Chapter

Gene Therapy for Heart Disease: Modified mRNA Perspectives

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Abstract

Ischemic heart disease (IHD) presents a gigantic clinical challenge that demands effective therapeutic approaches. With increasing knowledge of the basic molecular mechanisms guiding the progress of this disease, it is now possible to target the key pathological players through gene therapy. Modified mRNA-based gene delivery presents a promising alternative to traditional gene therapy, because modRNA approaches have high potency, non-immunogenicity, greater efficiency and controlled nucleic acid transfer to the body. However, until recently the therapeutic applications of mRNA have been limited, as naturally occurring mRNA is rapidly degraded and cleared from the circulation. In this chapter, we outline the compositional changes made to mRNA to enhance its translational capacity and discuss the available carrier molecules currently being employed to deliver modRNA to the heart. We provide a detailed overview of modRNA applicability for cardiac repair and regeneration and consider future directions for novel delivery methods that can facilitate its cardiac therapeutic use.

Keywords: cardiac repair, cardiac regeneration, gene therapy, modified mRNA, non-viral gene delivery

1. Introduction

1.1 Ischemic heart disease

Ischemic heart disease (IHD), a life-threatening cardiac condition, is the most common cause of heart failure, which is the leading cause of mortality worldwide, and in developed countries its burden is rivaled only by cancer. Systolic dysfunction and reduced cardiac output are the major hallmarks of ischemic heart failure (IHF). It is hard to determine the etiology of IHF, which is often associated with multiple underlying diseases, including ischemic cardiomyopathy, coronary artery disease or previous myocardial infarction (MI), that cause an imbalance between myocardial oxygen demand and supply. Decreased or complete loss of blood supply to an area of the myocardium for a prolonged period has detrimental effects on cardiomyocytes (CM). Myocardial ischemia progressively results in either reversible, via myocardial stunning and hibernation, or irreversible loss of CM. Following myocardial ischemia, a heavy burst of cell damage or death occurs in the infarct region, mostly within the first 24 hours [1]. However, low-grade cell death continues in the peri-infarct zone for months due to a complex cascade of molecular and cellular events [2]. Because the adult heart cannot regenerate, newer therapeutic approaches are needed to halt the ongoing damage that follows myocardial ischemia. Multiple studies have consistently

indicated that attenuating apoptotic cell death pathways (death receptor and mitochondrial apoptosis pathways) could be a viable therapeutic strategy for MI [3–6].

Soon after MI, a spectrum of clinical changes occurs in the heart and eventually results in heart failure. Billions of CMs die, triggering a multiphase reparative response known as cardiac remodeling that can be divided into three distinct but overlapping phases: the inflammatory phase, the proliferative phase and the maturation or healing phase [7]. The early inflammatory phase, characterized by immune cell recruitment and infiltration, clears dead cells and debris from the infarct region and prepares the injured region for the proliferative phase, during which mesenchymal reparative cells, mainly myofibroblasts and endothelial cells, proliferate and form granulation tissue. Myofibroblasts secrete excessive extracellular matrix proteins that play a critical role in preserving the structural integrity of the damaged heart. As myofibroblast and endothelial cell apoptosis ends, the maturation phase begins and heals the infarct into a collagen-rich fibrotic scar [8]. Post-ischemic myocardial remodeling replaces most of the dead necrotic infarct with fibrotic nonfunctional scar tissue, which increases the mechanical load on the adjacent myocardium and thus reduces the pumping capacity of the heart muscle. Informed by a detailed understanding of these mechanisms, gene-based therapy for IHD aims to upregulate the genes involved in (a) activating adult CM proliferation, (b) preventing cardiac cell apoptosis and necrosis, (c) reducing the innate and adaptive immune response and (d) inducing angiogenesis. Several studies utilize gene therapy strategies to investigate and treat IHD.

In the last two decades, emerging gene therapies have sought to improve heart function after MI. However, treatments involving traditional vectors have produced few meaningful results. Efforts to use DNA-based or viral gene delivery methods to induce cardiac repair processes post MI or in HF conditions have failed due to poor, uncontrolled gene delivery [9, 10]. Current pharmacological therapeutics help manage symptoms and improve quality of life, but these approaches lack organ specificity and cannot permanently remedy the disease. Synthetic modified mRNA (modRNA) is a promising gene delivery vector, and the Pfizer-BioNTech and Moderna vaccines against the novel coronavirus SARS-COV-2 have generated great optimism about its prospects in other areas. ModRNA therapeutics offer several advantages over conventional vectors, as they offer safe, non-immunogenic, efficient, transient, local, controlled delivery of an almost limitless range of nucleic acids to nearly any part of the human body. Over the last eight years, i.e. since the first time modRNA was delivered to the heart [11], various pre-clinical studies have explored using modRNA for gene introduction following IHD. In this chapter, we will focus on structural modifications that allow modRNA to escape the immune response, modRNA carrier systems in the heart and the use of modRNA for upregulating genes that support cardiovascular regeneration.

2. Exogenous mRNA translation

The process of gene expression begins with DNA transcription to messenger RNA (mRNA), which is then translated into a protein. Following the pre-mRNA transcript for synthesis, post-transcriptional or co-transcriptional modifications produce a mature, functional RNA molecule that can leave the nucleus to perform a variety of functions in the cell. These post-transcriptional modifications significantly alter the chemical structure of the RNA molecule in three major ways: (a) capping at 5′ end, (b) polyadenylation at 3′ end and (c) splicing introns in the coding region. These processes are vital to mature mRNA production and translation.

Capping is the first modification and involves adding N7-methylated guanosine to the first nucleotide at the 5′-end of growing nascent mRNA transcripts.

Synchronized with transcription, this modification takes effect immediately as the first 25–30 nucleotides of the nascent transcript are synthesized [12]. The 5′ cap not only protects nascent mRNA from 5′ to 3′ exonuclease cleavage and degradation but also facilitates mRNA splicing, polyadenylation and nuclear export. There is also evidence that 5′ untranslated region (UTR) plays a critical role in initiating protein synthesis [13]. Polyadenylation is another major post-transcriptional modification of the pre-mRNA transcript that occurs before the mature mRNA leaves the nucleus. As transcriptions terminate, polyadenylation begins when an endonuclease-protein complex releases the functional pre-mRNA by cleaving it between an AAUAAA consensus sequence and a GU-rich sequence at the 3′ end from the growing transcript. An enzyme called poly (A) polymerase (PAP), which is part of the endonuclease-protein complex, catalyzes the addition of an approximately 200-adenine-nucleotide string, also known as a polyA tail, to the 3′ end of cleaved pre-mRNA [14, 15]. Changes in these components of mRNA are discussed later the chapter.

These post-transcriptional pre-mRNA processes take place in the nucleus, and only the successfully processed mature mRNA molecules are exported out of the nucleus, whereas the faulty mRNAs and spliced-out introns are degraded by a specialized multi-subunit exosome complex. In the cytoplasm, the mature functional mRNA will be either directed to ribosomes for translation or shuttled to other compartments for sequestration and/or degradation [16].

3. Innate immune response

The innate immune system is the first line of defense against pathogens. It plays a critical role in initiating the protective immune response by detecting exogenous motifs, a process also known as recognizing pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs) present in many immune cell types, particularly sentinel APCs. Nucleic acids (DNA and RNA) have been documented as key PAMPs and are known to initiate a complex cascade of intracellular signaling pathways leading to the production of proinflammatory cytokines and chemokines. Therefore, synthetic or exogenously administered mRNA molecules are inherently immunogenic. Toll-like receptors (TLRs), the type I transmembrane proteins, are the major PRRs that contribute to RNA recognition in the endosomal compartment. TLR3 is localized in the endosomes of innate immune cells and recognizes the dsRNA. Upon binding to dsRNA, TLR3 is activated and recruits its downstream adaptor TRIF (TIR-domain containing protein inducing type 1 Interferon (IFN)-I), which facilitates the formation of TRIF signaling complex. This complex activates NF-κB and IRF3/IRF7 transcription factors that ultimately boost the transcription of proinflammatory cytokines and interferons (IFNs), respectively.

Alternatively, single-stranded RNA (ssRNA) and their degradative products in the endosomes are sensed by Toll-like receptor 7 (TLR7) and TLR8 [17], which recognize and bind to ssRNA, leading to confirmation changes in receptor dimers. These changes facilitate recruitment of downstream adaptor myeloid differentiation marker 88 (MyD88), which enables the formation of a multiprotein signaling complex, Myddosome. This complex activates downstream NF-κB and IRF7 signaling that induces the production of proinflammatory cytokines and IFNs, respectively [18], which activate antigen-presenting cells (APC). At the same time, higher INF levels activate the type I interferon (IFN-I) pathway which upregulates an array of genes including those involved in degrading cellular mRNA and ribosomal RNA-like oligoadenylate synthetase (OAS) [18] and inhibiting translation e.g. 2′-5′- and protein kinase R (PKR) [19, 20].

In the cytosol, unmodified mRNAs are detected by different sets of PRRs including retinoic acid-inducible gene-I-like receptors (RLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), oligoadenylate synthetase (OAS) receptors and RNA-dependent protein kinase (PKR). Almost all mammalian cell types express RLRs, which are the chief family of cytosolic RNA sensors [21]. The RLR family includes retinoic acid-inducible gene 1 (RIG-I), melanoma differentiation-associated-5 (MDA-5) and laboratory of genetics and physiology 2 (LGP2). Upon binding with the RNA, RIG-I and MDA5 undergo conformational changes and interact with mitochondrial antiviral-signaling protein (MAVS). MAVS plays an essential role in RLR signal transduction by recruiting a series of kinases which in turn elevate the expression of proinflammatory cytokines and IFN genes via IRF-3/7 and NF-kB pathways [22–24].

Given its immunostimulatory properties, synthetic modRNA has undergone various design modifications to ensure adequate escape from the innate immune response, thereby averting toxic overactivation and facilitating efficient translation of the exogenous mRNA.

4. mRNA alterations to escape immune response and enhance stability

As discussed above, the major obstacles to RNA therapeutics are their instability due to high RNAses activity and the higher immunogenicity of RNA molecules. The possible feedback loop between innate immune activation and reduced mRNA translation further decreases therapeutic mRNA potency. Thus, to push cardiac mRNA therapy towards clinical use, researchers seek to optimize the chemical structure of mRNA by changing its phosphate backbone, RNA terminals or nucleosides. Synthetic modRNA construction has been discussed previously [25]. In this section, we highlight recent progress in modifying RNA to regulate RNA stability.

4.1 Nucleotide modifications

Non-immunogenic mRNA is engineered by transcribing plasmid DNA in vitro. This flexible method can substitute uridine with modified nucleosides such as pseudouridine, 2-thiouridine, 5-methyluridine, 5-methylcytidine or N6-methyladenosine [26]. These modifications have been shown to bypass TLR7 and TLR8 activation. Specifically, completely replacing uridine with 1-Methylpseudouridine-5′-Triphosphate (N1m Ψ) enables more robust translation than other changes in mRNA structure [27]. Psuedouridine (Ψ) is a naturally occurring uridine isomer generated by a C-C, instead of N-C, bond to uridine. The bonding process entails breaking the glycosidic bond, rotating the base 180° and reforming bonds but retains the normal base pairing preference for adenosine [28]. Transcriptome-wide analyses have revealed thousands of Ψ sites in human mRNAs, demonstrating it is endogenous to the human body. Although Ψ-modified mRNA binds to RIG-I with high affinity post endocytosis, it fails to trigger the canonical RIG-I conformational changes associated with robust signaling, thus escaping the immune response.

4.2 Replacing mRNA capping

The 5′-cap is a hallmark of eukaryotic mRNA and plays an essential role in cap-dependent initiation of protein synthesis, stabilization of eukaryotic mRNA splicing, nuclear export and mRNA decay. This cap interacts with the cap binding

complex to trigger mRNA export from the nucleus. Once in cytoplasm, its interaction with the eukaryotic translation initiation factor 4E (eIF4E) causes 5′ to 3′ mRNA looping to recruit multiple ribosomes and enhance the protein translation rate. Chemically, the 5′-cap consists of an inverted 7-methylguanosine (m7G) linked to the first nucleotide of the RNA via a reverse 5′ to 5′ triphosphate bridge. As the abnormally capped (cap 0) or uncapped (5'ppp or 5'pp) mRNAs are recognized by PRRs, modifications like adding the 2′O methylation of +1 nucleotide (cap 1 or m7GpppNm) are central to the non-self-discrimination of the innate immune response against foreign RNA. However, Cap 1 can sometimes attach the mRNA strand in a reverse orientation, i.e., Gpppm7 instead of m7Gppp, which reduces translation efficiency. Thus, the second-generation capping system of anti-reverse cap analogs (ARCA) 3′-O- Mem7G(5′)ppp(5′)G, which cannot incorporate in the reverse orientation, is frequently used in vitro and in vivo to ensure correct capping orientation and higher translational efficiency [29, 30]. One downside of this chemical capping strategy is that the need for higher-ratio ARCA and GTP concentrations to produce a high percentage of capped mRNA significantly elevates the cost of modRNA production. Yet changing ratios of 5′ ARCA cap and N1m Ψ to favor ARCA over N1m Ψ greatly increases the yield per reaction, improves translation, reduces immunogenicity and lowers costs, making modRNA more affordable for basic and translational research [31].

4.3 Changes in untranslated regions

UTRs are important regulators of mRNA decay and translational efficiency as they serve as ribosome entry points during translation. The 5'UTR carries structural elements which are recognized by cell-specific RNA-binding proteins that may regulate translation initiation in a cap-dependent or cap-independent manner. The impact of the UTR on mRNA translation varies by species, cell type, and cell state; applying bioinformatics tools to diverse 5'UTR and 3'UTR combinatory libraries may be able to estimate mRNA translation efficiency. In vitro screening for optimized mRNA 5' UTR combinations has shown improved expression of arginase 1 (ARG1), a potential therapeutic mRNA target, with the 5'UTR for complement factor 3 (C3) and cytochrome p4502E1 (CYP2E1) [32]. Using a transcriptomic and proteomic analysis, Sultana et al. recently identified 5' UTR from the fatty acid metabolism gene carboxylesterase 1D (Ces1d) as an mRNA translation enhancer post cardiac and hepatic ischemic injuries. Ces1D belongs to the carboxylesterases family and plays vital roles in lipid metabolism. Thus, the fact that the heart switches to fatty acid metabolism under ischemic conditions may be why modRNA with Ces1D 5'UTR produces higher translation [33].

Like 5'UTR, 3'UTR located after ORF can also contribute to mRNA translation and controls multiple aspects of mRNA metabolism, including nuclear export, cytoplasmic localization, translational efficiency and mRNA stability [34]. Nevertheless, 3'UTR has proven conceptually more difficult to mechanistically regulate than 5'UTR. The 3'UTR length is a key regulator of mRNA expression: mRNAs with longer 3′ UTRs have a shorter half-life whereas mRNAs with shorter 3′ UTRs are less efficiently translated. To date, erythrocyte proteins, alpha [35] and beta [36] globin 3′UTR have primarily been used in modRNA production; their longer half-lives improve protein production and prolong modRNA expression. New cellular library screenings that explore cell-based selection processes to identify 3′ UTRs may further augment mRNA translation levels. Amino-terminal enhancer of split (AES), mRNA-mitochondrially encoded 12S rRNA (mtRNR1) and mtRNR1-AES-based 3′ UTRs can upgrade RNA translation in mRNA vaccination and mRNA-based reprogramming of human fibroblasts [37]. Apart from using stable mRNA

sequences, 3'UTR also offers the binding site for miRNA, which are responsible for mRNA degradation. Thus, either limiting the number of miRNA sites or artificially incorporating miRNA-binding sites in the mRNA sequence can reduce gene expression in nontargeted tissues and promote mRNA stability.

4.4 Optimizing polyA tail length

The final approach for controlling RNA stability and regulating translation are polyA tails, which are homopolymeric sequences at the 3′ ends of RNA molecules. Post transcription, about 20 factors work cohesively to recognize the polyadenylation site at the 3'end, cleave the pre-mRNA, add a polyA tail and trigger transcription termination. Beside adenosines, full-length polyA and mRNA sequencing reported the presence of non-A nucleotides, mostly cytosines, in the polyA tail region [38]. Conventionally, polyA tails were thought to be synthesized by adding ~250 adenosines to the 3'UTR; however, recent developments using the PAL-seq and TAIL-seq have shown that most mRNAs have much shorter tails, with a median between 50 and 100 nt. Additionally, tail length correlates negatively with expression, half-life and ribosome occupancy. In the case of modRNA expression in the heart, reporter gene comparisons have demonstrated higher translation levels for modRNA containing 173 polyA compared to 120 adenosine, thus suggesting that longer polyA tail length produces higher-translating modRNA [39].

5. Modified mRNA delivery systems in the heart

An efficient delivery system is critical for achieving therapeutic effects with modRNA. The delivery vehicle must protect the mRNA from RNAses and the innate immune system. For exogenous mRNA to be translated, it must pass the lipid bilayer barrier in order to reach the cytoplasm, a rather arduous process. Successful mRNA uptake greatly depends on the targeted cell type and the physiological properties of the mRNA delivery complexes. modRNA is a larger molecule than those used in other gene delivery methods, and its size makes diffusion through the cell membrane more challenging. Furthermore, because modRNA contains a negatively charged phosphate backbone, the cell membrane creates repulsion that influences cellular delivery and organ distribution. By enhancing stability and translation, the recent chemical modifications in modRNA components have led to encouraging progress in the development of various carrier systems that can deliver large mRNA molecules to hard-to-reach organs, especially the heart (**Figure 1**).

Lipid nanoparticle (LNP) complexes are a popular choice for in vivo modRNA delivery. Compared to other complexes, LNPs can encapsulate larger modRNA volumes, offer better protection against RNAses and renal clearance and induce higher cellular uptake. These carrier molecules are primarily composed of ionizable lipids to ensure self-assembly of this large mo.ecule (~100 nm), phospholipids to support the lipid bilayer, cholesterol, a stabilizing agent and polyethylene glycol (PEG) lipids that increase the half-life and hence the stability of the formulations. To efficiently deliver modRNA in the heart, Turnbull et al. used a custom formulation consisting of an epoxide-derived lipidoid (mixed in ethanol with the stabilizers DSPC, cholesterol and PEG-DMG) in addition to modRNA to generate formulated lipid nanoparticles (FLNPs) via nanoprecipitation. The custom-made FLNPs were more stable and showed faster translation than the traditional formulations [40, 41]. Even though LNPs are the most appealing and commonly used mRNA delivery vehicles, some remaining challenges need to be addressed. First, the lipid formulations may cause some toxicity in vivo, and second, the formulations need to be

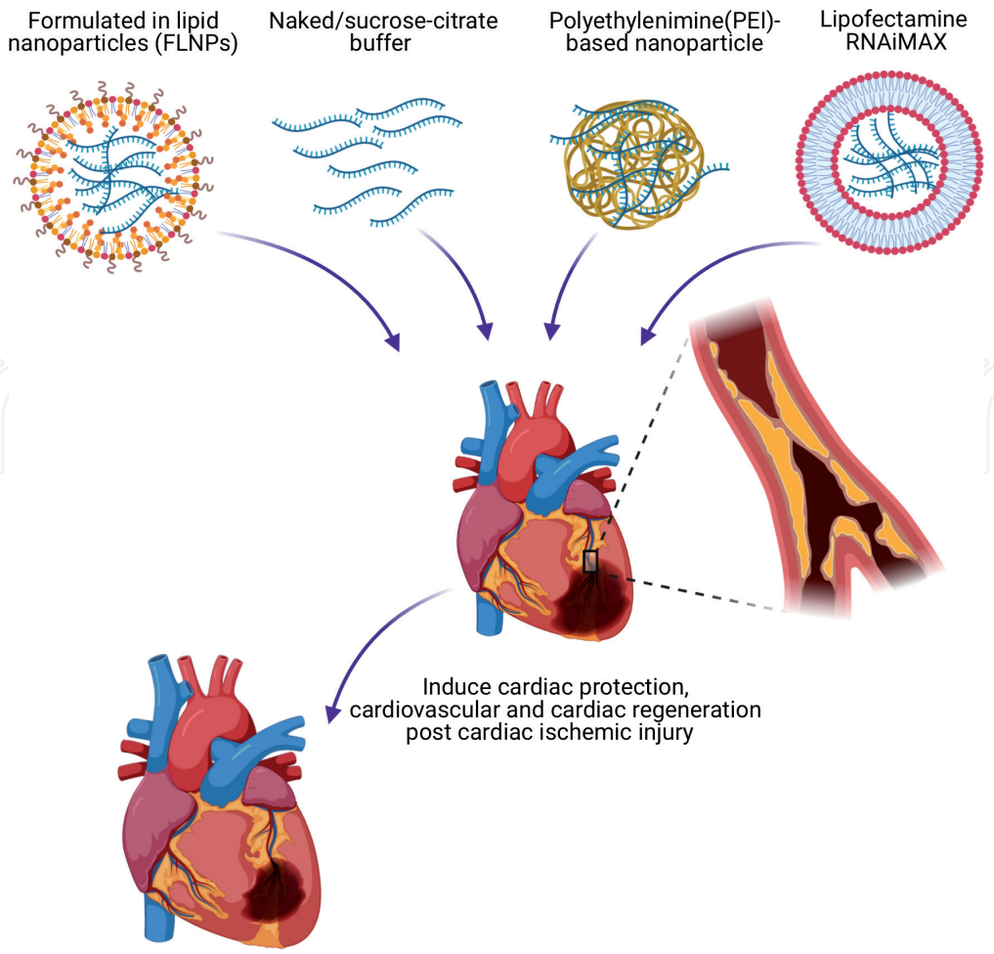

Figure 1.
Modified mRNA carrier systems for cardiac delivery.

adjusted depending on the administration route. An experimental study showed that reporter genes were expressed significantly more when delivered intradermally compared to intravenously [42]. Furthermore, the systemically delivered LNPs favor expression in the liver due to apolipoprotein E binding and subsequent receptor-mediated uptake by hepatocytes. Thus, fine-tuning LNP a component is pivotal to beneficial cardiac therapy [43].

Although developed for siRNA transfections, cationic lipid nanoparticles, commonly known as lipofectamine, also have promising outcomes for modRNA delivery in the heart. Lipofectamine particles consist of a 3:1 mixture of DOSPA (2,3-dioleoyloxy-N- [2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propaniminium trifluoroacetate) and DOPE (1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine) generating a cationic molecule that readily encapsulates the negatively charged modRNA and carries it across the plasma membrane due to the neutral co-lipid mediating fusion between the liposome and the cell membrane [44]. Kondrat et al. have formulated modRNA with RNAimax for myocardial delivery [25]. For in vitro and in vivo cardiac cell delivery, lipofectamine RNAimax and modRNA can be delivered in optiMEM medium and gel-based formulations. For instance, a mixture of Cre recombinase modRNA mixed with lipofectamine and polyacrylic acid successfully expressed the protein when painted on the surface of transgenic Rosa26^{mtmg} heart [45]. Further, Singh et al. reported alginate carrying microencapsulated modRNA (M^{3}RNA) can cause rapid protein expression in primary CMs and targeted expression in mouse and porcine hearts. Forming alginate matrix by crosslinking alignate in the presence of Ca^{2+} provides an excellent platform for modRNA delivery in infarcted hearts [46].

Delivery Method	Species	Administration Route	Protein Target	Results	Mechanism	Publication
Formulated lipidoid nanoparticles (FLNP)	Rat and Pig	Intramyocardial/ intracoronary	eGFP	Reported highly efficient, rapid and short-term mRNA expression in the heart	Epoxide-derived lipidoid complex enhanced eGFP mRNA levels in heart tissue	Turnbull et al. [40]
	Rat	Intramyocardial	EGFP	Protocol	Protocol	Turnbull et al. [41]
Alginate, nanomaterial encapsulated	Mouse and Pig	Intramyocardial/ intracoronary	FLuc, EGFP and mCherry	M^3RNA enabled rapid and targeted protein expression in mouse and porcine hearts.	The crosslinkage of alginate gel with Ca^{2+} at the infarcted site provided an in situ alginate matrix to encapsulate therapeutic mRNA	Singh et al. [46]
Polyethylenimine-based nanoparticle	Mouse	Intracardiac	IGF-1	Reduced cell apoptosis, enhanced cell survival	IGF1 increased the levels of Akt and Erk phosphorylation	Huang et al. [47]
Lipofectamine RNAimax	Mouse	Intracardiac	VEGFA	Induced angiogenesis, improved myocardial function and mice survival	Changed the fate of heart progenitor cells and induced vascular regeneration after MI	Zangi et al. [11]
	Mouse	Intracardiac	VEGFA	Endothelial specification, engraftment, proliferation and reduced apoptosis of the human Isl1+ progenitors in vivo	Induced $Isl1^+$ cells to endothelial cell fate, proliferation and survival of Isl1+ progenitors	Lui et al. [45]
	Mouse	Intracardiac/gel application	DN-IGF-1R, IGFR	Reduced cell differentiation into adipocytes after MI	Adipogenic differentiation ceased post MI	Zangi et al. [51]

Delivery Method	Species	Administration Route	Protein Target	Results	Mechanism	Publication
Sucrose-citrate buffer	Mouse	Intracardiac	mutated FSTL1	CM proliferation and limited cardiac remodeling	Replaced asparagine with glutamine in the N-glycosylation site at position 180 of FSTL1	Magadum et al. [52]
	Mouse	Intracardiac	Pkm2	CM cell cycle induction, decreased oxidative stress	PKM2 interacted with β-catenin and upregulated downstream targets Cyclin D1 and C-Myc	Magadum et al. [53]
	Mouse	Intracardiac	AC	Reduced cell apoptosis, increased cell survival, improved cardiac function	Decreased ceramide levels and lowered abundance of proinflammatory detrimental neutrophils	Hadas et al. [54]
	Mouse	Intracardiac	GFP, Luciferase	Optimized time and amount for efficient modRNA delivery in the heart	Optimized time and amount for efficient modRNA delivery in the heart	Sultana et al. [50]
	Human	Epicardial injection	VEGFA	Expected to increase blood flow in the heart	Increased angiogenesis	Moderna Therapeutics [55]
	Mouse	Intracardiac	Luciferase	Increased translation by replacing 5'UTR	Ces1D enhanced mRNA translation post cardiac and hepatic ischemic injury	Sultana et al. [33]
	Mouse	Intracardiac	Luciferase, Cre	Delivered modRNA in a MI model	Protocol	Kaur et al. [49]
	Minipig	Intracardiac	VEGFA	Promoted capillary density, improved heart function post MI, reduced fibrosis	Increased angiogenesis	Carlsson et al. [56]
	Mouse	Intracardiac injection	aYAP	Decreased CM necrosis, attenuated innate immune responses	YAP/TEAD1 suppressed toll like receptor pathway	Chen et al. [57]
	Mouse	Intracardiac injection	Cre	Specific modRNA translation system	L7Ae based modRNA delivery exclusively to CMs.	Magadum et al. [58]

Table 1.
Recent modRNA studies in the heart: Delivery system, target and findings.

To increase the efficiency of modRNA uptake in vivo, cationic polymers have occasionally been used to upregulate therapeutic genes in the heart. These chemically diverse and positively charged molecules are highly compatible with the negatively charged modRNA and thus hold great potential for functionalization. Polymers can be linear, branched or dendrimers; the latter comprise many branched repeats. One cardiac modRNA variant is polyethylenimine (PEI), which can efficiently promote gene transfection in vivo, as described by Huang et al. [47]. With a nitrogen atom at every third position along the polymer, PEI has a high charge density at reduced pH values, which may promote mRNA condensation and endosomal escape [48]. ModRNA transfection also depends on final formulations and reportedly works best at a nitrogen residue/phosphate (N/P) ratio of 6 of jetPEI/modRNA [47]. Despite PEI's initial use in modRNA delivery in mice, its molecular weight and linear versus branched form need to be optimized, as these are reportedly major determinants of transfection efficiency and cytotoxicity.

Lastly, the safest, most efficient modRNA delivery method to the heart, as presented by Kaur et al. [49], is naked delivery without conjugation of any nanoparticle. Unlike the nanoparticle formulations, naked modRNA in saline or sucrose-citrate buffer does not form any of the complexes that make its efficacy questionable in vitro. While the transfection reagent masks the negatively charged mRNA, allowing electromagnetic attachment to and endocytosis of the negatively charged cell membrane, these formulations are associated with increased cell death. While comparing various modRNA various delivery reagents, naked modRNA with sucrose-citrate buffer or saline yields higher protein translation compared to modRNA encapsulated in nanoparticles in the heart. This could be explained by the sucrose acting as a readily available energy source to enhance endocytosis of the modRNA. The composition of the sucrose-citrate buffer also provides appropriate viscosity and thus prevents clumping to ensure single-stranded mRNA enters the myocardium to achieve high translation and avoid activating the immune response. Further, encapsulating modRNA with RNAimax has proven to be detrimental in the heart and triggers apoptosis locally. Another study showed that delivering 100ug of Luciferase/Cre recombinase modRNA in sucrose-citrate buffer was sufficient to produce optimal gene translation, resulting in ~20% transfection of the left ventricle [50]. The studies using various modRNA delivery systems in the heart are compiled in **Table 1**.

6. Modified mRNA to induce cardiac repair

ModRNA holds enormous potential as a non-viral gene delivery method for cardiovascular diseases. Recent developments in the understanding of basic blood vessel formation mechanisms have shown it may be possible to use therapeutic vascular growth to treat heart disease. Traditional vectors have delivered several growth factors to achieve new vessel growth in the heart [59]. To circumvent the complications associated with recombinant protein- and gene transfer-based therapies, Zangi et al. published a landmark study using in vitro transcribed modRNA to efficiently deliver vascular endothelial growth factor A (VEGFA) into the heart with minimal induction of innate immune responses. Their work demonstrated that direct intramyocardial injection of synthetic mRNA containing pseudouridine and 5-methylcytidine, encoding VEGFA, promoted vascular growth, improved heart function and enhanced long-term survival following MI injury [11]. Furthermore, VEGFA also had a pro-survival effect on vascular and endothelial cells and enhanced endothelial proliferation of epicardial-derived progenitor cells [51].

Subsequent to this study in mice, Carlsson et al. established the efficacy of clinical-grade VEGFA modRNA, AZD8601 for heart regeneration after MI in porcine models. The group showed that the modRNA formulated in biocompatible citrate-buffered saline can transiently deliver genes into the heart. They also confirmed that intradermally or intravenously administered modRNA did not exhibit any signs of acute toxicity or inflammation in rats and cynomolgus monkeys. Epicardially injected AZD8601 administered 7 days after permanent ligation of the mid-left anterior descending coronary artery led to improved cardiac function in mini-pigs. The study demonstrated significant improvements in left ventricular ejection fraction, contractility and myocardial compliance with increased neovessel formation in the peri-infarct area and attenuated cardiac fibrosis 2 months after injection [56]. The promising results in the pig model led to the first clinical trial involving modRNA therapy for cardiac regeneration, by AstraZeneca (AZD8601) in collaboration with Moderna [55]. This phase 2a clinical trial (clinical trial number: NCT03370887) is expected to prove the safety and efficacy of epicardially injected VEGFA modRNA and authenticate improved myocardial blood flow in patients who underwent coronary artery bypass grafting surgery. Furthermore, the VEFGA modRNA, AZD8601 has progressed to a phase 1 clinical testing study in men with type 2 diabetes mellitus. The study involves delivering AZD8601 intradermally as a single dose into the forearm skin with the primary objective of assessing the safety and tolerability of this new therapeutic clinical trial number: NCT02935712). So far, the results have shown significantly increased local blood flow in the skin within 7 days of modRNA injection as well as elevated VEGFA protein concentration in cutaneous dialysate, thereby indicating VEGFA is a potential candidate for microvasculature therapy [60].

Following acute MI, ischemic stress is associated with LV deterioration, impaired cardiac function and higher ceramide levels. These elevated sphingolipid levels result from decreased ceramidase levels and are not induced by elevated sphingomyelinase activity. Further, the increased ceramides in the blood appear to be transient, as shown in a model of ischemic-reperfused myocardium in rats [60]. Thus, the pulse-like expression of mRNA encoding the enzyme acid ceramidase (AC) directly into the injured myocardium is sufficient to induce cardioprotection after MI [57]. Treatment with AC improved cardiac function, reduced LV scar size 28 days post MI and extended long-term survival. Huang et al. used modRNA delivery into mouse myocardium to evaluate the cytoprotective efficacy of insulin-like growth factor 1 (IGF1) on hypoxia-induced CM apoptosis. As expected, IGF1 secretion was observed 24 h after injection and peaked at 48 h. Note that a single dose of intramyocardially injected IGF1 post MI augmented Akt1 phosphorylation and decreased both Casp9 activity and TUNEL-positive cell levels within the border zone of infarcted mouse hearts. These changes reduced CM apoptosis and thus enhanced CM survival under hypoxic conditions [47]. The beneficial effects of the IGF1 signaling pathway in the MI model were evaluated by Zangi et al., who demonstrated that the IGF1 receptor triggers the formation of epicardial adipose tissue in the heart. Thus, the innovative approach of delivering modRNA by applying a gel comprising IGF1 receptor dominant-negative mutants to the heart surface reduced adipogenic marker expression and led to better survival for CMs and cardiac progenitors [51]. Additionally, introducing transcriptional co-activator yes-associated protein (aYAP) modRNA in the mouse heart produced promising outcomes in IR injury. Previously established for cell proliferation, aYAP effectively curtailed the innate immune inflammatory response and boosted CM survival in the damaged myocardium. This modRNA-mediated gene delivery led to reduced scarring, improved heart function and suppressed hypertrophic remodeling 4 weeks after intervention [52].

Another important aspect of cardiac repair is inducing the CM cell cycle. In this regard, delivering modRNA encoded for follistatin-like 1 (FSTL1, with glutamine substituted for asparagine in the N-glycosylation site at position 180) increased CM proliferation both in vitro and in a mouse model post MI without inducing CM hypertrophy. FSTL1 mutation of all three N-glycosilation sites present in hFSTL1 mimicked the glycosylation state of bacterially produced FSTL1 and was sufficient and essential to activate the CM cell cycle and cease cardiac remodeling post MI [53]. In another study, Magadum et al. showed modRNA-based CM cell cycle induction via upregulating pyruvate kinase muscle isoenzyme 2 (PKM2). This isoenzyme of the glycolytic enzyme pyruvate kinase can be detected in CMs during embryonic development and immediately after birth, but not during adulthood. Introducing PKM2 in CMs in vivo increased CM cell division, improved cardiac function and enhanced long-term animal survival. These results stem from PKM2 interaction with *β*-catenin in CM nuclei and upregulated downstream targets Cyclin D1 and C-Myc, which remove the brakes from cell cycle arrest, in addition to reduced oxidative stress damage through activation of anabolic pathways and β-catenin [61]. The study also described a unique system to achieve CM specificity by implementing archaeal ribosomal protein L7Ae in the modRNA constructs. The authors added kink-turn motif, a specific binding site for L7Ae, to the 5′UTR of PKM2 mRNA, and when the L7Ae recognized the kink-turn motif it cleaved the modRNA and hence blocked translation. Using this cell-specific system, modRNA encoding for PKM2 with kink-turn motif was co-transfected with modRNA encoding L7Ae, which included CM-specific microRNA recognition elements (miR1–1 and miR- 208a) within 3′UTR. This co-transfection prevented PKM2 expression in all cardiac cells except CMs [58, 61]. Collectively, these studies show the therapeutic potential and effectiveness of modRNA technology in cardioprotection and cardiac repair.

7. Concluding remarks and future directions

Current structural improvements that enhance modRNA stability and escalate its translational capacity, have spiked interest in modRNA for both basic research and clinical therapeutics. The innovative progress in modRNA design has facilitated rapid mass production, reduced costs and revealed the versatility of next-generation modRNA for future therapeutics. The recent success of modRNA vaccines against SARS-COV-2 has proven that modRNA can provide robust, safe and efficient gene transfer in humans and hence is considered a promising tool for treating various disorders, including heart failure [62]. However, the multifarious obstacles concerning modRNA's large size, charge, intrinsic instability and need to be administered through intracardiac injections hamper the translation of modRNA-based cardiac therapeutics to the clinic.

Significant advances in nanodelivery systems, especially in the field of LNPs, have shown the best prospects for developing modRNA-based therapies. The US Food and Drug Administration's recent approval of short interfering based patisiran to treat hereditary transthyretin amyloidosis has made LNPs an attractive potential non-toxic carrier system for modRNA delivery [63]. Despite developments in delivery systems, predictable, efficient delivery of nanoparticles to targeted tissues remains challenging. Changes in the internal and/or external charge of the nanoparticle is one key aspect of tuning tissue tropism. To address this, Cheng et al. developed a unique method of supplementing additional molecules to established LNP molar compositions in order to tune the internal charge for selective organ targeting. The resulting formulations were able to precisely target a variety of organs and enabled high levels of modRNA delivery. Although such studies have produced

highly promising results for modRNA delivery in lung, spleen and liver, more work remains to be done to create minimally invasive gene delivery models for the heart [64]. Developing such delivery models would also help resolve short span gene expression with modRNA delivery. Although transient gene delivery eliminates the risk of mutagenesis caused by protein overexpression, pulse-like expression of a gene might not be enough to induce substantial cardiac regenerative changes. Thus, the innovation of minimally invasive methods to deliver cardiac genes will mitigate the need for repeated modRNA transfections/injections to maintain effective protein levels for a longer but still controlled time period. As research on modRNA formulation and delivery rapidly accumulates, we are confident that modRNA therapy will soon become reality in the field of heart failure therapeutics.

Conflict of interest

The authors declare no conflict of interest.

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