up-regulation of β2-AR improved basal contractility and rescued left ventricular contractility after MI.[155] This difference has been explained by the ability of β2-AR to couple both Gi and the stimulatory guanine nucleotide-binding protein (Gs). In contrast, β1-AR couples only to Gs. The effects of the β2-AR on Gi proteins antagonize the contractile response controlled by the Gs proteins. Thus, the beneficial and pro-survival signalling derived from the β2-AR effect is mediated primarily by cAMP-PKA signalling activation and preferential activation of Gi during HF.[156]

Percutaneous-mediated intracoronary delivery of Ad vectors encoding β2-AR in rabbit models improved global left ventricular systolic and contractility performance.[103] In rat models of pressure-overloaded HF, β2-AR cDNA was transfected by intracoronary infusion using a liposomal delivery method. The results revealed an enhanced response to isoproterenol (β-adrenergic agonist) in failing hearts.[104] Recent evidence suggested that Ad vector-mediated β2-AR overexpression leads to enhanced endothelial cell proliferation and migration

compounded by VEGF production, which subsequently led to improved ischaemia-induced angiogenesis in an ischaemic hind limb model.[157] These results were extended to a rat model of post-MI via direct intramyocardial injection. Four weeks post-injection, rats exhibited improved left ventricular remodelling and cardiac function, increased capillary density, increased arteriolar length density, and enhanced *in vivo* myocardial blood flow and coronary reserve were observed, strengthening the role of β2-AR in the regulation of cardiac angiogenesis in the context of HF.[105]

Furthermore, homologous desensitization, a process by which kinases decrease the interaction between activated β receptors and their G proteins, is mediated by GRKs. Homologous desensitization reduces the neurohormonal response in the heart, which leads to worsening heart function during HF.[158]

Animal studies have shown that expression of a peptide inhibitor of GRK2 (βARKct) can improve the contractility of failing myocardium, promote reverse remodelling of the left ventricle, and improve outcome post-MI.[159–162] Translational studies in human cardiomyocytes showed that the delivery of βARKct using Ad vector-mediated gene transfer in ventricular cardiomyocytes from patients with end-stage HF improved contractile function and β-adrenergic responsiveness.[163] The long-term therapeutic impact and feasibility of the βARKct gene transfer was evaluated in a clinically relevant pig model of ischaemic cardiomyopathy using AAV6-mediated βARKct delivery.[164] Retrograde injection into the coronary veins resulted in efficient and long-term βARKct expression, with significant systolic performance improvement overall at 6 weeks post-treatment. The results also suggest a correction of the catecholaminergic overdrive in post-MI HF. Finally, long-term βARKct expression in this porcine FH model exhibited a positive effect on the adverse cardiac remodelling process after MI. The favourable efficacy and safety data in preclinical models support the use of βARKct gene therapy for future clinical trials.

3.2.5 Clinical trials for gene therapy to treat HF by targeting the myocyte Ca^{2+} handling/contractility pathway

The first-in-human Phase I/II clinical trial for the treatment of HF (*Table 4*), the 'calcium up-regulation by percutaneous administration of gene therapy in cardiac disease trial', (CUPID Trial) prompted renewed interest in gene therapy.[165] During Phase I, 9 patients with severe HF received an intracoronary delivery of AAV1 vector encoding SERCA2a cDNA in an open-label dose-escalation protocol (doses ranged from 1.4×10^{11} to 3×10^{12} vector particles per patient). After 6 months of follow-up, a tendency towards improvement in the functional, symptomatic, biomarker, and left ventricular/remodelling parameters was reported.[166] The Phase II trial is based on a randomized, double-blind, placebo-controlled, dose-escalation protocol. Thirty-nine patients with Class III/IV HF received intracoronary infusion of placebo ($n = 14$) or low-dose (6×10^{11} vector particles; $n = 8$), mid-dose (3×10^{12} vector particles; $n = 8$), or high-dose (1×10^{13} vector particles; $n = 9$) AAV1-SERCA2a vector via percutaneous intracoronary artery infusion. After 12 months of follow-up, improvement or stabilization was reported based on the Heart Failure Questionnaire, a 6-min walk test, peak maximum oxygen consumption, N-terminal pro-hormone brain natriuretic peptide levels, and left ventricular end-systolic volume.[167] Importantly, no adverse events could be attributed to the SERCA2a gene therapy and the AAV1-SERCA2a emerged as a viable therapeutic option for patients with severe HF with poor response to traditional pharmacologic treatments[140] with a general consensus that the CUPID results could constitute a basis for larger pivotal trials.[168] Recently, a 36-month follow-up analysis of the patients enrolled in the CUPID1 Phase IIa trial reported a lower number of cardiovascular events, including death in the treated groups compared with placebo.[169] Additionally, no adverse immune events related to the AAV1-SERCA2a treatment were apparent in a long-term assessment.[170]

Two new clinical trials are based on SERCA2a. The Phase II study 'Investigation of the safety and feasibility of AAV1-SERCA2a gene transfer in patients with chronic HF and a left ventricular assist device' (SERCA-LVAD; NCT00534703) evaluates the effects of SERCA2a in patients with chronic HF that have received previously a left ventricular assist device (LVAD). Up to 24 patients will be randomized to receive a unique dose of 1×10^{13} DRP (DNase resistant particles) of AAV1-SERCA2a or placebo by a percutaneous method. The first results are expected in mid-2016. The second Phase II study, 'AAV1-CMV-Serca2a GENe Therapy Trial in Heart Failure' (AGENT-HF; NCT01966887), is a double-blind, randomized, placebo-controlled, parallel study that aims to evaluate the effects of the AAV1-CMV-SERCA2a vector in the cardiac remodelling parameters of 44 patients with symptomatic HF NYHA IIb/IV and EF of 35% or less, after intracoronary infusion of a single dose of 1×10^{13} DRP of the vector. The first results are expected at the beginning of 2016.

Given the intrinsic limitations of the low number of patients in a Phase IIa study, a Phase IIb, double-blind, placebo-controlled, multinational, multicentre, randomized event-driven study was needed (CUPID2) to confirm the initial CUPID1 results in a large number of patients ($n = 250$).[171] CUPID2 is a randomized, double-blind, placebo-controlled, multinational trial evaluating a single, one-time, intracoronary infusion of the AAV1/SERCA2a vector vs. placebo added to a maximal, optimized HF drug and device regimen (NCT01643330). However, recently it was announced that this Phase 2b CUPID2 trial did not meet its primary and secondary endpoints (http://ir.celladon.com/releasedetail.cfm?releaseid=908592). In this study, the primary endpoint comparison of the AAV1/SERCA2a vector to placebo defined as HF-related hospitalizations or ambulatory treatment for worsening heart failure did not show a significant treatment effect. The secondary endpoint comparison of the AAV1-SERCA2a vector to placebo, defined as all-cause death, heart transplant or need for a mechanical circulatory support device, likewise failed to show a significant treatment effect. All other exploratory efficacy endpoints (improvement in NYHA classification, 6-min Walk Test, and Quality of Life) were also inconsistent with a treatment effect. No safety issues were noted, however. The exact reason for this outcome is not fully understood, and further studies are required to assess the efficiency of cardiac gene delivery using qPCR analysis on available patient biopsies. Intracoronary delivery of the AAV1 vector may not have resulted in efficient transduction of cardiomyocytes. This may reflect the relatively low transduction efficiencies in cardiomyocytes after anterograde delivery with AAV1-SERCA2a in porcine models,[91,172] consistent with the relatively low vector copy number determined by qPCR. Unfortunately, in these preclinical studies,[91] a placebo control group without AAV1-SERCA2a treatment was lacking. Consequently, since SERCA2a is normally also expressed in the heart, the contribution of the *de novo* expressed SERCA2a protein encoded by the AAV1-SERCA2a vector to the total SERCA2a protein levels could not be determined. Moreover, the presence of vector DNA in the myocardium does not necessarily imply bona fide gene transduction. In the CUPID2 trial, the vectors were administered by anterograde coronary delivery without any vessel balloon occlusion. The main advantage

of this delivery strategy is that it does not increase the risk of ischaemic events in an already functionally compromised heart. However, under those circumstances the vector rapidly disseminates via the circulation into distal tissues, diminishing the overall efficacy of cardiac transduction. Consequently, vector dissemination may result in the inadvertent transduction of non-target tissues, particularly the liver. The use of retrograde delivery methods may potentially increase cardiac transduction efficiencies.[172] Furthermore, the development of alternative AAV capsids by directed molecular evolution, permitting efficient transcytosis, and/or the use of reagents that enhance vector uptake may ultimately also overcome the limitations observed in the CUPID2 trial.

An alternative Phase I/II clinical gene therapy strategy for HF that is also based on Ca^{2+} handling has been initiated (NCT00787059). The objective of the AC6 trial for HF is to evaluate the safety and performance of human AC6 as a therapeutic option for patients HF. To achieve this goal, it is planned to include ~56 patients and deliver an Ad5 vector carrying the AC6 gene. In this dose-escalation study, patients with an ejection fraction <40% will receive the vector by intracoronary delivery. Five different doses (ranging from 3.2×10^9 to 3.2×10^{11}) will be compared with placebo. After 12 weeks of follow-up, patients will undergo exercise treadmill testing, and left ventricular functional parameters will be examined by echocardiography before and during the isoproterenol test.

4. Conclusions and future perspectives

The progress of unravelling the molecular pathways involved in cardiac function in normal and pathological states has increased efforts to develop co-adjuvant therapies as pharmacological and interventional options. Gene therapy is emerging as a suitable alternative, with substantial progress in preclinical models of CVD. Advances in therapies using angiogenic factors such as VEGF or FGF, the modification of β-adrenergic pathways, and molecules involved in cardiomyocyte Ca^{2+} cycling constitute clear examples of promising therapeutic alternatives. Additionally, an antagonist to the LDL receptor (LDLR), the proprotein convertase subtilisin/kexin type 9 (PCSK9) has been described as a promising therapeutic target for the prevention of CHD due to its role in the lipids metabolism,[8] a major risk factor for CVD. Mutations of the PCSK9 have been associated with significant reduction of LDL-C and a subsequent decrease in CVD risk. Ding *et al.*[8] demonstrated that using CRISP/Cas9 system was possible to induce a disruption in the PCSK9 gene and decrease blood cholesterol levels in mice up to 40% compared with control mice.

The advantages of the AAV vector-based therapeutic strategies over the other available recombinant vectors have positioned this delivery system as the preferred option for many of the gene therapy approaches for HF. However, the ability to obtain sustained expression of the gene of interest may not always be warranted and sometimes transient expression may be preferred based on safety considerations (e.g. angiogenic gene therapy). Though Ad vectors may at first glance seem ideally suited to achieve robust yet transient expression of a given therapeutic gene, this short-term expression results from immune complications intrinsic to this type of vectors. This inflammatory risk undermines the safety of adenoviral vector for CVD gene therapy and needs to be carefully assessed, even in the context of loco-regional catheter-mediated vector delivery into the myocardium.

The physiological and structural differences between animal models and humans and the development of immune response against the transgene products, the gene-modified cells, or the vectors themselves pose important challenges for clinical translation. The use of safer and more efficient gene delivery methods is warranted by improving the tissue specificity of the vectors using cardiac-specific enhancer/promoter to achieve better 'transcriptional targeting' and by using modified capsids to restrict vector entry into the desired target cells (*in casu* cardiomyocytes). For some gene therapy applications, it would be desirable to be able to control the duration and strength of the expression of the therapeutic gene, using clinically relevant approaches, which are now being developed.

Gene therapy clinical trials in ischaemic heart disease yielded limited results compared with preclinical models, with some improvement in secondary endpoints but no improvement in perfusion or myocardial function. The first clinical trial for HF reported substantial benefits in patients with severe angina, which justified the progression to larger clinical trials. In the future, consensus clinical and functional endpoints as well as the most appropriated measurement methods should be established to allow a clear and comparable understanding of the results between the different clinical trials. As gene therapy is currently also being used to treat non-lethal diseases in children, the hope is that the majority of patients who suffer from less severe heart disease may ultimately benefit from the advances in gene therapy.

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