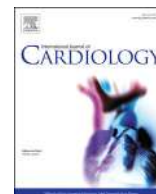




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## Review

Gene therapy for atrial fibrillation - How close to clinical implementation?<sup>☆</sup>Amar Trivedi, Jacob Hoffman, Rishi Arora<sup>\*</sup>

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## ABSTRACT

In this review we examine the current state of gene therapy for the treatment of cardiac arrhythmias. We describe advances and challenges in successfully creating and incorporating gene vectors into the myocardium. After summarizing the current scientific research in gene transfer technology we then focus on the most promising areas of gene therapy, the treatment of atrial fibrillation and ventricular tachyarrhythmias. We review the scientific literature to determine how gene therapy could potentially be used to treat patients with cardiac arrhythmias.

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## 1. Introduction

Invasive cardiac electrophysiology is a relatively young field in medicine. Modern ablative tools and techniques have been developed and refined over the last 50 years [1]. However, the treatment of cardiac arrhythmias is still suboptimal. Many diseases such as atrial fibrillation and ventricular tachycardia are difficult to cure with either invasive or pharmacological therapy [2]. Given the challenges of treating patients with heart rhythm disorders scientists are actively investigating new treatments paradigms such as the use gene therapy to treat cardiac arrhythmias. The field of gene therapy holds great promise to create a highly effective personalized treatment for cardiac arrhythmias. In this review we will begin by discussing the current state and advances in gene transfer technology. We will then examine that status of gene therapy for cardiac arrhythmias, with a particular focus on atrial fibrillation.

## 2. The basic components of myocardial gene transfer

For any potential cardiac gene therapy to be successful the gene(s) of interest must not only be delivered but also expressed at adequate concentrations in the myocardium. This is a two-step process in

which a vector is created to express the gene of interest; the vector is then delivered to the myocardium. There are two types of vectors which will be discussed, viral and non-viral vectors (Table 1).

## 2.1. Non-viral gene vectors

The basic non-viral gene vector is naked plasmid DNA directly injected into the myocardium. This type of vector has been used with success in cardiac gene therapy trials and is usually transduced to the cell via the process of lipid mediated transfection [3]. The benefits of plasmid DNA administration include scalability, limited cellular and antibody mediated immune response, and the ability to store a large DNA library with ease. A key advantage of plasmid vectors compared to other types of vectors is the lack of an antibody mediated immune response. This allows therapies to potentially be administered multiple times without causing adverse immune reactions.

The most pressing problem preventing the wide spread adoption of naked plasmid vectors is the low transfection rate when delivering genetic material [4]. Current lipid mediated transfection technology only allows a negligible amount of gene to enter the myocardium [5]. Complexing agents such as calcium phosphate have been shown to marginally improve the efficiency of gene uptake but their impact is limited [6]. One promising method of increasing drug delivery is sonoporation. Using ultrasound technology, energy can be non-invasively transmitted to a variety of structures throughout the myocardium. Genes can be placed in microbubbles that are “burst” with the application of ultrasound energy when the microbubbles pass through the region of interest. In the myocardium both naked

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**Table 1**

Table of available gene transfer methods. 1st section covers known methods of viral gene transfer, while the second section covers Non-viral gene transfer. For most viral vectors and non-viral methods of gene transfer, theoretical limits on transgene expression have not been determined experimentally as many trials find strong transgene expression after transduction for the duration of the trial. Many trials have gone on for months or even years without loss of expression.

	Transfer material	Diameter (nm)	Duration of expression	Packaging capacity	Cardiac specificity	Immunogenicity	Other features
Viral vector							
Adenovirus	dsDNA	70–100	1 month	36 kb	Non-specific	High	Previous adverse events
AAV 6	ssDNA	25	Max duration unknown	4 kb	High	Low	Induces immune tolerance
AAV 9	ssDNA	25	Max duration unknown	4 kb	Moderate	Low	Induces immune tolerance
Lentivirus	ssRNA	80–120	Max duration unknown	10 kb	Non-specific	Moderate	Integrating
Non-viral gene transfer							
Electroporation	Plasmid DNA	N/A	Max duration unknown	High	Local application	Very low	Technically challenging
Naked Plasmid	Plasmid DNA	N/A	Max duration unknown	High	Local application	Very low	Low efficiency

plasmid DNA as well as short interfering RNA (siRNA) has been transduced into the adult murine heart via sonoporation.

Our group uses non-viral DNA vectors with an alternative method to efficiently deliver gene therapy into the canine myocardium. Using a process known as electroporation guided gene delivery we can safely introduce high levels of gene products to specific locations of the myocardium. Electroporation is a two-step process that involves the injection of a non-viral gene vector into the myocardium followed by synchronized electrical pulses over short cycles of time which drives the target gene into the tissue (Fig. 1) [7]. The rate of gene uptake is 15–20 fold higher when electroporation is used vs standard plasmid DNA delivery [8]. Electroporation is the most effective and well developed method of non-viral gene vector delivery to date. Its advantages include relative ease of use, low cost of equipment and excellent ability to successfully transfer gene products into the myocardium. The main disadvantage is the need for at minimum a laparoscopic thoracic surgery to visualize the epicardium and effectively deliver gene therapy and electricity to the site of interest. Electroporation of non-viral vectors represents an exciting platform that can potentially allow the safe delivery of gene therapy to the human myocardium.

## 2.2. Viral gene vectors

Viral vectors are live, replication deficient viruses which have been genetically modified to allow the insertion of transgenes. Compared to non-viral plasmid insertion which must be directly injected into the tissue, viral vectors have the theoretical advantage of minimally invasive delivery via the blood stream.

The most commonly used viral vector is the retroviral vector. They have been used in numerous FDA approved clinical studies in both phase I and II including glioma and severe combined immunodeficiency. These studies have raised safety concerns about the risks of retroviral therapy, but advances in retroviral vector technology hope to mitigate these risks [9]. These vectors can integrate and express the transduced genes in the host DNA in a more stable manner [10]. The use of retroviral vectors in the myocardium, however, is limited. Retroviral vector technology requires active cellular reproduction to allow integration and expression of its transgene. This is problematic in terminally differentiated tissue like the myocardium. To overcome these obstacles, an area of active investigation is using lentiviral vectors in which the human immunodeficiency virus (HIV) machinery is used to transfer transgenes to post mitotic cells such as cardiac myocytes [11]. Lentiviral vectors are able to transfect intact nuclear membranes allowing transgene expression in terminally differentiated cells. A major advantage of lentiviral technology is what appears to be long term gene expression and multiple safeguards to protect against wild type reversion compared to other retroviral vectors [12]. In comparison to non-retroviral vectors, such as adenoviral vectors, lentivirus appears to have similar transfection efficiency when injected into the myocardium [11]. However, their safety and efficacy have yet to be demonstrated in any clinical trial of cardiac gene therapy to date.

The adenovirus (AD) and adeno-associated viruses (AAV) are currently the most commonly used viral vectors for cardiac gene therapy. The wild type adenovirus is a double stranded DNA virus that is one cause of the common cold. AD vectors have the 35 kB viral genome removed to allow delivery of moderate sized genes. This makes AD vectors simple to produce and distribute and easy to transduce with relative efficiency [13]. However, the AD vector has limited gene expression (2–4 weeks) and can cause intense immunological host responses that may lead to short term morbidity and organ damage [14]. This has led to disappointing short term results for the AD vector in clinical trials [15,16].

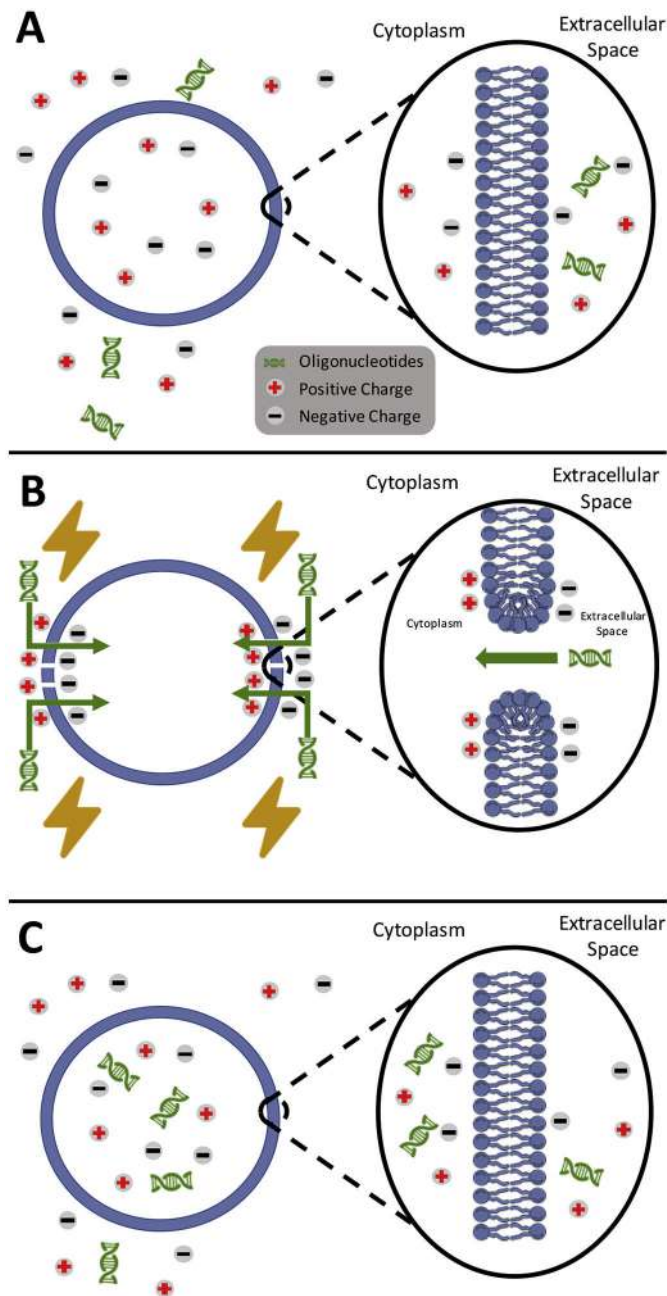
These limitations have led to the development of the adeno-associated virus (AAV) vector which share no relationship to the adenovirus and are a distinct class of virus. These vectors have multiple advantageous features including the capacity for long term gene expression. In some pre-clinical trials in skeletal muscle, AAV expression has persisted for prolonged periods of time, some for several years [17,18]. The primary disadvantage of AAV vectors is a limited gene insert size which makes it difficult to transduce larger genes and complex ion channels. Another disadvantage is delayed expression of the gene likely due to the need to convert the single stranded viral genome to the double stranded host genome [19]. Furthermore, transduction efficiency is more limited in larger mammal studies compared to the initial studies in rodents [20].

## 3. Gene delivery to the myocardium

Once a vector is chosen the next challenge is choosing the optimal method to deliver the vector to the target organ. In order to affect a wide variety of arrhythmia mechanisms gene therapy should provide dense, transmural and homogenous expression in the myocardial chamber(s) of interest. To date the ability to provide a delivery system that accomplishes the above goals remain one of the greatest challenges in translating molecular discoveries to actionable clinical therapies. There are three widely described methods to delivery gene vectors (both viral and non-viral) to myocardial tissue: direct injection, intracoronary perfusion, and epicardial gene painting.

Gene injection is the simplest and well-studied method. It involves direct injection of the gene vector into myocardial tissue. The vector can be injected epicardially or endocardially and produce a very high concentration of gene expression at the site of injection. However, gene expression only occurs within a few millimeters of the injection site making delivery to a large area of the myocardium technically challenging [21]. Though the use of electroporation as described previously improved the efficiency and depth of gene transfer, direct injection is still not practical to treat multiple chambers of the heart.

One method to overcome this limitation is delivering genetic vectors through the coronary vasculature, allowing the entire heart or selected regions of the left ventricle to be targeted. Intracoronary delivery can be used to deliver gene vectors via the coronary arteries; however, the conditions needed to optimize vector transduction are difficult to perform in humans [22,23]. Optimal



**Fig. 1.** Diagram of electroporative gene uptake. A. Before application of electrical energy, charges are evenly distributed and transgene-carrying plasmids are localized in the extracellular space. B. During electroporation, an electrical field is applied to the target area. Charge distribution becomes polarized resulting in a buildup of charge around the cell membrane. Micropores open in the membrane allowing the transgene to enter the cell. C. After electroporation micropores close and charges de-polarize, finalizing electroporative gene uptake. Transgene-containing plasmids are now localized in the cytoplasm.

conditions include hypothermia to 18 °C, disruption of normal coronary blood flow, and high doses of nitroglycerin, adenosine, and vascular endothelial growth factor. With optimization, intracoronary AAV vectors have been successfully delivered to the antero-septum of the left ventricle with high levels of gene expression [24]. The safety of intracoronary infusion was demonstrated in both the CUPID and CUPID 2 trials where an AAV vector carrying the SERCA2a gene was delivered to patients with advanced heart failure [25,26]. Both trials met their primary safety endpoints and demonstrated that gene therapy can be safely administered to patients with advanced heart failure.

Another approach to successfully deliver vectors to both atria in a transmural fashion is the process of epicardial gene painting (Fig. 2). First employed by Donahue and colleagues [27], this technique involves applying a combination of gene vector, poloxamer compound, and a protease (trypsin) to the atrial epicardium. The poloxamer compound improves vector contact with the atria, while trypsin is used to foster transmural penetration of the vectors. This method has been demonstrated to be safe and effective in animal models with minimal to no impact on atrial structure or function [24,27]. Epicardial gene painting has come closest to allowing a homogenous transmural application of gene therapy but is limited by requiring an epicardial approach that necessitates a surgical procedure. It is also unclear if this technique would also lead to unintended gene 'spillover' to the ventricle.

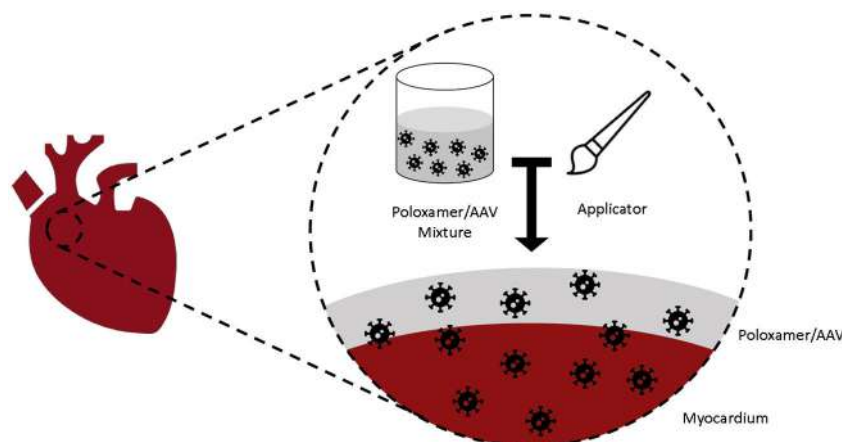
One further way to improve vector specificity of gene transfer is to insert genetic constructs under the control of cardiac specific promoters. In this way, accidental leakage of transduction to non-target tissues will not result in inappropriate transgene expression. Promoters for the human Troponin - T gene, human desmin, alpha MHC and MLC 2 genes have all been tested, with varying effectiveness reported [28–30]. While cardiac specific promoters can improve specificity, this may come at the cost of efficiency, as some cardiac specific promoters can drive lower levels of expression than comparable non-specific promoters such as CMV [28]. When designing a new gene therapy construct, care should be given to identify and test multiple promoters to find the one that has the optimal expression patterns for each individual therapy.

#### 4. Clinical outlook of gene vectors and delivery

The field of gene delivery continues to expand with new techniques and applications throughout the human body. While there is no “perfect” vector or delivery method that can target, integrate and express genes safely in the myocardium in a seamless manner, continued development of both viral and non-viral vectors will lead to safer and more effective methods. At the current time AAV vectors have been the most extensively studied in humans and demonstrated excellent safety profile in the CUPID trials. The safe delivery of target genes to the endocardial surface of the heart is another challenge that will need to be overcome prior to the widespread adoption of gene therapy for atrial fibrillation. Electroporation is currently being used by our group in animal models and is most feasible at the time of cardiac surgery. The gene painting technique is potentially another excellent approach to deliver target genes to the pulmonary veins and left atrium. With continued investigation an endocardial gene painting solution may be the most feasible way for electrophysiologists to deliver gene therapy. As progress continues to be made in gene vector and delivery technology much work is also being done to understand the appropriate target gene(s) needed to successfully treat atrial fibrillation.

#### 5. Gene therapy for atrial fibrillation

Of all the potential cardiac arrhythmias to be treated with gene therapy, atrial fibrillation (AF) is perhaps the most intensely researched. The development of atrial fibrillation increases a patient's risk of stroke, heart failure, dementia, and death [31,32]. There are many distinct genetic loci associated with AF identified through genome wide association studies [33]. There also appear to be other loci that have been found through familial linkage studies [34]. One of the most challenging aspects of atrial fibrillation treatment is the heterogeneous genetic, structural, and electrical abnormalities that lead to atrial fibrillation. Our current treatment strategies for restoring normal sinus rhythm in atrial fibrillation are limited by sub-optimal efficacy and potential morbidity and mortality. Pharmacological therapy with anti-arrhythmic drugs can have long term drug side effects [35,36]. AF ablation is increasingly being used to target patient with symptomatic AF but has limited efficacy in



**Fig. 2.** Epicardial gene painting. During surgery exposing the heart, a poloxamer/virus mixture is “painted” onto the heart. The viral vectors in the mixture transfect the heart, while the poloxamer acts as an immobilizing matrix that allows for increased duration of contact between virus and target tissue.

patients with persistent AF [37]. The procedure is also associated with significant morbidity including the risk of death [38,39]. In the following discussion we will highlight some of the more promising gene therapy approaches to preventing and treating atrial fibrillation. Thus far, all the gene therapy studies for AF have been performed in animal models. The vast majority of these have been performed in large animal models of AF, specifically pig and canine models. Since AF is predominantly a disease of the large, mammalian atrium, studies in these models have a major translational advantage, as compared to mouse models of AF. Indeed, most mechanistic studies in AF that demonstrate the role of electrical and structural remodeling in the genesis of AF have been performed in canine, goat and sheep models [40,41]. The nature of ion channel and structural remodeling in the atria of dogs, goats and sheep has been shown to parallel what is typically noted in patients with AF [42]. In the gene therapy studies performed in pigs, the nature of electrical remodeling noted in the atrium has been found to be similar to that noted in dogs and other large animal species [43,44]. Below we describe recent gene-based targeting of AF substrate in both pig and canine models of AF. Table 2 lists the main gene therapy studies performed in animal models in recent years.

## 6. Modifying electrical aspects of the atrial fibrillation substrate

### 6.1. Targeting ion channels

In atrial fibrillation a common mechanism of electrical remodeling is shortening of the action potential duration (APD) [45]. By shortening APD, re-entrant circuits are more easily inducible and maintained. Gene therapy has been used to prolong the APD in animal models by reducing the expression of the delayed rectifier

potassium channel  $IK_r$ . This occurs by inhibition of the  $KCNH2$  gene which is responsible for the alpha subunit of the  $IK_r$  channel. Amit et al. demonstrated in a pig model that APD could be prolonged via an epicardial gene painting of an adenovirus vector encoding a dominant negative mutation of  $KCNH2$  [46]. By interrupting the alpha subunit of the  $IK_r$  channel in the gene treated pigs, the investigators found an increase in the APD, resistance to burst pacing induced AF, and increased conversion from AF to sinus rhythm. These effects were reversed by 2 weeks which correlated with loss of gene expression. In another proof of concept study Soucek et al. used epicardial injection and electroporation to deliver the AdCERG-G627S transgene to disrupt  $KCNH2$  function [47]. They found that pigs who received gene therapy had markedly longer APD, and the development of persistent atrial fibrillation was delayed or prevented in a rapid atrial pacing model. Also of note, when compared to the control group, the pigs which received the transgene and developed persistent atrial fibrillation did not develop impaired left ventricular function [47]. Both studies were of too short of a duration to determine long term side effects of atrial  $IK_r$  suppression. Other studies have indicated that decreased L-type calcium channel density ( $I_{Ca,L}$ ) is implicated in AF [48]. Gene therapy to increase expression of these channels (either via up-regulation or by the addition of a highly expressed copy of the gene) could be effective in reversing this effect.

Kv 1.5 ion channels are another potential target for atrial fibrillation gene therapy. These ion channels are expressed preferentially in atrial myocytes and regulate the ultra rapid delayed rectifier current ( $IK_{ur}$ ) that is a major contributor to atrial repolarization after an action potential [49–51]. Inhibition of Kv1.5 leads to selective prolongation of the atrial, but not ventricular, action potential, and has thus been the target of a number of small molecule based therapies for AF [52]. Kv 1.5 knockdown (via a constitutively expressed siRNA or similar

**Table 2**  
Table of notable gene therapy studies in animal models of AF from 2005 to 2017.

Atrial fibrillation prevention/termination strategy	First author	Transgene	Vector	Delivery method	Model
Prolongation of the atrial action potential duration to suppress AF. Accomplished by introducing a dominant negative mutation to suppress $I_{Kr}$ current	Soucek et al. [38]	$KCNH2$ -G627S	Adenovirus	Electroporation and gene injection	Swine
	Kikuchi et al. [27]	$KCNH2$ -G628S	Adenovirus	Epicardial gene painting	Swine
	Liu et al. [72]	$KCNH2$ -G628S	Adenovirus	Epicardial gene painting	Swine
	Igarashi et al. [11]	CX43	Adenovirus	Epicardial gene painting	Swine
Increase expression of cardiac gap junction proteins CX43/CX40 to produce more homogenous atrial conduction	Bikou et al. [43]	CX43 + CX40	Adenovirus	Electroporation and gene injection	Swine
Inhibiting vagal signalling by disrupting Gi and/or Go proteins in the left atrium	Aistrup et al. [50]	Gi/Go terminal peptides	Plasmid	Electroporation and gene injection	Canine
Decreasing left atrial fibrosis to homogenize atrial conduction by introducing a dominant negative mutation of the TGF- $\beta$ receptor.	Kunamalla et al. [54]	TGF- $\beta$ Type II receptor	Plasmid	Electroporation and gene injection	Canine



oligonucleotide moieties) or knockout (via CRISPR mediated gene targeting) could have similar therapeutic effects without the need for repeated anti-arrhythmic treatments.

The Task-1 potassium channel is an atrial specific regulator of action potential duration. Its atrial specificity and strong regulatory effects on cardiac action potential make it an appealing target for anti-arrhythmic therapy [53]. Indeed, three different Task 1 mutations were shown to correlate with development of AF [54]. Patch clamp experiments and mathematical modeling have demonstrated that modulation of Task – 1 can alter the action potential of human cardiomyocytes [55]. The exact expression patterns of Task 1 in the context of AF have been investigated in large patient cohorts with persistent AF and have demonstrated enhanced atrial expression of TASK 1 [56,57]. While Task 1 inhibition contributes to an increase in action potential duration which would have potential positive effects on the AF disease state, in vivo porcine models have shown that in the context of dual AF and heart failure pathologies, Task 1 expression is downregulated [53]. This dual effect complicates the TASK 1 story and requires further consideration of TASK 1 before fully validating TASK 1 as a target for the treatment of AF.

### 6.2. Repairing dysregulated gap junctions

Gap junctions are key regulators of electrical conduction in the atria that regulate intracellular conduction velocity by connecting the cytoplasm of adjacent cells [58]. This connection is mediated by transmembrane ion channels called connexins. These connexins freely transport ions and small proteins between neighboring cells, allowing for electrical coupling and conduction. In human atria there are two connexin subunits that form gap junctions, CX40 and CX43 [58]. Due to the importance of these proteins in cell to cell coupling, altered expression of these molecules can have drastic effects on electrical conduction [59]. Additionally, altered connexin expression patterns can increase atrial refractoriness heterogeneity, contributing to the non-homogeneous conduction patterns that can lead to AF [58]. Indeed, it has been demonstrated in both animal models and human atrial tissue that decreased expression of CX 40 and 43 leads to AF associated remodeling [60]. Bikou et al. demonstrated that in pigs CX43 expression can be restored via epicardial direct injection/electroporation of an adenovirus encoding CX43 [61]. They found that gene treated pigs did not develop AF over 14 days of rapid atrial pacing while all control pigs developed AF. Another study in which epicardial painting restored expression of both connexins in a rapid atrial pacing pig model demonstrated improved conduction parameters with improved gap junction concentration [62].

### 6.3. Attenuation of parasympathetic signaling

Aberrant autonomic signaling, particularly cardiac parasympathetic signaling, is another factor that can contribute to the AF substrate [63]. The posterior left atrium (PLA), due to its denser innervation in comparison to other regions of the atrium has a parasympathetic profile that more readily contributes to AF [64]. Parasympathetic signaling is initiated via release of acetylcholine from vagal nerve endings, which then activate muscarinic type 2 receptors which interact with heterotrimeric  $G_i$  proteins [65].  $G_{Ai/o}$  subunits of this G protein go on to inhibit adenylate cyclase - cyclic AMP - protein kinase action, slowing down sinus rhythm and AV nodal conduction while shortening atrial refractoriness [66]. This shortening of refractoriness enhances propensity for re-entry, thereby contributing to the AF substrate [67]. Aistrup et al. showed that inhibition of  $G_{\alpha i}$  proteins by the addition of  $G_{Ai2/3}$  C-terminal peptides can attenuate vagal induced refractory period shortening and thereby lead to a decrease in vagal induced AF [68]. The same group then translated this finding into a gene-based therapy, demonstrating that the C-terminal  $G_{Ai/o}$  peptides expressed from a CMV expression plasmid following in vivo

electroporation could decrease vagal induced AF in a canine model [7].

## 7. Modifying structural aspects of the atrial fibrillation substrate

### 7.1. Targeting fibrosis

Basic molecular investigations have shown that AF is associated with an underlying pro-inflammatory state which leads to significant oxidative stress on the atria. This increase in inflammation and oxidative stress creates an imbalance in regulatory mechanisms which leads to increased cellular fibrosis and apoptosis. Extensive work in multiple organ systems has shown that organ fibrosis is intimately correlated with the upregulation of transforming growth factor (TGF- $\beta$ ) [69,70]. TGF- $\beta$  stimulates the production of collagen and other extracellular matrix proteins as well as the generation of reactive oxygen species [71]. In the myocardium the posterior left atrium has been found to have a unique role in the maintenance of AF due an increased susceptibility to fibrosis and heterogeneous conduction [72]. Our group has demonstrated that transduction of a transgene that interferes with TGF- $\beta$  signaling in the posterior left atrium can profoundly change the structure and electrophysiology of the posterior left atrium. Using direct injection and electroporation a potent dominant negative mutation of the TGF- $\beta$  receptor was transduced to the posterior left atrium of 12 canines. The canines who received gene injections had a significant reduction in atrial fibrosis. Reverse remodeling of the posterior left atrium, improved conduction, and reduction in pacing induced AF were seen after 3–4 weeks of rapid pacing. There was also a change in the restitution slope making the plasmid injected atrial tissue more resistant to AF. These electrical changes were correlated to reduced atrial fibrosis demonstrating a link between the atrial fibrillation substrate and conduction properties. These findings point to the ability of gene therapy to affect both the structure and function of atrial tissue by down regulating the inflammatory response that is seen in atrial fibrillation. This mechanistic insight may be useful for not only treating but also preventing the structural changes that foster the development of atrial fibrillation.

### 7.2. Targeting apoptosis

The increase in cellular apoptosis is another potential target for gene therapy. Dysregulation of the superoxide dismutase-1(SOD1) enzyme occurs in canine AF models. SOD1 plays an important role in cardiac apoptosis as well as oxidative stress signaling [73]. Zheng et al. demonstrated that silencing micro-RNA 206 could decrease susceptibility to AF by decreasing the activity of SOD1. They transduced an anti-MRI 206 lentivirus into the superior left ganglionated plexi in canines. These canines demonstrated a reduction in AF inducibility as well as a prolonged APD directly related to the effects of the anti-MRI 206 on the SOD1 enzyme. Another method of targeting apoptosis is through knocking down activity of caspase-3, a key apoptotic enzyme that can be inhibited with siRNA. In a porcine model treatment with an AD vector containing siRNA targeting caspase-3 led to a suppression of apoptotic activity within the atrium as well as a delayed onset of persistent atrial fibrillation [74].

### 7.3. Modifying both structural and electrical aspects of the AF substrate: Oxidative stress

The AF substrate is a complex interplay of cellular and ultrastructural factors; there is no single cause of AF. This complexity will likely require multiple points of contact to adequately modulate. The reactive oxygen species (ROS) generated by oxidative stress are ubiquitously reactive, interacting with biomolecules as common as protein, lipids and DNA [75,76]. This high reactivity translates to a vast array of

interfaces with the AF substrate; ROS have a high number of well documented interactions with a number of the known drivers of AF (Supplemental Fig. 1) [77]. By virtue of the many interfaces that ROS have with the AF substrate, their modulation has a high potential to exact outsized positive effects on the AF disease state.

Elevated levels of reactive oxygen species such as superoxide and  $H_2O_2$  are associated with the atrial fibrillation disease state [78]. This association is supported by secondary measures of oxidative stress, found in the serum of AF patients. AF patients have lower levels of nitric oxide bioavailability in addition to higher oxidized/reduced ratios of both glutathione and cysteine relative to patients without AF [79,80]. Hydroxyl radicals and peroxynitrate, two prominent reactive oxygen species, can cause oxidative damage to myofibrils, leading to some of the structural remodeling that characterizes AF [81]. ROS has also been linked to increased TGF- $\beta$  signaling, and the requisite fibrosis that is characteristic of structural remodeling in the AF substrate [82]. ROS can also damage mitochondrial DNA, causing cellular calcium overload and electrical remodeling that leads to AF [83]. Additionally, high ROS levels have been correlated with increased oxidation of CaMKII, which is linked to altered calcium handling and thus electrical remodeling in the atrial myocardium [77]. The current scientific evidence implicates ROS as a high level and compelling target to modify the disease course of atrial fibrillation.

#### 7.4. Targeting ROS production – NADPH oxidases

NADPH oxidases are membrane bound proteins found in a number of different tissue types; they catalyze the conversion of oxygen to superoxide [84]. The Nox family of NADPH oxidases are the primary generators of ROS in the healthy myocardium, and their contribution to overall ROS production is increased in the fibrillating atrium [78,85,86]. Though Nox activity is increased in the AF atrium, this effect appears, at least in some studies, to be independent of changes in Nox expression, indicating that higher levels of Nox activation, and not expression in the AF atrium are what drive increased ROS production [85]. Thus there are two ways to abrogate the amount of ROS being produced in the myocardium: lower Nox activation or reduce the total amount of Nox itself. To modulate Nox levels for therapeutic effect, we suggest two different gene based translational approaches. The first is through Nox inhibition via transgene mediated expression of inhibitory polypeptides. The second, and more straightforward route involves Nox knockdown either by CRISPR knockout or RNAi.

#### 7.5. Targeting *oxCaMKII* in AF

CaMKII (calmodulin dependent protein kinase II) is both a ROS sensor and pro-arrhythmic signal in the AF atrium [87]. CaMKII is also susceptible to oxidation at methionines 281 and 282, which locks it into a constitutively active formation associated with increased phosphorylation of RYR2 channels [88]. This phosphorylation leads to higher levels of SR calcium leakage, triggered action potentials, delayed after depolarizations (DADs) and ultimately AF [89]. Furthermore, this pro-fibrillatory effect was abrogated in knock in mice containing an oxidation resistant CaMKII variant indicating that *oxCaMKII* is an integral part of the ROS induced pro-fibrillatory pathway [89]. Gene therapy targeting *oxCaMKII* could potentially take one of two forms. The first is CaMKII knockdown, which can be achieved either by a CRISPR based system or RNAi. The second is gene replacement therapy, wherein CaMKII is replaced with an oxidation resistant form of the molecule to preserve normal functioning while decreasing the pro-fibrillatory capacity of the protein.

## 8. Future outlook

The treatment of atrial fibrillation at the substrate level with

gene therapy provides a revolutionary approach to a disease for which the current standard of care is at best limited. In recent years our understanding of the AF substrate has dramatically improved, creating attractive targets that can be precisely identified and thus targeted. As gene vectors and other gene delivery systems improve, the only limit to finding a gene based therapeutic for AF will be our understanding of the disease. As these two bodies of knowledge progress, we predict that a new paradigm in AF treatment will emerge, wherein human hearts are altered to be resistant to AF, improving quality of life for patients while reducing the burden on the healthcare system associated with AF co-morbidities.

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## Ethics statement

N/A.

## Conflict of interest

Dr. Arora has the following potential conflicts of interest: Rhythm Therapeutics (ownership interest).

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