Methods

Adenovirus vectors: AdlacZ contained the Escherichia coli *lacZ* gene driven by the human cytomegalovirus immediate early promoter. AdKCNH2-G628S contained the human cytomegalovirus immediate early promoter followed in sequence by the gene for enhanced green fluorescent protein, an independent ribosomal entry site and the KCNH2-G628S. Virus construction, expansion and quality control have been previously described.[1]

Myocardial infarction and defibrillator implantation: The Institutional Animal Care and Use Committee approved the experimental protocol. The animals used in this study were maintained in accordance with the guiding principles of the American Physiological Society. Thirty pigs weighing 25-35 kg were included in this study (7 infarct model creation, 5 gene transfer delivery testing, 15 gene transfer for VT study, 3 dofetilide). After inducing anesthesia and obtaining surgical access to the vessels, a 2.7 Fr balloon catheter was inserted through a Judkins left guide catheter into the middle portion of the left anterior descending coronary artery (LAD) immediately distal to the second diagonal branch. The balloon was expanded to 4 atm, and the LAD was occluded for 150 minutes. During the balloon occlusion, an implantable cardioverter-defibrillator (ICD) was inserted with lead in the right ventricular apex and generator in the left neck using conventional techniques. After completion of the balloon occlusion, catheters and sheaths were removed, and hemostasis was achieved by ligation of the vessel. In the post-operative period, the animals were treated with narcotics and non-steroidal anti-inflammatory drugs for pain management. Immediately after implant, the ICD was

activated to detect and treat any sustained arrhythmias. Otherwise, they received usual care.

Myocardial gene transfer: Three weeks after infarction, animals received aspirin 100mg and sildenafil 25mg orally 30 minutes before gene transfer. Balloon catheters were introduced into the middle portion of the LAD just distal to the second diagonal branch and into the adjacent site of the great cardiac vein. The LAD site approximated the position of the balloon catheter during the previous infarction procedure. The balloons were simultaneously expanded to 3 atm, and pre-treatment solution containing vascular endothelial growth factor 0.5 μg/ml, nitroglycerin 250 μg/ml, adenosine 5 mg/ml and 50 μM calcium concentration in 10 ml of Krebs' solution was infused into both vessels over 3 minutes. After pre-treatment, 5×10^9 pfu/ml of adenovirus was delivered in 12 ml of Krebs/nitroglycerin/adenosine/low calcium solution into both vessels over 2 minutes. After infusion, catheters and sheaths were removed and the hemostasis was obtained by ligation or repair of the vessel. The animals received usual care post-gene transfer. In addition, they were attached to a 3-lead ECG system on a daily basis to record 30 seconds of ECG output. Throughout the study period (before and after gene transfer) the animals were continuously monitored by the ICD for any sustained arrhythmias. Out of functional necessity, investigators were not blinded to treatment group, so hard endpoints were used for study results as much as possible.

Electrophysiology study (EPS): Animals underwent invasive EPS at the time of infarction, gene transfer and sacrifice. Non-invasive EPS was performed weekly, with exception of weeks where invasive EPS was performed.

Invasive EPS. After induction of anesthesia, sterile preparation, and surgical access to the neck or femoral vessels, a quadripolar EP catheter was place in the right ventricle. Programmed stimulation was performed from the right ventricular apex and outflow tract as follows: All pacing was performed at twice the pacing threshold. Extra stimuli were delivered after eight ventricular drive beats (pacing cycle length 250, 300, and 350 ms). The first extra stimulus (S2) was initially set 200-220 ms after the last pacing stimulus of the drive train (S1). S2 was then delivered at progressively shorter coupling intervals, scanning in 10 ms steps until the ERP is reached. If no arrhythmias were observed, S2 was reset to a point 30 ms outside the ERP. A second extra stimulus (S3) was then added 170-200 ms after S2 and scanning in 10 ms decrements was repeated until S2 and S3 were both refractory or equal to 140 ms. Again, if no arrhythmias were induced, a third extra stimulus (S4) was similarly introduced. After reaching a coupling interval of 140 ms or refractoriness with all extra stimuli, burst pacing was performed by delivering 20 beats at a cycle length of 280 ms. If no arrhythmias were observed, the burst cycle length was progressively decreased in 10 ms steps until arrhythmia induction, 2:1 conduction, or a minimum cycle length of 200 ms was reached (the minimum for the stimulator). If no arrhythmias were observed, the catheter was repositioned in the right ventricular outflow tract and the above pacing protocols were performed at that site. The endpoint of testing was either completion of the protocol to refractoriness or consistent induction of sustained monomorphic VT (at least twice with the same morphology). If VT was induced, a 64 electrode basket catheter was inserted through the arterial sheath into the LV. The site of earliest electrical activation was documented, and the corresponding

anatomical site was identified by fluoroscopic identification of the electrode with the earliest activation time.

After stimulation procedures, monophasic action potentials (MAPs) were recorded at each site while pacing through the ICD lead at a cycle length of 400ms. Local ERP was determined by pacing the local region from the MAP catheter. ERP was defined as the longest coupling interval of a premature stimulus that failed to capture the ventricle after an 8 beat drive train with a basic cycle length of 400ms. All pacing was performed at twice threshold voltage.

Non-invasive EPS: Prior to non-invasive EPS, animals were sedated with ketamine/xylazine/telazol. The indwelling ICD was interrogated using a conventional programmer. Before starting the pacing protocols, electrogram amplitude and pacing threshold were determined. Pacing protocols were performed at twice pacing threshold. Programmed stimulation and burst ventricular pacing were performed through the ICD system using the stimulation protocol described above.

Evaluation of gene transfer in the infarct border zone: The animals were sacrificed after the follow-up EP study. Tissue processing and transgene efficiency were evaluated as previously described.[1] Briefly, samples for RT-PCR were flash frozen *in situ* with use of liquid nitrogen-cooled clamps. The remaining sections of the hearts were removed and rinsed with ice-cold phosphate buffered saline (PBS). Gross sections were fixed with 2% formalin /0.5% glutaraldehyde for 15 minutes, rinsed with PBS, and stained with X-gal solution (1mg/ml 5-bromo-4-chloro-3-indonyl β-D-galactopyranoside, 5mM potassium ferricyanide, 5mM potassium ferrocyanide, 1mM $MgCl_2$ in PBS). For the microscopic examination, the anterior portion of distal ventricular septum was cut to 8 μm thickness,

and stained with X-gal and hematoxylin-eosin using conventional methods. X-gal staining was performed at pH 8.0 to minimize non-specific staining.[2] The percentage of cells expressing β-galactosidase was determined by counting cells in 5 randomly selected microscopic sections for each tissue (100 cells per field, 500 cells per animal).

RNA extraction and quantitative RT-PCR: Flash frozen tissue samples were kept at -80° C. Total RNA was extracted by RNeasy-Mini Kit (Qiagen), and reverse transcription used the Superscript First strand Synthesis system (Invitrogen). After reverse transcription, the amount of cDNA was adjusted to 10ng, and quantitative PCR was performed using SYBR green (PE Applied BioSystems), with ABIprism 7900HT (PE Applied BioSystems) and the following primers: KCNH2-F: *TGCTGAAGGAGACGGA-GGAG*, KCNH2-R: *CAGAGCCAGAGCCGAAGATG*, MYL2-F: *AGAGAGCAGATGGA-GCCAATTCCA*, MYL2-R: *TGCGTGGTCAGCATCTCCTTTACA*. The thermal profile was set as default (50 °C for 2 min and 95 °C for 10 min for activation, 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min). All measurement was duplicated.

For quantification of the starting copy number RT product for each sample, the standard curve was calculated using serial dilutions of purified plasmids (pcDNA3-KCNH2-G628S for KCNH2, and pGEMTeasy-MYL2 for MYL2). The measured KCNH2 copy number for each sample was normalized to the measured copy number of MYL2.

Cell isolation and patch clamp recording: Ventricular myocytes were isolated as previously described.[3] Cells expressing KCNH2-G628S were identified by green fluorescence, since the vector also contained the green fluorescent protein gene. Cells

were studied at 37°C under whole-cell patch clamp with a pipette solution containing (mmol/L) potassium aspartate 100, KCl 20, $CaCl_2$ 1, HEPES 5, MgATP 5, and EGTA 5, pH 7.20. The external solution contained NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, HEPES 11.8, and glucose 10, pH 7.40. Action potentials were recorded at a stimulation frequency of 0.5 Hz by current clamp using a patch-clamp amplifier (Axopatch 200B, Axon Instruments). Data acquisition was performed with a microcomputer using pCLAMP8 software and an A/D converter (Axon Instruments).

Statistical analysis: All parameters are summarized as mean ± standard error of mean. Between-group differences were compared by Student's t-test or analysis of variance for continuous variables, and chi-square tests for categorical variables. A $p < 0.05$ was considered statistically significant.

References

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