Connexin 43 gene therapy prevents persistent atrial fibrillation in a porcine model

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Aims
Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia, and effective treatment of AF still remains an unmet medical need. AF is associated with atrial conduction disturbances caused by electrical and/or structural remodelling. We hypothesized that AF suppresses expression of the gap junction protein connexin (Cx) 43 and that Cx43 gene transfer to both atria would prevent persistent AF. The first aim of this study was to assess whether AF is associated with connexin remodelling in a porcine model. A strategy to suppress persistent AF by gene therapy was then developed and evaluated in vivo.

Methods and results
AF was induced in domestic pigs via atrial burst pacing, causing a 62.4% reduction in atrial Cx43 protein. Adenoviruses encoding for Cx43 (AdCx43) or green fluorescent protein (AdGFP) were injected into both atria, followed by epicardial electroporation to enhance transgene expression. Combining direct injection of adenoviruses with electroportation achieved GFP reporter gene expression in ≏ 50% of atrial cells in vivo. AdCx43-treated animals exhibited a 2.5-fold increase in atrial Cx43 protein content and did not develop persistent AF during the observation period of 14 days. In contrast, control animals developed persistent AF within 7.4 ± 0.5 days. Rapid ventricular heart rates during AF led to deterioration of cardiac function in control pigs but not in pigs treated with AdCx43.

Conclusion
Our results highlight the contribution of Cx43 to the pathophysiology of AF and demonstrate the viability of gene therapy for prevention of atrial arrhythmias.

Keywords
Atrial fibrillation • Connexin 43 • Electrical remodelling • Gene therapy • Rhythm control

1. Introduction
Atrial fibrillation (AF) is the most common sustained arrhythmia, associated with high morbidity and mortality.1–3 AF is often observed in patients with structural heart disease but also occurs as lone AF.4 The co-existence of AF and cardiac disease is associated with a worse clinical outcome.5 The current treatment of symptomatic AF is based on drug therapy and ablative strategies. Antiarrhythmic drug therapy is limited by a relatively high recurrence rate and severe side effects including sudden cardiac death due to proarrhythmic effects.5 Ablative therapy focuses on elimination of electrical triggers located in pulmonary veins and in the left atrium that initiate and perpetuate AF.5 This may be achieved by electrical isolation of pulmonary veins, creation of linear lesions, ablation of complex-fractionated atrial electrograms, or ablation of autonomic ganglia. Pulmonary vein isolation suppresses paroxysmal AF in up to 80% of patients without structural heart disease.8 In patients with persistent AF or with concomitant structural heart disease, the relapse rate is increased and successful ablation is more difficult to achieve.9 In addition, AF ablation is associated with a risk of complications such as left atrial perforation, atrio-oesophageal fistula, pulmonary vein stenosis, or thrombo-embolic events.10,11

Pathophysiologically, AF is facilitated by atrial conduction disturbances and by shortening of repolarization.1,12,13 Irregular atrial conduction may be caused by structural and/or electrical remodelling of the atria. Ion channels and gap junction proteins are key regulators of conduction in the heart.14,15 In particular, intercellular communication via gap junctions influences electrical conduction velocity in cardiac tissue. Gap junctions are clustered channels consisting of
two hemichannels, each formed by six connexin (Cx) proteins which connect the cytoplasm of adjacent cells. Cx40 and Cx43 subunits form gap junctions in human atria, and mutations or polymorphisms in Cx40 and Cx43 genes have been linked to AF and reduced intercellular electrical coupling in humans and mice. However, there appears to be low penetrance and/or significant interindividual modulation of Cx40-mediated effects on atrial conduction and AF, as a previously described Cx40 promoter polymorphism was not found to influence Cx40 expression or risk of AF by different investigators. The complex interaction between connexin subunits is further illustrated by data, indicating that conduction velocity is determined by the individual ratio between Cx40 and Cx43 in atrial tissue. Previous work on connexin expression and function in animal models and humans primarily points towards AF-associated remodelling in which there is a decrease in Cx43 expression and slowing of electrical conduction. However, discrepancies regarding the impact of connexins on AF and on atrial conduction properties remain to be resolved. Specifically, clinical presentations of AF (i.e. paroxysmal, persistent, and permanent AF) may be associated with distinct connexin remodelling that changes with time. In addition to connexins, atrial conduction velocity depends on other ion channels and fibrosis. Furthermore, the electrophysiological mechanisms of AF depend on the underlying pathophysiological condition. Finally, complexity is added by combinations of structural and electrical proarrhythmic mechanisms leading to AF in individual patients.

In a candidate gene-based approach, we hypothesized that Cx43 gene transfer would prevent the development of persistent AF by increasing atrial conduction velocity in a clinically relevant porcine model. The present study consisted of three phases. The first aim was to assess whether AF is associated with remodelling of Cx43 in a porcine model. Next, an optimized hybrid method of atrial gene transfer was developed, combining direct virus injection and epicardial in vivo electroporation to increase gene transfer efficiency. In a third step, feasibility and efficacy of AF suppression by gene therapy targeting atrial Cx43 protein was evaluated in vivo.

2. Methods

2.1 AF/chronic heart failure animal model
Gene therapy for rate control was evaluated in an established porcine AF/chronic heart failure (CHF) model. Atrial pacing induced persistent AF. For the purpose of this study, persistent AF was defined as continuous AF without any evidence of intermittent sinus rhythm during daily surface ECG recordings for at least 48 h. This study was approved by the Institutional Animal Care and Use Committee at the University of Heidelberg and has been carried out 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 current version of the German Law on the Protection of Animals was followed.

2.2 Myocardial gene transfer
In order to establish and optimize the gene transfer method, two groups consisting of five animals each were observed for 1 week to assess gene transfer efficiency: (i) pacemaker implantation and atrial gene transfer followed by electroporation; and (ii) pacemaker implantation and atrial gene transfer without electroporation. Five additional animals were subjected to gene transfer and electroporation without prior pacemaker implantation to exclude effects of electroporation and atrial gene transfer on cardiac function. In the subsequent treatment phase of the present study, 10 additional animals were assigned to AF induction and atrial gene transfer of adenoviruses encoding for green fluorescent protein (GFP) \( n = 5 \) or Cx43 \( n = 5 \), followed by an observation period of 14 days after surgery during which atrial burst pacing was applied.

On the day of pacemaker implantation and gene transfer, pigs were sedated with ketamine (100 mg/kg; Roche, Grenzach-Wyhlen, Germany), anaesthetized with propofol (1 mL of a 1% solution; Astra Zeneca, Wedel, Germany), and ventilated with isoflurane (1–2%; Baxter, Unterschleißheim, Germany) in a 1:2 mixture of \( \text{O}_2 \) and \( \text{N}_2\text{O} \). To determine adequacy of anaesthesia, ventilation and oxygenation, cardiac electrical activity, and body temperature were monitored, and interdigital reflexes were tested. A median thoracotomy was performed and the pericardium was opened to expose the heart under sterile conditions. Adenoviruses were injected into both atrial appendages using a 22-G needle, carefully avoiding injections into the atrial cavity. In the first study phase evaluating the effect of electroporation on GFP gene transfer, the distance between injection sites was guided by a custom-made template of holes at 7 mm distances. With more advanced technical experience adenovirus injection was performed manually, achieving distances between injection sites of 7 ± 2 mm. Injection of adenoviruses was directly followed by electroporation that was carried out using a paddle-style quadripolar 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 pacemaker implantation and gene transfer, the burst pacing protocol was initiated after closure of the pericardium and thorax. A single prophylactic dose of penicillin (200 mg; aniMedica, Senden-Bönsensell, Germany) was given prior to thoracotomy, and the animals received buprenorphine (0.324 mg; Essex Pharma, Munich, Germany) for 1–3 days after surgery. All animals treated with AdCx43 survived. In contrast, two pigs died in the AdGFP group owing to heart failure on day 14 during anaesthesia, requiring the exclusion of these animals and assignment of two additional pigs to this group. These animals exhibited severe signs of heart failure (shortness of breath and slow, lethargic movements) prior to anaesthesia. In one of the animals, we detected bilateral pleural effusion and pulmonary oedema upon post-mortem examination.

2.3 Electrophysiological studies
Clinical observations and ECG recordings were performed daily during feeding in awake, alert animals. On the day of euthanization, the animals were anaesthetized and hearts were exposed by median sternotomy. In order to determine activation times and conduction velocities, we constructed an electrode patch (2 × 2 cm) consisting of 15 pairs of bipolar electrodes, forming a grid of three columns (5 mm distance) and five rows (3 mm distance) (see Supplementary material online, Figure S1). Constant atrial pacing at a basic cycle length of 400 ms was applied epicardially to both atria in longitudinal direction to the fibre orientation. Electrograms were recorded via the patch electrode during pacing (see Supplementary material online, Figure S1). The number of electrograms recorded simultaneously was limited to 12 owing to amplifier properties. Based on the activation time difference between the site of stimulation and the most distant electrode, conduction velocities were calculated.

2.4 Echocardiography
Two-dimensional echocardiography was performed on the day of pacemaker implantation and prior to euthanization (2 h after successful
electrical cardioversion). A detailed description of echocardiographic studies and analysis has been published previously.27,28

2.5 Western blot analysis
Euthanasia was carried out by intravenous application of KCl (1 M) in anaesthetized animals. Protein content was quantified in atrial tissue obtained from sham-operated animals (21 days), from control pigs exhibiting pacing-induced AF (21 days), from healthy animals during sinus rhythm, and from all animals subjected to gene transfer. Protein expression was normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) for quantification of optical density.

2.6 Immunohistochemistry
After data acquisition on day 7 (preliminary electroporation experiments) or day 14 (AdCx43 gene therapy evaluation), pigs were euthanized and the hearts were removed and rinsed with phosphate-buffered saline. The tissue was processed and transgene efficiency was evaluated as described.27,31

2.7 Statistical analyses
Data are presented as mean ± s.e.m. Statistical comparisons were performed with Origin software (OriginLab, Northampton, MA, USA) using paired and unpaired Student’s t-tests (two-tailed tests), where appropriate. A P-value of <0.05 was considered to be statistically significant.

3. Results

3.1 Reduced Cx43 expression in AF
Persistent AF was induced in domestic pigs by repetitive burst pacing of the right atrium via an implanted pacemaker. Due to excellent atrioventricular conduction properties, AF resulted in rapid ventricular rates, leading to a significant decrease in left ventricular ejection fraction (LVEF) and to clinical symptoms of CHF in previous studies.27,28 In the first phase of the present study, biochemical remodelling of Cx43 expression was evaluated in this large animal model. Groups of five animals each were either assigned to atrial burst pacing and AF induction or to an untreated control group exhibiting sinus rhythm. After a follow-up period of 21 days atrial Cx43 content was quantified in both groups by western blot analysis (Figure 1A and B). AF was associated with significant down-regulation of the gap junction protein Cx43 compared with animals in sinus rhythm (~62.4% reduction in total protein amount; n = 5 animals each). Phosphorylated Cx43 was reduced as well. However, there was no apparent change in the relative amount of phosphorylated Cx43 compared with total Cx43 (Figure 1A).

3.2 Optimized gene transfer efficacy achieved by local virus injection and electroporation
Based on these results, we hypothesized that targeted gene transfer of Cx43 to right and left atria would prevent persistent AF. Gene transfer techniques targeting both atria are challenging since direct arterial infusion is prevented by small vessel diameters. Alternative approaches include myocardial injection of adenoviruses or plasmids.31,32 However, epicardial injection causes inhomogenous gene expression, located predominantly in areas surrounding the injection site.33 We developed a novel and efficient technique of more homogenous atrial gene transfer to prevent AF in a porcine model of AF/CHF, employing local injections of adenoviruses followed by epicardial electroporation. This hybrid approach is based on the observation that electroporation increased expression of plasmid DNA in the skeletal muscle.29,34 In our model, local injection of adenoviruses encoding for GFP followed by epicardial electroporation markedly increased the expression of GFP in atrial myocytes after 1 week (see Supplementary material online, Table S1 and Figure S2).

3.3 Cx43 gene therapy prevents persistent AF
Next, we performed adenoviral gene transfer of Cx43 (AdCx43) to both atria to correct AF-associated remodelling of connexin expression. Control pigs treated with AdGFP developed persistent AF after 7.4 ± 0.5 days (n = 5; Figure 2A and B). While all animals in the AdGFP control group developed persistent AF, there were no cases of persistent AF in the AdCx43 group. Atrial gene transfer of AdCx43 completely inhibited the development of persistent AF defined as AF ≥ 48 h (n = 5; Figure 2A and B). Furthermore, inducibility of paroxysmal AF (defined as AF for 30 s to 48 h) or persistent AF (n = 5; Figure 2C) as well as the occurrence of any atrial arrhythmia (i.e. persistent AF, paroxysmal AF, or non-sustained runs of >2 supraventricular ectopic beats for <30 s; n = 5; Figure 2D) were significantly reduced in the AdCx43 treatment group. Paroxysmal AF was
observed in three animals treated with AdCx43, and non-sustained supraventricular ectopic beats were recorded in all pigs after Cx43 gene therapy. In control animals treated with AdGFP, mean ventricular rates of 151 ± 5 b.p.m. were recorded on day 14 during AF (n = 5). In contrast, pigs that received AdCx43 treatment displayed sinus rhythm with mean heart rates of 89 ± 5 b.p.m. (n = 5). During rapid atrial pacing, AdGFP treatment resulted in mean ventricular rates of 171 ± 4.4 b.p.m. (day 14; n = 5). Cx43 gene therapy caused a 5.3% reduction in mean heart rates during atrial pacing (162 ± 5.0 b.p.m.; n = 5). This difference was not significant, indicating that atrial AdCx43 gene therapy did not modify atrioventricular conduction properties.

Effective gene transfer and Cx43 protein expression in the right atrium after AdCx43 treatment was demonstrated by western blot analysis at the time of sacrifice (Figure 3A and B), revealing a 2.5-fold increase in total Cx43 protein (n = 5) compared with AdGFP-treated control animals (n = 5). Protein levels after AdCx43 treatment were not significantly different from expression levels during sinus rhythm (Figure 3A and B), indicating reconstitution of Cx43 expression by AdCx43 gene therapy. We could not detect any apparent consistent changes in Cx43 phosphorylation among groups. Atrial expression of Cx43 in infected pigs was further assessed by immunohistochemistry. We observed a 2.0-fold increase in Cx43 protein levels following AdCx43 gene therapy (n = 5), confirming successful overexpression (Figure 3C). Cx43 was primarily localized at intercellular junctions, and there were no apparent changes in Cx43 distribution after AdCx43 treatment. Cx43 gene transfer did not significantly affect the protein levels of non-targeted Cx40 and Cx45, respectively (n = 5) (see Supplementary material online, Figure S3).

Enhanced Cx43 expression resulted in accelerated atrial conduction. Electrical epicardial mapping (see Supplementary material online, Figure S1) performed on day 14 revealed that conduction velocity in right atria of AdCx43-treated pigs was significantly increased compared with AdGFP control animals, as expected (Table 1). In addition, we detected a tendency towards acceleration of left atrial conduction velocity that did not reach statistical significance (Table 1).

3.4 Beneficial effects of atrial AdCx43 gene therapy on left ventricular function

In order to evaluate effects of gene therapy on the development of heart failure, echocardiographic measurements were performed on the day of AdCx43 or AdGFP gene transfer and 14 days after infection (Figure 4A and B). Echocardiograms performed prior to study treatment revealed similar LVEFs among study groups (Figure 4A). Fourteen days after initiation of atrial burst pacing, we observed a significant reduction in LVEF in AdGFP-treated pigs (64.6 ± 3.3% on day 0 vs. 30.3 ± 4.1% on day 14; n = 5) owing to AF and ventricular tachyarrhythmia (Figure 4B). Compared with AdGFP animals, the

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cardiodepressive effect was significantly attenuated by Cx43 gene therapy (Figure 4B). Paired LVEF comparisons within each treatment group revealed significantly reduced ventricular function in the AdGFP control group (day 0, 64.6 ± 3.3%; day 14, 30.3 ± 4.1%; n = 5; Figure 4C). In contrast, LVEF values obtained from pigs in the AdCx43 group were not significantly different before and after burst pacing (day 0, 68.1 ± 5.3%; day 14, 55.3 ± 5.0%; n = 5; Figure 4D). To exclude potential effects of atrial electroporation on left ventricular function, five additional pigs were subjected to the experimental protocol as described, omitting pacemaker implantation and AF induction and performing euthanization 5 days after sham operation. In this independent series of control animals, electroporation did not significantly affect LVEF (71.2 ± 1.4% on day 0 vs. 75.5 ± 2.0% on day 5; n = 5 each).

Left ventricular end-systolic diameter (LVESD; Figure 5A) and left ventricular end-diastolic diameter (LVEDD; Figure 5C) were not significantly different at baseline. On day 14 after gene transfer, both LVESD and LVEDD were significantly increased in AdGFP animals compared with AdCx43-treated pigs (n = 5) (Figure 5B and D), corresponding to LVEF reduction. In addition, cardiac hypertrophy was evaluated by assessing diastolic interventricular septum thickness (IVSd). IVSd was similar in both groups prior to gene transfer (Figure 5E) and on day 14 after treatment (Figure 5F), respectively. There was a tendency towards increased IVSd in the AdGFP group that did not reach statistical significance.

4. Discussion

Safe and effective management of AF remains one of the greatest unmet medical needs. In search for novel treatment modalities, gene therapy offers greater selectivity than small molecule or interventional approaches. In this proof-of-concept large animal study, correction of AF-associated connexin remodelling by targeted atrial Cx43 gene transfer successfully prevented persistent AF and preserved LVEF in pigs. This approach could be used as a primary or supplementary AF treatment option after gene delivery optimization and following evaluation of long-term efficacy, safety, and toxicology.

4.1 Rhythm control and improved cardiac function achieved by atrial Cx43 gene therapy

AF caused a 62.4% reduction in atrial Cx43 protein (Figure 1). Cx43 gene therapy effectively suppressed the development of persistent AF. AdCx43-treated animals did not exhibit persistent AF at all during the
observation period of 14 days, whereas control animals treated with AdGFP showed persistent AF within 7.4 ± 0.5 days (Figure 2). The control pigs investigated here developed cardiomyopathy with decreased LVEF (Figure 4). These results correspond to clinical findings in AF patients and are consistent with tachycardiomyopathy in predisposed human subjects. Reduced LVEF in the AdGFP group is readily explained by high AF burden and associated tachyarrhythmia. In contrast, animals in the AdCx43 group did not exhibit persistent AF and were therefore protected from tachycardiomyopathy (Figure 4). Thus, preserved LVEF in the AdCx43 group was most likely caused by reduced ventricular rates during sinus rhythm compared with tachyarrhythmia in the AdGFP control group during AF. The residual tendency towards reduced LVEF despite prevention of persistent AF may be attributed to the experimental protocol (repetitive atrial burst pacing) and associated effects on ventricular function.

4.2 Molecular and electrophysiological mechanisms of genetic rhythm control
AF 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 the maintenance of persistent AF. In the present work, AF was associated with electrical remodelling of the atrial gap junction protein Cx43. Cx43 down-regulation is expected to result in slowed and heterogeneous atrial conduction. Gene therapy targeting atrial Cx43 expression effectively increased atrial Cx43 protein levels. In addition, there was an acceleration of conduction in both the right and left atria, as expected after overexpression of a key regulator of intercellular electrical communication. However, the increase in atrial conduction velocity was less pronounced and did not reach statistical significance in the left atrium. Gene transfer efficacy assessed using GFP (see Supplementary material online, Table S1) was similar in both atria, ruling out heterogeneous gene expression as the underlying cause. We may speculate that intercellular conduction is modulated differentially in the right and left atria (depending on specific electrophysiological remodelling independent of Cx43). One possible explanation is differential expression of Cx40. Cx40 displays higher expression levels in the right atrium vs. left atrium and has been shown to functionally interact with Cx43. We may assume that Cx43 expression determines atrial conduction velocity to a significant extent in the right atrium through interaction with Cx40, whereas in the left atrium, the relative contribution of Cx43 is limited. In summary, our data suggest that overexpression of Cx43 successfully inhibited the development of persistent AF by improving intercellular atrial conduction.

4.3 Study limitations
In this work, a correlation between atrial Cx43 expression, atrial conduction velocity, and inducibility of persistent AF is established. However, there is no definite evidence to prove that changes in Cx43 expression are primary factors determining the development of persistent AF. In addition, potential effects of AdGFP application on Cx43 expression cannot be excluded as no sinus rhythm control group treated with AdGFP in the absence of pacemaker implantation was included in this study. Remaining obstacles of
Cx43 gene therapy that need to be overcome include control over local gene distribution, potential tumorigenicity of vehicles, and prevention of local and systemic inflammatory responses. Safety and toxicology issues need to be solved prior to evaluation of antiarrhythmic gene therapy in humans. Adenoviral vectors were used in this work owing to their ability to induce peak expression within a short time (7–14 days) and to their high efficacy in infecting cardiomyocytes. An extension of the observation period beyond 14 days in order to associate diminished Cx43 expression with a potential increase in persistent AF induction was not performed in this proof-of-concept study, as longer follow-up prevents successful detection transgene expression by western blot and immunohistochemistry. To achieve long-lasting expression and to study long-term stability, efficacy, and safety of upstream gene therapy, the use of adeno-associated virus or lentivirus as a vector will be required.

The gene transfer technique employed in the present study shares most advantages and limitations of epicardial gene transfer using a poloxamer-trypsin gel described by Amit et al.\textsuperscript{38} Both gene application methods allow for gene therapy during open-chest cardiac surgery. Thoracotomy-related limitations and lack of long-term gene expression owing to adenovirus use are similar. While ‘gene painting’\textsuperscript{38} results in epicardial virus application and requires myocardial virus uptake that may limit transduction efficacy, our injection technique circumvents this barrier and directly transfers the adenovirus into cardiac tissue. Increased invasiveness of the procedure and associated cardiac micro-injuries caused by direct adenovirus injection may cause inflammatory responses. However, these may occur after epicardial tryptic digestion as well. Efficacy and safety of these approaches remain to be compared directly in a study employing both techniques.

### 4.4 Clinical implications and conclusion

This is the first report to demonstrate down-regulation of Cx43 as a factor contributing to inducibility of persistent AF in a clinically relevant large animal model. Correction of connexin remodelling by atrial Cx43 gene transfer successfully prevented persistent AF and improved LVEF in pigs. Our hybrid gene application technique combining local virus injection and electroporation could be readily performed during open-chest cardiac surgery. To further refine the gene transfer method, thoracotomy may be replaced in future studies by interventional, transvenous virus application via specific

![Figure 5](image-url)  
Left ventricular dilatation is reduced by AdCx43 gene therapy. LVESD and LVEDD were compared at baseline (A and C) and on day 14 following gene transfer (B and D) in groups of five animals. (E and F) Diastolic IVSd, a measure of cardiac hypertrophy, was not significantly different prior to treatment and after application of AdGFP (n = 4) or AdCx43 (n = 5), respectively. *p < 0.05 vs. AdGFP-treated pigs (unpaired Student’s t-test). Data are expressed as mean ± s.e.m.
catheters in combination with three-dimensional electro-anatomical
navigation that is currently used for radiofrequency ablation of
arrhythmias. After successful establishment of a minimally invasive
technique and following safety assessment, Cx43 gene therapy
could become a viable option to treat an arrhythmia associated
with high morbidity and mortality.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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