# Recent Advances in Gene Therapy for Atrial Fibrillation

Shin Yoo[1], Gail Geist[1], Anna Pfenniger[2], Markus Rottmann[1], and Rishi Arora[2]

[1]Northwestern University Feinberg School of Medicine
[2]Northwestern University

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**Abstract**

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**Authors:** Shin Yoo[1+], Gail Elizabeth Geist[1], Anna Pfenniger[1], Markus Rottmann[1], Rishi Arora[1+]

**Affiliations:** [1]Feinberg Cardiovascular and Renal Research Institute, Northwestern University Feinberg School of Medicine, Chicago, IL.

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[+] *Addresses for Correspondence* :

Rishi Arora, MD

Northwestern University-Feinberg School of Medicine

251 East Huron, Feinberg 8-503

Chicago, IL 60611

Fax: 312-926-6295; Phone: 312-503-3217

E-mail: r-arora@northwestern.edu

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Atrial fibrillation (AF) is the most common heart rhythm disorder in adults and a major cause of stroke. Unfortunately, current treatments for AF are suboptimal as they are not targeting the molecular mechanisms underlying AF. In this regard, gene therapy is emerging as a promising approach for mechanism-based treatment of AF. In this review, we summarize recent advances and challenges in gene therapy for this important cardiovascular disease.

**Key words:** Gene Therapy, Atrial Fibrillation

**Non-Standard Abbreviations and Acronyms**

AAV9 Adeno-associated virus serotype 9

ACh Acetylcholine

AF Atrial fibrillation

APD Action potential duration

CREM cAMP response element

Cxs Connexins

ERP Effective refractory period

GFP Green fluorescent protein

$I_{CaL}$ L-type $Ca^{2+}$ current

$I_{K1}$ Inward-rectifier $K^+$ current

$I_{KACh}$ Acetylcholine-dependent $K^+$ current

$I_{KH}$ Constitutively active form of$I_{KACh}$

NOX2 NADPH oxidase 2

RAP Rapid atrial pacing

ROS Reactive oxygen species

shRNA Short hairpin RNA

TASK-1 Tandem of P Domains in a Weak Inward Rectifying K+ Channel–Related Acid-Sensitive K+ Channel-1

# Introduction

Atrial fibrillation (AF) is the most common heart rhythm disorder, with an estimated prevalence of 12.1 million individuals in the US alone by 2030.[1] AF is a cause of significant morbidity and mortality, and because the incidence of AF increases with age, it is fast becoming an epidemic worldwide.[2,3] Despite its clinical importance, AF is a difficult condition to treat. Current therapies for AF include anti-arrhythmic drugs and ablation to electrically isolate the pulmonary veins.[4] Ablation is mostly effective for paroxysmal AF, with more limited efficacy in persistent AF, and is also associated with complications. Anti-arrhythmic drugs have limited long-term efficacy and can be associated with significant adverse effects, including pro-arrhythmia and effects on the nervous system.[5]

Given these challenges, researchers are actively investigating new treatments, including gene-based approaches to directly and specifically target the signaling pathways in the atrial myocardium that underlie the creation of electrical and structural remodeling in AF. In the preclinical stage, promising results having been obtained in animal models that parallel the electrical and structural remodeling seen in humans. While gene therapy holds great hope to produce a highly effective and personalized treatment for a diverse range of cardiac disorders, safe and successful clinical translation is in a nascent phase and therapies must be designed with careful attention to an ever-expanding body of knowledge.

In this review, we will begin by discussing the current state and advances in gene transfer and gene-editing technology, with a focus on the gene therapy vectors and methods for delivery of these vectors to the atrium. We will then examine molecular targets based upon AF mechanisms. Further, we will discuss the potential of novel AF mapping strategies to better target gene therapy delivery.

# Overall strategies of Myocardial Gene Transfer

The overarching concept of cardiac gene therapy is simple: replace or remove a disease-causing gene at the level of the myocardium, thereby eliminating a fundamental incipient for a given condition. In practice, there is an array of selection criteria and obstacles to consider. For any gene therapy to be successful, the gene(s) of interest must not only be delivered but also expressed at adequate concentrations in the target tissue bed. The tools used to accomplish this gene transfer are known as vectors. The ideal vector manifests tissue selectivity, low immunogenicity, adequate packaging capacity, and a durable level of gene expression. To date, current vector options incorporate some, but not all of these attributes. Vectors can be described in two broad categories: viral (gene transduction) and non-viral (transfection). Following selection, the vector of choice may be delivered to the myocardium through a variety of techniques over a spectrum of invasiveness and specificity. There is no single optimal combination of the above factors, rather, it is necessary to understand the appropriate applications and limitations of each.

# Gene Therapy Vectors

## Non-Viral Vectors

### Naked Plasmid DNA

While primarily used for *in vitro* gene transfer, plasmid DNA remains the most accessible tool for gene transfer *in vivo* . Plasmids are circular DNA constructs that can be customized with a versatile combination of transgenes and regulatory elements. Compared to other vectors, naked plasmids can hold significantly larger quantities of genetic information.[6,7] Plasmids are also easy to produce, with adequate infrastructure for clinical-grade plasmids already in place.[8] Naked plasmid DNA is non-immunogenic; while an immune response can be mounted against the foreign transgene product, there is no immune response generated against the plasmid itself.[9] This lack of vector-directed immune response enhances safety and allows for potential repeated administration of plasmid-based gene therapies.

However, naked plasmid alone transduces cells at therapeutically irrelevant levels,[10] and enhancement with transfection reagents is only marginally effective for gene uptake.[11,12] Overcoming this limitation requires select methods of administration, which will be discussed later in this review. In addition, plasmid DNA is not integrated in the genome, leading to a limited duration of expression. Prolongation of this expression is under investigation through numerous studies on select promotors. Our group has previously demonstrated expression of a dominant-negative TGFβ II receptor under the control of a long-acting polyubiquitin C (UBc) promoter for at least 3-4 weeks in a canine heart failure model of atrial fibrillation.[13] Similarly, intermediate term gene expression has been demonstrated by others in murine myocardium, bone, skeletal muscle, and lung.[14-18] While long term data has yet to be reported, the option of repeated rounds of plasmid gene therapy could compensate for loss of transgene expression over time.

### Nanoparticles

Another choice of non-viral vector for myocardial gene transfer are nanoscale liposomes. Lipid-based nanoparticles offer biocompatibility, good cellular uptake, and can be deployed with targeting ligands to enhance tissue specificity. The liposomal delivery mechanism for small molecule drugs is already in clinical use as a chemotherapeutic vehicle, and lipid-based nanoparticles containing a genetic construct have a demonstrated ability for transducing cardiac cells.[19,20] However, off-target tissue effects may still be encountered when nanoparticles are administered via systemic circulation, and charged lipid particles are subject to rapid clearance by the reticuloendothelial system. Future advances in liposomal stability, distribution, and release offer potentially exciting avenues for cardiac gene delivery.[20,21]

### Another non-viral vector, modified-mRNA (modRNA)

In the early 1990s, mRNA was successfully delivered to brain and skeletal muscle.[22,23] However, the use of mRNA as gene delivery vector to mammalian tissue did not evolve since then. This is mostly due to mRNA induced innate immune response via stimulation of Toll-like receptors.[24] Furthermore, mRNA is likely cleaved by RNase in vivo.[24] In 2005, Dr. Katalin Karikó, who contributed to recent development of COVID-19 mRNA vaccine, demonstrated that modifying mRNA's secondary structure by replacement of uridine with pseudouridine prevented innate immune system recognition and RNase degradation.[25]

Compared to DNA vectors, modRNA has advantages and disadvantages as a gene delivery tool. One advantage is that mRNA does not require localization of nucleus or transcription process. modRNA gene delivery has minimal risk of integration into the host genome.[26] modRNA has been shown to be highly efficient with robust transient expression with no sign of innate immune response.[27] ModRNA is translated within minutes and lasts up to 10 days in vivo.[28] The use of modRNA in heart is mainly for myocardial ischemia/reperfusion injury in ventricle because of its transient pharmacokinetic profile.[29,30]Disadvantages of modRNA are unstable modRNA generation and the need for repeated delivery due to its short expression pattern. To date, modRNA has not been tested in AF treatment yet. If translation efficiency of modRNA is improved, modRNA can be another non-viral vector for AF.

## Viral Vectors

Viral vectors are live, replication deficient viruses which have been genetically modified to replace the native viral genes with therapeutic transgenes. Any cell that the vector infects integrates the transgene payload to produce or inhibit a genetic product. Compared to non-viral plasmids which must be delivered directly to the tissue of interest, viral vectors have the theoretical advantage of minimally invasive delivery via the bloodstream. There are three main types of viral vectors used today in gene therapy, though the Adeno-associated virus (AAV) is currently best suited for cardiac gene therapy.

### Adeno-associated virus (AAV)

First isolated as an unrelated contaminant in adenovirus samples, AAV is a non-enveloped, non-integrating single-stranded DNA parvovirus. AAV emerged as a focus of gene therapy vector development due to low immunogenicity, potential for long duration of expression, and a robust safety profile.[31-33] AAV is capable of durable, possibly life-long transgene expression *in vivo* : no upper limit to duration of expression has been determined, with numerous studies showing transgene expression years after a single administration. Notably, AAV alone is incapable of productive replication and requires coinfection with a helper virus, usually adenovirus or herpesvirus. The lack of self-replication machinery increases the safety of AAV, but also limits the size of its genome to about 4.7kb.

The primary AAV serotypes are AAV 1-9. While more serotypes and variants have been characterized and silent infection is highly prevalent in humans, no associated pathogenicity has been identified.[34] Each serotype has a distinct capsid protein sequence correlating to variable tissue tropism, with AAV serotypes 1, 6, 8, and 9 exhibiting the highest cardiac tropism.[35,36] By engineering the makeup of the viral capsid proteins, it is possible to generate novel, chimeric AAVs with improved transduction efficiency and tropism in rodent models.[37-39] Tissue specificity can also be achieved with the use of site specific promoters to drive transgene expression only in the atria.[40] While transduction efficiency is often more limited in scale-up from rodent to large animal models, these emerging strategies are accompanied with recent FDA-approval for non-cardiac gene therapies and a number of clinical trials utilizing an AAV vector.[41-43]

The primary disadvantage of AAV vectors is limited transgene size. When including a cardiac-specific promoter, many transgenes exceed the maximal size for an AAV construct. Additionally, a clinical effect may be delayed as gene expression requires conversion of the single-stranded viral genome to the double stranded host genome.[44]AAV-mediated gene therapy is further hindered by the potential pre-existing neutralizing immune response generated against AAV capsid proteins, described in further detail in a following section.

### Adenovirus (Ad)

The wild-type Ad is a non-enveloped, non-integrating double stranded DNA virus, ubiquitous in the environment and one of the causative agents of the common cold. Ad vectors are simple to produce, transduce both dividing and non-dividing cells with high efficiency, and have a packaging capacity for moderate sized genes.[45]However, in the heart, gene expression after Ad vector transduction is robust but transient and Ads can trigger a strong innate immune response and toxicity due to viral gene products.[46] The use of Ads came into serious question in 1999 after the death of a patient with ornithine transcarbamylase deficiency due to a massive immune response following injection of Ad vector.[47]

Recombinant modifications have given rise to first, second, and third-generation Ad vectors, with key immunogenic components deleted. These vectors show promise for evading the host immune response and producing a prolonged gene expression, but are more difficult to produce.[48]

### Lentivirus (LV)

Lentiviral vectors are enveloped, integrating, single-stranded RNA retroviruses[42]. In gene therapy, LV vectors are usually derived from the HIV-1 virion, modified to be replication-defective to safeguard against off-target continued infection.[49,50] Retroviral vectors typically require active cellular division to integrate and express a transgene, but the machinery of HIV conveys an ability to transduce intact nuclear membranes in post-mitotic cells (such as cardiomyocytes), and accomplishes long-term gene expression with moderate packaging capacities.[51-53] Despite this attractive profile for efficacy, the LV apparatus of random genome integration with a preference for coding regions poses a clinical safety precedent for oncogenic transformation.[34,48] While terminally differentiated cardiomyocytes pose a lower mutagenic risk than mitotically active tissues, the safety and efficacy of lentiviral vectors for cardiac use have yet to be demonstrated in clinical trials.

### Immunogenicity of Viral Vectors

The promise of viral vectors is inseparable from the perennial obstacle of inherent immunogenicity. Viral capsids are targets for the innate and humoral immune responses, and foreign transgene products can trigger the adaptive immune response. Adenoviruses are most notoriously associated with immune provocation, resulting in declining use following adverse events in previous clinical trials.[54] Though the advent of AAV-mediated gene therapy has alleviated many of the safety concerns associated with the use of viral vectors, AAV infections are silently endemic to many human populations. A geographically variable but significant percentage (20 – 60%) of humans are predicted to have pre-existing neutralizing antibodies (NAbs) against one or more AAV capsids, rendering AAV-mediated treatments ineffective.[55,56] Furthermore, in naïve patients, initial exposure to an AAV therapy results in generation of NAbs against the AAV capsid, eliminating the potential for readministration of AAV vector-mediated gene therapy.[57] A complete understanding of the significance of AAV NAb titers and cross-reactivity between serotypes has yet to be established, posing a challenge for clinical study enrollments. Lentiviral vectors possess an advantageous ability to mostly evade the host immune system, however, the foreign transgene product itself can still incite an immune response and subsequent suppression. These altered proteins, while therapeutic, can present to the adaptive immune system as a potent neo-antigen. In an effort to overcome these sophisticated, protective host defenses, immunomodulation at the time of vector administration is an area of active research.[58]

## Gene Delivery to the Atrial Myocardium

A well-administered vector achieves homogeneous delivery at the affected tissue bed and demonstrates minimal accumulation at off-target sites. Vectors can be administered by a wide variety of techniques, but often with an inverse relationship between simplicity and specificity. Route consideration is imperative for patient safety and gene efficacy, and represents an area under active research in parallel with the vector itself.

## Intravenous administration (IV)

The least invasive method of administration is intravenous injection of the vector. While IV exposure should offer rapid transit to any well-vascularized target tissue, it is also the least specific route. Upon entering the intravascular compartment, the vector will be systemically dispersed and the tissue beds will be exposed in accordance with the blood flow to each site. AAV and nanoparticle studies have shown that numerous off-target organs, particularly the liver, are transduced following IV administration. This effect poses a clinically relevant concern for decreased efficacy and increased toxicity. To overcome this biodistribution obstacle, site-directed vector engineering including AAV capsid chimerism and nanoparticle targeting ligands may improve specificity and potency.[21,38]

## Cardiac perfusion

The common and well-refined clinical practice of coronary artery catheterization offers intracoronary perfusion as a minimally-invasive modification of IV administration. In this form of delivery, the cardiac vasculature is selectively isolated and perfused with the vector to maximize the potency of a single administration. While the vector still enters the systemic circulation, the cardiac tissues encounter the vector prior to attenuation by dilution or hepatic uptake. However, this high tissue dose is limited by permeability of the coronary endothelium and rapid blood flow clearance through the coronary circulation. These two factors are thought to have contributed to the negative outcome of the CUPID2 trial (AAV1/SERCA2a coronary injection in patients with heart failure).[59,60] Vascular permeability enhancers (substance P or thrombin) can be co-administered with a vector to enhance myocardial exposure, although any interference with the coronary arterial tree carries the risk of ischemia and could be unacceptable for patients with pre-existing cardiac disease.[34,61]

Retrograde infusion via coronary sinus injection may provide a myocardium-targeted approach without the ischemic risks or patient selection criteria of intracoronary techniques. Here, controlled infusion of the venous structures in the setting of obstructed outflow increases the capillary pressure gradient and drives the vector material into the tissue beds. Exposure time can be prolonged, as distribution is not dependent on arterial flow and the coronary sinus occlusion can be safely tolerated for an extended duration. Large animal studies have demonstrated efficacy for both drug and gene delivery utilizing this technique.[62-64] While coronary sinus cannulation is generally safe and commonly practiced in routine procedures, trauma to the delicate cardiac veins and myocardial edema are potential complications and necessitate careful injection pressure regulation.[34]

## Epicardial gene painting

Epicardial gene painting combines a vector with a protease and a polymer-forming gel to create a "paintable" gel that can be directly applied to the atrial epicardium.[65] Once applied, the polymer vehicle solidifies at body temperature and provides a substrate for strong adsorption of the vector to the tissue bed. The protease component of the paint facilitates transmural gene transfer in the thin atrial myocardium.[66]

Gene painting is safe and effective in animal models with no significant impact on atrial structure or function.[65,67] Though epicardial gene painting can yield homogenous and transmural transduction, the primary drawback is the invasiveness of the surgical procedure required to achieve epicardial access. In addition, structures that are difficult to access via the epicardium (posterior left atrium and pulmonary veins) may preclude delivery to the entire atria, and misapplication of the paint could theoretically result in unintended transmural gene delivery to the ventricle.

## Direct myocardial injection with or without reversible electroporation

As a simple and well-studied method, direct injection of vector into myocardial tissue has been extensively explored as a route of administration. Through a surgical approach, the vector can be precisely injected and an intense concentration of gene expression can be achieved. However, gene expression is highly localized to

within a few millimeters of the injection site. In this way, injection-mediated delivery to a large area of the myocardium is technically challenging and inefficient.[68]

Following direct injection, naked plasmid DNA gene therapy vectors require subsequent electroporation for effective myocardial transfection.[13,69,70] Electroporation acts by subjecting cells to synchronized electrical pulses, resulting in a transient electrical gradient that alters the structure of cell membranes and forms micropores at the cell surface. These micropores enable diffusion of surrounding plasmid into electroporated cells. The rate of gene uptake *in vivo* is 15-20 fold higher when electroporation is used versus standard plasmid DNA delivery alone.[71] Irreversible electroporation is a developing procedure used in clinical cardiac electrophysiology to ablate specific regions of the myocardium.[72] Modification of pre-existing irreversible electroporation techniques and equipment to reduce delivered current from an electroporation device could be used to transduce the myocardium with plasmid DNA.

# Targets for Gene Therapy

Effective gene therapy aims to identify and counteract AF mechanisms originating from or kindled by a genetic element.[73]Two principal driving mechanisms of AF are focal ectopic firing and re-entry. Both of these mechanisms are dependent on electrical and structural remodeling, autonomic nerve remodeling and $Ca^{2+}$-handling abnormalities.[74]Electrical remodeling is typically characterized by shortening of the atrial action potential duration (APD) through a decrease in the L-type $Ca^{2+}$ current and an increase in the inward-rectifier current ($I_{K1}$), and the emergence of constitutively active acetylcholine induced potassium current ($I_{KACh}$).[2] Structural remodeling results in left atrial enlargement, atrial fibrosis, and gap junction remodeling, culminating as slow and heterogeneous conduction.[75] In this review, we will limit ourselves to the current state of mechanistic targets utilizing aforementioned gene therapy vectors.

## Ion channels

Ion channels have long been a pharmacologic target for rhythm management, so it follows that gene therapy would pursue a similar path. Indeed, transfection of plasmid containing a clarithromycin-responsive variant of KCNE2 (Q9E), encoding the IKr regulatory subunit, hMiRP, lead to prolongation of the APD by administration of clarithromycin 2 weeks later.[76] Epicardial gene painting of adenovirus containing a dominant-negative variant of KCNH2-G628S (encoding alpha subunit of IKr) resulted in APD prolongation and reduction of AF burden and inducibility in a porcine model of AF.[77]Similarly, Soucek et al. confirmed prolongation of APD with myocardial injection and electroporation of adenoviruses expressing same KCNH2 variant in a canine model of AF.[78] Genetic suppression of TASK-1 (Tandem of P Domains in a Weak Inward Rectifying K+ Channel–Related Acid-Sensitive K+ Channel-1; K2P3.1) through transfection of AAV containing atrial anti–TASK-1 siRNA lead to reduction of expression of TASK-1 and prolongation of atrial APD and refractoriness.[79]

## $Ca^{2+}$ handling proteins

Abnormal sarcoplasmic reticulum (SR) $Ca^{2+}$ leak via the ryanodine receptor type 2 (RyR2) has been described in atrial cardiomyocytes from AF patients and in various AF models.[80,81] This disruption in calcium handling contributes to ectopic atrial activity and is implicated in the progression from paroxysmal to persistent AF. Phosphorylation at the residue site S2814 was shown to promote AF in mouse models, with mice harboring a phospho-resistant RyR2 form (S2814A) exhibiting a reduced susceptibility to AF.[82,83] This was demonstrated across two different mouse models of atrial arrhythmias: 1) mice lacking the RyR2-stabilizing subunit FKBP12.6, which causes spontaneous $Ca^{2+}$ waves and leads to a higher incidence of spontaneous and pacing-induced AF; and 2) mice exhibiting cardiac overexpression of the transcriptional repressor CREM-IbΔC-X (CREM-TG), which leads to atrial myopathy and spontaneous AF that progresses from paroxysmal

to persistent. Given these findings, gene therapy integrating a phospho-resistant form of RyR2, such as RyR2-S2814A may be indicated as a clinical target of interest.

Calmodulin (CaM) is an important regulator of RyR2. When bound to $Ca^{2+}$, CaM contributes to inactivation of RyR2. This regulatory property was investigated in a mouse model of catecholaminergic polymorphic ventricular tachycardia (CPVT), a syndrome where shortened refractoriness of RyR2 plays a dominant role.[84,85] Liu et al. engineered a form of CaM with slowed $Ca^{2+}$ dissociation (CaM M37Q, or therapeutic T-CaM).[86] They showed that injection of AAV9 T-CaM attenuated diastolic $Ca^{2+}$ waves and prevented ventricular tachycardias in a calsequestrin-associated mouse model of CPVT. It is conceivable that gene therapy with T-CaM in the atria would attenuate the SR $Ca^{2+}$ leak, and may therefore reduce atrial triggers and progression from paroxysmal to persistent AF.

## Autonomic nerve remodeling

The atria are highly innervated by the autonomic nervous system. Vagal stimulation results in shortening of the atrial effective refractory period (ERP) and increased vulnerability to AF.[87]Acetylcholine (ACh) released from parasympathetic nerves activates muscarinic type 2 receptors which interact with heterotrimeric G proteins: the $G\alpha_{i/o}$ subunits subsequently inhibit adenylate cyclase protein kinase, and the $G\beta\gamma$ subunit activates $I_{KACh}$.[88] Despite the apparent importance of the autonomic nervous system in AF, drug therapy studies using β-blockers and selective $I_{KACh}$ blockers have shown modest success.[89,90] Donahue et al. pioneered gene therapy targeting specific components of the G-protein autonomic pathway in the pig AV node as a rate control strategy for ventricular response in AF. In the study, an adenoviral vector encoding for the G-protein alpha inhibitory subunit 2 ($G\alpha_{i2}$) was delivered in the AV node of pigs, thereby mimicking increased vagal tone. There was a substantial increase in the local expression of $G\alpha_{i2}$ and a slowing of conduction through the AV node.[91]Similarly, Murata et al. overexpressed the ras-related small G-protein GEM in ovine AV node and showed slower conduction through AV node and reduction of overall heart rate during AF. [92]Conversely, another approach to AF rate control is the knockdown of the stimulatory G protein α subunit ($G\alpha_s$), which mimics beta-blockade. Lugenvil et al. found that genetic inhibition of $G\alpha_s$ protein using adenovirus containing siRNA against $G_{\alpha\varsigma}$ in the AV node reduced heart rate by 20% and prevented AF-associated cardiac dysfunction in a porcine model.[93] Our group also targeted of vagal signaling in the left atrium by inhibiting $G\alpha_i$ and $G\alpha_o$ in canine models.[94] Here, injection of plasmids encoding the inhibitory peptides of $G\alpha_i$ and $G\alpha_o$ to multiple sites in the posterior left atrium (PLA) lead to attenuation of vagal-induced shortening of ERP and diminished AF inducibility during vagal stimulation.[94]

## Gap Junction remodeling

Connexins (Cxs) are subunit transmembrane proteins that oligomerize to construct a connexon, composed of six Cxs. Gap junctions are formed as the connexons of two neighboring cells dock together, permitting direct cell-cell communication and bidirectional passage of ions and small molecules up to 1 kd.[95] Reduced expression or abnormal localization of Cx40 and Cx43 are associated with impaired electrical conduction in the atrium and an increased risk of developing AF.[96,97] Accordingly, gene transfer of both of these connexins using an epicardial painting approach significantly improved expression and localization of the proteins, and was associated with improved conduction and a reduction in arrhythmia burden in a porcine model of AF.[98] A separate study of Cx43 alone in the same type of model resulted in similar findings, with a marked reduction in the development of persistent AF.[99]

## Structural remodeling

Atrial fibrosis is also a well-known factor in the pathogenesis of AF, and may explain the increasing prevalence of this arrhythmia with age. A central feature of age-related fibrosis is up-regulation of transforming growth factor (TGF)-β.[100] The PLA has been found to play an important role in the maintenance of AF due to increase in susceptibility to fibrosis and inhomogeneous conduction.[101]

Our group evaluated the effect of a transgene that interferes with TGF-β signaling on structural remodeling in the PLA. Injection of a minigene expressing a dominant-negative type II TGF-β receptor in the PLA of a canine HF model of AF resulted in decreased fibrosis and reduction in pacing-induced AF in the treated animals.[13]

## Inflammation/Oxidative injury

AF is a multifactorial disease and there is an ample evidence supporting the involvement of inflammation and oxidative injury in the pathophysiology of AF.[102,103] Inflammatory processes have been shown to affect the electrical and structural properties of the atria.[104] The importance of the NLRP3 (NACHT, LRR and PYD domains-containing protein 3) inflammasome in the development of AF was recently established. The activity of NLRP3 inflammasomes is altered not only in patients with AF but also in canine RAP model and in a murine model of spontaneous AF (CREM-TG mice), suggesting a major role for NLRP3 inflammasome in AF pathophysiology in the context of different pathologies. Yao et al. found that pharmacological inhibition by MCC950, an AAV9-mediated shRNA delivery to knockdown NLRP3, or genetic inhibition by NLRP3 knockout prevented the development of AF.[105]

Oxidative injury results from the imbalance between the generation and neutralization of reactive oxygen species (ROS), is a major contributor for AF and a possible therapeutic target.[106,107] ROS generated in the cardiovascular system are primarily derived from NADPH oxidase (NOX), mitochondrial electron transport chain, xanthine oxidase and uncoupled nitric oxide (NO) synthase.[108,109]Despite considerable evidence that ROS play an important role in the generation of AF, clinical trials using conventional antioxidants for post-operative AF have been unsuccessful,[110,111]likely because antioxidants do not reach sufficient, localized concentrations to overcome kinetic limitations and allow for scavenging of highly reactive free radical species.[112] Our group recently demonstrated a clear causative role of NOX2-generated oxidative injury in the genesis as well as the maintenance of AF. We showed that oxidative injury contributes to electrical remodeling in AF by upregulating a constitutively active form of acetylcholine-dependent $K^+$ current ($I_{KAch}$) – called$I_{KH}$ - by a mechanism involving frequency dependent activation of protein kinase C epsilon ($PKC_\varepsilon$). Injection and electroporation of plasmids expressing shRNA against NOX2 in the atrium of a canine AF model not only delayed the time to onset of non-sustained AF more than 5 fold but also prevented the development of sustained AF for up to 12 weeks.[113]

## Apoptosis

Apoptosis is associated with inflammatory pathways which contribute to electrical and structural remodeling in AF.[104]Downregulation of caspase-3 in canine AF model indicated association of apoptosis with AF via inhibition of calpain, a intracellular $Ca^{2+}$ activated protease.[114] Genetic knockdown of caspase-3 by transfer of adenovirus containing siRNA against caspase-3 suppressed or delayed the onset of persistent AF by reduction in apoptosis and prevention of conduction delay in porcine model. [115]

MicroRNAs are a class of endogeneous non-coding small RNAs that are becoming more recognized to play an important role in pathogenesis of AF. Zhang et al. examined differential expression of miRNA in ganglionic plexus of a canine AF model and found expression of miR-206 was elevated 10 fold and lentiviral infection of miR-206 resulted in repression of superoxide dismutase-1 (SOD-1).[116] Anti-miR-206 infection with lentiviral vector, thus, lead to prolongation of ERP and reduction of AF inducibility.[116]

# Future Gene Therapeutic Solutions

## Targeted Delivery of Gene Therapy Using Activation Mapping and Imaging

Since the description of the role of structural remodeling in AF, there has been increasing research on atrial fibrosis in the pathophysiology of AF.[117] Imaging methods have been developed to detect, localize, and quantify atrial fibrosis, which correlated with outcomes such as stroke and recurrence of AF.[118,119] *In vivo* activation mapping methods may allow targeted therapy of AF-specific mechanisms and may detect atrial substrates and mechanisms initiating and/or maintaining AF.[120,121] Advances in percutaneous catheter-based techniques with fluoroscopic and electroanatomic guidance should allow a less invasive, transendocardial gene delivery. Importantly, electroanatomical mapping may be useful to allow clear delineation of the region of interest and targeted deployment of the therapeutic product.[122] This could be applicable not only in the ventricle (i.e. in the presence of chronic ischemic heart disease and subacute myocardial infarction) but also in the atria (i.e. targeting focal structural or electrical remodeling).

# Conclusion

This review article summarizes current gene therapy strategies for the treatment of atrial fibrillation. Further development of gene therapy for this condition is encouraged by the limited efficacy of pharmacological and catheter-based therapies for AF. While AF remains a complex and heterogeneous clinical entity, gene therapy targeting multiple signaling pathways show very promising results in pre-clinical models. Improved longevity of vectors and expansion of targeting and delivery of vectors may lead to the development of effective and long-lasting treatment for AF.

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