AAV6.βARKct cardiac gene therapy ameliorates cardiac function and normalizes the catecholaminergic axis in a clinically relevant large animal heart failure model

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Aims

G protein-coupled receptor kinase 2 (GRK2), which is markedly upregulated in failing human myocardium, has been implicated as a contributing factor or consequence of heart failure (HF). Importantly, cardiac-specific GRK2 knockout mice have recently proved the pathological nature of GRK2 in HF. Targeted inhibition of GRK2 is possible using a peptide inhibitor known as the βARKct, which has rescued several disparate small animal HF models. This study was designed to evaluate long-term βARKct expression in a clinically relevant large animal HF model, using stable myocardial gene delivery with adeno-associated virus serotype 6 (AAV6).

Methods and results

A porcine model of HF subsequent to left ventricular (LV) myocardial infarction (MI) was used to study the effects of retrograde injection into the anterior interventricular vein of either AAV6.βARKct or AAV6.luciferase as a control 2 weeks after MI. Echocardiography and LV hemodynamics were performed before and 6 weeks after gene transfer. Robust and long-term βARKct expression was found after AAV6-mediated delivery, leading to significant amelioration of LV hemodynamics and contractile function in HF pigs compared with AAV6.luciferase-treated control animals that showed a continued decline in cardiac function. Interestingly, the neurohormonal axis was virtually normalized in AAV6.βARKct-treated HF animals, represented by reductions in plasma norepinephrine levels, whereas AAV6.luciferase-treated pigs showed further increases in plasma catecholamine levels. As a result, LV remodelling and foetal gene expression was reversed by AAV6.βARKct gene therapy.

Conclusion

These data—showing sustained amelioration of cardiac function in a post-MI pig HF model—demonstrate the therapeutic potential of βARKct gene therapy for HF.

Keywords

Heart failure • Gene therapy • GRK2 • βARKct • Adeno-associated virus

Introduction

Despite recent advances in treatment strategies, heart failure (HF) remains a leading cause of morbidity, with annual patient mortality rates ranging from 25 to 30%.1 These facts highlight an important gap between current therapeutic approaches and key underlying biological processes relating to cardiomyocytes in the setting of chronic cardiac dysfunction. A major molecular hallmark of

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HF is deranged cardiac β-adrenergic receptor (β-AR) signalling. Abnormal β-AR inotropic responsiveness leads to an increased sympathetic drive with the upregulation of tissue and plasma catecholamines in an attempt to stimulate myocardial contractile function. This sustains a vicious cycle, as the chronic stimulation of cardiac β-ARs perpetuates a loss of signalling due in part to a marked upregulation of G protein-coupled receptor kinase 2 (GRK2), which desensitizes β-ARs in the heart through phosphorylation. Thus, a continual state of activation and abnormal signalling is created and chronic catecholamine bombardment of the heart can contribute to further cardiac deterioration. The successful use of β-AR blockers in clinical HF treatment demonstrates the effectiveness of an approach aimed at interrupting this vicious cycle of sympathetic tone, by blocking chronic β-AR activation; however, these agents do not address the molecular defects in failing cardiomyocytes directly.

Indeed, myocardial GRK2 activity appears to play a profound pathological role in cardiac function. Most recently, the pathological nature of GRK2 was directly demonstrated using inducible, cardiomyocyte-specific knockout mice where GRK2 was deleted in ventricular myocytes after myocardial infarction (MI), and there was a significant improvement in overall animal survival and cardiac function. This gave a clear indication that lowering GRK2 was therapeutic in HF and its upregulation was not a beneficial adaptive change due to compromised β-AR signalling and cardiac function. Since no suitable small-molecule GRK2 inhibitor exists for targeted GRK2 inhibition in HF, we have engineered a peptide inhibitor that blocks the membrane translocation and activation of GRK2. This peptide, known as the βARKct competes with endogenous GRK2 for membrane binding to the βγ subunits of activated heterotrimeric G proteins (Gβγ) and has been shown to inhibit GRK2 activity on several receptors, including β-ARs. Further, transgenic or viral (adenovirus and adeno-associated virus, AAV) expression of the βARKct to compromised myocardium has been found to rescue several models of experimental HF. Interestingly, all data with βARKct-mediated HF rescue include normalization of β-AR signalling, including receptor upregulation (to normal levels) and GRK2 lowering. Since long-term β-AR antagonism can promote receptor upregulation and GRK2 downregulation, improved β-AR signalling is also a natural consequence of clinical β-AR blocker use. Therefore, β-AR blocker use and molecular GRK2 inhibition may act synergistically. Indeed, this has been shown in several small animal models of GRK2 inhibition and HF.

Although data with cardiac βARKct expression, either in genetic mouse models of HF or in viral-mediated myocardial gene transfer, have been positive, studies completed to date have not been done in anything larger than a rabbit. Ultimately, to test whether βARKct and GRK2 inhibition is a potential therapeutic approach for treating HF patients, a large animal efficacy study is needed. In a clinically relevant large animal (swine) model of post-MI systolic HF, our current data demonstrate a sustained amelioration of cardiac function with adeno-associated virus serotype 6 (AAV6)-mediated βARKct delivery, with significant improvements of contractile function, reductions in plasma catecholamine levels, and subsequent reversal of cardiac remodelling and foetal gene expression. Thus, βARKct translation to human HF patients appears warranted.

### Methods

For a detailed description of Methods, we refer to Supplementary material online.

### Study protocol

The present investigation was carried out according to the Guide for the Care and Use of Laboratory Animals and was approved by the Animal Care and Use Committee of the state of Baden-Württemberg. Ischaemic cardiomyopathy/HF was created by left circumflex coronary artery (LCX) MI. Myocardial infarction was induced by occlusion of the LCX with a Maverick 3.0 × 12 mm PTCA balloon (Boston Scientific, Natick, MA, USA) for 2 h. Two weeks later, baseline cardiac function was determined (haemodynamics, echocardiography under basal and catecholamine-stressed conditions and blood sample collection for biomarker assessment) and animals were treated with AAV6.βARKct or AAV6.luciferase as control. Forty-two days later/56 days after MI, cardiac function was determined (haemodynamics under basal and catecholamine-challenging conditions, echocardiography, as well as blood sample collection for biomarker assessment). Animals were euthanized for standardized autopsy. Tissue samples were collected for molecular analyses.

### Gene transfer using retrograde injection into the coronary veins

Animals were anaesthetized with an intramuscular injection of ketamin 15 mg/kg body weight (ketamin 10%, Beta-Pharm, Augsburg, Germany), azaperone 10 mg/kg (Stresnil, Janssen-Cilag, Neuss, Germany), 1 mg of atropinsulphate (Braun, Melsungen, Germany), and 5 mg of midazolam (Hoffmann-La Roche, Grenzach-Wyhlen, Germany), followed by intravenous injection of 10 mg of midazolam and 0.1 mg of fentanyl (Janssen-Cilag). After intubation, animals were subjected to inhalation anaesthesia using 1.5% isoflurane (Baxter, Unterschleissheim, Germany) and catheter introducer sheaths were placed in the right carotid artery and the right internal jugular vein as reported previously. Blood samples were collected and evaluation of cardiac function was carried out (echocardiography and haemodynamics). For selective retrograde injection, the anterior interventricular vein (AIV) was catheterized using a 6F retroinfusion catheter. In all animals, a 7F guiding catheter was placed in the left coronary artery and the left anterior descending coronary artery (LAD) was wired. During retrograde delivery of the AAV vectors, the LAD was occluded by a PTCA balloon distal to the first diagonal branch in all groups. Animals were randomized to treatment (n = 10 control virus AAV6-Luciferase and n = 10 AAV6.βARKct). Adeno-associated virus serotype 6 vectors [1E13 vector genomes (vg) per animal] were diluted in 50 mL of 0.9% saline solution supplemented with 0.5 mg of substance P (Sigma Aldrich, St Louis, MO, USA) and 6 mg of adenosine (Sanofi-Aventis, Frankfurt, Germany). Vector solution was injected in three portions into the AIV over each 3 min, whereas the balloon at the tip of the retroinfusion catheter was inflated to block venous outflow and the PTCA balloon in the LAD was inflated to block arterial inflow. After each injection, both balloons were deflated for 3 min to allow reflow. At the end, all catheters and introducer sheaths were removed and animals recovered.

### Statistical analysis

Data are generally expressed as mean ± standard error of the mean (SEM). Data analysis was performed using SPSS 19 (SPSS, Inc., Chicago, IL, USA). An unpaired two-tailed t-test was performed when appropriate. A Kruskal–Wallis test in combination with a
Wilcoxon rank-sum test was used in case of very small sample size (at most four animals/group) where a Gaussian distribution could not be assumed. One-way analysis of variance (ANOVA) or a two-way repeated measurement ANOVA was performed for between-group comparisons. The Bonferroni method was used to adjust for multiple comparisons and the computed $P$-values were reported. $P$-values of $<0.05$ were considered statistically significant. The post hoc power of all ANOVAs showing significant results ranged between 0.7 and 0.98. In case of low power ($1 - \beta < 0.8$), this was indicated and absolute $P$-values were reported.

Results

Large animal model of post-myocardial infarction systolic heart failure

Since ischaemic cardiomyopathy is the primary cause of HF, we chose a porcine post-MI HF model to test the potential translation of our $\beta$ARKct gene therapy approach. In this pre-clinical large animal model, 2 h of LCX occlusion led to significant rise in serum high-sensitive troponin T (hsTnT) levels indicating significant loss of viable myocardium (Figure 1A and C). Four days after MI, an infarct size of $30.3 \pm 5.9\%$ (mean $\pm$ SEM; $n = 3$) of the left ventricle was observed (Figure 1B). Thus, this method of MI in the pig represents an appropriate HF model to evaluate potential therapeutics. For AAV6.$\beta$ARKct gene therapy, we delivered $1 \times 10^{13}$ vg to pigs 2 weeks after MI, and as a control AAV6.luciferase was delivered to a group of pigs. These groups were randomly assigned after post-MI cardiac functional assessment, and post-MI pigs in HF were followed for another 6 weeks after gene transfer.

Efficient and specific $\beta$ARKct transgene expression in the targeted myocardium

Gene delivery to post-MI pigs was done by retroinfusion into the AIV (Figure 2A; see Methods). Both transgenes ($\beta$ARKct and luciferase) were under the control of a CMV-enhanced myosin light chain promoter to target expression in a cardiac-selective manner. We found robust protein expression of both transgenes specifically in the left ventricular (LV) tissue up to 10 weeks post-gene transfer either via western blotting for the $\beta$ARKct (Figure 2B) or luciferase activity measurements (data not shown). Expression was restricted to the LAD target area because no expression was detected in the LCX territory (Figure 2B), and no expression was found in extracardiac tissue (data not shown). $\beta$ARKct-treated pigs showed that a large area of the LV free wall (LAD territory) from
Basal myocardium to the apex was transduced homogenously and expressing the βARKct peptide (Figure 2B). To further visualize transgene distribution, retrograde delivery of AAV6.βARKct in comparison with animals treated with AAV6.luciferase control virus. Direct EGFP immunofluorescence showing transgene (EGFP-) distribution in the non-targeted (left circumflex coronary artery control, C and E) and in the targeted (LAD) area (D and F) in an additional sham animal with retrograde delivery of AAV6-EGFP into the anterior interventricular vein 6 weeks post-gene delivery; scale bar: (C and D): 1 mm; (E and F): 50 µm.

Figure 2 Efficiency of βARKct gene delivery by retrograde coronary venous injection. (A) Representative delineation of the gene-transfected left anterior descending coronary artery territory (dashed arrows) by injection of contrast medium into the anterior interventricular vein (AIV); the retroinfusion catheter is blocked by a balloon at its tip, thereby preventing venous drainage. (B) βARKct transgene expression detected by western blotting in representative animals treated with AAV6.βARKct in comparison with animals treated with AAV6.luciferase control virus. Direct EGFP immunofluorescence showing transgene (EGFP-) distribution in the non-targeted (left circumflex coronary artery control, C and E) and in the targeted (LAD) area (D and F) in an additional sham animal with retrograde delivery of AAV6-EGFP into the anterior interventricular vein 6 weeks post-gene delivery; scale bar: (C and D): 1 mm; (E and F): 50 µm.

βARKct expression in the heart improves post-myocardial infarction global cardiac haemodynamic function

We assessed global cardiac function through catheterization of the LV and haemodynamic measurements (Figure 3, Table 1). Initially, haemodynamic cardiac function in pigs was analysed 2 weeks after MI and a significant loss of systolic and diastolic LV function

basal myocardium to the apex was transduced homogenously and expressing the βARKct peptide (Figure 2B). To further visualize transgene distribution, retrograde delivery of AAV6.EGFP into the AIV was performed in an additional sham animal. Interestingly, direct immunofluorescence 6 weeks post-gene delivery showed efficient EGFP expression in the targeted (LAD) area (Figure 2D and F), whereas EGFP expression was almost absent in the non-targeted (LCX) control region (Figure 2C and E).
was documented compared with sham-treated pigs that received no myocardial ischaemia (Figure 3A, C and E). No significant differences were observed in heart rate (Figure 3G). Post-MI pigs were then randomly assigned to receive either AAV6.βARKct or AAV6.luciferase as described above. Six weeks after retrograde gene delivery (56 days post-MI), the pigs were assessed again and these haemodynamic measurements revealed significant amelioration of global cardiac function by AAV6.βARKct treatment.

**Figure 3** Amelioration of left ventricular haemodynamics in post-myocardial infarction heart failure by AAV6.βARKct gene therapy. (A and B) Haemodynamic assessment of left ventricular contractility under basal conditions and β-adrenergic receptor stimulation with dobutamine (dP/dt max) pre (A) and 42 days post (B) gene delivery. (C and D) Haemodynamic assessment of left ventricular contractility under basal conditions and dobutamine stimulation at 40 mmHg (dP/dt max at P40 mmHg) pre (C) and 42 days post (D) gene delivery. (E and F) Haemodynamic assessment of left ventricular relaxation under basal conditions and dobutamine stimulation (dP/dt min) pre (E) and 42 days post (F) gene delivery. (G and H) Assessment of heart rate under basal conditions and dobutamine stimulation (dP/dt min) pre (G) and 42 days post (H) gene delivery. n = 10 animals/group.
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<th>Luciferase (Mean ± SEM)</th>
<th>βARKct (P-value vs. sham)</th>
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<th>Luciferase (Mean ± SEM)</th>
<th>βARKct (P-value vs. luciferase)</th>
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<td>46 ± 4</td>
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<td>58 ± 5</td>
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<td>Tau Mirsky (ms)</td>
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<td>FS (%)</td>
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<td>% Change of FS</td>
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<td>0.96</td>
<td>25 ± 14.1</td>
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n = 10 for each group. P values based on Bonferroni procedure for multiple comparisons. Significant P-values are shown in bold; non-significant P-values are italic.
Figure 4 Normalization of the catecholaminergic axis and myocardial β-adrenergic receptor responsiveness in heart failure post-myocardial infarction by AAV6.βARKct gene therapy. (A) Detected plasma levels of metanephrine and normetanephrine as direct degradation products of adrenalin and noradrenalin; blood samples were drawn 56 days post-myocardial infarction/42 days post gene therapy, n = 8 animals/group. (B) β-Adrenergic receptor density by radioligand binding assay 56 days post-myocardial infarction/42 days post-gene therapy, n = 3 animals/sham and AAV6.luciferase and n = 4 animals/AAV6.βARKct group.

Cardiac catheterization revealed preservation of β-adrenergic responsiveness in AAV6.βARKct-treated pigs represented by sustained LV contractility (LV + dP/dt\text{max}; LV + dP/dt\text{min}; Tau) and heart rate (Figure 3F, Table 1) and heart rate (Figure 3H). To correct for variations in heart rate, dP/dt\text{max} is plotted with heart rate in Supplementary material online, Figure S1.

**Cardiac AAV6.βARKct gene transfer preserves myocardial β-AR inotropic responsiveness in post-MI heart failure through normalization of the catecholaminergic axis**

To determine whether the preservation of β-adrenergic responsiveness and improved cardiac function was due to a lowering of sympathetic overdrive due to a negative feedback after GRK2 inhibition and less receptor desensitization, we measured plasma catecholamine levels at the end of the study period. Indeed, plasma metanephrine levels after AAV6.luciferase treated post-MI pigs β-AR contractile function worsened (Figure 3B and D). No significant differences were observed in LV relaxation (LV + dP/dt\text{min}; Tau) (Figure 3F, Table 1) and heart rate (Figure 3H). To correct for variations in heart rate, dP/dt\text{max} is plotted with heart rate in Supplementary material online, Figure S1.

βARKct expression in the post-myocardial infarction failing heart reverses adverse left ventricular remodelling

Heart failure is associated with adverse LV remodelling leading to the dilatation of the LV and maladaptive hypertrophy with an increase in the heart-to-body weight ratio. The heart-to-body weight ratio was measured 56 days post-MI (42 days post-gene therapy) and control HF pigs (treated with AAV6.luciferase) showed a significant increase in cardiac mass compared with sham animals (Figure 5A). Interestingly, AAV.βARKct-treated HF animals had hearts with significantly lower cardiac mass that was indistinguishable from sham controls (Figure 5A). In addition to cardiac mass, molecular markers of maladaptive cardiac hypertrophy and adverse LV remodelling were quantified by RT-PCR in myocardial tissue samples from the targeted non-infarcted (LAD) area harvested at the study end. Interestingly, markers of foetal gene activation were found to be significantly lower in HF pigs treated with AAV6.βARKct compared with luciferase-treated HF animals. This includes plasma BNP (brain natriuretic peptide) levels (Figure 5B), which indicates a reduction of LV volume overload and wall stress in AAV6.βARKct-treated pigs. In line with this, adverse remodelling gene expression as represented by marked upregulation of the foetal gene programme [atrial natriuretic factor (ANF) and β-myosin heavy chain (β-MHC)] was observed in AAV6.luciferase-treated pigs post-MI, whereas chronic βARKct expression resulted in reductions in ventricular ANF mRNA levels (Figure 5C) and β-MHC gene expression (Figure 5D), which indicates a reversal of foetal gene expression in post-MI HF.

Furthermore, cardiac fibrosis was addressed by determination of collagen 1α1 and collagen 3α1 mRNA levels from myocardial tissue samples from the targeted non-infarcted (LAD) area harvested at the study end. Interestingly, despite significant upregulation of these fibrosis markers in AAV6.luciferase-treated pigs,
Figure 5 AAV6.βARKct gene therapy inhibits adverse left ventricular remodelling in post-myocardial infarction heart failure. (A) Heart-to-body weight ratio 56 days post-myocardial infarction/42 days post-gene therapy. (B) Detected brain natriuretic peptide plasma levels 56 days post-myocardial infarction/42 days post-gene therapy. AAV6.βARKct gene therapy inhibits foetal gene expression in post-myocardial infarction heart failure; quantitative real-time PCR analysis on cardiac tissue isolated from the targeted non-infarcted left ventricular myocardium at day 56 post-myocardial infarction/day 42 post-gene therapy. (C) Atrial natriuretic factor mRNA molecules normalized to 18S molecules. (D) β-Myosin heavy chain mRNA molecules normalized to 18S molecules. (E) Collagen 1a1 mRNA molecules normalized to 18S molecules. (F) Collagen 3α1 mRNA molecules normalized to 18S molecules. (G) Determination of fractional shortening in non-targeted left ventricular areas (for the determination of global left ventricular function and remodelling) by echocardiography 14 days post-myocardial infarction (day of gene delivery) and 56 days post-myocardial infarction/42 days post-gene delivery and %change in fractional shortening 56 days post-myocardial infarction compared with 14 days post-myocardial infarction, n = 8 animals/group (A–F), n = 10 animals/group (G).
AAV6.βARKct treatment normalized expression of collagen 1α1 and collagen 3α1 mRNA (Figure 5E and F).

In addition to these molecular indices of reverse remodelling, echocardiography was used to determine cardiac function in the left ventricle. We specifically assessed cardiac function in the LV area that was not targeted for gene delivery (lateral short-axis M-Mode through septal and lateral walls) in order to gain a measure of regional LV remodelling distant from the MI and treatment area. Importantly, 2 weeks post-MI, echocardiography revealed a similar reduction in fractional shortening in both MI groups before gene delivery (Figure 5G). Six weeks after gene delivery (56 days post-MI), AAV6.βARKct treatment improved regional LV fractional shortening even in non-targeted areas (Figure 5G), whereas AAV6.luciferase-treated control hearts displayed impaired function in the same areas. As fractional shortening was determined in non-targeted areas, these results most likely indicate secondary inhibition of adverse LV remodelling through attenuated catecholamine overdrive. Taken together, these results demonstrate global repression of adverse LV remodelling by βARKct gene transfer in porcine post-MI HF.

Safety of AAV6.βARKct myocardial gene delivery

To delineate any potential systemic effects of our gene therapy approach, a broad clinical chemistry workup and blood count were performed. Interestingly, 42 days post-gene transfer, no difference was observed in AAV6.βARKct- or AAV6.luciferase-treated groups when compared with sham animals regarding blood count and serum clinical chemistry (see Supplementary material online, Table S1). Plasma hsTnT levels were only mildly elevated after AAV6.luciferase gene delivery; hsTnT levels after βARKct treatment were comparable with levels observed in sham animals. Thus, it appears that our method of AAV6 gene transfer does not adversely affect the animals.

Discussion

The aim of this study was to analyse the therapeutic potential of cardiac-targeted βARKct gene transfer in a clinically relevant large animal HF model. Retroinfection of AAV6 vectors harbouring a cDNA encoding the βARKct into the LAD area 2 weeks after MI (through LCX balloon occlusion) resulted in efficient and long-term βARKct expression in the target area, which was found to cause significant amelioration of cardiac function and LV haemodynamics, normalization of the catecholaminergic axis, and repression of adverse LV remodelling. Thus, our study represents a positive final step in the translation of GRK2 inhibition for HF therapy.

Transgenic or viral (adenovirus and AAV) expression of βARKct in the myocardium has led to a protective effect on the development of HF in different small animal models of HF including mice, rat, and rabbits.6-10 However, rodent and human cardiac physiologies differ significantly, thereby preventing extrapolation of data from rodent models to humans. The pig represents a large animal model that more closely approximates human cardiac physiology and has been used successfully to study gene therapy targets.14 Therefore, a post-MI porcine HF model was selected herein to test the therapeutic potential of the GRK2 inhibitory βARKct. A model of LCX posterolateral MI was chosen in order to leave viable tissue in the anterior and anteroseptal LV wall as target area for our gene delivery methodological approach.

Amelioration of left ventricular function by adeno-associated virus serotype 6-mediated βARKct gene delivery

Our study shows for the first time a robust therapeutic efficacy of βARKct expression in an experimental pre-clinical large animal HF model mimicking clinical conditions. Haemodynamic analysis revealed significant improvements of contractile/systolic cardiac performance 42 days after gene transfer, suggesting a long-term effect. Despite increased adrenergic drive under resting conditions, luciferase-treated animals revealed significantly decreased cardiac performance. Therefore, the beneficial effect in the βARKct group is most likely underestimated taking into account the bias of higher resting plasma catecholamine levels in MI luciferase-treated pigs. Future studies will potentially eliminate this limitation by studying single cardiac myocyte contractility from βARKct and control-treated pigs, thereby allowing comparable ambient conditions including adrenergic stimulation.

Overall, the degree of amelioration after βARKct treatment was significant, as systolic cardiac performance improved after βARKct delivery, whereas control animals revealed further deterioration over the 6 weeks of treatment period. Further, the beneficial effects of βARKct gene therapy were also present under β-AR challenge indicating sustained preservation of β-AR signalling. These results are similar to previous studies in smaller animal models after transgenic expression of the βARKct or after viral-mediated gene delivery.6-10 Thus, our findings are significant since they are now shown to occur in a clinically relevant HF model.

Reversal of heart failure-induced sympathetic overdrive by βARKct gene therapy

Neurohormones such as catecholamines usually elevate during chronic HF, drive cardiac remodelling, and contribute to enhanced morbidity and mortality.15 Sustained systemic sympathetic tone in chronic HF creates a vicious cycle perpetuating the release of catecholamines and further GRK2-driven dampening of cardiac β-AR signalling; these circumstances contribute further to cardiac deterioration.15,16 One question we specifically wanted to address in our study was whether improved cardiac performance in βARKct-treated pigs eliminates sympathetic overdrive? Indeed, another significant finding of our study is that long-term βARKct expression after AAV6-mediated gene transfer appeared to normalize the catecholaminergic overdrive in post-MI HF. In the present study, we have focused on measuring degradation products (normetanephrine and metanephrine) of plasma catecholamines (norepinephrine and epinephrine) as they offer higher stability and reflect long-term neurohumoral activity. Interestingly, circulating plasma levels of normetanephrine and metanephrine were virtually normalized by βARKct gene therapy, indicating interruption of this pathological neurohumoral cycle. Beyond systemic catecholamine levels, we
addressed local sympathetic tone in myocardial tissue by determination of β-AR density; interestingly, βARKct could preserve β-AR density at least to some extent, underscoring the notion that overall sympathetic tone is reduced contributing to the preservation of cardiac function. These findings are of utmost importance as they delineate βARKct gene therapy as the first molecular approach successfully normalizing the disturbed neurohormonal signals in a clinically relevant large animal HF model. Moreover, these results suggest that clinical β-AR blocker use and molecular GRK2 inhibition with the βARKct may act synergistically as previously shown in a mouse model of genetically caused cardiomyopathy and in a rat cryo-MI HF model.8

**Prevention of adverse left ventricular remodelling by βARKct gene therapy**

Long-term βARKct expression in the failing heart also had significant effects on cardiac post-MI adverse remodelling as indicated by a reduced heart-to-body weight ratio and repression of the activation of foetal genes, which underscores previous findings following conditional cardiac GRK2 knockdown in a mouse HF model8 and also after chronic AAV6,βARKct gene therapy in a rat model of HF.8 In addition, echocardiographic assessment of cardiac performance of the non-targeted LV areas revealed improvements in non-βARKct-treated myocardial tissue, representing indirect reverse remodelling effects. These observations could indirectly result from enhanced contractile function in the gene-targeted tissue of the heart, thereby reducing biomechanical overload and repressing the systemic sympathetic drive, which would allow reverse remodelling. Alternatively, βARKct might directly affect signal transduction pathways related to LV adverse remodelling effects. However, regardless of the mechanism, cardiac βARKct gene delivery to the post-MI heart beneficially affects LV remodelling. Future studies will address potential effects of cardiac βARKct gene therapy on additional neurohormonal signalling pathways like the renin–angiotensin–aldosterone system, which would allow reverse remodelling. Alternatively, βARKct might indirectly affect signal transduction pathways related to LV adverse remodelling effects.

**Adeno-associated virus serotype 6 vector, mode of gene delivery, and biosafety**

Previous studies in porcine models have identified AAV6 as candidate for a highly efficient cardiac gene transfer.13,17 Thus, there was a clear rationale for using AAV6 vectors, which enabled detection of a significant long-time βARKct protein expression in the target region compared with control myocardium. Regarding vector dose, we have chosen the highest dose currently used in humans in the CUPID trial, the first clinical cardiac gene therapy trial18 studying the safety of sarcoplasmic Ca2+ ATPase (SERCA2a) myocardial gene transfer (AAV1.SERCA2a).

A crucial point for the development of a gene therapeutic approach is the application mode of the vector. Cardiac vector delivery by selective retroinfusion through the coronary sinus13,17 is a promising and highly effective application method since it enables prolonged exposure of the vector to the endothelium and an increased intravascular pressure resulting in a homogenous gene delivery to the targeted area with all advantages of a percutaneous procedure like its minimally invasive nature. With this local delivery method in combination with the cardiac-specific promoter employed in our study, systemic transgene exposure was almost eliminated. The vector, dose, and mode of delivery chosen in our study resulted in efficient transgene expression limited to the LV target area.

In terms of safety, βARKct gene delivery and transgene expression showed no systemic side effects, as intensive blood workup and clinical chemistry disclosed no differences between sham and MI gene therapy groups. Especially, hsTnT was only mildly elevated at the study end in AAV6.luciferase-treated animals. No increase was found following AAV6.βARKct treatment. As hsTnT is a systemic marker for cardiac myocyte death, these results clearly demonstrate no harming effects on cardiac myocyte integrity by our gene therapy approach.

**Clinical potential of βARKct gene therapy**

AAV6.βARKct treatment resulted in similar effects on LV systolic function as previously observed in pigs receiving β-blocker therapy after MI.19 The latter study showed that β-blockade was associated with preserved myofilament phosphorylation and function in remote myocardium compared with the decrease in LV as well as myofilament function in the control MI group.

Furthermore, the βARKct construct showed comparable effects to the SERCA2a construct in rodent models.20–22 Kawase et al.14 recently tested SERCA2a gene therapy for HF in a preclinical pig model; in this model, SERCA2a revealed a positive isotropic effect along with beneficial effects on LV remodelling and BNP levels. Our present study could successfully establish βARKct as a novel gene therapeutic approach to treat the failing heart in a clinically relevant large animal HF model; beneficial effects of βARKct on cardiac function, LV remodelling, and neurohormonal signalling are significant and comparable with the SERCA2a construct. With the first clinical gene therapy study employing SERCA2a (CUPID trial),18 our present study builds the basis for further clinical investigation of βARKct gene therapy for HF treatment.

**Study limitations**

AAV6.βARKct treatment could ameliorate cardiac function in our preclinical ischaemic cardiomyopathy model although it could not completely restore cardiac function. Surprisingly, we found no effect of AAV6.βARKct treatment on LV relaxation. It is currently not clear whether this might be attributed to low test power (Figure 3F) or a limitation of our model itself, such as a potential influence of isoflurane anaesthesia on diastolic function or a limited effect of βARKct expression on LV relaxation. The latter might be explained by the mechanical allocation of βARKct effects to an increased L-type Ca-channel current,23 suggesting a predominant effect on systolic function.

Furthermore, our study shows two major limitations of gene therapy approaches of HF that need to be addressed in future studies. (i) Unlike moncausal genetic diseases, HF is a complex disease characterized by multiple intracellular signalling changes affecting the β-AR-adenyl cyclase-protein kinase A interactome, Ca2+ cycling, apoptosis, autophagy, etc. βARKct only affects parts of the disturbed intracellular signalling, which obviously is not enough to promote a complete rescue. Future studies combining βARKct gene therapy with other constructs affecting different
intracellular signalling pathways (for example, S100A1 or SERCA2a normalizing intracellular Ca\(^{2+}\) handling) might show potential therapeutic synergisms and might therefore obtain a more complete rescue.\(^{18,24}\) (ii) Gene transfer with retrograde delivery of AAV6 into the coronary vein allows an efficient and safe mediated gene transfer in combination with selective retroinfusion.\(^{18,24}\) Nevertheless, our study shows for the first time that AAV6-mediated gene transfer in combination with selective retroinfusion into the coronary vein allows an efficient and safe βARKct gene therapy in a clinically relevant model. Thus, our approach may be a step towards translation of this promising therapeutic strategy into improved patient care.

**Supplementary material**

Supplementary material is available at European Heart Journal online.

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**References**