

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

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

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	follicle-stimulating-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 Zacchigna⁴; 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 Giacca⁷ 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 AAV2⁹ or AAV9 serotypes.¹⁰

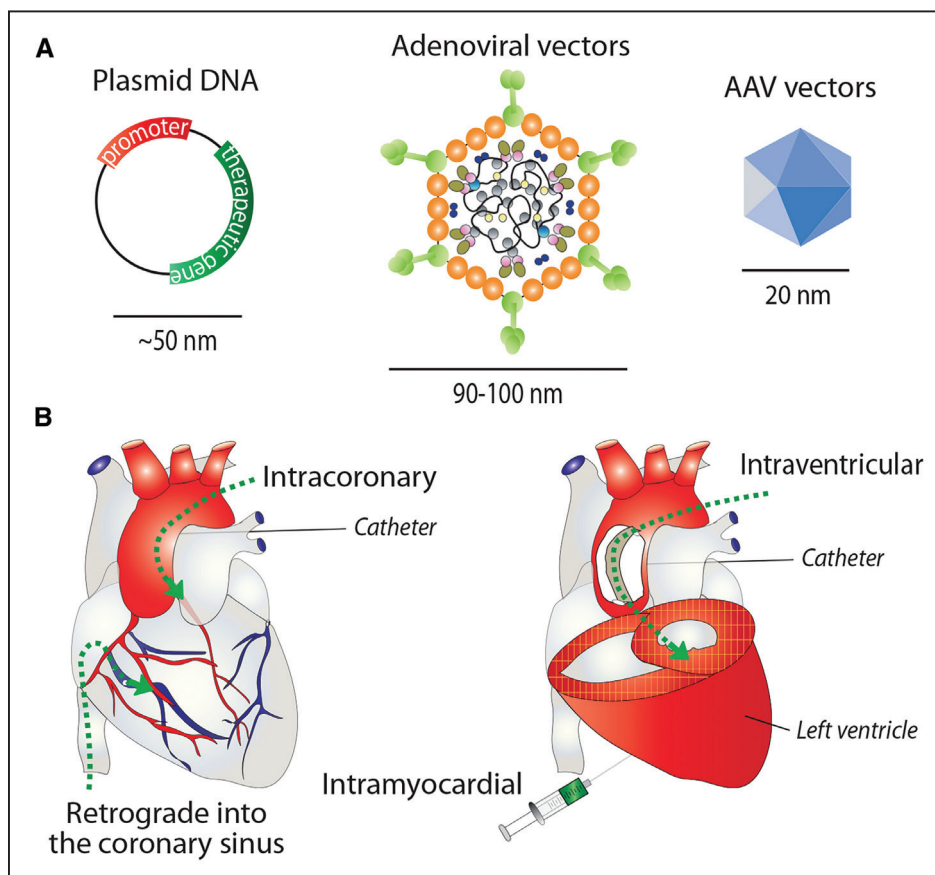


Figure 1. Delivery vehicles and routes for cardiac gene therapy.

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 Grimm¹¹). 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 transepical delivery, or by intraventricular injection using a percutaneous catheter, to achieve transendocardial delivery (Figure 1B). The transepical 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),

and Stiletto (Boston Scientific, Natick, MA).¹⁴⁻¹⁷ The only transepical, 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 electro-mechanical 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 transepical 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 over-expression 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)-A¹⁶⁵, one of the master regulator of the angiogenesis process (reviewed in study by Giacca and Zacchigna⁴). Some positive results from these applications are largely anecdotal, as these had an open-labeled design 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 EUROINJECT^{1,26} 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-A₁₆₅ (the VIF-CAD study²⁹) and in one small study aimed at increasing VEGF-A¹⁶⁵ 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-A₁₂₁ 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 trial³²; 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 trial³⁴); 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

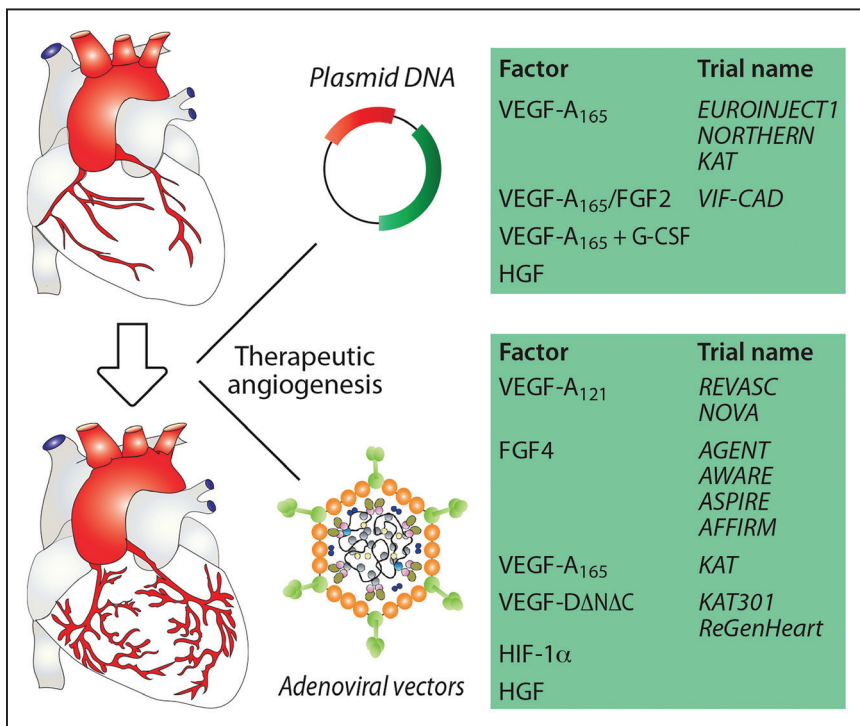


Figure 2. Clinical trials for therapeutic angiogenesis.

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 Genex, 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]; NCT01550614³⁷). 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-A₁₆₅.²⁸ 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 transcatheter injections; myocardial perfusion was reported increased at 1 year from treatment (the KAT301 trial⁴¹). 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 isoforms⁴³ 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 al³¹). 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

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 Teerlink⁴⁶ 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+} ATPase 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 (NCT01966887⁵¹) and the SERCA-LVAD study (Sarcoplasmic/Endoplasmic Reticulum Ca^{2+} -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 vector⁵³ 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 adenylylase (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

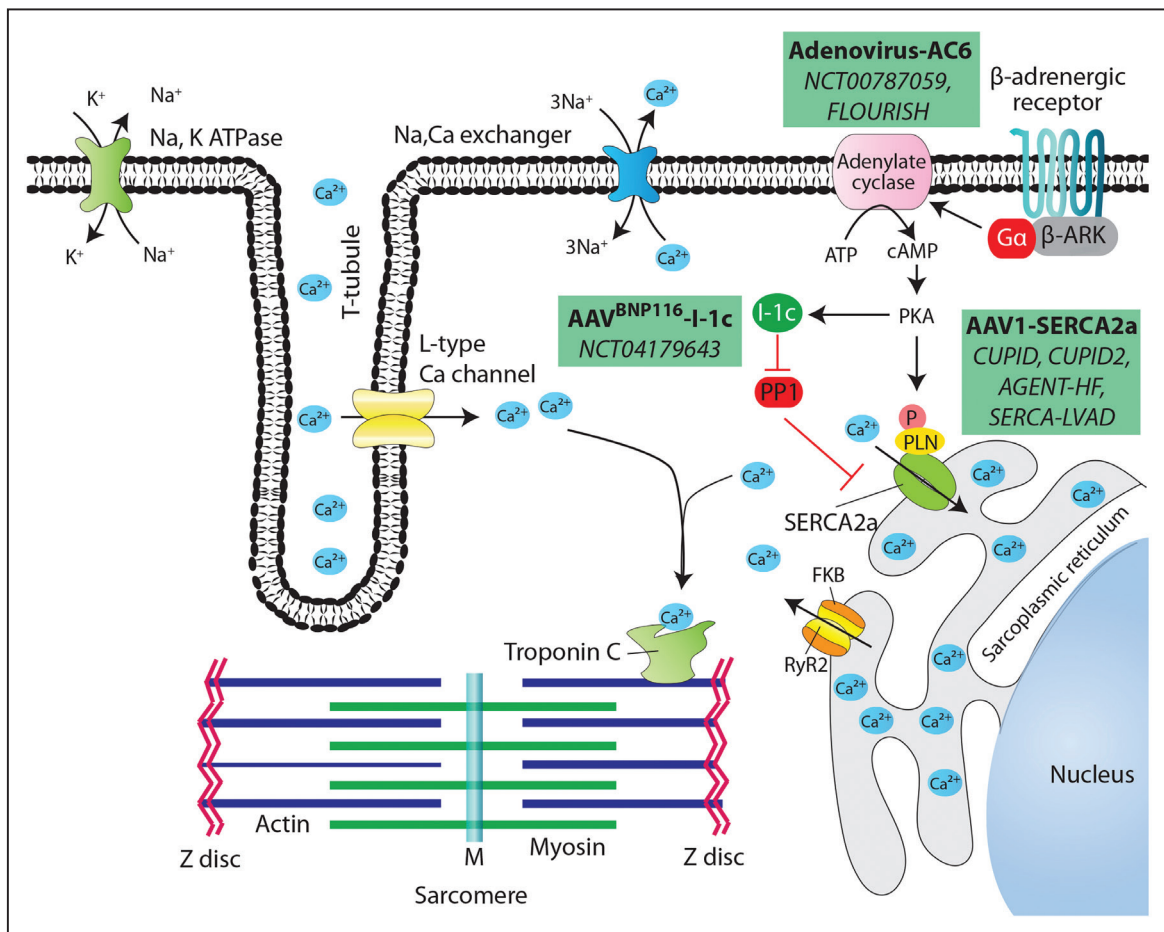


Figure 3. Clinical trials for heart failure.

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 FKBP12.6 protein. 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 $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX). The activity of the SERCA2a pump is controlled by association of this protein with PLN (phospholamban). In its nonphosphorylated form, PLN inhibits SERCA2a, while phosphorylation blocks this inhibition. The main kinase phosphorylating PLN 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 FKBP12.6; and (3) PLN, blocking its inhibitory activity on SERCA2a. These modifications amplify the efficacy of 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 transferring 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)-1 α .⁶⁴ Treatment with SDF1 α 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

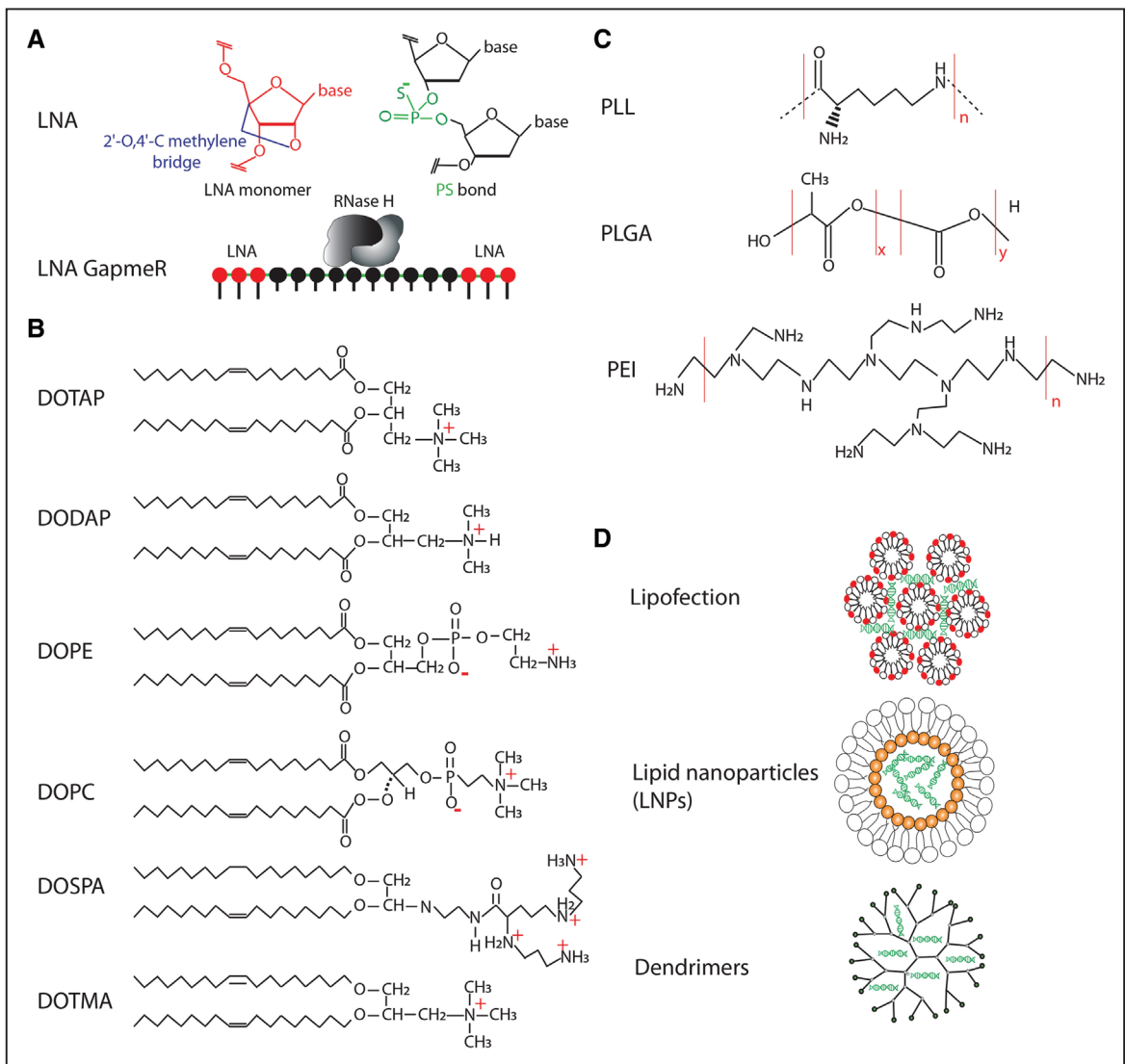


Figure 4. Molecules and methods for the delivery of RNA therapeutics.

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 accessibility 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 SDF1 α 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 models⁶⁸ and using AAV

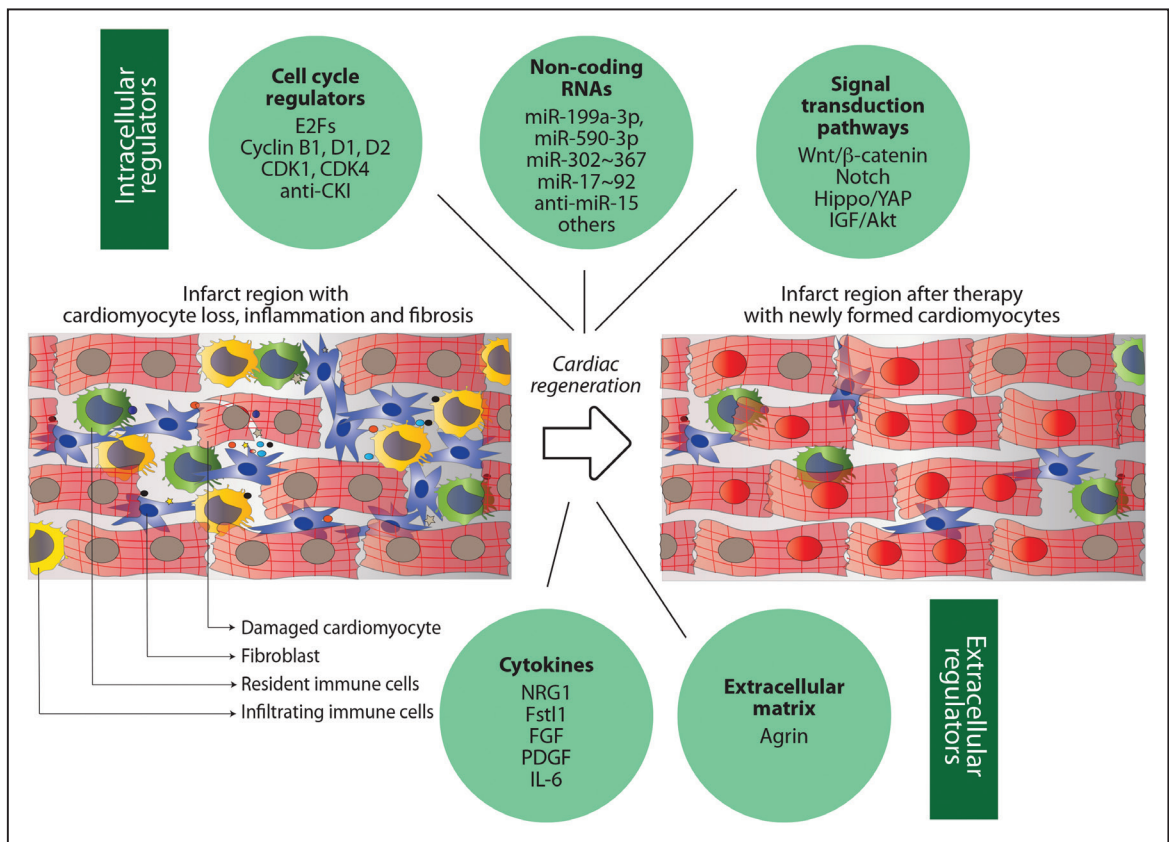


Figure 5. Molecules, genes, and RNAs shown to stimulate cardiomyocyte proliferation and cardiac regeneration.

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.

vectors⁶⁹ 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 administration⁷¹). 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 al⁶).

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 as 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 DNA⁵⁰ (as a reference, DNA from 1×10^6 mononucleated diploid cells weights $\approx 6 \mu\text{g}$). 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

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 SDF1 α 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 cells⁷⁸ 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 Gerritsen⁷⁹ 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, SDF1 α and VEGF-A¹⁶⁵ in patients with an implanted left ventricular assist device, either as a bridge to transplant or destination

therapy. Major unknowns of such an approach, however, remain the poor efficiency of plasmid gene transfer and the efficacy of SDF1 α .

Finally, upstream transcriptional regulators of the response to ischemia might be better positioned to drive a functional angiogenic response. Of these, however, HIF-1 α 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 (\approx 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 variegated 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 ventricle⁸⁴ but also occurs in most cardiac diseases (reviewed in study by Braga et al⁸⁵). 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

dysfunction are governed by one or multiple lncRNAs (reviewed in study by Hobuß et al,⁹⁴ Salamon et al,⁹⁵ and Lucas et al⁹⁶).

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 Behlke⁹⁷ and van Rooij and Olson⁹⁸). 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 GapmeRs¹⁰⁰). 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-32¹⁰³ 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 mice¹⁰⁷ whereas regional administration is effective in pigs¹⁰⁸; inhibition of miR-199a-5p reverses hypertrophy and fibrosis in HF in mice¹⁰⁹; antagonizing endothelial miR-24 limits MI size by preventing endothelial apoptosis¹¹⁰; 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 dysfunction¹¹³; against Chast both prevents and attenuates TAC-induced pathological cardiac remodeling¹¹⁴; 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 al⁹⁶.

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-octadecenyl)-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-octadecenyl)-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

by mutations in the transthyretin gene.¹²⁴ At least other 8 lipid nanoparticles formulations 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 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 exosomes¹³³ 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 Lee¹³⁷).

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 al⁸⁵ 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 Hippo¹⁴⁰ 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 down¹⁴²). 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,

overexpression of YAP using an AAV9 vector¹⁴⁹ or knock-out of the Mst1 co-factor Salvador in mouse hearts with post-MI HF¹⁵⁰ 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 D2¹⁵²) 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 activators¹⁵⁵; 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/19b¹²¹ 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 rodents¹⁵⁶ 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-15b¹⁶² and miR34a¹⁶³ 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 al⁸⁵).

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 superfamily¹⁶⁴ 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-3p¹⁵⁶ 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

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 al⁸⁵).

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 vector¹⁷⁰ or oligonucleotides for exon skipping for DMD¹⁷¹ or titin-associated DCM,¹⁷² transduction of gene modifiers (such as miR-669a for sarcoglycan dysfunction¹⁷³) and induction of trans-splicing (for lamin A/C mutations¹⁷⁴). 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 dogs¹⁷⁵ and pigs¹⁷⁶ 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 gene¹⁷⁸ 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 defect¹⁸⁰ or TALEN [transcription activator-like effector nuclease] exonuclease-mediate correction of phospholamban¹⁸¹).

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

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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None.

REFERENCES

- Rosenberg SA, Aebbersold P, Cornetta K, Kasid A, Morgan RA, Moen R, Karson EM, Lotze MT, Yang JC, Topalian SL. Gene transfer into humans—immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. *N Engl J Med*. 1990;323:570–578. doi: 10.1056/NEJM199008303230904
- High KA, Roncarolo MG. Gene therapy. *N Engl J Med*. 2019;381:455–464. doi: 10.1056/NEJMra1706910
- Jaroff L. Fixing the genes. *Time*. 1999;153:68–70, 73.
- Giacca M, Zacchigna S. VEGF gene therapy: therapeutic angiogenesis in the clinic and beyond. *Gene Ther*. 2012;19:622–629. doi: 10.1038/gt.2012.17
- St George JA. Gene therapy progress and prospects: adenoviral vectors. *Gene Ther*. 2003;10:1135–1141. doi: 10.1038/sj.gt.3302071
- Zacchigna S, Zentilin L, Giacca M. Adeno-associated virus vectors as therapeutic and investigational tools in the cardiovascular system. *Circ Res*. 2014;114:1827–1846. doi: 10.1161/CIRCRESAHA.114.302331
- Zacchigna S, Giacca M. Extra- and intracellular factors regulating cardiomyocyte proliferation in postnatal life. *Cardiovasc Res*. 2014;102:312–320. doi: 10.1093/cvr/cvu057
- Grimm D, Büning H. Small but increasingly mighty: latest advances in AAV vector research, design, and evolution. *Hum Gene Ther*. 2017;28:1075–1086. doi: 10.1089/hum.2017.172
- Müller OJ, Kaul F, Weitzman MD, Pasqualini R, Arap W, Kleinschmidt JA, Trepel M. Random peptide libraries displayed on adeno-associated virus to select for targeted gene therapy vectors. *Nat Biotechnol*. 2003;21:1040–1046. doi: 10.1038/nbt856
- Varadi K, Michelfelder S, Korff T, Hecker M, Trepel M, Katus HA, Kleinschmidt JA, Müller OJ. Novel random peptide libraries displayed on AAV serotype 9 for selection of endothelial cell-directed gene transfer vectors. *Gene Ther*. 2012;19:800–809. doi: 10.1038/gt.2011.143
- Domenger C, Grimm D. Next-generation AAV vectors—do not judge a virus (only) by its cover. *Hum Mol Genet*. 2019;28:R3–R14. doi: 10.1093/hmg/ddz148
- Sarcar S, Tulalamba W, Rincon MY, Tipanee J, Pham HQ, Evens H, Boon D, Samara-Kuko E, Keyaerts M, Loperfido M, et al. Next-generation muscle-directed gene therapy by in silico vector design. *Nat Commun*. 2019;10:492. doi: 10.1038/s41467-018-08283-7
- Sherman W, Martens TP, Viles-Gonzalez JF, Siminiak T. Catheter-based delivery of cells to the heart. *Nat Clin Pract Cardiovasc Med*. 2006;3(suppl 1):S57–S64. doi: 10.1038/ncpcardio0446
- Marshall DJ, Palasis M, Lepore JJ, Leiden JM. Biocompatibility of cardiovascular gene delivery catheters with adenovirus vectors: an important determinant of the efficiency of cardiovascular gene transfer. *Mol Ther*. 2000;1:423–429. doi: 10.1006/mthe.2000.0059
- Hill JM, Dick AJ, Raman VK, Thompson RB, Yu ZX, Hinds KA, Pessanha BS, Guttman MA, Varney TR, Martin BJ, et al. Serial cardiac magnetic resonance imaging of injected mesenchymal stem cells. *Circulation*. 2003;108:1009–1014. doi: 10.1161/01.CIR.0000084537.66419.7A
- Losordo DW, Vale PR, Symes JF, Dunnington CH, Esakof DD, Maysky M, Ashare AB, Lathi K, Isner JM. Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia. *Circulation*. 1998;98:2800–2804. doi: 10.1161/01.cir.98.25.2800
- Vale PR, Losordo DW, Milliken CE, Maysky M, Esakof DD, Symes JF, Isner JM. Left ventricular electromechanical mapping to assess efficacy of phVEGF(165) gene transfer for therapeutic angiogenesis in chronic myocardial ischemia. *Circulation*. 2000;102:965–974. doi: 10.1161/01.cir.102.9.965
- Dib N, Menasche P, Bartunek JJ, Zeiher AM, Terzic A, Chronos NA, Henry TD, Peters NS, Fernández-Avilés F, Yacoub M, et al; International Society for Cardiovascular Translational Research. Recommendations for successful training on methods of delivery of biologics for cardiac regeneration: a report of the International Society for Cardiovascular Translational Research. *JACC Cardiovasc Interv*. 2010;3:265–275. doi: 10.1016/j.jcin.2009.12.013

19. Gepstein L, Hayam G, Ben-Haim SA. A novel method for nonfluoroscopic catheter-based electroanatomical mapping of the heart. In vitro and in vivo accuracy results. *Circulation*. 1997;95:1611–1622. doi: 10.1161/01.cir.95.6.1611
20. Corti R, Badimon J, Mizsei G, Macaluso F, Lee M, Licato P, Viles-Gonzalez JF, Fuster V, Sherman W. Real time magnetic resonance guided endomyocardial local delivery. *Heart*. 2005;91:348–353. doi: 10.1136/hrt.2004.034363
21. Sheng CC, Zhou L, Hao J. Current stem cell delivery methods for myocardial repair. *Biomed Res Int*. 2013;2013:547902. doi: 10.1155/2013/547902
22. Ishikawa K, Aguero J, Naim C, Fish K, Hajjar RJ. Percutaneous approaches for efficient cardiac gene delivery. *J Cardiovasc Transl Res*. 2013;6:649–659. doi: 10.1007/s12265-013-9479-7
23. Raake P, von Degenfeld G, Hinkel R, Vachenaue R, Sandner T, Beller S, Andrees M, Kupatt C, Schuler G, Boekstegers P. Myocardial gene transfer by selective pressure-regulated retroinfusion of coronary veins: comparison with surgical and percutaneous intramyocardial gene delivery. *J Am Coll Cardiol*. 2004;44:1124–1129. doi: 10.1016/j.jacc.2004.05.074
24. Boekstegers P, von Degenfeld G, Giehl W, Heinrich D, Hullin R, Kupatt C, Steinbeck G, Baretton G, Middeler G, Katus H, et al. Myocardial gene transfer by selective pressure-regulated retroinfusion of coronary veins. *Gene Ther*. 2000;7:232–240. doi: 10.1038/sj.gt.3301079
25. Risau W. Mechanisms of angiogenesis. *Nature*. 1997;386:671–674. doi: 10.1038/386671a0
26. Kastrup J, Jørgensen E, Rück A, Tägil K, Glogar D, Ruzyllo W, Bøtker HE, Dudek D, Drvota V, Hesse B, et al; Euroinject One Group. Direct intramyocardial plasmid vascular endothelial growth factor-A165 gene therapy in patients with stable severe angina pectoris A randomized double-blind placebo-controlled study: the Euroinject One trial. *J Am Coll Cardiol*. 2005;45:982–988. doi: 10.1016/j.jacc.2004.12.068
27. Stewart DJ, Kutryk MJ, Fitchett D, Freeman M, Camack N, Su Y, Della Siega A, Bilodeau L, Burton JR, Proulx G, et al; NORTHERN Trial Investigators. VEGF gene therapy fails to improve perfusion of ischemic myocardium in patients with advanced coronary disease: results of the NORTHERN trial. *Mol Ther*. 2009;17:1109–1115. doi: 10.1038/mt.2009.70
28. Hedman M, Hartikainen J, Svänne M, Stjernvall J, Hedman A, Kivelä A, Vanninen E, Mussalo H, Kauppila E, Simula S, et al. Safety and feasibility of catheter-based local intracoronary vascular endothelial growth factor gene transfer in the prevention of postangioplasty and in-stent restenosis and in the treatment of chronic myocardial ischemia: phase II results of the Kuopio Angiogenesis Trial (KAT). *Circulation*. 2003;107:2677–2683. doi: 10.1161/01.CIR.0000070540.80780.92
29. Kukuła K, Chojnowska L, Dąbrowski M, Witkowski A, Chmielak Z, Skwarek M, Kądziała J, Teresińska A, Małecki M, Janik P, et al. Intramyocardial plasmid-encoding human vascular endothelial growth factor A165/basic fibroblast growth factor therapy using percutaneous transcatheter approach in patients with refractory coronary artery disease (VIF-CAD). *Am Heart J*. 2011;161:581–589. doi: 10.1016/j.ahj.2010.11.023
30. Ripa RS, Wang Y, Jørgensen E, Johnsen HE, Hesse B, Kastrup J. Intramyocardial injection of vascular endothelial growth factor-A165 plasmid followed by granulocyte-colony stimulating factor to induce angiogenesis in patients with severe chronic ischaemic heart disease. *Eur Heart J*. 2006;27:1785–1792. doi: 10.1093/eurheartj/ehl117
31. Wang LS, Wang H, Zhang QL, Yang ZJ, Kong FX, Wu CT. Hepatocyte growth factor gene therapy for chronic diseases. *Hum Gene Ther*. 2018;29:413–423. doi: 10.1089/hum.2017.217
32. Rosengart TK, Lee LY, Patel SR, Sanborn TA, Parikh M, Bergman GW, Hachamovitch R, Szulc M, Kliffeld PD, Okin PM, et al. Angiogenesis gene therapy: phase I assessment of direct intramyocardial administration of an adenovirus vector expressing VEGF121 cDNA to individuals with clinically significant severe coronary artery disease. *Circulation*. 1999;100:468–474. doi: 10.1161/01.cir.100.5.468
33. Stewart DJ, Hilton JD, Arnold JM, Gregoire J, Rivard A, Archer SL, Charbonneau F, Cohen E, Curtis M, Buller CE, et al. Angiogenic gene therapy in patients with nonrevascularizable ischemic heart disease: a phase 2 randomized, controlled trial of AdVEGF(121) (AdVEGF121) versus maximum medical treatment. *Gene Ther*. 2006;13:1503–1511. doi: 10.1038/sj.gt.3302802
34. Kastrup J, Jørgensen E, Fuchs S, Nikol S, Bøtker HE, Gyöngyösi M, Glogar D, Kornowski R. A randomised, double-blind, placebo-controlled, multicentre study of the safety and efficacy of BIOVAPASS (AdGVVEGF121.10NH) gene therapy in patients with refractory advanced coronary artery disease: the NOVA trial. *EuroIntervention*. 2011;6:813–818. doi: 10.4244/EIJV6I7A140
35. Grines C. The AGENT clinical trials programme. *Eur Heart J*. 2004;6(suppl):E18–E23.
36. Henry TD, Grines CL, Watkins MW, Dib N, Barbeau G, Moreadith R, Andrasfay T, Engler RL. Effects of Ad5FGF-4 in patients with angina: an analysis of pooled data from the AGENT-3 and AGENT-4 trials. *J Am Coll Cardiol*. 2007;50:1038–1046. doi: 10.1016/j.jacc.2007.06.010
37. Kaski JC, Consuegra-Sanchez L. Evaluation of ASPIRE trial: a Phase III pivotal registration trial, using intracoronary administration of Genex (Ad5FGF4) to treat patients with recurrent angina pectoris. *Expert Opin Biol Ther*. 2013;13:1749–1753. doi: 10.1517/14712598.2013.827656
38. Hedman M, Muona K, Hedman A, Kivelä A, Svänne M, Eränen J, Rantala A, Stjernvall J, Nieminen MS, Hartikainen J, et al. Eight-year safety follow-up of coronary artery disease patients after local intracoronary VEGF gene transfer. *Gene Ther*. 2009;16:629–634. doi: 10.1038/gt.2009.4
39. Muona K, Mäkinen K, Hedman M, Manninen H, Ylä-Herttuala S. 10-year safety follow-up in patients with local VEGF gene transfer to ischemic lower limb. *Gene Ther*. 2012;19:392–395. doi: 10.1038/gt.2011.109
40. Rutanen J, Rissanen TT, Markkanen JE, Gruchala M, Silvennoinen P, Kivelä A, Hedman A, Hedman M, Heikura T, Ordén MR, et al. Adenoviral catheter-mediated intramyocardial gene transfer using the mature form of vascular endothelial growth factor-D induces transmural angiogenesis in porcine heart. *Circulation*. 2004;109:1029–1035. doi: 10.1161/01.CIR.0000115519.03688.A2
41. Hartikainen J, Hassinen I, Hedman A, Kivelä A, Saraste A, Knuuti J, Husso M, Mussalo H, Hedman M, Rissanen TT, et al. Adenoviral intramyocardial VEGF-DANAC gene transfer increases myocardial perfusion reserve in refractory angina patients: a phase I/IIa study with 1-year follow-up. *Eur Heart J*. 2017;38:2547–2555. doi: 10.1093/eurheartj/ehx352
42. Kilian EG, Sadoni S, Vicol C, Kelly R, van Hulst K, Schwaiger M, Kupatt C, Boekstegers P, Pillai R, Channon K, et al. Myocardial transfection of hypoxia inducible factor-1alpha via an adenoviral vector during coronary artery bypass grafting. - a multicenter phase I and safety study. *Circ J*. 2010;74:916–924. doi: 10.1253/circj.09-0594
43. Kaminsky SM, Quach L, Chen S, Pierre-Destine L, Van de Graaf B, Monette S, Rosenberg JB, De BP, Sondhi D, Hackett NR, et al. Safety of direct cardiac administration of AdVEGF-All6A+, a replication-deficient adenovirus vector cDNA/genomic hybrid expressing all three major isoforms of human vascular endothelial growth factor, to the ischemic myocardium of rats. *Hum Gene Ther Clin Dev*. 2013;24:38–46. doi: 10.1089/humc.2013.054
44. Yang ZJ, Zhang YR, Chen B, Zhang SL, Jia EZ, Wang LS, Zhu TB, Li CJ, Wang H, Huang J, et al. Phase I clinical trial on intracoronary administration of Ad-hHGF treating severe coronary artery disease. *Mol Biol Rep*. 2009;36:1323–1329. doi: 10.1007/s11033-008-9315-3
45. Meng H, Chen B, Tao Z, Xu Z, Wang L, Weizhu J, Hong Y, Liu X, Wang H, Wang L, et al. Safety and efficacy of adenovirus carrying hepatocyte growth factor gene by percutaneous endocardial injection for treating post-infarct heart failure: a phase IIa clinical trial. *Curr Gene Ther*. 2018;18:125–130. doi: 10.2174/1566523218666180404162209
46. Metra M, Teerlink JR. Heart failure. *Lancet*. 2017;390:1981–1995. doi: 10.1016/S0140-6736(17)31071-1
47. Kranias EG, Hajjar RJ. Modulation of cardiac contractility by the phospholamban/SERCA2a regulome. *Circ Res*. 2012;110:1646–1660. doi: 10.1161/CIRCRESAHA.111.259754
48. Jaski BE, Jessup ML, Mancini DM, Cappola TP, Pauly DF, Greenberg B, Borow K, Dittrich H, Zsebo KM, Hajjar RJ; Calcium Up-Regulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID) Trial Investigators. Calcium upregulation by percutaneous administration of gene therapy in cardiac disease (CUPID Trial), a first-in-human phase 1/2 clinical trial. *J Card Fail*. 2009;15:171–181. doi: 10.1016/j.cardfail.2009.01.013
49. Jessup M, Greenberg B, Mancini D, Cappola T, Pauly DF, Jaski B, Yaroshinsky A, Zsebo KM, Dittrich H, Hajjar RJ; Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID) Investigators. Calcium upregulation by percutaneous administration of gene therapy in cardiac disease (CUPID): a phase 2 trial of intracoronary gene therapy of sarcoplasmic reticulum Ca²⁺-ATPase in patients with advanced heart failure. *Circulation*. 2011;124:304–313. doi: 10.1161/CIRCULATIONAHA.111.022889
50. Greenberg B, Butler J, Felker GM, Ponikowski P, Voors AA, Desai AS, Bernard D, Bouchard A, Jaski B, Lyon AR, et al. Calcium upregulation by percutaneous administration of gene therapy in patients with cardiac disease (CUPID 2): a randomised, multinational, double-blind, placebo-controlled, phase 2b trial. *Lancet*. 2016;387:1178–1186. doi: 10.1016/S0140-6736(16)00082-9
51. Hulot JS, Salem JE, Redheuil A, Collet JP, Varnous S, Jourdain P, Logeart D, Gandjbakhch E, Bernard C, Hatem SN, et al; AGENT-HF Investigators. Effect of intracoronary administration of AAV1/SERCA2a on ventricular

- remodelling in patients with advanced systolic heart failure: results from the AGENT-HF randomized phase 2 trial. *Eur J Heart Fail*. 2017;19:1534–1541. doi: 10.1002/ehfj.826
52. Pathak A, del Monte F, Zhao W, Schultz JE, Lorenz JN, Bodi I, Weiser D, Hahn H, Carr AN, Syed F, et al. Enhancement of cardiac function and suppression of heart failure progression by inhibition of protein phosphatase 1. *Circ Res*. 2005;96:756–766. doi: 10.1161/01.RES.0000161256.858333.fa
 53. Fish KM, Ladage D, Kawase Y, Karakikes I, Jeong D, Ly H, Ishikawa K, Hadri L, Tilemann L, Muller-Ehmsen J, et al. AAV9-I-1c delivered via direct coronary infusion in a porcine model of heart failure improves contractility and mitigates adverse remodeling. *Circ Heart Fail*. 2013;6:310–317. doi: 10.1161/CIRCHEARTFAILURE.112.971325
 54. Ishikawa K, Fish KM, Tilemann L, Rapti K, Agüero J, Santos-Gallego CG, Lee A, Karakikes I, Xie C, Akar FG, et al. Cardiac I-1c overexpression with reengineered AAV improves cardiac function in swine ischemic heart failure. *Mol Ther*. 2014;22:2038–2045. doi: 10.1038/mt.2014.127
 55. Watanabe S, Ishikawa K, Fish K, Oh JG, Motloch LJ, Kohlbrenner E, Lee P, Xie C, Lee A, Liang L, et al. Protein phosphatase inhibitor-1 gene therapy in a swine model of nonischemic heart failure. *J Am Coll Cardiol*. 2017;70:1744–1756. doi: 10.1016/j.jacc.2017.08.013
 56. Lai NC, Roth DM, Gao MH, Fine S, Head BP, Zhu J, McKirman MD, Kwong C, Dalton N, Urasawa K, et al. Intracoronary delivery of adenovirus encoding adenylyl cyclase VI increases left ventricular function and cAMP-generating capacity. *Circulation*. 2000;102:2396–2401. doi: 10.1161/01.cir.102.19.2396
 57. Lai NC, Roth DM, Gao MH, Tang T, Dalton N, Lai YY, Spellman M, Clopton P, Hammond HK. Intracoronary adenovirus encoding adenylyl cyclase VI increases left ventricular function in heart failure. *Circulation*. 2004;110:330–336. doi: 10.1161/01.CIR.0000136033.217774D
 58. Hammond HK, Penny WF, Traverse JH, Henry TD, Watkins MW, Yancy CW, Sweis RN, Adler ED, Patel AN, Murray DR, et al. Intracoronary gene transfer of adenylyl cyclase 6 in patients with heart failure: a randomized clinical trial. *JAMA Cardiol*. 2016;1:163–171. doi: 10.1001/jamacardio.2016.0008
 59. Tan Z, Giamouridis D, Lai NC, Kim YC, Guo T, Xia B, Gao MH, Hammond HK. Cardiac-directed expression of adenylyl cyclase catalytic domain (C1C2) attenuates deleterious effects of pressure overload. *Hum Gene Ther*. 2019;30:682–692. doi: 10.1089/hum.2018.176
 60. Gao MH, Lai NC, Giamouridis D, Kim YC, Tan Z, Guo T, Dillmann WH, Suarez J, Hammond HK. Cardiac-directed expression of adenylyl cyclase catalytic domain reverses cardiac dysfunction caused by sustained beta-adrenergic receptor stimulation. *JACC Basic Transl Sci*. 2016;1:617–629. doi: 10.1016/j.jaccbts.2016.08.004
 61. Pepe M, Mamdani M, Zentilin L, Csizsar A, Qanad K, Zacchigna S, Ungvari Z, Puligadda U, Moimas S, Xu X, et al. Intramyocardial VEGF-B167 gene delivery delays the progression towards congestive failure in dogs with pacing-induced dilated cardiomyopathy. *Circ Res*. 2010;106:1893–1903. doi: 10.1161/CIRCRESAHA.110.220855
 62. Woitek F, Zentilin L, Hoffman NE, Powers JC, Ottiger I, Parikh S, Kulczycki AM, Hurst M, Ring N, Wang T, et al. Intracoronary cytoprotective gene therapy: a study of VEGF-B167 in a pre-clinical animal model of dilated cardiomyopathy. *J Am Coll Cardiol*. 2015;66:139–153. doi: 10.1016/j.jacc.2015.04.071
 63. Plegler ST, Shan C, Ksienzyk J, Bekeredjian R, Bookstegers P, Hinkel R, Schinkel S, Leuchs B, Ludwig J, Qiu G, et al. Cardiac AAV9-S100A1 gene therapy rescues post-ischemic heart failure in a preclinical large animal model. *Sci Transl Med*. 2011;3:92ra64. doi: 10.1126/scitranslmed.3002097
 64. Penn MS, Pastore J, Miller T, Aras R. SDF-1 in myocardial repair. *Gene Ther*. 2012;19:583–587. doi: 10.1038/gt.2012.32
 65. Penn MS, Mendelsohn FO, Schaefer GL, Sherman W, Farr M, Pastore J, Rouy D, Clemens R, Aras R, Losordo DW. An open-label dose escalation study to evaluate the safety of administration of nonviral stromal cell-derived factor-1 plasmid to treat symptomatic ischemic heart failure. *Circ Res*. 2013;112:816–825. doi: 10.1161/CIRCRESAHA.111.300440
 66. Chung ES, Miller L, Patel AN, Anderson RD, Mendelsohn FO, Traverse J, Silver KH, Shin J, Ewald G, Farr MJ, et al. Changes in ventricular remodeling and clinical status during the year following a single administration of stromal cell-derived factor-1 non-viral gene therapy in chronic ischaemic heart failure patients: the STOP-HF randomized phase II trial. *Eur Heart J*. 2015;36:2228–2238. doi: 10.1093/eurheartj/ehv254
 67. Shishebor MH, Rundback J, Bunte M, Hammad TA, Miller L, Patel PD, Sadanandan S, Fitzgerald M, Pastore J, Kashyap V, et al. SDF-1 plasmid treatment for patients with peripheral artery disease (STOP-PAD): randomized, double-blind, placebo-controlled clinical trial. *Vasc Med*. 2019;24:200–207. doi: 10.1177/1358863X18817610
 68. Dor Y, Djonov V, Abramovitch R, Itin A, Fishman GI, Carmeliet P, Goelman G, Keshet E. Conditional switching of VEGF provides new insights into adult neovascularization and pro-angiogenic therapy. *EMBO J*. 2002;21:1939–1947. doi: 10.1093/emboj/21.8.1939
 69. Tafuro S, Ayuso E, Zacchigna S, Zentilin L, Moimas S, Dore F, Giacca M. Inducible adeno-associated virus vectors promote functional angiogenesis in adult organisms via regulated vascular endothelial growth factor expression. *Cardiovasc Res*. 2009;83:663–671. doi: 10.1093/cvr/cvp152
 70. Lehrman S. Virus treatment questioned after gene therapy death. *Nature*. 1999;401:517–518. doi: 10.1038/43977
 71. Mingozi F, High KA. Immune responses to AAV vectors: overcoming barriers to successful gene therapy. *Blood*. 2013;122:23–36. doi: 10.1182/blood-2013-01-306647
 72. Zacchigna S, Tasciotti E, Kusmic C, Arsic N, Sorace O, Marini C, Marzullo P, Pardini S, Petroni D, Pattarini L, et al. In vivo imaging shows abnormal function of vascular endothelial growth factor-induced vasculature. *Hum Gene Ther*. 2007;18:515–524. doi: 10.1089/hum.2006.162
 73. Karvinen H, Pasanen E, Rissanen TT, Korpisalo P, Vähäkangas E, Jazwa A, Giacca M, Ylä-Herttuala S. Long-term VEGF-A expression promotes aberrant angiogenesis and fibrosis in skeletal muscle. *Gene Ther*. 2011;18:1166–1172. doi: 10.1038/gt.2011.66
 74. Mingozi F, Anguela XM, Pavani G, Chen Y, Davidson RJ, Hui DJ, Yazicioglu M, Elkouby L, Hinderer CJ, Faella A, et al. Overcoming preexisting humoral immunity to AAV using capsid decoys. *Sci Transl Med*. 2013;5:194ra92. doi: 10.1126/scitranslmed.3005795
 75. Sorop O, Heinonen I, van Kranenburg M, van de Wouw J, de Beer VJ, Nguyen ITN, Octavia Y, van Duin RWB, Stam K, van Geuns RJ, et al. Multiple common comorbidities produce left ventricular diastolic dysfunction associated with coronary microvascular dysfunction, oxidative stress, and myocardial stiffening. *Cardiovasc Res*. 2018;114:954–964. doi: 10.1093/cvr/cvy038
 76. Hinkel R, Howe A, Renner S, Ng J, Lee S, Klett K, Kaczmarek V, Moretti A, Laugwitz KL, Skroblin P, et al. Diabetes mellitus-induced microvascular destabilization in the myocardium. *J Am Coll Cardiol*. 2017;69:131–143. doi: 10.1016/j.jacc.2016.10.058
 77. Askari AT, Unzek S, Popovic ZB, Goldman CK, Forudi F, Kiedrowski M, Rovner A, Ellis SG, Thomas JD, DiCorleto PE, et al. Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. *Lancet*. 2003;362:697–703. doi: 10.1016/S0140-6736(03)14232-8
 78. Eschenhagen T, Bolli R, Braun T, Field LJ, Fleischmann BK, Frisén J, Giacca M, Hare JM, Houser S, Lee RT, et al. Cardiomyocyte regeneration: a consensus statement. *Circulation*. 2017;136:680–686. doi: 10.1161/CIRCULATIONAHA.117.029343
 79. Gerritsen ME. HGF and VEGF: a dynamic duo. *Circ Res*. 2005;96:272–273. doi: 10.1161/01.RES.0000157575.66295.e0
 80. Creager MA, Olin JW, Belch JJ, Moneta GL, Henry TD, Rajagopalan S, Annex BH, Hiatt WR. Effect of hypoxia-inducible factor-1alpha gene therapy on walking performance in patients with intermittent claudication. *Circulation*. 2011;124:1765–1773. doi: 10.1161/CIRCULATIONAHA.110.009407
 81. Hinkel R, Trenkwalder T, Petersen B, Husada W, Gesenhues F, Lee S, Hannappel E, Bock-Marquette I, Theisen D, Leitner L, et al. MRTF-A controls vessel growth and maturation by increasing the expression of CCN1 and CCN2. *Nat Commun*. 2014;5:3970. doi: 10.1038/ncomms4970
 82. He L, Liu Q, Hu T, Huang X, Zhang H, Tian X, Yan Y, Wang L, Huang Y, Miquerol L, et al. Genetic lineage tracing discloses arteriogenesis as the main mechanism for collateral growth in the mouse heart. *Cardiovasc Res*. 2016;109:419–430. doi: 10.1093/cvr/cvw005
 83. Kocjan T, Rehman M, Colliva A, Leban M, Vodret S, Volf N, Zucca G, Cappelletto A, Bin Z, Adams R, et al. Genetic lineage tracing reveals poor angiogenic potential of cardiac endothelial cells. *Cardiovasc Res*. 2020. In press. doi: 10.1093/cvr/cvaa012
 84. Murry CE, Reinecke H, Pabon LM. Regeneration gaps: observations on stem cells and cardiac repair. *J Am Coll Cardiol*. 2006;47:1777–1785. doi: 10.1016/j.jacc.2006.02.002
 85. Braga L, Ali H, Secco I, Giacca M. Non-coding RNA therapeutics for cardiac regeneration. 2019. In press. doi: 10.1093/cvr/cvaa071
 86. Denegri M, Bongianino R, Lodola F, Boncompagni S, De Giusti VC, Avelino-Cruz JE, Liu N, Persampieri S, Curcio A, Esposito F, et al. Single delivery of an adeno-associated viral construct to transfer the CASQ2 gene to knock-in mice affected by catecholaminergic polymorphic ventricular tachycardia is able to cure the disease from birth to advanced age. *Circulation*. 2014;129:2673–2681. doi: 10.1161/CIRCULATIONAHA.113.006901
 87. Bongianino R, Priori SG. Gene therapy to treat cardiac arrhythmias. *Nat Rev Cardiol*. 2015;12:531–546. doi: 10.1038/nrcardio.2015.61

88. Donahue JK. Current state of the art for cardiac arrhythmia gene therapy. *Pharmacol Ther*. 2017;176:60–65. doi: 10.1016/j.pharmthera.2017.06.005
89. Trivedi A, Hoffman J, Arora R. Gene therapy for atrial fibrillation - how close to clinical implementation? *Int J Cardiol*. 2019;296:177–183. doi: 10.1016/j.ijcard.2019.07.057
90. Liu N, Olson EN. MicroRNA regulatory networks in cardiovascular development. *Dev Cell*. 2010;18:510–525. doi: 10.1016/j.devcel.2010.03.010
91. Porrello ER. microRNAs in cardiac development and regeneration. *Clin Sci (Lond)*. 2013;125:151–166. doi: 10.1042/CS20130011
92. Boon RA, Dimmeler S. MicroRNAs in myocardial infarction. *Nat Rev Cardiol*. 2015;12:135–142. doi: 10.1038/nrcardio.2014.207
93. Kopp F, Mendell JT. Functional classification and experimental dissection of long noncoding RNAs. *Cell*. 2018;172:393–407. doi: 10.1016/j.cell.2018.01.011
94. Hobuß L, Bär C, Thum T. Long non-coding RNAs: at the heart of cardiac dysfunction? *Front Physiol*. 2019;10:30. doi: 10.3389/fphys.2019.00030
95. Salamon I, Saccani Jotti G, Condorelli G. The long noncoding RNA landscape in cardiovascular disease: a brief update. *Curr Opin Cardiol*. 2018;33:282–289. doi: 10.1097/HCO.0000000000000507
96. Lucas T, Bonauer A, Dimmeler S. RNA therapeutics in cardiovascular disease. *Circ Res*. 2018;123:205–220. doi: 10.1161/CIRCRESAHA.117.311311
97. Lennox KA, Behlke MA. Chemical modification and design of anti-miRNA oligonucleotides. *Gene Ther*. 2011;18:1111–1120. doi: 10.1038/gt.2011.100
98. van Rooij E, Olson EN. MicroRNA therapeutics for cardiovascular disease: opportunities and obstacles. *Nat Rev Drug Discov*. 2012;11:860–872. doi: 10.1038/nrd3864
99. Elmén J, Lindow M, Schütz S, Lawrence M, Petri A, Obad S, Lindholm M, Hedtjörn M, Hansen HF, Berger U, et al. LNA-mediated microRNA silencing in non-human primates. *Nature*. 2008;452:896–899. doi: 10.1038/nature06783
100. Fluiter K, Mook OR, Vreijling J, Langkjaer N, Højland T, Wengel J, Baas F. Filling the gap in LNA antisense oligo gapmers: the effects of unlocked nucleic acid (UNA) and 4'-C-hydroxymethyl-DNA modifications on RNase H recruitment and efficacy of an LNA gapmer. *Mol Biosyst*. 2009;5:838–843. doi: 10.1039/b903922h
101. Carè A, Catalucci D, Felicetti F, Bonci D, Addario A, Gallo P, Bang ML, Segnalini P, Gu Y, Dalton ND, et al. MicroRNA-133 controls cardiac hypertrophy. *Nat Med*. 2007;13:613–618. doi: 10.1038/nm1582
102. Thum T, Gross C, Fiedler J, Fischer T, Kissler S, Bussen M, Galuppo P, Just S, Rotbauer W, Frantz S, et al. MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. *Nature*. 2008;456:980–984. doi: 10.1038/nature07511
103. Ucar A, Gupta SK, Fiedler J, Eriki E, Kardasinski M, Batkai S, Dangwal S, Kumarswamy R, Bang C, Holzmann A, et al. The miRNA-212/132 family regulates both cardiac hypertrophy and cardiomyocyte autophagy. *Nat Commun*. 2012;3:1078. doi: 10.1038/ncomms2090
104. Boon RA, Iekushi K, Lechner S, Seeger T, Fischer A, Heydt S, Kaluza D, Tréguer K, Carmona G, Bonauer A, et al. MicroRNA-34a regulates cardiac ageing and function. *Nature*. 2013;495:107–110. doi: 10.1038/nature11919
105. van Rooij E, Sutherland LB, Thatcher JE, DiMaio JM, Naseem RH, Marshall WS, Hill JA, Olson EN. Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. *Proc Natl Acad Sci USA*. 2008;105:13027–13032. doi: 10.1073/pnas.0805038105
106. Wahlquist C, Jeong D, Rojas-Muñoz A, Kho C, Lee A, Mitsuyama S, van Mil A, Park WJ, Sluijter JP, Doevendans PA, et al. Inhibition of miR-25 improves cardiac contractility in the failing heart. *Nature*. 2014;508:531–535. doi: 10.1038/nature13073
107. Bonauer A, Carmona G, Iwasaki M, Miome M, Koyanagi M, Fischer A, Burchfield J, Fox H, Doebele C, Ohtani K, et al. MicroRNA-92a controls angiogenesis and functional recovery of ischemic tissues in mice. *Science*. 2009;324:1710–1713. doi: 10.1126/science.1174381
108. Hinkel R, Penzkofer D, Zühlke S, Fischer A, Husada W, Xu QF, Baloch E, van Rooij E, Zeiher AM, Kupatt C, et al. Inhibition of microRNA-92a protects against ischemia/reperfusion injury in a large-animal model. *Circulation*. 2013;128:1066–1075. doi: 10.1161/CIRCULATIONAHA.113.001904
109. da Costa Martins PA, Salic K, Gladka MM, Armand AS, Leptidis S, el Azzouzi H, Hansen A, Coenen-de Roo CJ, Bierhuizen MF, van der Nagel R, et al. MicroRNA-199b targets the nuclear kinase Dyrk1a in an auto-amplification loop promoting calcineurin/NFAT signalling. *Nat Cell Biol*. 2010;12:1220–1227. doi: 10.1038/ncb2126
110. Fiedler J, Jazbutyte V, Kirchmaier BC, Gupta SK, Lorenzen J, Hartmann D, Galuppo P, Kneitz S, Pena JT, Sohn-lee C, et al. MicroRNA-24 regulates vascularity after myocardial infarction. *Circulation*. 2011;124:720–730. doi: 10.1161/CIRCULATIONAHA.111.039008
111. Halkein J, Tabruyn SP, Ricke-Hoch M, Haghikia A, Nguyen NQ, Scherr M, Castermans K, Malvaux L, Lambert V, Thiry M, et al. MicroRNA-146a is a therapeutic target and biomarker for peripartum cardiomyopathy. *J Clin Invest*. 2013;123:2143–2154. doi: 10.1172/JCI64365
112. De Majo F, De Windt LJ. RNA therapeutics for heart disease. *Biochem Pharmacol*. 2018;155:468–478. doi: 10.1016/j.bcp.2018.07.037
113. Piccoli MT, Gupta SK, Viereck J, Foinquinos A, Samolovac S, Kramer FL, Garg A, Remke J, Zimmer K, Batkai S, et al. Inhibition of the cardiac fibroblast-enriched lncRNA Meg3 prevents cardiac fibrosis and diastolic dysfunction. *Circ Res*. 2017;121:575–583. doi: 10.1161/CIRCRESAHA.117.310624
114. Viereck J, Kumarswamy R, Foinquinos A, Xiao K, Avramopoulos P, Kunz M, Dittrich M, Maetzig T, Zimmer K, Remke J, et al. Long noncoding RNA chast promotes cardiac remodeling. *Sci Transl Med*. 2016;8:326ra22. doi: 10.1126/scitranslmed.aaf1475
115. Micheletti R, Plaisance I, Abraham BJ, Sarre A, Ting CC, Alexanian M, Maric D, Maison D, Nemir M, Young RA, et al. The long noncoding RNA Wisper controls cardiac fibrosis and remodeling. *Sci Transl Med*. 2017;9:eaai9118. doi: 10.1126/scitranslmed.aai9118
116. Matsui M, Corey DR. Non-coding RNAs as drug targets. *Nat Rev Drug Discov*. 2017;16:167–179. doi: 10.1038/nrd.2016.117
117. Stein CA, Castanotto D. FDA-approved oligonucleotide therapies in 2017. *Mol Ther*. 2017;25:1069–1075. doi: 10.1016/j.jymthe.2017.03.023
118. Filion MC, Phillips NC. Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells. *Biochim Biophys Acta*. 1997;1329:345–356. doi: 10.1016/s0005-2736(97)00126-0
119. Ito Y, Kawakami S, Charoensit P, Higuchi Y, Hashida M. Evaluation of proinflammatory cytokine production and liver injury induced by plasmid DNA/cationic liposome complexes with various mixing ratios in mice. *Eur J Pharm Biopharm*. 2009;71:303–309. doi: 10.1016/j.ejpb.2008.09.005
120. Beg MS, Brenner AJ, Sachdev J, Borad M, Kang YK, Stoudemire J, Smith S, Bader AG, Kim S, Hong DS. Phase I study of MRX34, a liposomal miR-34a mimic, administered twice weekly in patients with advanced solid tumors. *Invest New Drugs*. 2017;35:180–188. doi: 10.1007/s10637-016-0407-y
121. Gao F, Kataoka M, Liu N, Liang T, Huang ZP, Gu F, Ding J, Liu J, Zhang F, Ma Q, et al. Therapeutic role of miR-19a/19b in cardiac regeneration and protection from myocardial infarction. *Nat Commun*. 2019;10:1802. doi: 10.1038/s41467-019-09530-1
122. Tian Y, Liu Y, Wang T, Zhou N, Kong J, Chen L, Snitow M, Morley M, Li D, Petrenko N, et al. A microRNA-Hippo pathway that promotes cardiomyocyte proliferation and cardiac regeneration in mice. *Sci Transl Med*. 2015;7:279ra238. doi: 10.1126/scitranslmed.3010841
123. Kulkarni JA, Cullis PR, van der Meel R. Lipid nanoparticles enabling gene therapies: from concepts to clinical utility. *Nucleic Acid Ther*. 2018;28:146–157. doi: 10.1089/nat.2018.0721
124. Adams D, Gonzalez-Duarte A, O'Riordan WD, Yang CC, Ueda M, Kristen AV, Tourneir I, Schmidt HH, Coelho T, Berk JL, et al. Patisiran, an RNAi therapeutic, for hereditary transthyretin amyloidosis. *N Engl J Med*. 2018;379:11–21. doi: 10.1056/NEJMoa1716153
125. Ray KK, Landmesser U, Leiter LA, Kallend D, Dufour R, Karakas M, Hall T, Troquay RP, Turner T, Visseren FL, et al. Inclisiran in patients at high cardiovascular risk with elevated LDL cholesterol. *N Engl J Med*. 2017;376:1430–1440. doi: 10.1056/NEJMoa1615758
126. Christian DA, Cai S, Bowen DM, Kim Y, Pajeroski JD, Discher DE. Polymersome carriers: from self-assembly to siRNA and protein therapeutics. *Eur J Pharm Biopharm*. 2009;71:463–474. doi: 10.1016/j.ejpb.2008.09.025
127. Bellera N, Barba I, Rodriguez-Sinovas A, Ferrer E, Asin MA, Gonzalez-Alujas MT, Pérez-Rodon J, Esteves M, Fonseca C, Toran N, et al. Single intracoronary injection of encapsulated antagomir-92a promotes angiogenesis and prevents adverse infarct remodeling. *J Am Heart Assoc*. 2014;3:e000946. doi: 10.1161/JAHA.114.000946
128. Conde J, Oliva N, Atilano M, Song HS, Artzi N. Self-assembled RNA-triple-helix hydrogel scaffold for microRNA modulation in the tumour microenvironment. *Nat Mater*. 2016;15:353–363. doi: 10.1038/nmat4497
129. Boca S, Gulei D, Zimta AA, Onaci A, Magdo L, Tigu AB, Ionescu C, Irimie A, Buiga R, Berindan-Neagoe I. Nanoscale delivery systems for microRNAs in cancer therapy. *Cell Mol Life Sci*. 2020;77:1059–1086.
130. MacDiarmid JA, Brahmabhatt H. Minicells: versatile vectors for targeted drug or si/shRNA cancer therapy. *Curr Opin Biotechnol*. 2011;22:909–916. doi: 10.1016/j.copbio.2011.04.008

131. van Zandwijk N, Pavlakis N, Kao SC, Linton A, Boyer MJ, Clarke S, Huynh Y, Chranowska A, Fulham MJ, Bailey DL, et al. Safety and activity of microRNA-loaded minicells in patients with recurrent malignant pleural mesothelioma: a first-in-man, phase 1, open-label, dose-escalation study. *Lancet Oncol*. 2017;18:1386–1396. doi: 10.1016/S1470-2045(17)30621-6
132. Jiang L, Vader P, Schiffelers RM. Extracellular vesicles for nucleic acid delivery: progress and prospects for safe RNA-based gene therapy. *Gene Ther*. 2017;24:157–166. doi: 10.1038/gt.201778
133. Mathiyalagan P, Sahoo S. Exosomes-based gene therapy for MicroRNA delivery. *Methods Mol Biol*. 2017;1521:139–152. doi: 10.1007/978-1-4939-6588-5_9
134. Ohno S, Kuroda M. Exosome-mediated targeted delivery of miRNAs. *Methods Mol Biol*. 2016;1448:261–270. doi: 10.1007/978-1-4939-3753-0_19
135. Hashimoto H, Olson EN, Bassel-Duby R. Therapeutic approaches for cardiac regeneration and repair. *Nat Rev Cardiol*. 2018;15:585–600. doi: 10.1038/s41569-018-0036-6
136. Tzahor E, Poss KD. Cardiac regeneration strategies: staying young at heart. *Science*. 2017;356:1035–1039. doi: 10.1126/science.aam5894
137. Uygur A, Lee RT. Mechanisms of cardiac regeneration. *Dev Cell*. 2016;36:362–374. doi: 10.1016/j.devcel.2016.01.018
138. Singh AP, Umbarkar P, Guo Y, Force T, Gupte M, Lal H. Inhibition of GSK-3 to induce cardiomyocyte proliferation: a recipe for in situ cardiac regeneration. *Cardiovasc Res*. 2019;115:20–30. doi: 10.1093/cvr/cvy255
139. Pedrazzini T. Control of cardiogenesis by the notch pathway. *Trends Cardiovasc Med*. 2007;17:83–90. doi: 10.1016/j.tcm.2007.01.003
140. Xiao Y, Leach J, Wang J, Martin JF. Hippo/yap signaling in cardiac development and regeneration. *Curr Treat Options Cardiovasc Med*. 2016;18:38. doi: 10.1007/s11936-016-0461-y
141. Bassat E, Mutlak YE, Genzelinakh A, Shadrin IY, Baruch Umansky K, Yifa O, Kain D, Rajchman D, Leach J, Riabov Bassat D, et al. The extracellular matrix protein agrin promotes heart regeneration in mice. *Nature*. 2017;547:179–184. doi: 10.1038/nature22978
142. Engel FB, Hsieh PC, Lee RT, Keating MT. FGF1/p38 MAP kinase inhibitor therapy induces cardiomyocyte mitosis, reduces scarring, and rescues function after myocardial infarction. *Proc Natl Acad Sci USA*. 2006;103:15546–15551. doi: 10.1073/pnas.0607382103
143. Collesi C, Zentilin L, Sinagra G, Giacca M. Notch1 signaling stimulates proliferation of immature cardiomyocytes. *J Cell Biol*. 2008;183:117–128. doi: 10.1083/jcb.200806091
144. Campa VM, Gutiérrez-Lanza R, Cerignoli F, Díaz-Trelles R, Nelson B, Tsuji T, Barcova M, Jiang W, Mercola M. Notch activates cell cycle reentry and progression in quiescent cardiomyocytes. *J Cell Biol*. 2008;183:129–141. doi: 10.1083/jcb.200806104
145. Felician G, Collesi C, Lusic M, Martinelli V, Ferro MD, Zentilin L, Zacchigna S, Giacca M. Epigenetic modification at Notch responsive promoters blunts efficacy of inducing notch pathway reactivation after myocardial infarction. *Circ Res*. 2014;115:636–649. doi: 10.1161/CIRCRESAHA.115.304517
146. D'Uva G, Aharonov A, Lauriola M, Kain D, Yahalom-Ronen Y, Carvalho S, Weisinger K, Bassat E, Rajchman D, Yifa O, et al. ERBB2 triggers mammalian heart regeneration by promoting cardiomyocyte dedifferentiation and proliferation. *Nat Cell Biol*. 2015;17:627–638. doi: 10.1038/ncb3149
147. Xin M, Kim Y, Sutherland LB, Murakami M, Qi X, McAnally J, Porrello ER, Mahmoud AI, Tan W, Shelton JM, et al. Hippo pathway effector Yap promotes cardiac regeneration. *Proc Natl Acad Sci USA*. 2013;110:13839–13844. doi: 10.1073/pnas.1313192110
148. Heallen T, Morikawa Y, Leach J, Tao G, Willerson JT, Johnson RL, Martin JF. Hippo signaling impedes adult heart regeneration. *Development*. 2013;140:4683–4690. doi: 10.1242/dev.102798
149. Lin Z, von Gise A, Zhou P, Gu F, Ma Q, Jiang J, Yau AL, Buck JN, Gouin KA, van Gorp PR, et al. Cardiac-specific YAP activation improves cardiac function and survival in an experimental murine MI model. *Circ Res*. 2014;115:354–363. doi: 10.1161/CIRCRESAHA.115.303632
150. Leach JP, Heallen T, Zhang M, Rahmani M, Morikawa Y, Hill MC, Segura A, Willerson JT, Martin JF. Hippo pathway deficiency reverses systolic heart failure after infarction. *Nature*. 2017;550:260–264. doi: 10.1038/nature24045
151. Kakiuchi-Kiyota S, Schutten MM, Zhong Y, Crawford JJ, Dey A. Safety considerations in the development of hippo pathway inhibitors in cancers. *Front Cell Dev Biol*. 2019;7:156. doi: 10.3389/fcell.2019.00156
152. Collesi C, Giacca M. Gene transfer to promote cardiac regeneration. *Crit Rev Clin Lab Sci*. 2016;53:359–369. doi: 10.1080/10408363.2016.1176987
153. Di Stefano V, Giacca M, Capogrossi MC, Crescenzi M, Martelli F. Knockdown of cyclin-dependent kinase inhibitors induces cardiomyocyte re-entry in the cell cycle. *J Biol Chem*. 2011;286:8644–8654. doi: 10.1074/jbc.M110.184549
154. Ebel T, Hufnagel N, Neuhaus P, Neuhaus H, Gajawada P, Simm A, Müller-Werdan U, Werdan K, Braun T. Divergent siblings: E2F2 and E2F4 but not E2F1 and E2F3 induce DNA synthesis in cardiomyocytes without activation of apoptosis. *Circ Res*. 2005;96:509–517. doi: 10.1161/01.RES.0000159705.17322.57
155. Mohamed TMA, Ang YS, Radzinsky E, Zhou P, Huang Y, Eifenbein A, Foley A, Magnitsky S, Srivastava D. Regulation of cell cycle to stimulate adult cardiomyocyte proliferation and cardiac regeneration. *Cell*. 2018;173:104–116.e12. doi: 10.1016/j.cell.2018.02.014
156. Eulalio A, Mano M, Dal Ferro M, Zentilin L, Sinagra G, Zacchigna S, Giacca M. Functional screening identifies miRNAs inducing cardiac regeneration. *Nature*. 2012;492:376–381. doi: 10.1038/nature11739
157. Diez-Cuñado M, Wei K, Bushway PJ, Maurya MR, Perera R, Subramaniam S, Ruiz-Lozano P, Mercola M. miRNAs that induce human cardiomyocyte proliferation converge on the hippo pathway. *Cell Rep*. 2018;23:2168–2174. doi: 10.1016/j.celrep.2018.04.049
158. Chen J, Huang ZP, Seok HY, Ding J, Kataoka M, Zhang Z, Hu X, Wang G, Lin Z, Wang S, et al. mir-17-92 cluster is required for and sufficient to induce cardiomyocyte proliferation in postnatal and adult hearts. *Circ Res*. 2013;112:1557–1566. doi: 10.1161/CIRCRESAHA.112.300658
159. Porrello ER, Johnson BA, Aurora AB, Simpson E, Nam YJ, Matkovich SJ, Dorn GW II, van Rooij E, Olson EN. MiR-15 family regulates postnatal mitotic arrest of cardiomyocytes. *Circ Res*. 2011;109:670–679. doi: 10.1161/CIRCRESAHA.111.248880
160. Aguirre A, Montserrat N, Zacchigna S, Nivet E, Hishida T, Krause MN, Kurian L, Ocampo A, Vazquez-Ferrer E, Rodriguez-Esteban C, et al. In vivo activation of a conserved microRNA program induces mammalian heart regeneration. *Cell Stem Cell*. 2014;15:589–604. doi: 10.1016/j.stem.2014.10.003
161. Porrello ER, Mahmoud AI, Simpson E, Johnson BA, Grinsfelder D, Canseco D, Mammen PP, Rothermel BA, Olson EN, Sadek HA. Regulation of neonatal and adult mammalian heart regeneration by the miR-15 family. *Proc Natl Acad Sci USA*. 2013;110:187–192. doi: 10.1073/pnas.1208863110
162. Hullinger TG, Montgomery RL, Seto AG, Dickinson BA, Semus HM, Lynch JM, Dalby CM, Robinson K, Stack C, Latimer PA, et al. Inhibition of miR-15 protects against cardiac ischemic injury. *Circ Res*. 2012;110:71–81. doi: 10.1161/CIRCRESAHA.111.244442
163. Yang Y, Cheng HW, Qiu Y, Dupee D, Noonan M, Lin YD, Fisch S, Unno K, Sereti KI, Liao R. MicroRNA-34a plays a key role in cardiac repair and regeneration following myocardial infarction. *Circ Res*. 2015;117:450–459. doi: 10.1161/CIRCRESAHA.117.305962
164. Borden A, Kurian J, Nickoloff E, Yang Y, Dupee D, Troupes CD, Ibbett J, Lucchese AM, Gao E, Mohsin S, Koch WJ, et al. Transient introduction of miR-294 in the heart promotes cardiomyocyte cell cycle reentry after injury. *Circ Res*. 2019;125:14–25. doi: 10.1161/CIRCRESAHA.118.314223
165. Gabisonia K, Prosdocimo G, Aquaro GD, Carlucci L, Zentilin L, Secco I, Ali H, Braga L, Gorgodce N, Bernini F, et al. MicroRNA therapy stimulates uncontrolled cardiac repair after myocardial infarction in pigs. *Nature*. 2019;569:418–422. doi: 10.1038/s41586-019-1191-6
166. Lesizza P, Prosdocimo G, Martinelli V, Sinagra G, Zacchigna S, Giacca M. Single-dose intracardiac injection of pro-regenerative MicroRNAs improves cardiac function after myocardial infarction. *Circ Res*. 2017;120:1298–1304. doi: 10.1161/CIRCRESAHA.116.309589
167. van Gent DC, Hoeijmakers JH, Kanaar R. Chromosomal stability and the DNA double-stranded break connection. *Nat Rev Genet*. 2001;2:196–206. doi: 10.1038/35056049
168. Doetschman T, Georgieva T. Gene editing with CRISPR/Cas9 RNA-directed nuclease. *Circ Res*. 2017;120:876–894. doi: 10.1161/CIRCRESAHA.116.309727
169. McNally EM, Mestroni L. Dilated cardiomyopathy: genetic determinants and mechanisms. *Circ Res*. 2017;121:731–748. doi: 10.1161/CIRCRESAHA.116.309396
170. Wasala NB, Shin JH, Lai Y, Yue Y, Montanaro F, Duan D. Cardiac-specific expression of ΔH2-R15 mini-dystrophin normalized all electrocardiogram abnormalities and the end-diastolic volume in a 23-month-old mouse model of Duchenne dilated cardiomyopathy. *Hum Gene Ther*. 2018;29:737–748. doi: 10.1089/hum.2017.144
171. Nguyen Q, Yokota T. Antisense oligonucleotides for the treatment of cardiomyopathy in Duchenne muscular dystrophy. *Am J Transl Res*. 2019;11:1202–1218.
172. Gramlich M, Pane LS, Zhou Q, Chen Z, Murgia M, Schötterl S, Goedel A, Metzger K, Brade T, Parrotta E, et al. Antisense-mediated exon skipping:

- a therapeutic strategy for titin-based dilated cardiomyopathy. *EMBO Mol Med*. 2015;7:562–576. doi: 10.15252/emmm.201505047
173. Quattrocelli M, Crippa S, Montecchiani C, Camps J, Cornaglia AI, Boldrin L, Morgan J, Calligaro A, Casasco A, Orlacchio A, et al. Long-term miR-669a therapy alleviates chronic dilated cardiomyopathy in dystrophic mice. *J Am Heart Assoc*. 2013;2:e000284. doi: 10.1161/JAHA.113.000284
174. Azibani F, Brull A, Arandel L, Beuvin M, Nelson I, Jollet A, Ziat E, Prudhon B, Benkhelifa-Ziyyat S, Bitoun M, et al. Gene therapy via trans-splicing for LMNA-related congenital muscular dystrophy. *Mol Ther Nucleic Acids*. 2018;10:376–386. doi: 10.1016/j.omtn.2017.12.012
175. Amoasii L, Hildyard JCW, Li H, Sanchez-Ortiz E, Mireault A, Caballero D, Harron R, Stathopoulou TR, Massey C, Shelton JM, et al. Gene editing restores dystrophin expression in a canine model of Duchenne muscular dystrophy. *Science*. 2018;362:86–91. doi: 10.1126/science.aau1549
176. Moretti A, Fonteyne L, Giesert F, Hoppmann P, Meier AB, Bozoglu T, Baehr A, Schneider CM, Sinnecker D, Klett K, et al. Somatic gene editing ameliorates skeletal and cardiac muscle failure in pig and human models of Duchenne muscular dystrophy. *Nat Med*. 2020;26:207–214. doi: 10.1038/s41591-019-0738-2
177. Marian AJ, Braunwald E. Hypertrophic cardiomyopathy: genetics, pathogenesis, clinical manifestations, diagnosis, and therapy. *Circ Res*. 2017;121:749–770. doi: 10.1161/CIRCRESAHA.117.311059
178. Mearini G, Stimpel D, Geertz B, Weinberger F, Krämer E, Schlossarek S, Mourot-Filiatre J, Stoehr A, Dutsch A, Wijnker PJ, et al. Mybpc3 gene therapy for neonatal cardiomyopathy enables long-term disease prevention in mice. *Nat Commun*. 2014;5:5515. doi: 10.1038/ncomms6515
179. Yadav S, Yuan CC, Kazmierczak K, Liang J, Huang W, Takeuchi LM, Kanashiro-Takeuchi RM, Szczesna-Cordary D. Therapeutic potential of AAV9-S15D-RLC gene delivery in humanized MYL2 mouse model of HCM. *J Mol Med (Berl)*. 2019;97:1033–1047. doi: 10.1007/s00109-019-01791-z
180. Salvarani N, Crasto S, Miragoli M, Bertero A, Paulis M, Kunderfranco P, Serio S, Forni A, Lucarelli C, Dal Ferro M, et al. The K219T-Lamin mutation induces conduction defects through epigenetic inhibition of SCN5A in human cardiac laminopathy. *Nat Commun*. 2019;10:2267. doi: 10.1038/s41467-019-09929-w
181. Karakikes I, Stillitano F, Nonnenmacher M, Tzimas C, Sanoudou D, Termglinchan V, Kong CW, Rushing S, Hansen J, Ceholski D, et al. Correction of human phospholamban R14del mutation associated with cardiomyopathy using targeted nucleases and combination therapy. *Nat Commun*. 2015;6:6955. doi: 10.1038/ncomms7955
182. Johansen AK, Molenaar B, Versteeg D, Leitoguinho AR, Demkes C, Spanjaard B, de Ruiten H, Akbari Moqadam F, Kooijman L, Zentilin L, et al. Postnatal cardiac gene editing using CRISPR/Cas9 with AAV9-mediated delivery of short guide RNAs results in mosaic gene disruption. *Circ Res*. 2017;121:1168–1181. doi: 10.1161/CIRCRESAHA.116.310370
183. Carroll KJ, Makarewich CA, McAnally J, Anderson DM, Zentilin L, Liu N, Giacca M, Bassel-Duby R, Olson EN. A mouse model for adult cardiac-specific gene deletion with CRISPR/Cas9. *Proc Natl Acad Sci USA*. 2016;113:338–343. doi: 10.1073/pnas.1523918113
184. Ma H, Marti-Gutierrez N, Park SW, Wu J, Lee Y, Suzuki K, Koski A, Ji D, Hayama T, Ahmed R, et al. Correction of a pathogenic gene mutation in human embryos. *Nature*. 2017;548:413–419. doi: 10.1038/nature23305