Suppression of persistent atrial fibrillation by genetic knockdown of caspase 3: a pre-clinical pilot study

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Aims
Atrial fibrillation (AF) is linked to cardiomyocyte apoptosis, leading to atrial remodelling and reduction in electrical conduction velocity. We hypothesized that genetic suppression of an apoptotic key enzyme, caspase 3, would prevent the development of persistent AF by reducing apoptosis which may serve as an arrhythmogenic substrate.

Methods and results
Atrial fibrillation was induced in domestic pigs by atrial burst pacing via an implanted cardiac pacemaker. Study animals were then assigned to receive either Ad-siRNA-Cas3 gene therapy to inactivate caspase 3 or green fluorescent protein (Ad-GFP) as a control. Adenoviruses were applied using a hybrid technique employing right and left atrial virus injection followed by epicardial electroporation to increase expression of plasmid DNA. In pigs treated with Ad-siRNA-Cas3, the onset of AF was suppressed or significantly delayed compared with controls (10.3 ± 1.2 days vs. 6.0 ± 1.6 days; \( P = 0.04 \)). Electrical mapping revealed prolonged atrial conduction in the control group that was prevented by Ad-siRNA-Cas3 gene therapy. On the molecular level, Ad-siRNA-Cas3 application resulted in down-regulation of caspase 3 expression and suppression of apoptotic activity.

Conclusion
Knockdown of caspase 3 by atrial Ad-siRNA-Cas3 gene transfer suppresses or delays the onset of persistent AF by reduction in apoptosis and prevention of intra-atrial conduction delay in a porcine model. These results highlight the significance of apoptosis in the pathophysiology of AF and demonstrate short-term efficacy of gene therapy for suppression of AF.

Keywords
Apoptosis • Atrial fibrillation • Electrophysiology • Gene therapy • Remodelling

Introduction
Atrial fibrillation (AF), the most common sustained cardiac arrhythmia, contributes significantly to population morbidity and mortality and is associated with severe complications such as chronic heart failure and stroke.1–3 The mechanistic understanding of pathophysiological pathways underlying AF has advanced profoundly over the past two decades. Atrial fibrillation is maintained by electrical reentrant circuits resulting from shortening of effective refractory periods and from localized deceleration of intra-atrial conduction.3–4 Electrical and structural alterations referred to as atrial remodelling play an essential role in development and maintenance of AF.3 In particular, increased apoptosis (programmed cell death) was detected in fibrillating atria in humans and in a canine model.5–8 resulting in atrial fibrosis which is considered a fundamental mechanism in the perpetuation of AF.9–13 Caspases, a group of cysteine proteases that are activated specifically in apoptotic cells, are responsible for execution of the apoptotic pathway.14 Caspase 3 is a key downstream enzyme in the apoptotic process that directly cleaves apoptotic substrates. Increased caspase 3 expression has been detected in human and canine fibrillating atria.5–8 The goal of this study was...
to develop a gene therapy strategy to achieve rhythm control in AF by genetic suppression of atrial caspase 3 using an adeno-virus encoding for a respective silencing RNA (Ad-siRNA-Cas3). We demonstrate that inhibition of atrial caspase 3 reduces cardiomyocyte apoptosis, reverses deceleration of atrial conduction, and suppresses the development of persistent AF in a clinically relevant porcine AF model.\textsuperscript{15,16}

**Methods**

**Atrial fibrillation animal model**

We used an established AF model\textsuperscript{15,16} to evaluate antiarrhythmic gene therapy. A cardiac pacemaker (Frontier II, St Jude Medical, St Paul, MN, USA) was implanted in domestic pigs (20–30 kg body weight) to induce AF. Anaesthesia and analgesia were performed using ketamine (100 mg/kg; Roche, Grenzbach-Wyhlen, Germany), midazolam (15 mg/kg; Roche), isoflurane (1–2%; Baxter, Unterschleißheim, Germany), and buprenorphine (10 μg/kg; Essex Pharma, Munich, Germany). A bipolar active fixation pacing lead (4042 CapSure SP, Medtronic, Minneapolis, MN, USA) was inserted via the right external jugular vein and fixed in the right atrial (RA) appendage under fluoroscopic guidance. The lead was connected to the pacemaker unit positioned in a subcutaneous pocket in the neck. Following gene delivery, the pacemaker was programmed to a sinus detection rate of 180/min.

After pacemaker implantation and injection of Ad-siRNA-Cas3 or adenovirus containing green fluorescent protein (Ad-GFP), daily six-lead ECG recordings were performed during feeding to analyse heart rhythm. Animals were awake and alert at consistent levels during all ECG measurements during the observation period of 14 days. Persistent AF was defined as continuous AF without any evidence of intermittent sinus rhythm. The present study was performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health (NIH publication No. 86-23, revised 1985). The European Commission Directive 86/609/EEC was followed, and approval was granted by Heidelberg University ethics board.

**Adenoviruses**

An adenovirus encoding for caspase 3 siRNA (Ad-siRNA-Cas3) was used to knock down atrial caspase 3 expression (siRNA sense sequence: 5′-GCAGTAGAATTGCAGTCCTTT-3′). In the control group, a recombinant Ad-GFP was used. Ad-siRNA-Cas3 and Ad-GFP were constructed by Sirion Biotech (Martinsried, Germany) using the AdMax system (Microbix Biosystems, Toronto, Canada). Quality control of virus stocks included virus infective particle titre quantification by plaque assay, transgene expression confirmation by western blot analysis after transduction of HEK cells, and confirmation of absence of replication-competent virus by polymerase chain reaction (Sirion Biotech). Virus concentration was determined using a Rapid Titer Kit (Clontech, Mountain View, CA, USA).

**HL-1 cell culture and in vitro gene transfer**

HL-1 cells, a cardiac muscle cell line derived from the AT-1 mouse atrial myocyte tumour lineage which maintain the morphological, biochemical, and electrophysiological phenotype of adult myocytes in culture, were kindly provided by Dr William Claycomb (New Orleans, LA, USA).\textsuperscript{17} Spontaneous contractions of the cells were observed. HL-1 cells were plated on culture dishes coated with gelatin and fibronectin (both from Sigma-Aldrich, St Louis, MO, USA) and maintained at 37°C in an atmosphere of 95% humidified air and 5% CO\textsubscript{2} in Claycomb Medium (Sigma-Aldrich) supplemented with 10% foetal bovine serum, 2 mM l-glutamine, 0.1 mM norepinephrine (Sigma-Aldrich), penicillin, and streptomycin as published.\textsuperscript{17,18} The medium was changed approximately every 24–48 h. Cells were passaged regularly and subcultured prior to treatment. When confluence was reached, cells were dissociated using 0.05% trypsin/ethylenediaminetetraacetic acid (EDTA) (Gibco BRL) and resuspended in complete Claycomb Medium. Gene transfer was performed when cells were 70% confluent by adding 0.2 mL solution containing Ad-GFP (3 × 10\textsuperscript{9} plaque-forming units) or Ad-siRNA-Cas3 (1.3 × 10\textsuperscript{9} plaque-forming units) to 15 mL HL-1 culture media per 75 mL cell culture flask. Cells were harvested 48 h after virus application.

**In vivo gene delivery**

Atrial gene transfer was performed immediately following pacemaker implantation. Gene transfer by epicardial injection causes inhomogenous gene expression, located predominantly in areas surrounding the injection site.\textsuperscript{19} Here, we applied a novel and efficient technique of homogenous atrial gene transfer, employing local injections of adenoviruses followed by epicardial electroporation (EPO) to optimize vector uptake. This hybrid approach is based on the observation that electroporation increases expression of plasmid DNA into the cardiac and skeletal muscle.\textsuperscript{20,23} Median thoracotomy and pericardiectomy were performed, and the animals were randomized to receive either Ad-siRNA-Cas3 or Ad-GFP treatment. Then, 1 mL solution containing Ad-siRNA-Cas3 or Ad-GFP (5 × 10\textsuperscript{9} plaque forming units) was injected in aliquots of 0.1 mL into the RA and left atrial (LA) appendage wall. Adenoviruses were injected into both atrial appendages using a 22 G needle, carefully avoiding injections into the atrial cavity. Initially, the distance between injection sites was guided by a custom-made template of holes at 5 mm distances. With more advanced technical experience, adenovirus injection was performed manually. Injection of adenoviruses was directly followed by electroporation that was carried out using a paddle-style quadrupolar rectangular array of 2 × 2 stainless steel electrodes (electrode length, 5 mm; gap size, 15 mm). The electric field introduced by the electrodes causes transient pores to form in the cells of the atrial tissue, allowing adenovirus uptake into cells. Electroporation was applied to both atria. Three different positions (anterior, posterior, and atrial appendage) of each atrium were targeted, and five square wave applications were carried out at each position (20 V/100 ms; ECM 830, BTX Harvard Apparatus, Holliston, MA, USA). During electroporation, the atria were isolated from the ventricles using a custom-made rubber pad (3 × 3 cm). Following approximation of the pericardium and closure of the thorax, the burst pacing protocol was initiated as described.

**Epicardial mapping**

On the day of euthanization, the animals were anaesthetized and hearts were exposed by median sternotomy to perform epicardial mapping during sinus rhythm. In animals exhibiting AF, electrical cardioversion preceded epicardial mapping studies by at least 30 min. Atrial conduction properties were determined using a custom-made multi-electrode patch (2 × 2 cm) consisting of 3 × 4 bipolar electrodes with respective inter-electrode distances of 5 mm (Figure 1A). The mapping array was positioned at the free wall of the RA appendage. Usually more than 90% of electrodes recorded adequate signals with...
sufficient amplitude and no apparent motion artefacts. Right atrial epicardial pacing was performed at a basic cycle length of 400 ms in longitudinal and transversal direction to the fibre orientation (Cerablate Easy 30D Classic 110L, OSYPKA AG, Rheinfelden-Herten, Germany), and bipolar electrograms were recorded (Figure 1B). Fibre orientation was confirmed histologically. In order to assess intra-atrial conduction times, the activation time difference between the site of stimulation and the most distant electrode (row or column, respectively) was measured. Electrophysiological recordings and analyses were carried out using the EP-Lab System (Bard Electrophysiology Division C. R. Bard, Lowell, MA, USA).

**Echocardiography**

Echocardiographic examinations (Sonos 5500, Philips Healthcare, Hamburg, Germany) were performed on the day of pacemaker implantation and before euthanization. Animals were sedated and anaesthetized as described, and AF was electrically converted to sinus rhythm prior to examination. Sizes of LAs and left ventricles were measured in M-mode. Left ventricular ejection fraction (LVEF) was calculated from M-mode measurements using the Teichholz formula ($V = [7/(2.4 + LVid)] \times [LVid]^3$).

**Western blot analysis**

Protein immunodetection was performed by SDS gel electrophoresis and western blotting. Cardiac sections were dissected, rapidly frozen in liquid nitrogen, and stored at −80°C. Tissue samples were homogenized (Yellow Line DI 18 basic homogenizer, IKA, Essex, UK), followed by lysis in a buffer containing 1 M Tris–HCl, 50% NP-40, 10% sodium-deoxycholate, 5 M NaCl, 0.5 M EDTA, 0.1 M Na VO4, 0.5 M NaF, and protease inhibitors (Complete, Roche, Indianapolis, IN, USA). HL-1 cells were solubilized for 1 h at 4°C in lysis buffer containing 1% Triton X-100 and ‘Complete’ protease inhibitors (Roche Diagnostics). Protein concentrations were determined with the BCA method (Pierce, Rockford, IL, USA). Samples were normalized for protein content and proteins were separated on 6–10% gradient SDS polyacrylamide gels. Proteins were then transferred to nitrocellulose membranes and detected by sequential exposure to blocking buffer (5% dry milk), rabbit primary monoclonal antibodies directed against caspase 3 (9665; Cell Signaling Technology, Danvers, MA, USA), transforming growth factor β1 (polyclonal antibody; sc-146; Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphorylated homolog of the Drosophila protein ‘mothers against decapentaplegic’ SMAD2/3 (polycional antibody; sc-11769-R; Santa Cruz Biotechnology), and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH; G8140-11; US Biological, Swampscott, MA, USA), and HRP-conjugated goat anti-rabbit secondary antibodies (ab 6721, Abcam). Signals were developed using the enhanced chemiluminescence assay (Amersham, ECL Western Blotting Reagents, Buckinghamshire, UK) and quantified using ImageJ 1.41 software (National Institute of Health, USA). Protein content was normalized to GAPDH for quantification of optical density.

**Immunohistochemistry and direct fluorescence visualization**

Right atrial sections (4 µm thickness) embedded in paraffin wax were dewaxed in xylene, rehydrated through a graded alcohol series, and rinsed in phosphate-buffered saline (PBS). Formalin-fixed tissue was subjected to heat-induced epitope retrieval prior to immunohistochemical staining using 0.01 M sodium citrate buffer (pH 6.0). After inhibition of endogenous peroxidase activity with 3% H2O2 in methanol for 20 min to reduce background staining, non-specific-binding sites were blocked using 0.5% Triton X-100 in bovine serum albumin (1% in PBS) for 1 h. Sections were then incubated with polyclonal rabbit anti-caspase 3 antibodies (9662; Cell Signaling Technology). Antigen–antibody complexes were visualized with HRP-conjugated goat anti-mouse IgG (sc-2004, Santa Cruz Biotechnology) for 1 h. Peroxidase activity was detected with diaminobenzidine (DAB) using the SK4100 kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer’s instructions. In addition, haematoxylin and eosin staining was performed, and images were acquired with an AX70 microscope (Olympus, Hamburg, Germany). Ten sections were analysed from each animal for immunodetection of caspase 3, terminal deoxyribonucleotide transferase-mediated dUTP

![Figure 1](https://academic.oup.com/eurheartj/article-abstract/34/2/147/433304/433304) Epicardial mapping. (A) Multielectrode array used to record bipolar electrograms from right atrium during sinus rhythm. (B) Representative right atrial electrograms recorded using the electrode array from (A). Electrical stimulus and 11 resulting electrograms are indicated by arrows.
nick end labelling (TUNEL) assays, and quantification of fibrosis, respectively. The percentage of HL-1 cells expressing GFP was determined 48 h after Ad-GFP gene transfer with direct fluorescence visualization by counting cells in 10 randomly selected sections.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling staining
Apoptosis was detected by TUNEL. Sections of RA tissue were embedded in paraffin and cut into 4 μm thickness. Embedded tissue was dewaxed and rehydrated. After washing with PBS for 10 min, sections were post-fixed in ice-cold acetone, rinsed with PBS, and incubated in 200 mL of 0.1 M citrate buffer (Sigma-Aldrich) and 0.1% Triton X-100 (Roche Applied Science, Mannheim, Germany) (pH 6.0). Then, 300 μL TUNEL reaction mixture (285 μL label solution + 15 μL enzyme solution; Hoechst, Frankfurt am Main, Germany) was added to the sections and slides were incubated for 60 min at 37°C. After removal of the TUNEL reagent, slides were rinsed with PBS and stained with blue nuclear stain (Hoechst 33258, Sigma-Aldrich) to visualize all nuclei. Sections incubated with control solution alone (TUNEL kit, Hoechst) and sections incubated with DNAse I (Sigma Aldrich) served as negative and positive controls, respectively. Termi nal deoxynucleotidyl transferase-mediated dUTP nick end labelling-positive cells were counted using a fluorescence microscope (IX 50, Olympus) to calculate the percentage of TUNEL-positive cells in randomly selected fields.

Electrophysiological studies
Electrophysiological studies were performed during sinus rhythm prior to pacemaker implantation and before euthanization using the EP-Lab system (Bard Electrophysiology Division). In the case of AF, an electrical cardioversion preceded the examination, and studies were performed at least 30 min after cardioversion. A quadrupolar diagnostic catheter (Supreme CRD-2, St Jude Medical) was introduced via the jugular vein and placed in high RA position. To determine atrial effective refractory periods (AERP), repeated trains of 10 stimuli at a fixed cycle length of 400 ms were applied, followed by a single programmed premature stimulus. The coupling interval between the last basic stimulus and the premature stimulus was decreased in 10 ms steps until no atrial response was detected.

Evaluation of fibrosis
Right atria were dissected from the heart, and diffuse fibrotic material was removed. Sections for microscopic analysis were fixed in 10% formalin, embedded in paraffin, cut to 7 μm thickness, and stained with Masson’s trichrome to identify interstitial fibrosis. Sections were examined at 64-fold magnification by three independent and blinded observers using an Olympus Provis AX 70 microscope (Olympus Life Science Europe GmbH, Hamburg, Germany). The extent of fibrosis was graded using an arbitrary scale from 1 to 4, where 1 reflects normal cardiac tissue and 4 indicates near-complete fibrosis. Reported scores reflect average values of n = 3 blinded observers.

Statistical analysis
A total of 11 pigs were investigated in this study. Data are presented as mean ± SEM. Statistical differences in continuous variables were determined with Origin 6 software (OriginLab, Northampton, MA, USA) using paired and unpaired Student’s t tests (two-sided tests) where appropriate. A value of P < 0.05 was considered statistically significant. Multiple comparisons were performed using one-way ANOVA. If the hypothesis of equal means could be rejected at the 0.05 level, pair wise comparisons of groups were made and the probability values were adjusted for multiple comparisons using the Bonferroni correction.

Results
In vitro efficacy of virus transduction
To assess transduction efficacy in vitro, mouse atrial cardiac myocytes (HL-1 cells) were subjected to Ad-GFP gene transfer. The percentage of cells exhibiting green fluorescence as a measure of transduction efficacy was 33.6 ± 2.6% (n = 10 randomly selected sections) 48 h after gene transfer (Figure 2A), indicating moderate gene transduction efficacy in the absence of electroporation in vitro. In addition, significant reduction in caspase 3 protein was demonstrated in vitro by western blot analysis 48 h after Ad-siRNA-Cas3 treatment (−58.8%; n = 3; P = 0.001) compared with untreated HL-1 cells (Figure 2B and C). Adenovirus containing green fluorescent protein application did not significantly affect caspase 3 expression in HL-1 cells (Figure 2B and C).
Anti-caspase 3 gene therapy suppresses atrial fibrillation

Gene transfer was performed in vivo employing a hybrid approach combining right and LA adenovirus injection and EPO to increase plasmid DNA expression. Electroporation increases expression of plasmid DNA in the skeletal and cardiac muscle. During the follow-up period of 14 days, persistent AF was induced by repetitive atrial burst pacing in all control animals treated with Ad-GFP after 6.0 ± 1.6 days (n = 5; Figure 3A, C, and D). In contrast, genetic inactivation of caspase 3 by Ad-siRNA-Cas3 therapy suppressed AF in two animals (33.3%; Figure 3C) and delayed the mean onset of AF to 10.3 ± 1.2 days (n = 6; P = 0.04; Figure 3B-D). Mean ventricular heart rates during AF were not significantly different between both groups [Ad-GFP, Day 14, 272 ± 4.6 b.p.m.; (n = 5); Ad-siRNA-Cas3, Day 14, 263 ± 6.3 b.p.m.; (n = 4); P = 0.25], suggesting that Ad-siRNA-Cas3 gene therapy did not alter atrioventricular conduction properties.

Effective attenuation of cardiomyocyte apoptosis by caspase 3 knock down

Atrial fibrillation is linked to apoptosis, leading to atrial fibrosis, delay of intra-atrial conduction, and perpetuation of AF. Significant suppression of caspase 3 protein expression in RA appendage wall after Ad-siRNA-Cas3 treatment (−31.7%; n = 5; P = 0.01) was demonstrated by western blot analysis at the time of sacrifice (Figure 4A and B). In western blots, antibodies against unfragmented caspase 3 display a distinct double band with molecular weights of ~31 and 33 kDa. It is not known whether the two forms are the result of pre- or post-translational modification. Immunohistochemistry and subsequent quantification of cells exhibiting brown staining was carried out to provide additional evidence for reduced caspase 3 expression (~73.3%; n = 5; P = 0.003; Figure 4C and D). To assess whether genetic inactivation of caspase 3 and suppression of AF were associated with reduced programmed cell death, apoptotic activity was evaluated by TUNEL fluorescence of RA tissue sections (Figure 4E and F).

Figure 3 Suppression of atrial fibrillation by anti-caspase 3 gene therapy. (A) Representative ECG recordings from control animals (Ad-GFP) showing sinus rhythm on Day 1 and persistent AF on Day 14. In contrast, sinus rhythm was still observed on Day 14 in two animals receiving Ad-siRNA-Cas3 treatment, while others had developed persistent AF (B). (C and D) Onset of persistent AF (C) and mean duration of freedom from AF (D); Ad-GFP, n = 5; Ad-siRNA-Cas3, n = 6), illustrating that the development of persistent atrial fibrillation was suppressed or delayed by anti-caspase 3 gene therapy. Data are given as mean ± SEM; *P < 0.05 vs. Ad-GFP-treated animals.
We found that Ad-siRNA-Cas3 gene therapy decreased apoptosis rates from $11.6 \pm 1.6\%$ ($n = 4$) in the control group to $1.3 \pm 0.2\%$ ($n = 5$; $P = 0.0004$).

**Correction of intra-atrial conduction heterogeneity after gene therapy**

To evaluate the effects of caspase 3 suppression and reduction in apoptosis on intra-atrial conduction properties, conduction times were assessed by epicardial mapping using a multipolar patch electrode. Analyses were performed on RA appendage tissue during sinus rhythm following electrical cardioversion if required. We detected conduction heterogeneity in the control group when activation times in longitudinal and transversal orientation to the muscle fibre direction were compared (GFP long, $49.4 \pm 4.3\ms$; GFP trans, $33.8 \pm 6.7\ms$; $n = 5$; $P = 0.016$; Figure 5A), consistent with previous reports. Ad-siRNA-Cas3 treatment corrected conduction heterogeneity (Cas3 long, $32.8 \pm 1.9\ms$; Cas3 trans, $32.8 \pm 4.9\ms$; $n = 5$; $P = 0.76$; Figure 5B) through a significant reduction of longitudinal conduction compared with control animals treated with Ad-GFP ($P = 0.006$). This beneficial effect of Ad-siRNA-Cas3 gene therapy is expected to remove local areas of slow conduction that maintain persistent AF. Left atrial tissue was not readily accessible owing to the surgical access (median sternotomy), preventing in vivo electrical mapping of the LA.

Changes in conduction velocity may be accompanied by altered AERP. Atrial effective refractory periods were obtained from high RA during electrophysiological studies on Day 1 (Figure 5C) and on the day of sacrifice (Day 14; Figure 5D). We observed significant AERP prolongation after initiation of the repetitive burst stimulation protocol in Ad-GFP animals (Figure 5D). Ad-siRNA-Cas3 gene therapy did not significantly modulate AERP (Figure 5D). In addition, AF is associated with increased interstitial fibrosis. Reduced longitudinal conduction velocity may be attributed to beneficial effects of gene therapy on atrial interstitial fibrosis. On the other hand targeted atrial gene therapy may induce local fibrosis. To assess the degree of RA fibrosis (Figure 6), hearts of four animals from each study group were subjected to histological analysis using Masson’s trichrome stain (Figure 6A). Control animals (Ad-GFP) exhibited only mild fibrosis after 14 days. Relative to controls, we detected no significant differences in fibrosis in response to Ad-siRNA-Cas3 gene therapy (Day 14; Figure 6B).
Consistent with this observation, profibrotic factors TGFβ1 (transforming growth factor β1) and phosphorylated homolog of the Drosophila protein 'mothers against decapentaplegic' (SMAD2/3) exhibited similar expression levels between both study groups (Figure 6C–F).

Functional effects of Ad-siRNA-Cas3 gene therapy

Suppression of persistent AF may be associated with beneficial effects on cardiac function. To assess changes in LA and left ventricular function, echocardiographic exams were performed before pacemaker implantation and after 14 days. All measurements were carried out after restoration of sinus rhythm, where appropriate.

Repetitive atrial burst pacing and persistent AF resulted in pronounced dilation of the LA, resembling findings in humans. In the control group (Figure 7A and B), LA diameter increased from 21.5 ± 3.3 mm (Day 1) to 35.9 ± 4.8 mm (Day 14; n = 5; P = 0.039). Pigs treated with Ad-siRNA-Cas3 exhibited atrial dilation as well (Day 1, 16.9 ± 2.2 mm; Day 14, 26.6 ± 2.7 mm; n = 6; P = 0.018). On Day 14, we observed a tendency towards reduced LA diameters in the Ad-siRNA-Cas3 group (Figure 7B); however, this difference did not reach statistical significance (P = 0.109). Echocardiograms performed to evaluate ventricular function on Day 1 during sinus rhythm revealed similar LVEFs among both study groups (Figure 7C). Mean LVEF values yielded 66.0 ± 2.2% (Ad-siRNA-Cas3) and 62.9 ± 1.3% (Ad-GFP) (n = 5; P = 0.27). At the time of sacrifice (Day 14), LVEF was reduced in control animals (50.2 ± 2.2%; n = 5; P = 0.0011), consistent with impaired cardiac function observed in a subset of AF patients. In pigs treated with Ad-siRNA-Cas3, LVEF was reduced compared with Day 1 similar to the control group (52.1 ± 1.8%; n = 6; P = 0.006; Figure 7D).

Discussion

Medical therapy represents the standard initial treatment for most AF patients. However, pharmacotherapy is limited by reduced efficacy, side effects, and safety issues in a significant number of patients. Non-pharmacological therapy is improving, but only a restricted number of patients can be treated by pulmonary vein isolation. In search for novel treatment modalities to overcome these limitations, gene therapy offers greater selectivity than small-molecule approaches.

To further explore this emerging field, we sought to suppress AF by specifically preventing AF-associated remodelling via targeted atrial gene therapy. We present a genetic approach to rhythm control using siRNA-mediated inactivation of a key apoptotic enzyme, caspase 3, in a clinically relevant porcine AF model. We demonstrate that AF-associated apoptosis was suppressed by targeted gene therapy. As a result, deceleration of atrial conduction was prevented and the development of persistent AF was inhibited or delayed.
In vivo effects of genetic caspase 3 silencing on atrial fibrillation and cardiac function

Gene therapy delayed the onset of persistent AF during the follow-up period of 14 days from 6 to 10 days (Figure 3). In two pigs treated with Ad-siRNA-Cas3, persistent AF was not inducible at all by RA pacing. The follow-up period was limited to 14 days to avoid confounding the results by loss of gene expression after 2 weeks that is known to occur with first-generation adenoviral vectors. Similar to AF patients, an increase in mean LA diameter was detected after initiation of burst pacing. Animals treated with Ad-siRNA-Cas3 exhibited a tendency towards less LA dilation compared with controls (Figure 7), indicating beneficial effects of gene therapy on macroscopic atrial remodelling. Consistent with tachyarrhythmia in predisposed human subjects, the pigs investigated here developed decreased LVEF. Although there were significant differences in AF burden between the Ad-siRNA-Cas3 group and control pigs, all animals exhibited similar LVEF reduction. The tendency towards reduced LVEF independent of suppression of persistent AF is readily explained by the experimental protocol (i.e. repetitive atrial burst pacing and associated rapid ventricular rates). In summary, genetic suppression of atrial caspase 3 protein expression and the resulting protection against AF did not significantly attenuate the moderate cardiodepressive effect of AF and/or atrial burst pacing in vivo under the given experimental conditions.

Molecular and electrophysiological mechanisms of genetic rhythm control

Atrial fibrillation may result from a variety of pathophysiological processes, leading to electrical and structural remodelling. The generation of substrates that support slow conduction, shortening
of atrial refractory periods, and electrical reentry is particularly relevant as it provides the basis for maintenance of persistent AF.\textsuperscript{4,34} Apoptosis is a critical factor in AF-associated structural remodelling, slowing atrial conduction and promoting reentry.\textsuperscript{3,5–7,9,35}

The rationale of this short-term proof-of-concept study was to suppress persistent AF by reducing atrial apoptosis, thereby targeting substrate development. First, we found that pacing-induced AF was associated with apoptosis in control animals (Figure 4). Next, a significant decrease in atrial caspase 3 expression was achieved in vivo by myocardial Ad-siRNA-Cas3 gene therapy, which caused reduction in apoptotic activity and ultimately resulted in suppression of AF triggered by RA burst pacing. Atrial fibrillation suppression may either be achieved by elimination of electrical triggers (e.g. by pulmonary vein isolation) or by prevention of electrical reentry.\textsuperscript{3} Here, electrical mapping revealed that anti-caspase 3 gene therapy effectively suppressed AF by prevention of atrial conduction delay (Figure 5), a mechanism that prevents reentry (Figure 8). To our knowledge, this is the first study demonstrating an improvement of atrial conduction by targeting structural remodelling. Prolongation of atrial refractoriness which has proven effective during experimental pharmacotherapy\textsuperscript{36} was not observed. In contrast to studies that demonstrated AERP shortening associated with AF,\textsuperscript{37,38} AERP was prolonged in our study. This finding may be attributed either to electrical remodelling resulting from atrial stretch as described in heart failure models\textsuperscript{39,40} or to repetitive burst pacing. Furthermore, recent studies indicate that myocardial interstitial fibrosis is a fundamental mechanism in the perpetuation of AF.\textsuperscript{10} We detected mild fibrosis in RAs of both study groups (Figure 6). Of note, there was no significant difference between animals treated with Ad-siRNA-Cas3 compared with controls despite similar expression of profibrotic factors TGF\textbeta{}\textsubscript{1} or phosphorylated SMAD2/3 (Figure 6), suggesting that indirect electrophysiological remodelling induced by expression of caspase 3 rather than attenuated fibrosis contributed to reduction in longitudinal conduction velocity. In particular, cardiac fibroblasts have been shown to produce paracrine factors that reduce cardiac conduction velocity through down-regulation of cardiac ion channels SCN5A (I\textsubscript{Na}), KCNJ2 (I\textsubscript{K1}), and KCND3 (I\textsubscript{to}).\textsuperscript{41} After ruling out specific effects of Ad-siRNA-Cas3 gene therapy on atrial interstitial fibrosis or AERP, we hypothesize that our approach targets apoptosis at an early stage, when paracrine electrophysiological effects of cardiac fibroblasts suppress the development of induced persistent AF but before significant structural damage is apparent.

**Study limitations**

The goal of the present work was to assess the feasibility and short-term efficacy of antiarrhythmic gene therapy to treat persistent AF. In this pre-clinical, proof-of-concept study, reduction in apoptosis by genetic suppression of caspase 3 expression successfully prevented the onset of persistent AF in pigs. It is important to recognize that the sample size in this pilot study was small due to the animal model and that additional studies should be conducted in larger groups of animals with extended observation periods prior to evaluation of antiarrhythmic gene therapy in humans. The Ad-GFP control group used in this study allows for comparison of caspase 3 knock down (Ad-siRNA-Cas3) with GFP gene...
transfer that is not expected to induce electrophysiological effects. Additional control groups allowing for a precise assessment of unspecific effects of virus application or of gene transfer were not included in the study concept. The reduction in caspase 3 expression and prevention of apoptosis was observed in Ad-siRNA-Cas3 animals but not in pigs treated with Ad-GFP that were subjected to adenovirus injection and control gene transfer. Thus, we conclude that potential non-specific effects of virus application or gene transfer did not significantly affect apoptosis and antiarrhythmic effects of Ad-siRNA-Cas3 gene therapy. At the molecular level, Ad-siRNA-Cas3 treatment did not significantly affect the extent of fibrosis in atrial tissue, despite significant reduction in apoptosis. Paracrine modifiers may account for altered electrical conduction velocity in the Ad-siRNA-Cas3 group; however, the precise molecular mechanism remains to be elucidated. Remaining obstacles of antiarrhythmic Ad-siRNA-Cas3 gene therapy that need to be overcome include optimized control over local gene distribution, potential tumorigenicity of vehicles and siRNA application, and prevention of local and systemic inflammatory responses. These potential safety issues need to be solved prior to application of siRNA-based antiarrhythmic gene therapy in humans. Adenoviral vectors were used in this work owing to their ability to induce peak expression within a short time and to their high efficacy in infecting cardiomyocytes. For long-term applications and to study long-term stability, efficacy, and safety of upstream gene therapy, the use of adeno-associated virus or lentivirus as a vector would be more suitable.

Clinical implications and conclusions
Antiarrhythmic drug approaches to treat AF have limited effectiveness. Basic research has revealed that the ability of an antiarrhythmic intervention to suppress AF depends on its capacity to suppress the underlying mechanisms. Thus, reversal of atrial remodelling by targeting substrate development has become the focus of attempts at therapeutic intervention (so-called upstream therapy). The present pre-clinical study confirms the role of cardiomyocyte apoptosis in the development of an AF substrate. Based upon these mechanistic data, we demonstrate the efficacy of gene therapy targeting apoptosis in the suppression of AF in a clinically relevant large animal model.

Our hybrid gene application technique combining local virus injection and electroporation for anti-remodelling treatment could be readily performed during open-chest cardiac surgery in humans to suppress post-operative AF. To further refine the gene transfer method, thoracotomy may be replaced in future studies by interventional, transvenous virus application via specific catheters. After successful establishment of a minimally invasive technique and following safety assessment, antiarrhythmic gene therapy could become a valuable option to treat an arrhythmia associated with high morbidity and mortality. In summary, we demonstrate that AF suppression by targeted biological modification of upstream substrate development represents a promising therapeutic approach. We conclude that antiarrhythmic gene therapy may prove to be an effective strategy to eliminate the most debilitating of arrhythmias in the future.

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