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REVIEW

Gene Therapy for the Heart

Lessons Learned and Future Perspectives

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ABSTRACT: While clinical gene therapy celebrates its first successes, with several products already approved for clinical use and several hundreds in the final stages of the clinical approval pipeline, there is not a single gene therapy approach that has worked for the heart. Here, we review the past experience gained in the several cardiac gene therapy clinical trials that had the goal of inducing therapeutic angiogenesis in the ischemic heart and in the attempts at modulating cardiac function in heart failure. Critical assessment of the results so far achieved indicates that the efficiency of cardiac gene delivery remains a major hurdle preventing success but also that improvements need to be sought in establishing more reliable large animal models, choosing more effective therapeutic genes, better designing clinical trials, and more deeply understanding cardiac biology. We also emphasize a few areas of cardiac gene therapy development that hold great promise for the future. In particular, the transition from gene addition studies using protein-coding cDNAs to the modulation of gene expression using small RNA therapeutics and the improvement of precise gene editing now pave the way to applications such as cardiac regeneration after myocardial infarction and gene correction for inherited cardiomyopathies that were unapproachable until a decade ago.

Key Words: gene editing ■ heart failure ■ microRNA ■ nanoparticles ■ regeneration

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It was 1989 when Steven Rosenberg and his team at the National Cancer Institute in Bethesda performed the first approved genetic modification in humans.[1] These investigators used a retroviral vector to genetically mark the lymphocytes that were retrieved from the tumor mass of a patient with melanoma before their reinfusion into the same patient. The purpose of the study was to prove that tumor infiltrating lymphocytes, once isolated and expanded in vitro, maintain the capacity to home to the tumor and its metastasis. It was not only a key finding for immunotherapy but also the official birth of the field of gene therapy.

In the 30 years that followed, according to the Gene Therapy Clinical Trials Worldwide database (http://www.abedia.com/wiley/, updated September 2019), ≈3000 clinical trials have been approved, conducted, or initiated in most fields of clinical medicine. Success has taken a long time. Eventually, however, there are now seven products already approved by the Food and Drug Administration (FDA) or European Medicines Agency, and a few more hundreds are in the final stages of the clinical approval pipeline.[2] Given the rapid pace of progress of gene therapy product development, the FDA has recently (January 2020) released 6 new guidelines for the Chemistry, Manufacturing, and Control information for human gene therapy investigational new drug applications (https://www.fda.gov/vaccines-blood-biologics/biologics-guidances/cellular-gene-therapy-guidances). These streamlined and standardized guidelines are expected to further speed clinical application in this area.

While gene therapy celebrates its clinical successes, it is quite surprising that not a single application targets any of the diseases affecting the heart, while these represent a most prevalent cause of morbidity, disability, and mortality in the world. This void reflects the many hurdles that are intrinsic to the development of gene therapy in this area.

This article reviews the past experience gained in cardiac gene therapy clinical trials and highlights the problems that still need to be solved before success. It also

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Nonstandard Abbreviations and Acronyms

AAV	adenoassociated virus
AC	adenylate cyclase
ASO	antisense oligonucleotide
CRISPR	clustered regularly interspaced short palindromic repeats
DCM	dilated cardiomyopathy
EGFR	epidermal growth factor receptor
FGF	fibroblast growth factor
FSTL	follistatin-like 1
G-CSF	granulocyte colony stimulating factor
HCM	hypertrophic cardiomyopathy
HF	heart failure
HR	homologous recombination
I-1c	inhibitor-1c
IL-6	interleukin-6
MI	myocardial infarction
miRNA	microRNAs
LNA	locked nucleic acid
lncRNA	long noncoding RNA
MRTF	myocardin-related transcription factor
NRG	neuregulin
PDGF	platelet-derived growth factor
PKA	protein-kinase A
PLN	phospholamban
PP1	protein phosphatase 1
RNAi	RNA interference
SDF	stromal cell–derived factor
TALEN	transcription activator-like effector nuclease
VEGF	vascular endothelial growth factor

presents 3 new areas (small RNA therapeutics, cardiac regeneration, and cardiac gene editing) that hold great promise for future application.

DELIVERY, DELIVERY, DELIVERY

In the late 1990s, when gene therapy, after an initial excitement phase, had suffered tremendous drawbacks, a journalist interviewed Inder Verma, a gene therapy pioneer, asking him which were the 3 major unsolved issues according to his opinion. Verma's answer was adamant: "There are only 3 problems in gene therapy: delivery, delivery and delivery".[3] This anecdote well reflects the problems that this discipline had to face over the years in all fields. Cardiac gene therapy has not been immune from this issue.

Efficiency of nucleic acid delivery essentially relies on 2 parameters, the gene vehicle and the route of administration. Early attempts at gene therapy to induce therapeutic angiogenesis in the 1990s aimed at injecting large amounts of naked DNA plasmids coding for angiogenic factors into the myocardium based on the assumption that cardiomyocytes could spontaneously internalize extracellular DNA. Double-blinded clinical trials have clearly indicated that this approach is largely unsatisfactory (reviewed in study by Giacca and Zacchigna[4]; cf. also later). Not different from other cell types, the nonpolar and hydrophobic nature of the cardiac sarcolemma represents a formidable barrier to negatively charged nucleic acids.

Extensive experimentation in small and large animals has instead indicated that at least 2 viral vector systems are effective at cardiac gene transfer, those based on adenovirus and the adenoassociated virus (AAV); Figure 1A. First generation adenoviral vectors, in which a therapeutic gene expression cassette substitutes the viral E1 and/or E3 regions, have advanced to clinical trials for applications in both therapeutic angiogenesis and heart failure. However, these vectors still retain a number of adenoviral genes, the expression of which stimulates important inflammatory and immune responses, which raise safety and efficacy concerns.

A vector family that is now considered the gold standard for cardiomyocyte gene transfer is based on the small, defective, and nonpathogenic parvovirus AAV, especially because of the specific tropism of these vectors for postmitotic cells in vivo, most notably cardiomyocytes. Compared with adenoviral vectors, for which transgene expression returns to baseline within a few weeks from administration,[5] AAV transgenes are expressed indefinitely. We have previously reviewed extensively the properties of these vectors.[6] Despite their already satisfactory efficiency in transducing postmitotic tissues, AAV vectors are still amenable to significant improvement in terms of transduction efficiency, packaging capacity, tissue specificity, and immune evasion. In particular, the AAV capsid proteins play key roles in all these processes. The field of designing AAV capsids with improved characteristics has evolved from rationale design of selective *cap* gene mutants, to the generation of randomly mutated or shuffled libraries, which are then selected and evolved in vivo for organ specificity and liver de-targeting. Some of the generated variants display improved cardiac selectivity. These include the AAV2i8, AAV2i8G9, and AAV-SASTG chimeras, some AAV serotype 9 variants, or vectors obtained through the screening of peptide display libraries or DNA-shuffled libraries (reviewed in study by Zacchigna and Giacca[7] and Grimm and Büning.[8] Besides targeting cardiomyocytes, these capsid engineering approaches can also be applied to endothelial cells. A random peptide library display screening has revealed peptide motifs that permit targeted delivery into coronary artery endothelial cells using the AAV2[9] or AAV9 serotypes.[10]

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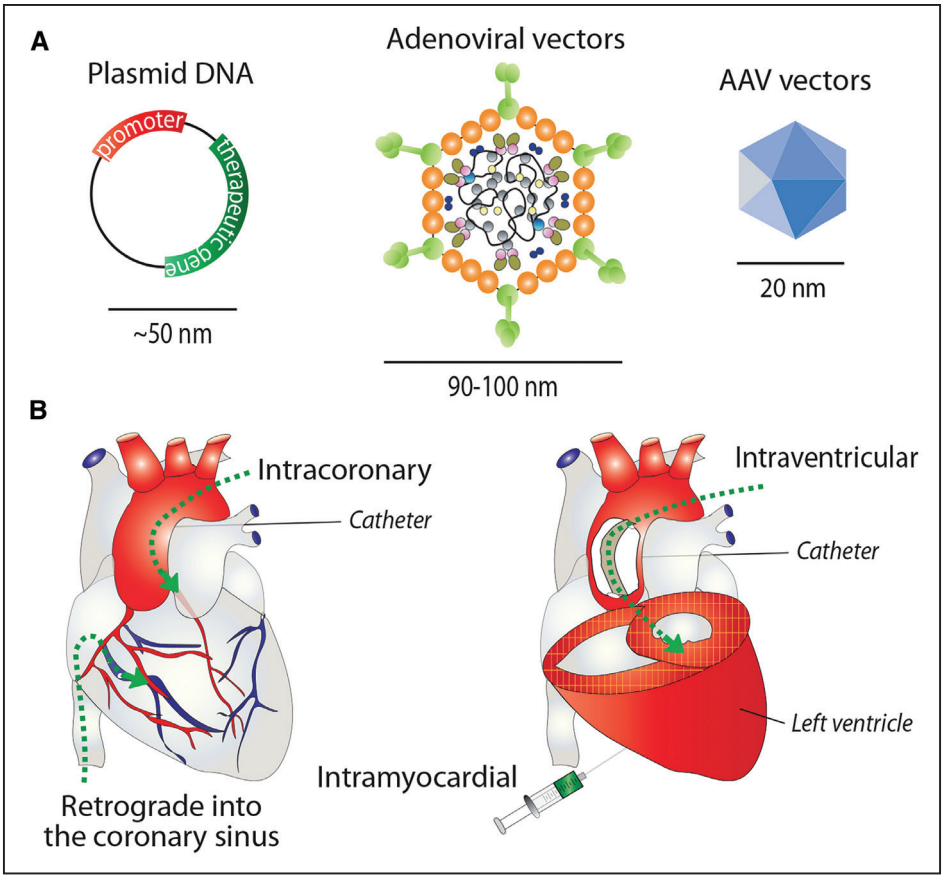

Figure 1. Delivery vehicles and routes for cardiac gene therapy.
A, Schematic representation of the main delivery strategies for cardiac gene therapy (injection of naked plasmid DNA or gene transduction using adenoviral or adenoassociated virus [AAV]-based vectors). The approximate size of the delivery vehicle is indicated. **B**, Main delivery routes to reach the heart. These include injection into the coronary artery as during standard percutaneous coronary intervention or retrograde into the coronary sinus, on the left side panel; or intramyocardial, on the right side panel, through either direct injection after minithoracotomy or during bypass surgery, or after percutaneous catheterization to reach the left ventricle, followed by transendocardial delivery.

In this regard, however, it needs to be pointed out that expanding the repertoire of available vectors by capsid engineering does not necessarily translate into improved efficacy, since this is the ultimate outcome of a number of molecular steps (in particular, de-capsidation, nuclear transport, single-stranded to double-stranded DNA conversion and genome concatemerization) that extend beyond virion internalization and Cap function. Thus, capsid engineering remains just an aspect of improved tissue-specific tropism.

A second area amenable to AAV vector improvement pertains to the design of promoters with improved characteristics. The rational design or selection of synthetic regulatory sequences that provide cardiomyocyte or endothelial cell specificity and detarget the liver has been the subject of investigation over the last few years (reviewed in study by Domenger and Grimm[11]). The search of such tissue-specific promoters can follow a similar approach to that performed in skeletal muscle, which ended in the generation of AAV vectors carrying a synthetic promoter with a 400-fold enhanced, muscle-specific gene expression, which could efficiently correct the dystrophic phenotype in mice.[12]

In addition to gene transfer efficiency, a second most important variable affecting overall efficacy of cardiac gene therapy is the route of administration. A most common and effective technique for cardiac gene transfer is the direct intramyocardial injection. This can be either through a surgical access, that is, mini-thoracotomy followed by transepicardial delivery, or by intraventricular injection using a percutaneous catheter, to achieve transendocardial delivery (Figure 1B). The transepicardial approach is straightforward in terms of myocardial access; however, it is fraught with the problems related to cardiac surgery. Conversely, the percutaneous approach, which is a relatively common procedure performed in several routine cardiac catheterizations, is less invasive and is feasible in stable patient. Several catheters have been tested and approved for human application by either FDA or EMA, or both.[13] While these catheters were indicated for cell therapy, they might also be used to inject gene therapy products. The most commonly used devices are the Helix (BioCardia, Inc, South San Francisco, CA), MyoCath (Bioheart, Inc, Sunrise, FL), Myostar (Biologics Delivery Systems, Diamond Bar, CA),

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and Stiletto (Boston Scientific, Natick, MA).[14–17] The only transepicardial, percutaneous, FDA-approved device, the TransAccess Delivery System (Medtronic Vascular, Santa Rosa, CA), can be advanced into the cardiac venous system through a femoral vein approach and, using a trans-vascular needle guided by intravascular ultrasound, effectively advanced to deliver the desired treatment from the epicardial layer.[13] The site of injection can be identified preoperatively by different imaging techniques and confirmed during the procedure by either electromechanical mapping using the NOGA system (Biosense Webster, Diamond Bar, CA), intravascular ultrasound or 2-dimensional and 3-dimensional cardiac imaging.[18–20]

Over 30 cell therapy clinical studies have taken advantage of intracoronary administration,[21] as this procedure is routinely performed for left heart catheterization and invasive percutaneous coronary intervention. The 2 main techniques employed for treatment delivery are either infusion after complete balloon occlusion of the coronary flow or infusion during a sub or nonocclusive procedure. Despite the complication rate is relatively low, the main disadvantage of this approach is that the infused treatment has to compete with the coronary flow. Improvements in this procedure might be achieved using agents increasing permeability or by retrograde, instead of anterograde, vector administration.[22]

An alternative approach to anterograde coronary infusion is retrograde infusion through the vein system. This approach, which is commonly used in cardiac surgery to deliver effective cardioplegia, can increase transduction efficacy, especially in a transepicardial fashion. However, it requires balloon occlusion of both the coronary artery and the respective vein, and its efficacy is proportional to the occlusion time.[23,24] Vein system anatomy, presence of cardiac resynchronization devices, and a relatively high risk of coronary sinus or vein rupture might hinder the widespread applicability of this technique.

GENE THERAPY CLINICAL STUDIES TO INDUCE CARDIAC THERAPEUTIC ANGIOGENESIS

The birth of gene therapy for cardiovascular disorders in the mid 1990s coincided with a series of attempts at inducing therapeutic angiogenesis in patients with coronary or peripheral artery disease. These applications were fueled by the notion that new blood vessel formation is a cytokine-driven process and that the overexpression of some of the angiogenic cytokines was sufficient to solve acute conditions of ischemia in animal models. Yet, after over 150 clinical trials, there is not a single successful application to achieve therapeutic angiogenesis in humans.

New blood vessel formation in adult life occurs through capillary sprouting from preexisting vessels, a process that is triggered and maintained by secreted factors first acting on endothelial cells and later inducing maturation of the newly formed capillaries.[25] Over 20 clinical applications in the late 1990s and early 2000s have entailed the intramyocardial injection of plasmids encoding VEGF (vascular endothelial growth factor)-A^{165}, one of the master regulator of the angiogenesis process (reviewed in study by Giacca and Zacchigna[4]). Some positive results from these applications are largely anecdotal, as these had an open-labeled designed and the placebo effect is notoriously strong in angiogenic therapies. When randomized, double-blinded, placebo-controlled trials with naked plasmid DNA injection were performed, these failed to show a major positive impact on either clinical outcome or symptoms (the EUROINJECT1,[26] NORTHERN [NOGA Angiogenesis Revascularization Therapy: Assessment by RadioNuclide Imaging],[27] and KAT [Kuopio Angiogenesis Trial][28] trials; Figure 2). Unsatisfactory results were also reported for a bi-cistronic plasmid encoding both FGF2 and VEGF-A$_{165}$ (the VIF-CAD study[29]) and in one small study aimed at increasing VEGF-A^{165} plasmid efficacy by the administration of G-CSF (granulocyte colony stimulating factor).[30] Additional clinical studies with intramyocardial plasmid delivery are ongoing for hepatocyte growth factor, based on a previous, small clinical Phase I experimentation.[31]

Compared with plasmid DNA, much more effective cDNA delivery vehicles for cardiac gene therapy are vectors based on adenovirus. One of the first adenoviral vectors to be used clinically was a vector expressing the VEGF-A$_{121}$ cDNA under the control of the strong CMV enhancer/promoter, named Ad$_{GV}$VEGF121.10NH (commercial name: BIOBYPASS), which was used in a series of studies in patients with coronary artery disease. The vector was delivered into the myocardium during coronary bypass artery grafting in an open-labeled, noncontrolled trial[32]; via mini-thoracotomy in a Phase II, randomized, nonplacebo, controlled trial in no-option patients, performed at multiple clinical sites in North America (the REVASC trial [Randomized Evaluation of VEGF for Angiogenesis][33]); and by transendocardial delivery using the NOGA system in 2 studies performed in Europe (the NOVA trial[34]); Figure 2. While REVASC reported durable improvements in cardiac function despite a lack of objective proof of increased perfusion,[33] the NOVA trial was prematurely terminated due to a sponsoring company's portfolio decision, after no difference was detected in the treated patients.[34] A similar fate (premature termination) also occurred for the AGENT (Angiogenic Gene Therapy) clinical trial program, which was aimed at exploring the potential benefit of the intracoronary delivery of an adenovirus 5 vectors expressing FGF4, named Ad5FGF-4.[35] AGENT and AGENT-2 were randomized, double-blinded, placebo-controlled trials in patients with chronic stable angina. AGENT-3 and -4 trials were planned to determine the efficacy and safety

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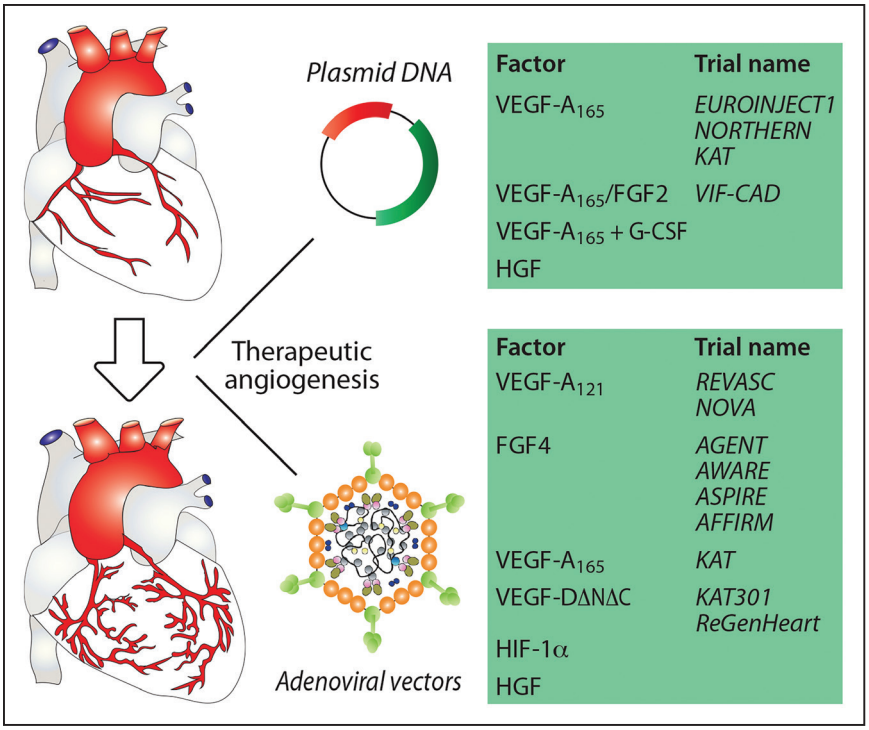

Figure 2. Clinical trials for therapeutic angiogenesis.
The figure summarizes the main clinical trials for therapeutic angiogenesis, grouped according to the delivery method used (naked plasmid DNA, top or adenoviral vectors, bottom), along with the indication of the therapeutic gene and the trial name. VEGF-A indicates vascular endothelial growth factor-A.

of Ad5FGF-4 in selected doses in larger populations of patients. After >500 patients were overall recruited, an interim review of the data from AGENT-3, while indicating no safety concerns, showed that differences were unlikely to reach significance, and further enrolment in the trials was stopped.

A subsequent pooled analysis from AGENT-3 and -4 indicated that treatment with Ad5FGF-4 was associated with improvements of surrogate end points of myocardial perfusion, specifically in postmenopausal women.[36] Based on these findings, an additional clinical study with the same vector was planned in women with refractory angina who are not candidates for revascularization (the AWARE trial [Angiogenesis in Women With Angina Pectoris Who Are Not Candidates for Revascularization]; NCT00438867). The recruitment status of this trial is currently not reported. Finally, a follow-up of the AGENT studies was an application in 6 centers in Russia, which assessed myocardial perfusion using SPECT in 11 patients in whom the same Ad5FGF4 vector, under the tradename of Generx, was delivered intracoronary (the ASPIRE trial [Efficacy and Safety of Ad5FGF-4 for Myocardial Ischemia in Patients With Stable Angina Due to Coronary Artery Disease]; NCT01550614[37]). This study run between 2012 and 2016; no results have been reported yet. A further Phase III clinical study in 320 patients with refractory angina, which was filed in 2016, is still planned to start recruitment in 2020 (the AFFIRM trial; NCT02928094).

Additional experimentations have continued to take advantage of adenoviral vectors. One was the already mentioned KAT trial, which also contained an arm with patients receiving an adenovirus expressing VEGF-A_{165}.[28] This study reported improvement in perfusion; however, no significant difference in mortality or incidence of major adverse events in the long term.[38,39] A first-generation adenoviral vector was also at the basis of a recent Phase I/IIa randomized, controlled, angiogenesis trial aimed at delivering a proteolytically processed, mature form of VEGF-D (VEGFDΔNΔC), which possesses angiogenic activity.[40] The vector was delivered to the ischemic myocardium by NOGA-mediated transendocardial injections; myocardial perfusion was reported increased at 1 year from treatment (the KAT301 trial[41]). This is currently followed by the ReGenHeart trial, a randomized, double-blinded, placebo-controlled, multicenter Phase II study with adenoviral delivery of VEGF-D using a NOGA catheter system, which will be conducted at 6 centers in the European Union (NCT03039751; https://www.regenheart.eu/study-design). Finally, a small, placebo-controlled, Phase I study reported the safety of the intramyocardial administration of an adenoviral vector expressing HIF-1α (hypoxia-inducible factor).[42]

Ongoing clinical studies based on adenoviral vectors also include a Phase I/II trial based on the expression of 3 different VEGF-A isoforms[43] via mini-thoracotomy, to be started in 2020 (NCT01757223) and a series of Phase II trials expressing hepatocyte growth factor, performed in China (reviewed in study by Wang et al[31]). After a small, open-label clinical study,[44] a more recent report on 15 patients who received percutaneous endocardial adenovirus-hepatocyte growth factor gene transfer revealed a modest but significant improvement of

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cardiac function compared with a matched control group at a 6-month follow-up.[45]

When these experimentations are analyzed collectively, it seems clear that gene therapy for therapeutic angiogenesis has not fulfilled the expectations it raised over 20 years ago. Lack of clinical success well highlights the intrinsic difficulties in translating animal results into patients and can be attributed to a number of different reasons, which are critically discussed later.

GENE THERAPY CLINICAL STUDIES FOR HEART FAILURE

Development of novel therapies for heart failure (HF) are sorely needed, as this condition has reached epidemic proportions, now affecting 2% of the adult population worldwide and over 10% of those older than 75 years (see study by Metra and Teerlink[46] and citations therein).

One main target for intervention in HF is the modulation of Ca^{2+} handling in cardiomyocytes, since this is pivotal in ensuring normal cardiac function (Figure 3). A key factor for Ca^{2+} reuptake by the sarcoplasmic reticulum is the Ca^{2+} ATPse SERCA2a, which is inhibited by de-phosphorylated PLN (phospholamban). The main kinase phosphorylating PLN in cardiomyocytes is the cAMP (cyclic adenosine monophosphate)-dependent PKA (protein-kinase A), which is under the control of β-adrenergic stimulation. Blockade of I-1c (inhibitor-1c) binding to PP1 (protein phosphatase 1) leads to increased PLN phosphorylation and thus increased SERCA2a activity. Over the last 2 decades, a number of preclinical studies in animal models have explored the beneficial effect of modulating these mechanisms by gene transfer to improve cardiac function in HF.

Three of the proposed gene therapy strategies have reached clinical experimentation (Figure 5); a summary of the main HF gene therapy clinical studies is shown in the Table.

The first, and more extensive trial, was based on the transfer of the SERCA2a cDNA, based on the observation that this sarcoplasmic Ca^{2+} ATPase is downregulated in failing hearts and that restoration of its levels improves heart function in both mice and swine.[47] A Phase I/II trial followed these experimental findings, showing safety of intracoronary infusion of an AAV1 vector expressing the human SERCA2a cDNA.[48] Based on the results of this study, the CUPID trial (Calcium Upregulation by Percutaneous Administration of Gene Therapy in Patients With Cardiac Disease; NCT00454818) was the first clinical attempt to use AAV gene therapy to treat HF. CUPID was a Phase II, blinded, randomized, placebo-controlled, multicenter study enrolling 39 patients with symptomatic HF, severely reduced ejection fraction, and implantable cardioverter defibrillator for primary prevention of sudden cardiac death. Patients were randomized into 4 arms to receive intracoronary infusion of either placebo or different doses of AAV1.SERCA2a.[49] The result of this study, which showed reduction in HF hospitalization and significant improvement in both symptoms and functional parameters, paved the way to a larger, Phase IIb, randomized, double-blinded, multicenter trial, CUPID2 study (NCT01643330). This trial enrolled 250 patients with stable symptomatic HF, of both ischemic and nonischemic etiology, with severely depressed ejection fraction, randomized in a 1:1 fashion to receive intracoronary infusion of either placebo or 1×10^{13} AAV1.SERCA2a viral particles.[50] Unfortunately, this treatment failed to improve clinical outcomes. This discouraging finding also halted 2 other related clinical studies using the same vector, the AGENT-HF study, evaluating efficacy of AAV1.SERCA2a in patients with left ventricular assist devices (NCT01966887[51]) and the SERCA-LVAD study (Sarcoplasmic/Endoplasmic Reticulum Ca2+-ATPase/Left Ventricular Assist Device), assessing the potential of the same vector to modify LV remodeling in subjects with HF (NCT00534703). Both studies were prematurely terminated in 2016.

A second proposed approach for HF gene therapy is aimed at modulating Ca^{2+} reuptake by overexpressing of a constitutively active form of I-1c, which binds PP1. The ultimate effect of this intervention is to increase the levels of the SERCA2a and restore proper β-adrenergic stimulation.[52] After experimental success of I-1c gene transfer in rodents, large animal work was performed by the intracoronary administration of an AAV9 vector[53] or an AAV vector with a chimeric AAV2/AAV8 capsid, which was reported to traverse the blood vasculature and selectively transduce cardiac and skeletal muscle while de-targeting the liver and the lungs (BNP116[54,55]). Following this experimentation, a small, Phase I clinical study is scheduled in 2020, entailing intracoronary infusion of BNP116.sc-CMV.I1c (under the name of NAN-101) in 12 patients with NYHA Class III HF (NCT04179643).

A third approach to improve cardiac function in HF by gene transfer is to restore the normal Ca^{2+} homeostasis by blocking the maladaptive response due to altered β-adrenergic receptor stimulation. Signal transduction from these receptors is mediated by a heterotrimeric G protein that activates an AC (adenylate cyclase) located on the cytosolic side of the receptor complex, which catalyzes conversion of ATP to cAMP, which in turn activates PKA. Preclinical experimentation has shown that overexpression of isoform 6 of adenylate cyclase (AC6) has a beneficial effect on failing hearts.[56,57] These positive results prompted a randomized, double-blinded, placebo-controlled, Phase II clinical study that assessed the safety of 5 doses of an adenoviral vector expressing AC6 (Ad5.hAC6, investigation name RT-100) administered intracoronary.[58] The results of this trial showed that this treatment partially ameliorated systolic function, mostly in patients with nonischemic HF. A larger, Phase

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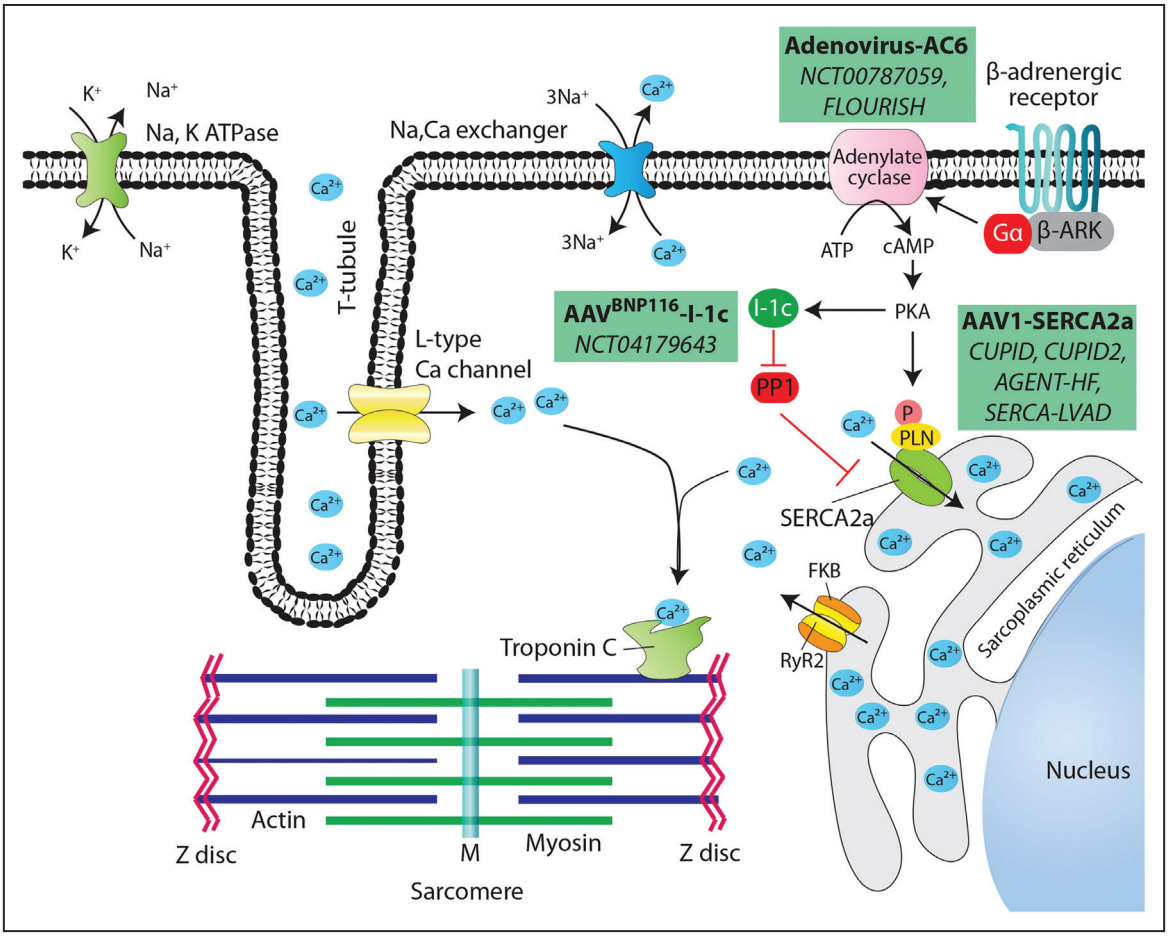

Figure 3. Clinical trials for heart failure.
The figure shows 3 current gene therapy approaches targeted to cardiac excitation-contraction coupling through the cardiomyocyte Ca^{2+} cycle. The depolarization of the cardiomyocyte plasma membrane induces the opening of membrane L-type voltage-dependent Ca^{2+} channels, with permit entry of a small quantity of Ca^{2+} into the cytosol; this in turn determines release of Ca^{2+} from the sarcoplasmic reticulum stores through the RyR2 ryanodine receptor. Massive entry of Ca^{2+} into the cytosol triggers biochemical coupling between actin and myosin, which is mediated by Ca^{2+} binding to troponin C, and subsequent contraction. In the relaxation phase, RyR2 is inhibited by the FKBP12.6protein. The released Ca^{2+} is in part re-convoyed into the sarcoplasmic reticulum by the ATPase SERCA2a, and in part eliminated outside the cell by the Na^+/ Ca^{2+} exchanger (NCX). The activity of the SERCA2a pump is controlled by association of this protein with PLN (phospholamban). In its nonphosphorylated form, PLB inhibits SERCA2a, while phosphorylation blocks this inhibition. The main kinase phosphorylating PLB in cardiomyocytes (followed by pump activation) is the cAMP (cyclic adenosine monophosphate)-dependent PKA (protein-kinase A), which is under the control of β-adrenergic stimulation. In particular, engagements of β-adrenergic receptors with their ligands activates an associated, heterotrimeric G protein, which in turn leads to activation of an AC (adenylate cyclase) located on the cytosolic side of the receptor complex, which catalyzes conversion of ATP to cAMP. This in turn activates PKA, which phosphorylates (1) the L-type Ca^{2+} channels, thus determining further Ca^{2+} entry each depolarization cycle; (2) RyR2, causing dissociation of the inhibitory protein FKB12.6; and (3) PLB, blocking its inhibitory activity on SERCA2a. These modifications amplify the efficacy of Ca Ca^{2+} release and re-uptake every cardiac cycle. Conversely, dephosphorylation of PLN leads to SERCA2a inactivation. This is mainly carried out by PP1 (protein phosphatase-1), which is inhibited by I-1c (inhibitor-1c). The cartoon shows the 3 main clinical approaches to modulate these pathways in heart failure by transfering the cDNAs coding for SERCA2a, AC6, or I-1c (rectangular green boxes), with the indication of the vector used and the name of the clinical studies (in italic).

III clinical trial was planned to begin enrollment in 2018; however, it was later withdrawn due to clinical recruitment plans and strategy being re-evaluated (FLOURISH; NCT03360448). Recent experimental work indicates that adenylyl cyclase type 6 (AC6) activity can be mimicked by a fusion protein of the intracellular C1 and C2 segments of the protein, which can fit into the backbone of AAV vectors,[59,60] possibly improving efficacy and safety of gene delivery.

An alternative manner to sustain a failing heart is through the delivery of genes coding for extracellular factors that exert a beneficial function. Over the years, a number of cytokines and growth factors has proved effective in large animal HF models, most notably VEGF-B,[61,62] S100A1,[63] and SDF (stromal cell–derived factor)-1α.[64] Treatment with SDF1α reached Phase I clinical experimentation in patients with ischemic cardiomyopathy using a naked DNA plasmid, named JVS-100.[65] However, a randomized, double-blind, placebo-controlled study Phase II study (STOP-HF; NCT01643590) failed to meet its primary composite end point, despite reporting improvements in selected

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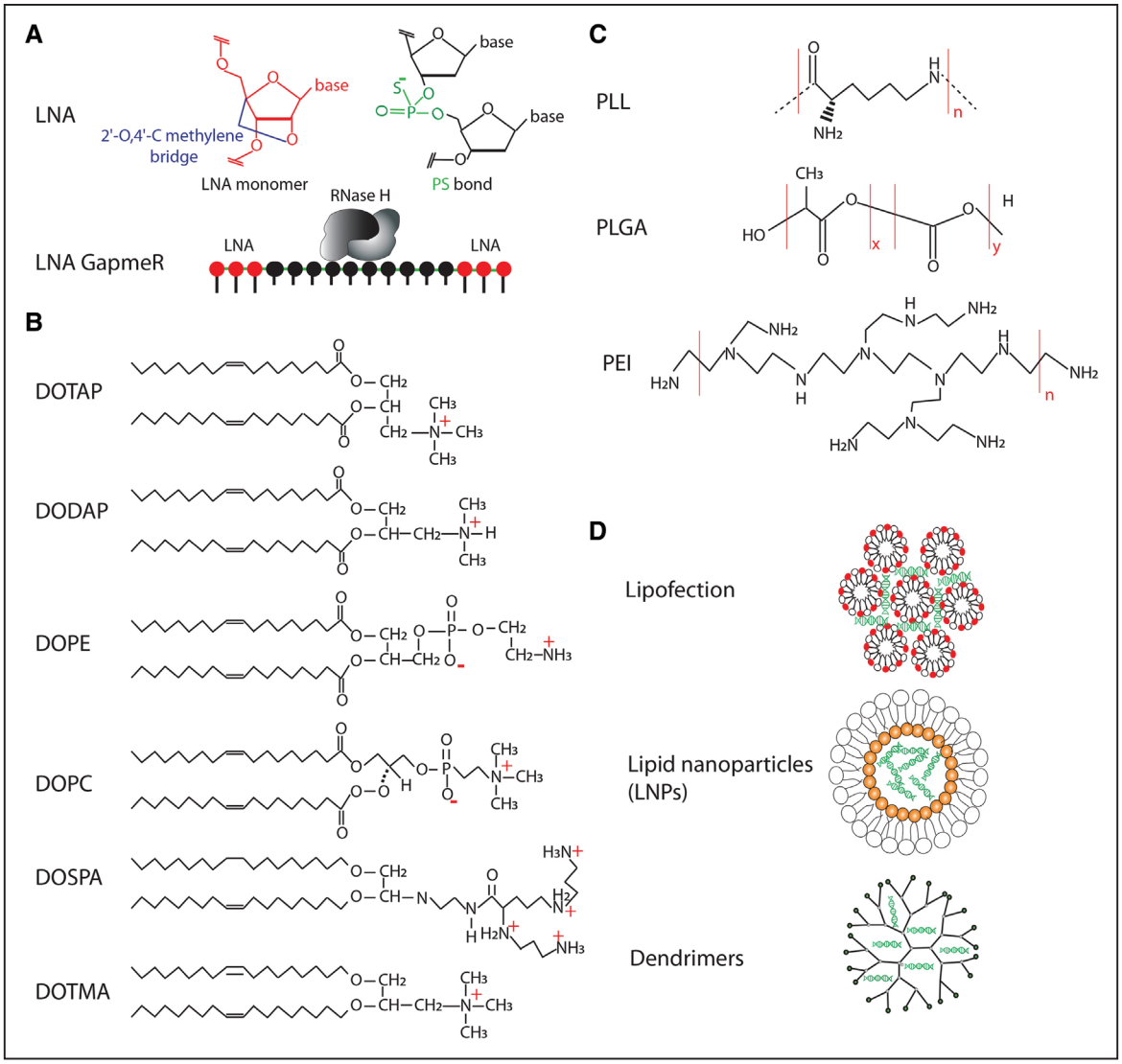

Figure 4. Molecules and methods for the delivery of RNA therapeutics.
A, Chemical structure of a locked-nucleic acid (LNA) nucleotide (upper left) and of a phosphorothioate bond (PS, upper right). The lower part of the panel shows the structure of an LNA gapmer, in which LNA-modified nucleotides are positioned at the 2 extremities of the oligonucleotide to allow RNase H accessibiltity to the central part once the duplex with the target RNA is formed. **B**, Chemical structure of the main cationic and neutral lipids used for lipofection and lipid nanoparticle formation. **C**, Main polymers used for polyplexes formation (poly(ethylenimine) [PEI], poly-L-lysine [PLL], poly(lactic-co-glycolic acid) [PLGA]). **D**, Schematic representation of DNA-nanoparticle structures.

groups of patients.[66] A parallel clinical study of the same SDF1α plasmid in patients with crucial limb ischemia (STOP-PAD; NCT02544204) also failed.[67] A third Phase I/II trial with the same plasmid administered by retrograde delivery in patients with heart failure (RETRO-HF; NCT01961726) was initiated in 2014 but has not reported yet.

CLINICAL TRIALS FOR GENE THERAPY OF CARDIAC DISORDERS—THE LESSONS WE LEARNED

While the overall clinical outcome of the cardiac gene therapy applications seems unsatisfactory, still a large amount of information was learned, which can guide future development in this area.

Efficiency of Gene Delivery

A foremost problem of gene therapy remains the efficiency of in vivo nucleic acid delivery. As anticipated, plasmid transfection is simple and not fraught with major safety concerns; however, the efficiency of naked DNA uptake by cardiomyocytes remains poor. In addition, measurable levels of gene expression are only maintained for the first couple of weeks after naked plasmid injection, a condition that might not be sufficient to exert a therapeutic effect. This seems particularly relevant for therapeutic angiogenesis, since experimentation in both genetic models[68] and using AAV

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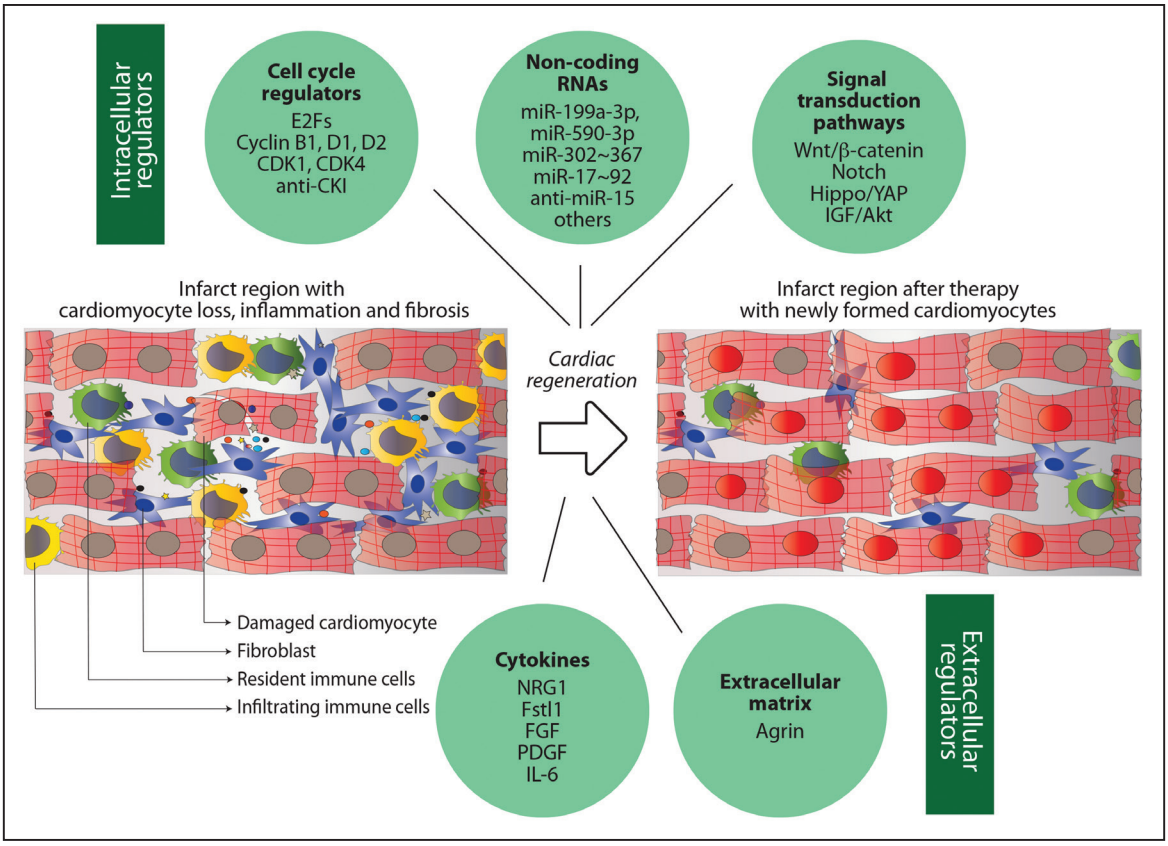

Figure 5. Molecules, genes, and RNAs shown to stimulate cardiomyocyte proliferation and cardiac regeneration.
The upper part of the figure lists a series of cell cycle proteins, microRNAs, and the main signal transduction pathways acting inside the cardiomyocytes to control their proliferation. The lower part of the figure shows cytokines and other molecules acting from outside these cells. See text for further explanation. PDGF indicates platelet-derived growth factor.

vectors[69] has shown that prolonged (several weeks) cytokine stimulation is required to form a stable vasculature.

A most efficient manner for gene transfer is by using adenoviral vectors. These vectors, however, elicit robust immune and inflammatory responses, which raise safety concerns. This problem became dramatically apparent in 1999, when a young patient recruited in a gene therapy trial for the treatment of a rare metabolic disorder of the liver died because of a systemic inflammatory response to the vector.[70] Despite no specific safety issues related to vector delivery were reported in the adenovirus-based trials for cardiac gene therapy, the decision to stop some of the applications (among which the AGENT trials) were temporally coincident with the progressive appreciation of these problems, which de facto limit the dose of vector that can be injected.

In contrast to adenovirus, AAV is poorly immunogenic (or its immunogenicity can be easily controlled by pharmacological treatment at the time of vector administration[71]). However, the use of these vectors is only indicated when permanent expression of the transgene is desirable. This is not the case, for example, of therapeutic angiogenesis, when continuous angiogenic factor expression might be deleterious over time.[69,72,73] Unfortunately, no efficient system for the transcriptional or posttranscriptional control of gene expression in vivo is currently available (discussed in study by Zacchigna et al[6]).

Given the still relative inefficiency of cardiac gene transfer, applications based on the production of secreted proteins are more likely to succeed than those requiring extensive cardiomyocyte transduction, such was the delivery of the SERCA2a cDNA in the CUPID trials. When the amount of AAV1.SERCA2a DNA was assessed in cardiac samples from treated patients who deceased or underwent transplantation or mechanical support device implantation, this turned out to be 43 copies per microgram total DNA[50] (as a reference, DNA from 1×10^6 mononucleated diploid cells weights ≈6 µg). This was definitely too low considering that SERCA2a acts intracellularly. While vector intracoronary injection, such as in the CUPID trials, is a minimally invasive procedure, extravasation from the intact endothelium (paracellular permeability) is inefficient for particles having a size of ≈20 nm diameter, such as AAV virions.

An additional, delivery-related factor that might have hampered efficacy of AAV1-SERCA2a in the CUPID2 trial was the reported lower amount of empty viral vectors compared with the original CUPID study (25% versus 90%, respectively). While empty capsids are commonly considered detrimental for transduction, they could still act as decoys for serum neutralizing antibodies.[74]

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Table. Main Clinical Studies of Gene Therapy for Heart Failure

Phase	Gene	Trial Name	Study Design	No. of Patients	Delivery Method	Mean LVEF	NYHA III/IV	Ischemic	Follow-Up, mo	Primary End-Point	Main Findings
I	SDF-1	n/a	Open-label, dose-escalation study	17	Intramyocardial injection	33%	100%	100%	12	Number of major adverse cardiac events	Safe
II	SDF-1	STOP-HF	Double-blind, randomized, placebo-controlled	93	Intramyocardial injection	28%	68%	100%	12	Functional: 6MWD and MLWHFQ at 4 mo post-dosing	Neutral
I / II	SDF-1	RETRO-HF	Randomized, open label	72	Coronary sinus infusion	n/a	n/a	100%	4	Functional: 6 MWD	Results not reported
II	AC6	AC6	Double-blind, randomize, placebo-controlled	56	Intracoronary injection	31%	55%	48%	12	LVEF changes and functional changes	Slight increase in LVEF, no changes in symptoms
I / II	SERCA2A	CUPID	Open label	39	Intracoronary infusion	25%	100%	100%	12	Composite: symptoms, exercise, BNP, echo, clinical outcomes	Functional improvement
IIb	SERCA2A	CUPID 2	Double-blinded, randomized, placebo-controlled	243	Intracoronary infusion	23%	82%	52%	12	Time to recurrent event (hospitalization/worsening heart failure/cardiovascular events)	Neutral
I	I-1-c	NAN-101	Prospective, multicenter, open-label	12	Intracoronary infusion	n/a	100%	n/a	12	Number of major adverse cardiac events	Recruiting

6MWD indicates 6-min walk distance; BNP, B-type natriuretic peptide; LVEF, left ventricular ejection fraction; and MLWHFQ, Minnesota Living with Heart Failure Questionnaire.

Inappropriate Animal Models

A second, important lesson learned from the outcome of several of the gene therapy clinical trials is the likely inappropriateness of most of the available preclinical models. All the therapeutic angiogenesis clinical studies were based on very convincing preclinical results in small and large animals, to then fail in humans. However, there is no adequate preclinical model to mimic a chronic condition such as human HF. This usually develops on the basis of either extensive coronary disease or heart muscle dysfunction and develops over year-long periods, often in the context of multiple comorbidities, most notably hypertension and diabetes mellitus. Despite a few, notable exceptions,[75,76] most of the large animal experimentations have been performed in young and healthy animals and the induced disease has been commonly of short duration. Similar considerations also apply to the available models of HF in large animals. Thus, caution must be exercised in translating large animal findings to patients, especially when the detected treatment effects are statistically significant but biologically miniscule, as, for example, is the case for several plasmid treatments.

Choice of Therapeutic Gene

A third set of considerations relates to the efficacy of the therapeutic gene. In some instances, the choice of the gene itself is problematic. For example, one of the proposed mechanisms of action of SDF1α for HF gene therapy was the promotion of cardiac homing of both bone marrow–derived and cardiac stem cells to the sites of ischemic injury.[77] However, the recognition that no cells in either the bone marrow or the heart can act as true cardiac stem cells[78] questions the root of this assumption.

In the therapeutic angiogenesis studies, the lack of clinical success might be consequent to the complexity of the vascularization process, which requires a precisely dosed and temporally scheduled combination of different factors acting on both endothelial cells and vessel mural cells, as well as requires the generation of both a capillary network and larger conductance vessels. No less than 30 different factors are known to take part in physiological vessel formation; thus, it is possible that the expression of any one of these factors alone might not be sufficient to trigger or complete the process. For example, VEGF-A is a powerful inducer of endothelial cell proliferation and sprouting; however, the vasculature that is formed in response to this factor alone is leaky and not functional.[72] Analogous considerations also apply to the combination of VEGF and hepatocyte growth factor (see study by Gerritsen[79] and citations therein). The delivery of gene cocktails, obviously, increases the technical difficulties of clinical translation.

In terms of gene combinations, at the end of 2019, the completion of enrollment in a Phase I clinical trial was announced for a triple gene therapy (INXN-4001, NCT03409627) entailing the retrograde coronary sinus infusion of a plasmid expressing S100A1, SDF1α and VEGF-A[165] in patients with an implanted left ventricular assist device, either as a bridge to transplant or destination

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therapy. Major unknowns of such an approach, however, remain the poor efficiency of plasmid gene transfer and the efficacy of SDF1α.

Finally, upstream transcriptional regulators of the response to ischemia might be better positioned to drive a functional angiogenic response. Of these, however, HIF-1α has failed to improve clinical end points in a peripheral artery disease gene therapy study,[80] while MRTF-A (myocardin-related transcription factor-A), which was shown to provide benefit in pigs with chronic cardiac ischemia,[81] still awaits confirmation from studies in large animals with co-morbidities.

Study Design

A component to the failure of some of the gene therapy clinical trials might also relate to the type of study design. For example, the failure of the CUPID2 trial compared with CUPID might be partially attributed to the fact that the inclusion criteria in CUPID comprised several symptomatic patients in NYHA functional class III or IV, while CUPID2 enrolled patients who had lower NYHA classes (≈20% of patients were in NYHA class II[49,50]). Even more relevant, it is becoming progressively apparent that the definition of HF itself comprises a variegate set of pathological conditions, of both ischemic and nonischemic etiology. This variability might represent an important confounding factor.

Are We Missing Something?

Besides the above learned lessons, it seems possible that we are still missing some specific aspect of cardiac biology, in particular, related to the unexpected difficulties at inducing therapeutic angiogenesis. Different but still scattered evidence indicates that the adult heart is a poorly angiogenic environment. It is one of the few organs where the growth of tumors, which require extensive angiogenesis, is suppressed. Additionally, collateral vessels formation in the heart after myocardial infarction (MI) occurs by direct arteriogenesis and not through angiogenesis.[82] Finally, AAV vectors expressing VEGF are very efficient at driving an angiogenic response in the skeletal muscle, however, fail to do so in the heart.[83] Thus, it might be conceived that cardiac endothelial cells are impaired at mounting an efficient angiogenic response, which might explain the lack of efficacy of angiogenic growth factor delivery. A better understanding of the cardiac endothelium biology is clearly needed.

Conceptually similar considerations also apply to HF. All the available gene therapy approaches are aimed at improving individual cardiomyocyte function, however, fail to address the problem that the total number of cardiomyocytes survived in a damaged heart might not be sufficient to sustain global organ function. Numerous lines of evidence indeed indicate that cardiomyocyte loss is a major cause of HF. This is clear after MI, when often 1 billion cardiomyocytes die in a few hours, representing as much as 25% of the total number of these cells in the left ventricle[84] but also occurs in most cardiac diseases (reviewed in study by Braga et al[85]). None of the available therapies address the problem of limiting cardiomyocyte loss or increasing cardiomyocyte number. How gene therapy can stimulate cardiac regeneration is specifically discussed later.

GENETIC THERAPIES IN THE PIPELINE

In this chapter, we focus onto 3 therapeutic areas—noncoding RNA therapeutics, cardiac regeneration, and gene editing—in which gene therapy has progressed at a rapid pace over the last few years and in which it has already entered or is rapidly approaching clinical experimentation. Other very interesting applications, such as gene therapy for arrhythmogenic disorders—for example, allele-specific gene silencing in dominant arrhythmias, delivery of dominant negative proteins for atrial fibrillation, or the generation of a biological pacemaker for sinus node dysfunction—are not considered here, as their clinical translation, with a few exceptions such as gene therapy of catecholaminergic polymorphic ventricular tachycardia,[86] remains more challenging. Excellent reviews on these experimental gene therapy applications have been published recently.[87–89]

Noncoding RNA Therapeutics for Heart Disease

The discovery that virtually all aspects of cardiac cell function (including cardiomyocytes, fibroblasts, endothelial cells, and resident macrophages) are controlled by a vast series of noncoding RNAs paves the way to developing innovative therapeutic approaches. Noncoding RNAs of interest for gene therapy essentially fall into one of 2 classes, microRNAs (miRNAs) and long noncoding RNAs (lncRNAs).

The latest release of the miRBase database (miRBase 22.1; http://www.mirbase.org/index.shtml) reports information for 1917 annotated precursor and 2654 mature miRNAs encoded from the human genome. These are 21 to 23 nt-long, double-stranded RNAs that regulate abundance of target mRNAs by base pairing to partially complementary sequences. Since target recognition is promiscuous, each microRNA (miRNA) can simultaneously control the production of several tens or hundreds of proteins to fine-tune cell functions. Extensive reviews are available covering the function of the miRNA network in the heart in both normal and pathological conditions.[90–92] LncRNAs are instead defined as a class of functional, nontranslated RNA molecules of >200-nt. There is a broad range of estimates for the number of lncRNAs in humans, ranging from <20 000 to >100 000.[93] Again, most aspects of cardiac function and

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dysfunction are governed by one or multiple lncRNAs (reviewed in study by Hobuß et al,[94] Salamon et al,[95] and Lucas et al[96]).

Inhibition of endogenous miRNA and lncRNA levels can be achieved by administering short, antisense oligonucleotides or deoxy-oligonucleotides (ASOs) complementary to their sequences. To avoid rapid degradation and facilitate cellular uptake, a number of chemical modifications can be introduced into the nucleic acid backbone or bases (reviewed in study by Lennox and Behlke[97] and van Rooij and Olson[98]). One of the most effective of these modifications now seems to be the inclusion of locked nucleic acid (LNA) nucleotides, in which the sugar contains an extra bond connecting the 2' and 4' carbons (2'-*O*,4'-*C* methylene bridge)[99]; Figure 4A. These LNA-containing oligonucleotides can be designed with a steric block approach, by which a central LNA segment is flanked by non-LNA gaps (LNA GapmeRs[100]). This structure improves stability while still permits recruitment of RNAse H to the duplex for target RNA degradation.

A number of laboratories have already taken advantage of microRNA inhibition using ASOs with different chemistries in various experimental models. These include, for example, inhibition of miR-133,[101] miR-21,[102] or miR-32[103] to prevent cardiac hypertrophy or pathological remodeling, of miR-34 to assist recovery after MI,[104] of miR-29 to contrast cardiac fibrosis,[105] of miR-25 to improve contractility in failing hearts.[106] Systemic administration of anti-miR-92a LNA ASOs enhances blood vessel growth and functional recovery of damaged tissue in mice[107] whereas regional administration is effective in pigs[108]; inhibition of miR-199a-5p reverses hypertrophy and fibrosis in HF in mice[109]; antagonizing endothelial miR-24 limits MI size by preventing endothelial apoptosis[110]; inhibition of miR-146a is beneficial in a peripartum cardiomyopathy mouse model.[111] Several additional anti-miR antisense oligonucleotide applications were recently reviewed in study by De Majo and De Windt.[112]

Antisense inhibition using ASOs also apply to lncRNAs. For example, GapmeR antisense oligonucleotides against the cardiac fibroblast-enriched lncRNA Meg3 prevents cardiac fibrosis and diastolic dysfunction[113]; against Chast both prevents and attenuates TAC-induced pathological cardiac remodeling[114]; against Wisper counteracts MI-induced fibrosis and cardiac dysfunction.[115] Other applications of the anti-lncRNA antisense technology are reviewed in Hobuß et al,[94] Salamon et al,[95] and Lucas et al[96].

On a final note, the clinical development of ASOs for cardiovascular applications can take advantage of over 30-year antisense oligonucleotide development, for which over 100 Phase I clinical trials have been performed, of which 25% have reached Phases II or III; 6 antisense RNA therapies have already obtained approval for commercial use.[116,117]

The delivery of small double-stranded RNA therapeutics, such as miRNAs and siRNAs, can take advantage from facilitating their intracellular uptake using various chemical vehicles. A first generation of lipid-mediated transfection (lipofection) reagents was based on nucleic acid entrapment by mixtures of a cationic lipid molecule (such as 1,2-di-O-octadecenyl-3-trimethylammonium propane or N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate, the positive charge of which neutralizes the negative charge of DNA), formulated with a neutral co-lipid helper (such as 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine); Figure 4B. These lipoplexes include the very popular, commercial Lipofectamine reagent (a 3:1 formulation of the cationic lipid DOSPA and 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine) and its derivatives. Lipoplexes are efficient tools for cell transfection with siRNAs or miRNAs in vitro; however, their large particle size (often >1 μm) and the positive charge imparted by the cationic lipid results in rapid plasma clearance, toxicity, and inflammation once administered systemically.[118,119] The first miRNA mimic tested in a clinical trial was miR-34a, formulated in a pH-dependent lipid vehicle, called MRX34, and delivered in a Phase I trial to patients with liver cancer or other advanced primary cancers. The lipid carrier, named Smarticle, was composed of amphoteric lipids having an overall anionic charge at physiological pH, while becoming cationic in the acidic tumor environment. Development of the drug, however, was terminated due to severe immune-related side effects.[120]

Some of the drawbacks of charged lipoplexes can be overcome by the use of neutral lipids, which show improved biodistribution and reduced clearance from the circulation. An example of these neutral lipids is the commercial preparation MaxSuppressor In Vivo RNA-LANCEr II—composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine, squalene oil, polysorbate 20, and an antioxidant—which was used to form a neutral lipid emulsion to deliver various miRNAs in the cancer and cardiovascular fields.[121,122]

A more significant progress in this area was however represented by the introduction of ionizable cationic lipids, such as 1,2-dioleoyl-3-dimethylammonium-propane, and of methods to load nucleic acids into small (<100 nm diameter) lipid nanoparticles with neutral surface charge. These ionizable cationic lipids are positively charged at low pH and are thus efficient to encapsulate negatively charged nucleic acids. Subsequently, when the pH is raised to physiological values, the surface of the lipid nanoparticles becomes neutral.[123] One of these lipid nanoparticles was used to develop patisiran, the first RNA interference therapeutic to reach the market in August 2018 in both the United States and Europe. Patisiran lowers the hepatic levels of transthyretin for the treatment of hereditary transthyretin-induced amyloidosis, a rapidly progressive and often fatal disease caused

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by mutations in the transthyretin gene.[124] At least other 8 lipid nanoparticlesformulations of siRNAs and miRNAs are currently undergoing clinical experimentation.[123] In the cardiovascular field, one of these is inclisiran, an siRNA targeting PCSK9, administered as a subcutaneous injection in patients at high risk for cardiovascular disease who have elevated LDL cholesterol levels.[125]

An alternative to the use of lipid mixtures is the formulation of miRNAs or siRNAs with synthetic or natural polymers, such as poly(ethylenimine), poly-L-lysine and poly(lactic-co-glycolic acid)[126]; Figure 4C. These polyplexes have usually large size, which however might not be relevant or even be desirable when the target is the vasculature. For example, recent evidence indicates that the intracoronary administration of an antagomiR-92 encapsulated in poly(lactic-co-glycolic acid) microspheres of >9 μm diameter promotes angiogenesis and improves cardiac function after MI in pigs.[127]

Another class of synthetic molecules that are of interest for small RNA delivery are dendrimers (Figure 4D). These are highly branched polymers containing cationic groups on their branches, which associate with negatively charged small nucleic acids. Similar to poly(ethylenimine), dendrimers facilitate endosomal escape by acting as proton sponges once in the endosomes. PAMAM dendrimers were reported to successfully deliver an RNA-triple-helix structure comprising 2 miRNAs and an antagomiR to breast cancer cells in a mouse model.[128] A major limitation of these molecules remains their toxicity in vivo. Other classes of nanoscale delivery systems for the delivery of noncoding RNA therapeutics are reviewed in study by Boca et al.[129]

Finally, a newer avenue for miRNA delivery is represented by the use of biological carriers. In an open-label trial carried out in in Australia, patients with pleural mesothelioma were administered intravenously with miR-16 TargomiRs. These are bacterially derived minicells with 400 nm in diameter, which are produced as a result of a mutations in genes that control bacterial cell division, loaded with a miR-16 mimic and targeted to EGFR (epidermal growth factor receptor) to suppress tumor growth.[130] Preliminary results from this phase I clinical trial indicate that this therapy has an acceptable safety profile.[131] Another biological miRNA delivery system takes advantage of endogenous vesicles such as exosomes, namely 30 to 100 nm extracellular vesicles involved in cell-to-cell communication through the transfer of bioactive material, such as lipids, proteins, and nucleic acids.[132] Methods can be devised for the enrichment of selected miRNAs into exosomes[133] and for the inclusion, within the exosome membrane, of specific ligands for cell targeting.[134] These technologies, however, are still immature, as loading efficiency of miRNA is relatively low, and the circulating exosomes tend to accumulate in the liver.

Gene Therapy for Cardiac Regeneration

A specific therapeutic area that has gained momentum recently is based on the possibility of achieving cardiac regeneration by stimulating the proliferation of cardiomyocytes by gene transfer. Cell loss in the heart contrasts with their very limited renewal capacity in adult organisms, which was estimated in the order of 1% per year in both humans and mice. This percentage increases slightly after damage, however, to an extent that remains below the threshold to be clinically relevant. Evidence in neonatal mice and pigs indicates that cardiac damage immediately after birth is repaired through cardiomyocyte proliferation, similar to what occurs throughout the entire life in salamander and fish. These observations suggest that cardiac regeneration in adult mammals could be obtained by stimulating adult cardiomyocytes to enter the cell cycle and divide (reviewed in study by Braga et al,[85] Hashimoto et al,[135] Tzahor and Poss,[136] and Uygur and Lee[137]).

Over the last years, significant progress has been made in understanding which are the mechanisms that control cardiomyocyte proliferation and can thus be exploited for regeneration purposes (Figure 5). A few cytokines are known to stimulate cardiomyocyte proliferation during development and at birth. These include members of the FGF (fibroblast growth factor) family, PDGF (platelet-derived growth factor), IL-6 (interleukin-6), Fstl1 (follistatin-like 1), and NRG1 (neuregulin-1; see study by Braga et al[85] and citations therein). Cardiomyocyte replication also results from sensing the extracellular environment surrounding cardiomyocytes. This involves activation of at least four signaling pathways. Three of these (the Wnt/β-catenin,[138] Notch,[139] and Hippo[140] pathways) regulate cardiomyocyte proliferation during embryonic development. A fourth mechanism links the extracellular matrix to cardiomyocyte proliferation through the protein agrin.[141]

Modulation of cardiomyocyte proliferation and cardiac regeneration can be achieved by overexpressing the positive regulators of these pathways or antagonizing the inhibitors, however, with some caveats that still prevent clinical translation. For example, stimulation of cardiomyocyte proliferation by FGF1 is modest and requires intracellular co-stimulation (eg, by p38 knock down[142]). Overexpression of the Notch implantable cardioverter defibrillator by AAV- or adenovirus-mediated gene transfer drives the expansion of neonatal cardiomyocytes.[143,144] However, this pathway is switched off in adult hearts due to suppressive epigenetic modifications at Notch-responsive promoters.[145] Stimulation of cardiomyocyte proliferation by NRG1 is powerful during development, but it loses efficacy at 1 week after birth in mice due to the downregulation of its c-ErbB2 receptor.[146] Transgenic mice overexpressing activated YAP or lacking the inhibitory Mst1 kinase repair myocardial injury through regeneration instead of fibrosis[147,148]; in addition,

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overexpression of YAP using an AAV9 vector[149] or knockout of the Mst1 co-factor Salvador in mouse hearts with post-MI HF[150] induce effective cardiac regeneration. However, genetic manipulation of the Hippo pathway for therapeutic purposes needs to be taken with caution, given the broad tumor suppressor role that this pathway exerts for several cancers.[151]

Similar to all cell types, cell cycle regulation in cardiomyocytes is also governed by a series of positive and negative regulators that converge on Cyclin/CDK regulation. Past work has shown that the overexpression of positive regulators (including E2F family members, cyclin D1, cyclin D2 [152]) or silencing of negative regulators (eg, the cyclin-dependent kinase inhibitors $p21^{WAF1/CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$[153]) can stimulate cardiomyocyte proliferation, in agreement with a series of studies in transgenic animals. However, single gene overexpression or downregulation is often ineffective at driving full cell replication and cytokinesis and often results in mitotic catastrophe and cell apoptosis.[154] Recent work shows that bona fide cardiomyocyte replication can instead be achieved by the simultaneous delivery of multiple cell cycle activators[155]; however, this remains poorly practical for therapeutic applications.

A very appealing alternative to achieve cardiac regeneration by manipulating the cardiomyocyte proliferative potential is to resort to the microRNA network. A large body of evidence over the last decade indicates that a number of miRNAs control, either positively or negatively, the rate of cardiomyocyte proliferation.[85] Several of these miRNAs were identified in 2 large screenings that analyzed 988 miRNAs encoded by the human genome in both rodent and human cells.[156,157] Collectively, these miRNAs can be classified into one of three categories. The first corresponds to miRNAs that are highly expressed in embryonic stem cells and are required to maintain pluripotency of these cells. These include members of the largely correlated miR-302~367 and miR-miR-290 family, which share the same seed sequence. In mice, reactivation of the miR-302-367 cluster induces cardiac regeneration.[122] A second class of proproliferative miRNAs include a series of miRNAs regulating the cell cycle and involved in tumorigenesis. These comprise the miR-17~92 cluster and its paralogues miR-106b~25 and miR-106a~363. Transgenic cardiac expression of miR-17~92,[158] or delivery of miR19a/19b[121] belonging to the same miRNA group, induce cardiomyocyte proliferation in embryonic, postnatal, and adult hearts and exerts a therapeutic effect after MI. A third group includes several unrelated miRNAs, among which the human miR-199a-3p, which was originally identified as one of the most effective proregenerative miRNAs in rodents[156] while acting as a tumor suppressor for several human malignancies.[85]

Other microRNAs are instead known to be physiologically expressed in cardiomyocytes and to suppress their proliferation. Two important groups of these inhibitory miRNAs include the let-7 family miRNAs, which suppress the cell cycle during development and stem cell differentiation, and the miR-15 family, which participates in the withdrawal of cardiomyocytes from the cell cycle after birth.[159] The forced inhibition of these miRNAs after MI in mice exerts regenerative effects.[160–162]

Of interest for therapeutic applications, miRNAs regulate mRNA stability and translation by base-pairing to partially complementary sequences. As a consequence, these molecules can target tens or hundreds of different transcripts, thus qualifying as broad regulators of complex biological functions, as is the replication of highly structured and electrically connected cells such as cardiomyocytes. Endogenously expressed, inhibitory miRNAs can be silenced using antisense nucleic acids. AntimiR LNAs against miR-15b[162] and miR34a[163] were shown to exert a beneficial effect after MI in rodents. Alternatively, sponges sequestering suppressive miRNAs can be expressed using AAV vectors, as shown in the case of let-7 and miR-99/100 miRNAs and for a few recently discovered, inhibitory lncRNAs (reviewed in study by Braga et al[85]).

The efficacy of the antisense or sponge approach strictly depends on the levels of expression of the inhibitory ncRNAs and their relative relevance in controlling the regeneration phenotype. A more tempting strategy is to impart a proliferative phenotype to cardiomyocytes with small RNAs, irrespective whether the molecules that are administered take part in the normal physiology of the heart. In infarcted mice, the expression of miR-199a or miR-590a,[156] miR-294, a member of the miR-302 superfamily[164] and the miR-17~92 cluster member miR-19a/19b,[121] in all cases using AAV vectors, were all shown to stimulate cardiomyocyte proliferation and restoration of cardiac function after MI.

Expression of miRNAs using AAV vectors, however, is fraught with problems in view of clinical application. These vectors persist indefinitely in the transduced cells, while expression of their transgenes cannot readily be regulated by the currently available promoters.[6] This creates general safety issues for vectors expressing proproliferative genes and specific cardiac concerns, in light of the need, for cardiomyocytes, to partially de-differentiate to undergo replication. Additionally, AAV vectors deliver the pri-miRNA gene, which is then processed by the RNA interference machinery to eventually generate the mature miRNA duplex. Thus, both miRNA strands (5p and 3p) are produced upon gene transfer, which might result in unwanted effects. This became evident in one large animal study so far conducted for cardiac regeneration, in which an AAV6 vector expressing the miR-199a gene (encoding the pro-proliferative miR-199a-3p[156] and the complementary strand miR-199-5p) was tested after MI infarction in pigs. While cardiac regeneration and improvement in cardiac function was clearly evident

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one month after MI and treatment, several of the treated pigs developed fatal arrhythmias at longer times.[165] This finding is also consistent with observations in transgenic mice overexpressing the miR-302~367 cluster, in which cardiac regeneration was observed after MI, however, followed by dysfunction due to cardiomyocyte hyperproliferation and de-differentiation in the long term.[122]

The possibility of delivering therapeutic nucleic acids transiently opens a new translational perspective for clinical cardiac regeneration. Current experimental evidence already shows that, in mice, a single intramyocardial injection miR-199a-3p or miR-590-3p mimics using a cationic lipid formulation is sufficient to stimulate a regenerative response.[166] Consistent results were observed, again after MI in mice, by the intramyocardial administration of miR-19a/19b mimics using a neutral lipid delivery reagent or using the same formulation for the daily intravenous administration of miR302b/c, miR-19a/19b, or miR-708 mimics, or the intracardial delivery of cholesterol-modified miR-302b/c mimics using a hydrogel (reviewed in study by Braga et al[85]).

Further studies are clearly needed to improve the formulation of these ncRNA therapeutics and test their efficacy and safety in large animals, before moving to clinical experimentation. The concept that cardiac regeneration could be achieved by stimulating the endogenous capacity of cardiomyocytes to proliferate using noncoding RNA therapeutics remains however exciting.

Gene Editing for Inherited Cardiac Diseases

The repair of specific DNA mutations through precise gene editing remains one of the ultimate goals of gene therapy. This would allow to insert, eliminate, or modify gene sequences in the genome aiming at correcting inherited mutations. For decades, however, precise gene editing has been considered too inefficient in vivo, especially in postmitotic cells.

The use of programmable endonucleases able to introduce site-specific, double-stranded DNA breaks has opened a route to possible clinical applications. Their application stems from the long-standing notion that the presence of a double-stranded DNA breaks in the correspondence of a desired DNA sequence markedly increases recruitment of the cellular DNA damage repair machinery to that specific sequence. Then, DNA damage repair can act in essentially 2 manners, one entailing the introduction of small insertions or deletions through the error-prone non homologous end joining pathway while the other one employing an homologous DNA sequence as a template for homology directed repair, which recreates a normal genome sequence.[167]

The discovery of bacterial nucleases capable of recognising specific genomic sequences through Watson-Crick base pairing of a guide RNA (the clustered regularly interspaced short palindromic repeats [CRISPR]/Cas9 system), instead of protein-DNA recognition as in other gene editing systems, has given momentum to the field. For the heart, this holds a concrete promise for the genetic correction of inherited cardiomyopathies (in particular, dilated cardiomyopathy [DCM] and hypertrophic cardiomyopathy [HCM], respectively).[168]

The genetic background of DCM is particularly heterogeneous. Pathogenic variants have been identified in >40 genes encoding for proteins of different cellular compartments.[169] In some DCM preclinical models, more conventional gene therapy has proven successful, to a variable extent, using truncated proteins or by taking advantage of indirect approaches rather than straight gene replacement. These include, as examples, gene therapy for Duchenne muscular dystrophy-associated cardiomyopathy using a minigene-carrying vector[170] or oligonucleotides for exon skipping for DMD[171] or titin-associated DCM,[172] transduction of gene modifiers (such as miR-669a for sarcoglycan dysfunction[173]) and induction of trans-splicing (for lamin A/C mutations[174]). The application of the new gene editing technologies now offers unprecedented possibilities to this field. In particular, CRISPR/Cas9-mediated mutagenesis has already shown remarkable efficiency in inducing cardiac and skeletal muscle exon skipping in both dogs[175] and pigs[176] carrying frameshift mutations in Duchenne muscular dystrophy exons 50 and 52, respectively.

As far as HCM is concerned, >1400 different pathogenic mutations in sarcomeric proteins have been related to the development of this condition, 80% of which are in the MYH7 and MYBPC3 genes.[177] Here, in only a few cases, standard gene therapy was shown possible in animal models, for example, by overexpressing the normal MYBPC3 gene[178] or a phosphomimetic variant of the myosin regulatory light chain—the MYL2 protein.[179]

In both DCM and HCM, canonical replacement gene therapy faces important hurdles. This is because, especially in DCM, the cDNAs of several of the genes that cause the disease are too large to fit into conventional AAV vectors. In HCM then, the mutations causing the disease most often act in a dominant manner. Similar considerations also apply to several arrhythmogenic disorders, in which ion channel and desmosome protein mutations are often dominant. For all these conditions, gene editing would seem to be an ideal therapeutic strategy. Indeed, a number of recent studies performed in patients' induced pluripotent stem cell-derived cardiomyocytes have led to phenotypic correction ex vivo (eg, CRISPR/Cas9-mediated correction of a Lamin A/C defect[180] or TALEN [transcription activator-like effector nuclease] exonuclease-mediate correction of phospholamban[181]).

Translation of these findings to the clinic, however, can be problematic. For a dominant condition, inactivation of the deleterious allele through non homologous end joining requires high efficiency discrimination of the mutant and normal sequences, which can be not

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straightforward with all gene sequences. In addition, allele inactivation should not be followed by haploinsufficiency. Previous work has indeed shown that gene inactivation in vivo can be achieved in the heart using AAV vectors to deliver the required CRISPR/Cas9 components.[182,183] However, the impossibility of modulating the resulting phenotype renders this method an outstanding tool for gene function investigation but a still an immature approach for clinical application.

In contrast to non homologous end joining gene correction by homology directed repair requires the activation of the cellular homologous recombination machinery. Recently, the germline mutations of a patient with HCM caused by a GAGT-deletion in exon 16 of the MYBPC3 gene was corrected in human preimplantation embryos by CRISPR/Cas9 gene editing, using the normal allele as a template for homology directed repair.[184] However, this requires cell cycling and is thus possible in ex vivo cultured cells but not in cardiomyocytes in the heart. This problem could be overcome by the identification of ways of inducing expression of the homologous recombination machinery in postmitotic cells in vivo, to nudge the correction system toward homology directed repair, or by coupling gene repair with cardiomyocyte replication, for example, using one of the treatments described earlier.

CONCLUSIONS

Despite over 30 years have passed since gene therapy was first conceived and no successful application has yet been developed for the heart, this remains a young discipline with many additional arrows in its quiver. In particular, the transition from gene addition studies using protein-coding cDNAs to the modulation of gene expression using RNA therapeutics and the improvements in precise gene editing seem to offer new avenues for this discipline, paving the way to applications in myocardial infarction, heart failure, and inherited cardiac diseases that were unapproachable until a decade ago.

ARTICLE INFORMATION

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Disclosures

None.

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