Electrophysiological engineering of heart-derived cells with calcium-dependent potassium channels improves cell therapy efficacy for cardioprotection

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We have shown that calcium-activated potassium (KCa)-channels regulate fundamental progenitor-cell functions, including proliferation, but their contribution to cell-therapy effectiveness is unknown. Here, we test the participation of KCa-channels in human heart explant-derived cell (EDC) physiology and therapeutic potential. TRAM34-sensitive KCa3.1-channels, encoded by the KCNN4 gene, are exclusively expressed in therapeutically bioactive EDC subfractions and maintain a strongly polarized resting potential; whereas therapeutically inert EDCs lack KCa3.1 channels and exhibit depolarized resting potentials. Somatic gene transfer of KCNN4 results in membrane hyperpolarization and increases intracellular [Ca2+]i, which boosts cell-proliferation and the production of pro-healing cytokines/nanoparticles. Intramyocardial injection of EDCs after KCNN4-gene overexpression markedly increases the salutary effects of EDCs on cardiac function, viable myocardium and peri-infarct neovascularization in a well-established murine model of ischemic cardiomyopathy. Thus, electrophysiological engineering provides a potentially valuable strategy to improve the therapeutic value of progenitor cells for cardioprotection and possibly other indications.
Despite significant advances in the management of cardiac diseases, heart failure remains one of the top killers worldwide. In response, stem cell therapy has emerged as a potential approach to prevent the progression of heart failure and, among the number of candidates proposed for cardiac cell therapy, heart explant-derived cells (EDCs) have been developed as a promising paracrine-based cell therapeutic. Preclinical work has shown that EDCs are intrinsic cardiac CD105+ cells that reduce pathological cardiac remodeling and improve myocardial function. Akin to noncardiac cell products, clinical comorbidities may attenuate the regenerative potency of EDCs but very few markers of product identity have been shown to clearly predict an inert cell product, which makes clinical reliability and translation challenging.

In this study, we focus on the regulation of membrane potential (V_m) and intracellular Ca^{2+}, which can exert significant influence over stem cell properties. We recently showed that the function of bone-marrow-derived mesenchymal stem cells and resident cardiac c-Kit+ cells is critically governed by the intermediate-conductance Ca^{2+}-activated K+ channel KCa3.1 (encoded by the KCNN4 gene). In both adult progenitor cell types, KCa3.1 channels open in response to store-operated Ca^{2+}-entry (SOCE) to hyperpolarize the cell membrane, increase the driving force for Ca^{2+} entry, and enhance transmembrane Ca^{2+} flux. KCa3.1-channel inhibition decreased SOCE, which suggested that Ca^{2+}-induced increases in KCa3.1 are necessary to optimize membrane potential during Ca^{2+} entry.

Based on these insights, we hypothesized that KCa3.1-channel activity might influence the behavior of therapeutically relevant cells, and that KCNN4 overexpression might improve therapeutic efficacy by optimizing V_m during SOCE. Here, we test this hypothesis and provide evidence that tailoring plasma-membrane ion-channel function influences the therapeutic efficacy of ex vivo expanded human heart cells using a promising adult cell therapeutic within an established immunodeficient mouse model of ischemic cardiomyopathy.

Results
Distinct endogenous currents exist in human EDCs. EDCs were obtained from patients with characteristics shown in Supplementary Table 1. Intriguingly, recent evidence has suggested that the major antigenic population found within ex vivo expanded heart-derived cells (the CD90 negative population, CD90−) constitutes the therapeutically active fraction within these heart-derived cell preparations and is responsible for most of the functional benefits associated with cell transplantation. Thus, to characterize endogenous Ca^{2+}-activated K+ (KCa) channels within physiologically relevant subpopulations, CD90+ cells were fluorescently labeled before starting patch-clamp experiments. Under conditions that allow the activation of Ca^{2+}-dependent current, we recorded outwardly rectifying voltage-dependent currents in both CD90+ and CD90− cells (Fig. 1a). The reversal potential (E_rev) of these currents averaged −51 ± 5 mV and −75 ± 5 mV (P < 0.01) for CD90+ and CD90− cells, respectively (Fig. 1b). Although whole-cell ion-current density was found to be of the same order in both cell types (Fig. 1c), the differences in E_rev hinted that the two subpopulations possess distinct bioelectrical properties, and thus different profiles of plasma-membrane ion channels.

EDCs contain 2 types of Ca^{2+}-dependent K+ currents with distinct contributions to V_m. Since currents recorded in EDCs displayed some biophysical characteristics (like outward rectification) of the large-conductance Ca^{2+}-dependent K+ current (I_BKCa), we tested the response to the selective BKCa-blocker paxilline. The outwardly rectifying current–voltage (I–V) relationship of EDCs was strongly affected by paxilline, leaving a substantial inwardly rectifying current in CD90− cells and a smaller residual outwardly rectifying current in CD90+ cells (Fig. 1d). The paxilline-sensitive current representing I_KCa3.1 was large and similar in both cell types (Supplementary Fig. 1a). V_m recorded with perforated-patch methods to maintain the physiological intracellular milieu was significantly less negative in CD90− cells versus CD90+ cells (−25 ± 2 mV vs. −64 ± 7 mV; P < 0.001), and was unaffected by paxilline (Supplementary Fig. 1b), suggesting that it is governed by another charge carrier(s). The differences in paxilline-resistant current between the 2 cell types was due to a TRAM-34-sensitive component, since their I–V relationships became indistinguishable after exposure to the selective KCa3.1-blocker TRAM-34 (Fig. 1e). In contrast to the substantial KCa3.1 currents (TRAM-34-sensitive; I_KCa3.1) in the CD90− subpopulation, I_KCa3.1 was negligible in CD90+ cells (Fig. 2a). Blocking I_KCa3.1 with TRAM-34 substantially depolarized V_m of CD90− EDCs without altering V_m of CD90+ cells (Fig. 2b). In the presence of TRAM-34, the V_m of CD90− EDCs became similar to that of CD90+ cells. These results indicate that the V_m of EDCs is largely determined by TRAM-34 sensitive KCa3.1 conductance, which is substantial in CD90− cells and undetectable in CD90+ cells.

V_m is controlled by Ca^{2+}-dependent K+ currents during Ca^{2+}-entry. One potential role for I_KCa3.1 in EDCs would be hyperpolarization of V_m to optimize Ca^{2+}-inflow during SOCE, as previously demonstrated for cardiac-derived c-Kit+ cells and bone-marrow-derived mesenchymal stem cells. To address this possibility, we induced SOCE in EDCs and monitored V_m (Fig. 2c–d). In the presence of very low intracellular [Ca^{2+}] resulting from perfusion with nominally Ca^{2+}-free extracellular solution, V_m was −25 mV in both CD90− and CD90+ cells. Following store depletion, the addition of 1.8 mM/L extracellular Ca^{2+} induced a strong hyperpolarization in CD90− cells (from −25 ± 3 mV to −67 ± 3 mV, P < 0.001), but had no effect on V_m in CD90+ cells. The addition of TRAM-34 during SOCE strongly inhibited the SOCE-associated hyperpolarization (Fig. 2d), indicating a key role for I_KCa3.1 in governing V_m in CD90− cells during activation by SOCE.

KCNN4-gene transfer hyperpolarizes V_m and increases intracellular Ca^{2+}. These observations are consistent with the notion that the differential expression of KCNN4 contributes to the reduced regenerative performance of CD90− cells compared to CD90− while, preserving a potential target to enhance the effectiveness of EDC therapy. We therefore engineered EDCs (mixed CD90+/CD90− population) to overexpress KCa3.1 current in EDCs prior to in vivo cell delivery. Lentivirus-mediated KCNN4-gene transfer increased KCNN4-gene expression about 20-fold compared to the empty vector (EV) (Fig. 3a). KCNN4 overexpression conferred a much more homogeneous electrophysiological phenotype to EDCs: KCNN4-transfer increased I_KCa3.1 density and hyperpolarized V_m to about the same level in both CD90− and CD90+ cells (−77 ± 2 mV vs. −81 ± 2 mV; Fig. 3b–c). Because V_m hyperpolarization is known to facilitate Ca^{2+} entry through voltage-independent channels, we investigated whether KCNN4-transfer would affect intracellular Ca^{2+}. Consistent with our hypothesis, we found that intracellular [Ca^{2+}] was significantly higher under resting conditions in KCNN4-transfered EDCs compared to EV-control (Fig. 3d). Taken together, these results indicate that KCa3.1 overexpression enhances Ca^{2+} signaling in EDCs.
Overexpression of KCNN4 increases proliferation. When EDCs were exposed to in vitro culture conditions designed to mimic the harsh post-infarct environment (1% oxygen/H$_2$O$_2$/nutrient deprived), both EV- and NT-EDC numbers decreased from baseline, whereas overexpression of KCNN4 significantly increased proliferation (Fig. 4a and Supplementary Fig. 2a, b) without altering resistance to apoptosis (Fig. 4b and Supplementary Fig. 2c). The importance of intracellular Ca$^{2+}$ in controlling EDC proliferation was underscored by the observation that exposure of KCNN4-EDCs to the cell permeant Ca$^{2+}$ chelator BAPTA-AM greatly reduced EDC cell numbers and proliferation (Fig. 4c and Supplementary Fig. 3). Despite ongoing constitutive expression of KCNN4, somatic gene transfer did not reduce the cardiogenic potential of EDCs (Supplementary Fig. 4).

Transplantation of KCNN4-engineered EDCs improves cardiac function after myocardial infarction. Male NOD/SCID IL2R$^\gamma$ mice underwent left coronary artery (LCA) ligation, which was followed 1 week later by randomization to echocardiographic

**Fig. 1** Endogenous ion currents expressed in human EDCs. a Original whole-cell currents from CD90$^+$ (upper panel) and CD90$^-$ EDCs (lower panel) under various experimental conditions, with a 1000-ms depolarizing voltage-ramp protocol from $-120$ to $+60$ mV, with a holding potential of $-40$ mV, before and after the addition of KCa inhibitors. Baseline = black, paxilline = red, paxilline + TRAM-34 = blue. b Reversal potential of whole-cell ion currents recorded in CD90$^+$ and CD90$^-$ EDCs in the presence of 300 nmol/L free-[Ca$^{2+}$]. c–e I-V relationship of whole-cell ion currents (recorded with 1000 ms voltage-ramps from $-120$ to $+60$ mV with a holding potential of $-40$ mV) in CD90$^+$ and CD90$^-$ EDCs under c basal conditions, d after addition of 1 µmol/L paxilline, or e after addition of 1 µmol/L paxilline and 1 µmol/L TRAM-34. Open circles = CD90$^-$, closed circles = CD90$^+$. Two-sided non-paired student $t$-test (panel b); two-way repeated-measures ANOVA showing significance of group effect; n/N = cells/cell lines per group. EDC explant-derived cell. Data in b–e are shown as individual data points along with mean and SEM. *$P<0.05$; ****$P<0.0001$ vs CD90$^-$ EDCs. For precise $P$ values, see Supplemental Source Data and Statistics file.
guided intramyocardial injection of EV-EDCs, KCNN4-EDCs, NT-EDCs, or vehicle (Fig. 5a). Laboratory staff were blinded to treatment allocations and all outcome assessment and analysis was conducted by individuals blinded to group allotment. The study had very little mortality; replacement of subjects was therefore not necessary and not performed (Supplementary Fig. 5). As shown in Fig. 5b and Supplementary Table 2, all animals demonstrated equivalent degrees of pretreatment cardiac dysfunction and chamber dimensions 1 week after LCA ligation. Mice that received EV- or NT-EDCs demonstrated similar improvements in echocardiographic (Fig. 5b and Supplementary Table 2, $P < 0.05$ vs. vehicle) and hemodynamic (Fig. 5b, Supplementary Fig. 6 and Supplementary Table 3) measures of cardiac function 4 weeks after LCA ligation, suggesting that lentiviral transduction per se had no effect on cell-mediated improvement of ischemic injury. Animals that received KCNN4-transferred EDCs demonstrated significantly improved cardiac function (Fig. 5b–c, Supplementary Fig. 6 and Supplementary Tables 2 and 3) and showed smaller infarctions (Fig. 6a) 4 weeks after LCA ligation as compared to animals that received vehicle, EV- and NT-EDCs. As shown in Supplementary Fig. 7 and Supplementary Table 4, a separate experimental cohort revealed that the observed differences persisted over the following weeks and did not represent a transient improvement in heart function. Cell treatment had no detectable effect on the ejection fraction of control, noninfarcted mice (Supplementary Fig. 8 and Supplementary Table 5).

Fig. 2 Functional endogenous KCa3.1 current and membrane-potential changes during SOCE in EDCs. a. $I$–$V$ relationship of $I_{\text{Ca3.1}}$ (TRAM-34–sensitive current) in CD90$^+$ and CD90$^-$ EDCs. Data are mean ± SEM; n/N = cells/cell lines per group. b. Resting potential of EDCs before and after exposure to 1 µmol/L TRAM-34. Two-way repeated-measures ANOVA with individual-mean comparisons by Bonferroni-corrected two-sided $t$-tests; n/N = cells/cell lines per group. Data are individual points along with mean and SEM. c. Original current-clamp recording from one CD90$^+$ cell (left panel) and mean ± SEM data for $V_{\text{mem}}$ changes induced by SOCE (resulting from intracellular Ca$^{2+}$-depletion by exposure to nominally Ca$^{2+}$-free extracellular Ca$^{2+}$ (0 [Ca$^{2+}$]o) and then reintroduction of 1.8 mmol/L Ca$^{2+}$ (1.8 [Ca$^{2+}$]o)), with subsequent exposure to 1-µmol/L TRAM-34 (right panel). d. Original current-clamp recording from one CD90$^-$ cell (left panel) and mean ± SEM data for $V_{\text{mem}}$ changes induced by SOCE and subsequent exposure to 1-µmol/L TRAM-34 (right panel). One-way ANOVA with individual-mean comparisons by Bonferroni-corrected two-sided $t$-tests; numbers shown on bars are numbers of cells studied; n/N = cells/cell lines per group; Open circles = CD90$^-$, closed circles = CD90$^+$. EDC explant-derived cell, min minutes, $V_{\text{mem}}$ membrane potential. ***$P < 0.0001$. For precise $P$ values, see Supplemental Source Data and Statistics file.
Histological analysis revealed that treatment with KCNN4-EDCs reduced infarct size (Fig. 6a) and increased both infarct wall thickness and the amount of preserved living tissue within the ischemic area (Fig. 6b). These salutary changes in cardiac structure were paralleled by increases in blood vessel density (Fig. 6c and Supplementary Figs. 9 and 10), healing-promoting M2 macrophages (Supplementary Fig. 11) and proliferative BrdU+ cells (Fig. 6d and Supplementary Fig. 12), all of which might have contributed to the reduced expression of apoptotic markers (Supplementary Fig. 13) and of proinflammatory M1 macrophages (Supplementary Fig. 14) seen within the remodeling peri-infarct area. Consistent with the increased in vitro proliferation that we observed, overexpression of KCNN4 boosted the long-term retention of EDCs by 3 ± 1 (according to human nuclear antigen immunohistochemical profiling; P < 0.05, Supplementary Fig. 15) or 7 ± 2 (per retained human alu sequences; P < 0.01; Fig. 6e) fold compared to intramyocardial injection of NT-EDCs. By surface telemetry, EDC-transplantation had no effect on ECG-indices of heart rate, cardiac conduction or repolarization (Supplementary Table 6). Programmed electrical stimulation induced ventricular arrhythmias with 2 extrastimuli in a single vehicle-treated animal (Supplementary Fig. 16a) while no
EDC-treated animals showed inducible arrhythmias; EDC-transplantation had no detectable effect on cardiac electrophysiology in vivo (Supplementary Fig. 16b). Thus, increasing $I_{\text{KCa3.1}}$ within EDCs boosts endogenous repair without affecting electrophysiological function or increasing susceptibility to malignant ventricular arrhythmias.

![Graph](image)

**Fig. 4** Effect of KCNN4 overexpression on EDC phenotype after 24 h exposure to ischemia-mimicking media culture conditions. **a** Increasing $I_{\text{KCa3.1}}$ boosts EDC cell numbers ($n = 5$ biological replicates/group). *$P < 0.05$ vs. EV- and NT-EDCs. **b** KCNN4 overexpression increases the proportion of EDCs actively proliferating ($n = 5$ biological replicates/group). *$P < 0.05$ vs. EV- and NT-EDCs. **b** Increasing $I_{\text{KCa3.1}}$ had no effect on the ability of EDCs to withstand apoptosis as indicated by expression of apoptotic (Annexin V$^+$/7AAD$^-$; $n = 5$ biological replicates/group except KCNN4-EDCs where $n = 4$ biological replicates) and necrotic (Annexin V$^+$/7AAD$^+$) markers ($n = 5$ biological replicates). 7AAD 7-aminoactinomycin. **c** Calcium chelation using BAPTA-AM reduces EDC cell numbers ($n = 5$ biological replicates/group) and the proportion of EDCs actively proliferating ($n = 5$ biological replicates/group). All data are presented as individual and mean values ± SEM. Significance is indicated in all panels using *$P < 0.05$ vs. KCNN4-EDCs exposed to BAPTA-AM, †$P < 0.05$ vs. NT-EDCs exposed to BAPTA-AM. For precise $P$ values, see Supplemental Source Data and Statistics file. All data were analyzed using a one-way ANOVA with individual-mean comparisons by Bonferroni’s multiple two-tailed comparisons test. Blue filled symbols = KCNN4-transduced EDCs, blue open symbols = EV-EDCs, gray filled symbols = NT-EDCs. EDC explant-derived cell, EV empty vector.

![Graph](image)

**Fig. 5** Effects of KCNN4 overexpression on myocardial function. **a** Schematic of in vivo experiments comparing the effect of KCNN4-engineered human EDCs (KCNN4-EDCs) to empty vector human EDCs (EV-EDCs), nontransduced human EDCs (NT-EDCs) and vehicle using a NOD/SCID IL2R$^\gamma$ model of ischemic injury. LCA, left coronary ligation. **b** Effects of KCNN4-EDCs ($n = 12$ biological replicates), EV-EDCs ($n = 12$ biological replicates), NT-EDCs ($n = 13$ biological replicates), or vehicle ($n = 14$ biological replicates) injection on echocardiographic ejection fraction 4 weeks after LCA ligation. **c** Effects of KCNN4-EDCs ($n = 12$ biological replicates), EV-EDCs ($n = 12$ biological replicates), NT-EDCs ($n = 13$ biological replicates) or vehicle ($n = 14$ biological replicates) injection on invasive hemodynamic measures of myocardial function 4 weeks after LCA ligation. All data are presented as individual and mean values ± SEM. Significance is indicated in all panels using *$P < 0.0001$ vs. vehicle-treated mice, †$P < 0.05$ vs. EV- and NT-EDCs treated mice. For precise $P$ values, see Supplemental Source Data and Statistics file. All data was analyzed using a one-way ANOVA with individual-mean comparisons by Bonferroni multiple two-tailed comparisons test. Blue filled symbols = KCNN4-transduced EDCs, blue open symbols = EV-EDCs, gray filled symbols = NT-EDCs, gray open symbols = Vehicle. EDC explant-derived cell, EV empty vector.

EDC-treated animals showed inducible arrhythmias; EDC-transplantation had no detectable effect on cardiac electrophysiology in vivo (Supplementary Fig. 16b). Thus, increasing $I_{\text{KCa3.1}}$ within EDCs boosts endogenous repair without affecting electrophysiological function or increasing susceptibility to malignant ventricular arrhythmias.

Given that depleting the CD90$^+$ fraction enhances the functional benefit of heart-derived cells$^{11}$ and that increasing...
IKCa3.1 boosts cell-mediated repair, we hypothesized that the targeted overexpression of KCNN4 within CD90\(^+\) EDCs alone might suffice to enhance EDC-mediated repair of injured myocardium. To test this idea, NOD/SCID IL2R\(\gamma\)mice underwent coronary ligation and were randomized 1 week later to receive intramyocardial injection of KCNN4 CD90\(^+\) EDCs, KCNN4 CD90\(^-\) EDCs, NT CD90\(^+\) EDCs, NT CD90\(^-\) EDCs, recombined KCNN4-EDCs, recombined NT-EDCs or vehicle (Supplementary Figs. 17, 18, and 19; Supplementary Table 7). Interestingly, depletion of CD90\(^+\) cells alone did not suffice to improve EDC treatment outcomes (NT CD90\(^+\) cells \(\approx\) NT CD90\(^-\) cells). Overexpression of KCNN4 did not significantly improve the regenerative performance of isolated cells, be it CD90\(^-\) or CD90\(^+\). Only intramyocardial injection of the recombined KCNN4 cells (i.e., KCNN4 CD90\(^-\) and KCNN4 CD90\(^+\)) improved cell-treatment outcomes, suggesting that important synergies must exist within the mixed cell product that cannot be recapitulated by a single cell type or overcome using genetic engineering. Further work is clearly needed to clarify the underlying mechanisms.

Overexpression of KCNN4 increases cytokine and nanoparticle secretion. The cytokine signature of EDCs was profiled using an unbiased proteomic array capable of detecting 102 cytokines within conditioned media after 48 h of cell culture in nutrient-deprived hypoxic media (1% oxygen) conditions (Fig. 7a). Although KCNN4 overexpression did not significantly increase the number of cytokines within conditioned media as compared to EV-EDCs (39 vs. 42 cytokines, respectively; Chi-square 0.33, \(P = 0.56\)), KCNN4 overexpression increased the production of cytokines already found within EDC conditioned media (6 vs. 1 cytokine levels increased, respectively; Chi-square 3.78, \(P = 0.05\)). Interestingly, among the cytokines increased via KCNN4 overexpression several implicated in angiogenesis (vascular endothelial...
growth hormone)\textsuperscript{14,15}, post-infarct healing (angiogenin\textsuperscript{16,17}, insuln-like growth factor-binding protein \textsuperscript{3}], stromal derived factor 1 alpha)\textsuperscript{19,20} and immune modulation (intercellular adhesion molecule 1)\textsuperscript{21} were found (Fig. 7b).

Given recent evidence supporting a critical role for extracellular vesicles in the salutary benefits conferred by heart-derived cell therapy\textsuperscript{22}, the effect of KCNN4 overexpression on extracellular vesicle production was probed. Media conditioned by \textit{KCNN4}-EDCs demonstrated a 1.6 ± 0.2 fold increase (\textit{P} < 0.05 vs. EV or NT-EDCs; Fig. 7c) in nanoparticles with a size typical for extracellular vesicles (133 ± 5 nm, \textit{P} = ns vs. EV or NT-EDCs).

Fig. 7 Effects of \textit{KCNN4} overexpression on the paracrine profile of EDCs. \(\mathbf{a}\) Direct comparison of cytokine proteomic expression within 39 cytokines found to be elevated within media conditioned by EV- or \textit{KCNN4}-EDCs. \(\star \text{P} < 0.05 \) vs. EV- or \textit{KCNN4}-EDCs, Fisher’s exact test. BAFF B-cell activating factor, BDNF brain-derived neurotrophic factor, CC C5/C5a complement Component C5a, Dkk-1 Dickkopf-related protein 1, DPP IV Dipeptidyl peptidase-4, ENA-78 epithelial-neutrophil activating peptide, FGF-9 fibroblast growth factor 19, GDF-15 growth/differentiation factor 15, GROx growth-regulated oncogene alpha, HGF hepatocyte growth factor, ICAM-1 intercellular adhesion molecule 1, IGBP-2 insulin-like growth factor-binding protein, IGBP-3 insulin-like growth factor-binding protein, IL-6 interleukin-6, IL-8 Interleukin-8, IL-11 interleukin-11, IL-17A interleukin-17A, IL-19 interleukin-19, IL-22 interleukin-22, MCP-1 monocyte chemoattractant protein 1, MIF macrophage migration inhibitory factor, RBP-4 retinol binding protein 4, TFF3 Trefoil factor 3, uPAR urokinase-type plasminogen activator receptor, VEGF vascular endothelial growth factor, Vitamin D BP Vitamin D binding protein. (\(n = 4\) biological replicates). Data are shown as mean and SEM.

\(\mathbf{b}\) Relative abundance of the top 10 cytokines produced by \textit{KCNN4} and EV-EDCs. 

\(\mathbf{c}\) Comparison of the size distribution of extracellular vesicles within EDC conditioned media (\(n = 5\) biological replicates). Effects of \textit{KCNN4} overexpression relative to the miRNA expression profile of NT-EDCs (\(d\) \(n = 3\) biological replicates) or EV-EDCs (\(e\) \(n = 3\) biological replicates). Data in \(c\)–\(e\) are shown as individual points, along with mean and SEM. Significance is indicated in all panels using \(\text{\textsuperscript{\star}P} < 0.05\) vs. EV- or \textit{KCNN4}-EDCs. For precise \textit{P} values, see Supplemental Source Data and Statistics file. All data were analyzed using a one-way ANOVA with individual-mean comparisons by Bonferroni adjusted two-sided \textsc{t}-test. Blue = \textit{KCNN4}-transduced EDCs, gray = EV-EDCs, black = NT-EDCs. EDC explant-derived cell, EV empty vector, min minutes, Vmem membrane potential.
abundant miRNAs found within adherent cultured EDC nanoparticles were associated with cardiomyocyte proliferation (e.g., miR-199a-5p), cardiomyocyte salvage (e.g., miR-125b-5p), protection against oxidative stress (e.g., miR-21-5p), or reducing cardiac fibrosis (e.g., miR-22-3p; Supplementary Table 8). Perhaps surprisingly, somatic gene transfer of KCNN4 decreased the expression of only 4 miRNAs (KCNN4-EDCs vs. NT-EDCs, fold change > 1.5, P-adjusted < 0.05, Fig. 7d, Supplementary Table 9). The targets of these miRNAs typically clustered within TGF-β signaling, Hippo signaling, apoptosis, steroid biosynthesis, RNA transport, protein processing, or cytoskeleton regulation (Supplementary Fig. 20a). In contrast to lentiviral transduction alone, KCNN4 overexpression altered the expression of 16 miRNAs (fold change > 1.5; P-adjusted < 0.05, Fig. 7e and Supplementary Table 10) involved in the regulation of stem cell pluripotency, extracellular matrix turnover, cell-cycle regulation, transforming growth factor beta signaling, and Hippo signaling (Supplementary Figs. 20b, c). This, akin to effects seen on cytokine production, KCNN4 activity increases nanoparticle production and alters miRNA expression to improve the healing-promotion signature of the EDC secretome.

Discussion
In this study, we found that the therapeutic potential of EDCs derived from human hearts is governed by the expression and function of the intermediate-conductance Ca2+-activated channel KCa3.1. Although Ca2+-activated K+ channels were identified in all EDCs, KCa3.1 channels were exclusively expressed in the CD90+ subpopulation that is thought to be responsible for most of the functional benefits associated with heart-derived cell therapy11,12. Genetic manipulation to enhance KCa3.1-channel expression increased the proliferation and paracrine profile of EDCs in vitro. Compared to nonmodified cells, electrophysiologically engineered EDCs were superior in improving cardiac function in association with a combination of enhanced angiogenesis, myocardial salvage and cardiomyogenesis when transplanted into a clinically relevant mouse model that mirrored post-infarct healing. Our study provides the first evidence in the literature that manipulation of plasma-membrane ion channels can enhance the therapeutic benefits of progenitor cells.

Ion channels provide the basis for generating bioelectric signals that control cell function24,25. This important role is underscored by the observation that KCa channels are widely distributed in many adult stem cell types and play a key role during embryonic development10,26–30. Until recently, their contribution to the regulation of Vmem and Ca2+ dynamics has been uncertain—a knowledge gap, which may stem from a myriad of differences in cell culture or technical methods31. While BKCa channels have a large conductance, they show strong outward rectification and carry relatively little current in EDCs near the K+ reversal potential, which explains why they do not contribute to EDC resting potential (Supplementary Fig. 1).

As shown in mesenchymal stem cells32–34, embryonic stem cells35 or myoblasts36,37, proliferation and differentiation are tightly controlled by changes in Vmem. In contrast to other cardiac cell types (cardiac fibroblasts38 or myocytes39), the membrane potential of EDCs is not determined by Kir-channels. Rather, we found that the EDC resting membrane potential is largely governed by the intermediate-conductance Ca2+-activated channel KCa3.1. This finding mirrors our prior observation in expanded bone-marrow-derived mesenchymal stem cells10, in which KCa3.1 channels control EDC membrane potential during SOCE to maintain the electrical gradient for Ca2+ influx. We now show here that in heart-derived cells, KCa3.1 channels play a pivotal role at the crossroad between Ca2+ influx, outward ion fluxes and Vmem to provide critical internal signaling, which modulate cell growth, migration, differentiation, and paracrine signaling40. When considered within the context of KCa channel function defining cardiogenesis in both embryonic and induced pluripotent stem cells41–43, our results highlight the important physiological role of KCa3.1 regulation of Vmem and hint that channel expression may provide an independent phenotypic marker of cell product “potency”.

Somatic gene transfer of KCNN4 increased proliferation and ultimately improved the long-term engraftment of transplanted cells. Given that KCNN4 overexpression had negligible effects on resistance to apoptosis, it is unlikely that the acute retention or resistance to toxic metabolites was increased. We hypothesize that the increase in EDC proliferation resulting from KCNN4-transfer affords retained human cells the opportunity to multiply and more effectively release cytoprotective molecules. In addition to producing a larger transient pool of transplanted cells within the myocardium that increases paracrine stimulation of endogenous repair mechanisms, long-term cell retention was also greater. However, given the trivial number of transplanted cells that were ultimately retained 3 weeks after injection (597 ± 200 vs. 69 ± 22 for KCNN4- vs. NT-EDCs, respectively), it is unlikely that differentiation into working myocardium played any role in the benefits seen after intramyocardial injection.

It is widely agreed that most of the benefits conferred by adult cell therapy occur through paracrine stimulation of angiogenesis, cardiomyogenesis, immunomodulation, and myocardial salvage5,44. Consistent with the notion that paracrine engineering enhances cell-treatment outcomes20,45, we found that KCNN4 overexpression improved the paracrine repertoire of EDCs. Since IKCa3.1-mediated increases in intracellular Ca2+ are necessary for the production of inflammatory chemokines and cytokines by various immune cells46,47, it is reasonable to believe that a similar mechanism occurs in EDCs. It follows that the marked functional improvement in cardiac function observed after KCNN4-EDC transplant is likely mediated, in part, via increased cytokine and nanoparticle stimulation at the site of injection resulting from a combination of increased absolute cell numbers and per-cell paracrine output within the myocardium.

Our findings have potentially significant practical applications, as identifying a heretofore unappreciated mediator of cell transplant outcome has the potential to improve the inconsistent clinical record of cardiac cell therapy2. In comparison to expensive, more invasive strategies that repeatedly administer more of the same potentially ineffective cell product, strategies that optimize endogenous ionic-current flows have the potential to regulate more than cell excitability, as evidenced by the marked improvements in cell behavior48. Our study is the first to elucidate the important participation of KCa3.1 channels in controlling the therapeutic capacity of progenitor-cell preparations. Given the role of these channels in bone-marrow-derived progenitor cells10, our findings may have implications beyond simply cardiac disease. The ability to control cell function by modulating bioelectric properties is a potentially powerful tool to enhance the effectiveness of other progenitor cell types toward therapeutic application.

This is one of the first studies to examine the effect of electrophysiologically engineering for cardiac cell therapy. The only prior work in the field that examined the effect of modifying the electrophysiological properties of a cell treatment aimed to reduce arrhythmogenicity (not effectiveness) of therapy with skeletal myoblasts. As these noncardiac cells lack connexin-43, tissue inhomogeneities arise in the form of electrically uncoupled grafts that provide a ready substrate for re-entrant arrhythmias49. Their clinical use caused a high incidence of life-threatening ventricular

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arrhythmias. Although therapy to increase expression of connexin-43 increased electrical coupling and decreased arrhythmogenicity, equivocal clinical benefits (i.e., no enhancement in left ventricular ejection fraction 6 months after skeletal myoblast injection) combined with ongoing concerns regarding pro-arrhythmia combined to reduce enthusiasm and stall work on skeletal myoblast-mediated cardiac repair. Unlike the skeletal myoblast experience, our approach focuses on a key regulator, a Ca++–dependent K’–current that controls Ca++–entry and cell function, within a cell product undergoing clinical investigation for several diseases (cardiomyopathy secondary to Duchenne Muscular Dystrophy, NCT02485938 & NCT03406780; heart failure with preserved ejection fraction, NCT02941765; pulmonary hypertension, NCT03145298). There has been no signal for adverse electrophysiological effects in the preclinical (50 + independent labs) or clinical (two phase 1, two phase 1/2 and one phase 2) trials, with cardiac EDCs to date. Based on our prior work, we suspect the KCNN4-engineering approach may be generalizable to other cell types, but this remains to be shown.

In comparison to other genetically modified cell types used for post-ischemic cardiac repair, several studies have used direct genetic modification to modify stem cell properties before transplantation. One such study demonstrated reduced apoptotic cell clearance and increased long-term retention of cardiac c-Kit+ cells by overexpressing a modulator of Akt signaling, Pim-1 kinase. In the past, we have taken a more direct approach by overexpressing cytokines implicated in myocardial repair by direct paracrine engineering. Parallel efforts using genetic overexpression within noncardiac cell types have been made to improve cell survival (SDF-1, Bcl-2), differentiation (TGF-β), migration (CXC4, cNOS), and vasculogenesis (HIF-1, VEGF). KCNN4 overexpression differs from this previous work as it focuses on a fundamental electrophysiological property, endogenously present within many different types of progenitor cells, that generates replicative function. Boosting electrophysiological function enhanced proliferation, extracellular vesicle production/potency, and cell-treatment outcomes.

Although KCNN4 gene transfer clearly enhanced the therapeutic benefits of cardiac cell therapy, the detailed molecular mechanisms by which boosting KCa3.1-channel-function improved cell function will require further investigation. In this study, we used a viral vector to transfer the genetic material into cells. Lentiviruses are a useful tool for the engineering of adult stem cells because of their transduction efficiency toward slowly proliferating cells, and the absence of detrimental cellular effects. Despite the utility of such vectors to establish proof of concept, integration of genetic material into proto-oncogenic sites is a potential risk associated with the use of retroviruses that limits clinical translation. Other presently available viral vectors will need to be harnessed for clinical translation. This investigation also studied human cells transplanted into immunodeficient mice, making extrapolation to immune competent clearance of allogeneic cells uncertain. Finally, for this proof of principle experiment, we chose to inject cells 1 week after LCA ligation (analogous to ~1 month after a clinical myocardial infarction). Future work will be needed to evaluate the effect of KCNN4 overexpression on cell-treatment outcomes when therapies are delivered into the extensively scarred and remodeled myocardium.

**Methods**

**Cell culture and lentiviral transduction.** Human EDCs were cultured from left atrial appendages donated by patients undergoing clinically indicated heart surgery after obtaining informed consent under a protocol approved by the University of Ottawa Heart Institute Research Ethics Board. As shown in Supplementary Table 1, there were no significant differences in the characteristics of patient cell lines used in vitro or in vivo experiments. Briefly, cardiac biopsies were minced, digested (Collagenase IV, Thermo Fischer Scientific) and plated within MSC to define global ion currents (Biologic LFP, 5% oxygen). Once a week for 4 weeks, EDCs that spontaneously emerged from the plated biopsy were collected using TrypLE Select (Thermo Fischer Scientific). Cells were transduced with lentivirus to overexpress KCNN4 (Hs68053-21357357, GE Healthcare Dharmacoll), empty backbone (25880, Addgene). Two days later, cells were harvested using TrypLE Select for direct experimentation.

Cardiogenic profiling was performed after exposure to inductive media conditions (Dulbecco’s Modified Eagle Medium Low Glucose, 40% MCDB-201, 0.75% dimethylsulfoxide, 0.1% 10 mmol/l L-ascorbic acid, 0.01% ITS liquid media) to define global ion currents (Biologic LFP, 5% oxygen). 0.01% Pro Start (10 mmol/l potassium L-dihydrogen phosphate, 0.001% 2-mercaptoethanol, 10 ng/ml recombinant mouse fibroblast growth factor 8b, 100 ng/ml fibroblast growth factor 4, 10 ng/ml recombinant human protein rhDDK-1, and 10 ng/ml recombinant human bone morphogenetic protein 2) for 1 week,20,21,28,29. Total RNA was isolated at day 1 and 7 (Quagen) for qPCR analysis (Roche) using commercial primers (Integrated DNA Technologies; TTN2T; nm_001041314; ACTA2: nm_0010613; VWF: nm_000352).

Protein expression was evaluated using flow cytometry for cTNT (ab8295, Abcam), a-SMA, (ab32575, Abcam), VWF (ab8822, Abcam) with appropriate isotype controls (Supplementary Figure 4). A minimum of 30,000 events was collected with fluorescence compensation performed using single labeled controls (GuavaSoft v2.7, Millipore).

**RNA extraction and gene expression analysis.** Messenger RNA were isolated from cultured EDCs using the NucleoSpin RNA Mini kit (Ref 740955.50, Macherey Nagel) and purified mRNA was quantified using a NanoDrop 1000 spectrophotometer. Retrotranscription was performed on 25 ng of RNA with the High-Capacity cDNA Reverse Transcription Kit (Ref 4368841, Thermofisher) using streptavidin-coated magnetic beads at 25 °C. Annealing was done at 37 °C for 10 min. RT-qPCR was done using SYBR Green primers for GAPDH and KCNN4 (Supplementary Table 1). SYBR Select Master Mix (Ref 4472930, Thermofisher) was added to primers and 1 ng cDNA per well. RT-qPCR was run on a StepOnePlus Real-Time PCR System (4376600, Thermofisher) with an initial denaturation 95 °C step for 10 min, followed by 40 cycles (Denaturation 95 °C 15 sec—Annealing and Extension 60 °C for 1 min). The KCNN4 Ct values were normalized to GAPDH and data were analyzed using the 2−ΔΔCt method.

**Ion-current and Vmem recording.** All in vitro recordings were obtained at 37 °C. Whole-cell perforated-patch and tight-seal techniques were used to record membrane potential (Vmem, current-clamp mode) and ion-currents (voltage-clamp mode). Borosilicate glass electrodes (tip resistances 2–3 MΩ) when filled with pipette solution) were connected to a patch-clamp amplifier (Axopatch 200B, Axon Instruments). In standard (normal) Tyrode solution containing (mmol/L) NaCl 136, CaCl2 1.8, KCl 5.4, MgCl2 1, NaHPO4 0.33, dextrose 10, and HEPES 5, titrated to pH 7.4 with NaOH. For nominally Ca++–free Tyrode solution experiments, Ca++ was omitted and 1 mM EGTA was added. The pipette solution used contained (mmol/L) NaCl 140, KCl 5, MgCl2 1.2, and HEPES 5, titrated to pH 7.4 with NaOH. For nominally Ca++–free Tyrode solution experiments, Ca++ was omitted and 1 mM EGTA was added. The pipette solution used contained (mmol/L) NaCl 140, KCl 5, MgCl2 1, and HEPES 5, pH 7.4 (KOH). The amount of CaCl2 required to achieve 300 nmol/L free-[Ca++], was calculated using the WebMaxC's Web-based software (http://net.physiology.org/docs/webmaxc/). Junction potentials between bath and pipette solution were adjusted to match bath and pipette solution averaged 10 mV and were corrected before recording, for both Vmem and ion-current measurements. Currents are expressed as densities (pA/pF) to control for cell size/capacitance differences. To study the effect of SOCE on Vmem (Ca++), was depleted passively by bathing cells in Ca++–free Tyrode solution for at least 30 min, and then cell contents were dialyzed by tight-seal attachment with pipette solution containing (mmol/L) KCl 140, NaCl 5, MgCl2 1, and HEPES 10 (pH 7.2, KOH). The amount of CaCl2 required to achieve 300 nmol/L free-[Ca++] was calculated using the WebMaxC's Web-based software (http://net.physiology.org/docs/webmaxc/). Junction potentials between bath and pipette solution were adjusted to match bath and pipette solution averaged 10 mV and were corrected before recording, for both Vmem and ion-current measurements. Currents are expressed as densities (pA/pF) to control for cell size/capacitance differences. To study the effect of SOCE on Vmem (Ca++), was depleted passively by bathing cells in Ca++–free Tyrode solution for at least 30 min, and then cell contents were dialyzed by tight-seal attachment with pipette solution containing (mmol/L) KCl 140, NaCl 5, MgCl2 1, and HEPES 10 (pH 7.2, KOH).

After whole-cell configuration and cell-dialysis were established, extracellular (Ca++–) was restored and Vmem changes were recorded. Before starting patch-clamp experiments, a fluorescently-tagged antibody (AB2606G, R&D Systems) was used to labeled the CD90+ subpopulation.

**Ca++–imaging.** EDCs genetically engineered to overexpress KCNN4 (KCNN4-EDCs) and empty backbone-transduced EDCs (EV-EDCs) were loaded with Fluo-4-acetoxymethyl ester (10 μmol/L; Invitrogen) in complete growth medium in the presence of Pluronic F-127 (20% solution in dimethylsulfoxide, 2.5 μg/mL) for 30 min at 37 °C in a humidified incubator under standard cell culture conditions. Chamber slides were positioned on the stage of a confocal microscope; cells were incubated with Tyrode solution and maintained for 15 min at room temperature before experimental protocols to allow deesterification of Fluo-4-acetoxymethyl ester. Images were acquired with a confocal microscope (Olympus IX81). Images were acquired with ImageJ software (http://imagej.net/). Images were analyzed with ImageJ software. Data are presented as mean fluorescence intensity relative to EV-EDCs.
Colorimetric, flow cytometric, immunohistochemical, proteomic, and nanoparticle evaluation. EDC proliferation was measured in conditions designed to mimic the myocardium environment of the heart (1% calf serum, 10 U/mL human recombinant EGF (Thermo Scientific; basal media without growth factors) and in the presence of BPAT-AM (B1205, Fisher). Relative cell counts were evaluated using a colorimetric WST-8 assay (Cell Counting Kit-8, Dojindo) with confirmatory manually cell counts and random field analysis for Ki67/DAPI expression (12 visual field per-cell line; ab156902, Abcam). The quantification of expression of miRNA within extracellular vesicles of culture media in 1% calf serum and growth factors conditions, EDCs and the culture medium were collected for analysis. Flow cytometry (Guava easyCyte, EMD Milipore) was used to evaluate cell viability by propidium iodide (PI) and 7-AAD (TSA, ThermoFisher) and using the propidium iodide exclusion method. The percentage of viable cells was calculated. The supernatant was then centrifuged at 10,000 × g for 5 min at 4 °C (5810 R centrifuge; Eppendorf) to remove cell debris. The supernatant was then centrifuged at 100,000 × g for 90 min at 4 °C (Optima XE-90 ultracentrifuge; Beckman Coulter) to remove large vesicles and debris. The supernatant was then centrifuged at 100,000 × g for 4 h at 4 °C to pellet extracellular vesicle.

In vivo protocol. This protocol was reviewed and approved by the University of Ottawa Animal Care Committee. The detailed protocol was registered a priori within the Open Science Framework (https://osf.io/nx2ck/). Male NOD/SCID IL2Rγc (8–9 weeks old; Charles River) were pretreated with buprenorphine and anaesthetized with isoflurane under northermotic temperature control, for surgical LCA ligation. Seven days after LCA ligation, animals were randomized to be treated with echocardiographically guided intramyocardial injection of 100,000 unmodified EDCs (NT-EDCs), 100,000 KCNN4-EDCs, 100,000 EV-EDCs or saline (i.e., vehicle-treated group). All animals underwent echocardiographic imaging 7 and 28 days after LCA prior to sacrifice for histological evaluation of infarct size (Mason-Trichrome).

Statistical analysis. Clamplt 10.4 (Axon Instruments) and GraphPad Prism 6.0 were used for data analysis. Experiments and analyses were performed blinded to treatment allocation. Animals were allocated randomly to experimental groups as follows: A series of sealed envelopes were prepared that contained allocation to each treatment. Envelopes were evenly distributed throughout the surgery days. Four to eight surgeries were performed each day. Lab staff prepared 1–2 treatments of each type (e.g., KCNN4-EDCs, EV-EDCs, or vehicle) per lab day. The animal surgeon opened the envelope and administered the treatment. All data are presented as mean ± SEM. All statistical comparisons were with two-sided tests. Multiple group comparisons were obtained with one-way ANOVA for nonrepeated measures analyses in experiments involving more than two groups and two-way repeated-measures ANOVA for all multi-group analyses involving repeated measured variables. In all cases, normality was confirmed prior to further post hoc testing. If ANOVA showed significant differences, Bonferroni’s multiple comparisons test was used to compare individual means. Differences in categorical measures were compared using Fischer’s exact test. p < 0.05 was considered statistically significant. All raw data supporting the findings from this study are available from the corresponding authors upon reasonable request.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The data supporting the findings from this study are available within the article and its supplementary information. Any remaining raw data will be available from the corresponding authors upon reasonable request. Source data are provided with this paper.

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**Author contributions**

P.V. performed all the in vitro work, designed the study in collaboration with S.N. and D.R.D., coordinated all the work and wrote the first draft of the paper. S.P., P.K., and C.M. performed the in vivo work and associated assays under the direct supervision of D.R.D. S.N. supervised the overall study, handled most of the funding, and worked closely with P.V. and D.R.D. on finalizing the paper.

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**Competing interests**

P.V., S.P., D.R.D., and S.N. are co-inventors for a patent requested regarding the concept of KCNN4-gene transfer to boost stem cell therapeutic response. The intellectual property belongs to the University of Ottawa and Université de Montréal. The remaining authors declare no competing interests.

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