Molecular therapies for cardiac arrhythmias
Boink, G.J.J.

Citation for published version (APA):
Boink, G. J. J. (2013). Molecular therapies for cardiac arrhythmias

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
EFFECT OF SKELETAL MUSCLE NA+ CHANNEL DELIVERED VIA A CELL PLATFORM ON CARDIAC CONDUCTION AND ARRHYTHMIA INDUCTION

Gerard J. J. Boink1,3,4,*, Jia Lu5,*, Helen E. Driessen1; Lian Duan1; Eugene A. Sosunov1; Evgeny P. Anyukhovsky1; Iryna N. Shlapakova1; David H. Lau1; Tove S. Rosen1,2; Peter Danilo1; Zhiheng Jia5; Nazira Ozgen1; Yevgeniy Bobkov1; Yuanjian Guo5; Peter R. Brink5; Yelena Kryukova1; Richard B. Robinson1; Emilia Entcheva6; Ira S. Cohen1; Michael R. Rosen1,2,5

1Department of Pharmacology, Center for Molecular Therapeutics; 2Department of Pediatrics, Columbia University, New York, NY, USA; 3Interuniversity Cardiology Institute of the Netherlands (ICIN), Utrecht, the Netherlands; 4Heart Failure Research Center, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands; 5Department of Physiology and Biophysics, Institute for Molecular Cardiology, Stony Brook University, Stony Brook, NY, USA; 6Department of Biomedical Engineering, Stony Brook University, Stony Brook, NY, USA; *contributed equally as first author

Circulation: Arrhythmia and Electrophysiology 2012; 5:831-40
ABSTRACT

Background: In depolarized myocardial infarct epicardial border zones, the cardiac sodium channel is largely inactivated, contributing to slow conduction and reentry. We have demonstrated that adenoviral delivery of the skeletal muscle Na+ channel (SkM1) to epicardial border zones normalizes conduction and reduces induction of ventricular tachycardia/ventricular fibrillation. We now studied the impact of canine mesenchymal stem cells (cMSCs) in delivering SkM1.

Methods and Results: cMSCs were isolated and transfected with SkM1. Coculture experiments showed cMSC/SkM1, but not cMSC alone, maintained fast conduction at depolarized potentials. We studied 3 groups in the canine 7d infarct: sham, cMSC, and cMSC/SkM1. In vivo epicardial border zones electrograms were broad and fragmented in sham, narrower in cMSCs, and narrow and unfragmented in cMSC/SkM1 (P<0.05). During programmed electrical stimulation of epicardial border zones, QRS duration in cMSC/SkM1 was shorter than in cMSC and sham (P<0.05). Programmed electrical stimulation–induced ventricular tachycardia/ventricular fibrillation was equivalent in all groups (P>0.05).

Conclusion: cMSCs provide efficient delivery of SkM1 current. The interventions performed (cMSCs or cMSC/SkM1) were neither antiarrhythmic nor proarrhythmic. Comparing outcomes with cMSC/SkM1 and viral gene delivery highlights the criticality of the delivery platform to SkM1 antiarrhythmic efficacy.
INTRODUCTION
Reentry causes most life-threatening cardiac arrhythmias in ischemic heart disease.\textsuperscript{1,2} Antiarrhythmic drugs and surgery terminate reentrant arrhythmias by creating bidirectional conduction block, depressing conduction, and/or prolonging refractoriness.\textsuperscript{3} Normalization of conduction in depressed pathways might be an antiarrhythmic alternative to blocking conduction. Yet, the only tools to effect such outcomes have been norepinephrine and acetylcholine, which hyperpolarize cell membranes and whose toxicities render their use here impractical.

Clinical Perspective on Page 277
We recently reported a novel means for speeding/normalizing conduction in settings associated with depolarized membrane potentials, leading to low availability of cardiac Na\textsuperscript{+} channels.\textsuperscript{4,5} The skeletal muscle Na\textsuperscript{+} channel (SkM1, Nav1.4) gene has a 10 mV more depolarized midpoint of inactivation\textsuperscript{4,5} than the cardiac isoform (SCN5A, Nav1.5). Computer simulations indicated that SkM1 but not SCN5A expression preserves conduction velocity in depolarized environments.\textsuperscript{4} When administered via adeno-viral vector into ventricular myocardium, SkM1 increases action potential $V_{\text{max}}$ and conduction velocity and reduces the incidence of ventricular tachycardia (VT)/ventricular fibrillation (VF) initiated by programmed electrical stimulation (PES) in healing infarcts\textsuperscript{4} or occurring spontaneously during ischemia/reperfusion.\textsuperscript{5}

Because viral gene delivery is not innocuous, 1 objective of the present study was to explore an alternative delivery system. We have successfully introduced hyperpolarization-activated cyclic nucleotide-gated channel 2 (HCN2), a pacemaker channel gene, into canine myocardium in vivo using adult human mesenchymal stem cells (MSCs) as a delivery platform. Human MSCs express cardiac connexins (Cx40 and Cx43),\textsuperscript{6} electrically couple with myocytes, and carrying overexpressed HCN2 channels create biological pacemakers in canine ventricle.\textsuperscript{7} These outcomes suggested feasibility of cell-based gene delivery. Therefore, we selected canine MSCs (cMSCs) as a delivery platform in the present study.

Preliminary in vitro experiments\textsuperscript{8} demonstrated superior effects of SkM1 over SCN5A on the maximal action potential upstroke velocity ($V_{\text{max}}$) and conduction in a cell line. We now report the isolation of cMSCs, SkM1 properties in this delivery platform, and their impact on conduction, arrhythmia induction, and action potential $V_{\text{max}}$ in healing canine infarcts.

MATERIALS AND METHODS
Protocols were performed per American Physiological Society recommendations and reviewed and approved by the Columbia and Stony Brook University Institutional Animal Care and Use Committees.
Unless otherwise indicated, chemicals were from Sigma Chemical Co (St. Louis, MO). An expanded version of the Materials and Methods and supporting data is provided in the Data Supplement at the end of this chapter.

**Cell isolation and transfection**

After euthanasia for other purposes, 4-mL canine bone marrow was aspirated from the iliac crest of 1-year old dogs. cMSC isolation was proceeded by the standard techniques, and transfection of the cells with SkM1 and SCN5A constructs was performed via electroporation using nucleofector technology (Amaxa Lonza, Gaithersburg, MD). Transfection efficiency was 30% to 45%.

**In vitro studies**

Whole-cell patch clamp with a signal amplifier (Model Axopatch-1B; Axon Instruments Inc, Inverurie, Scotland, United Kingdom) was used to measure single cell membrane current. Electrode resistances were 3 to 4 MΩ. The liquid junction potential (≈8 mV between bath and electrode solutions) was not corrected because exchange between pipette and cell is never complete.

Neonatal Sprague-Dawley rats were euthanized, and ventricular myocytes were isolated by an approved Stony Brook University Institutional Animal Care and Use Committee protocol as previously described. Isolated ventricular myocytes were replated at 4×10⁵ cells per cm² for the control group and 3.5×10⁵ cells per cm² for the coculture groups at a 20:1 ratio, with cMSCs set onto grooved scaffolds. Cultures were maintained for 4 to 5 days before making functional measurements.

For immunocytochemistry, cMSCs were loaded with quantum dots (Qdot 655; Invitrogen, Carlsbad, CA) before coculture. After 4 days of coculture on plastic cover slips, samples were stained with mouse anti-Cx43 (Invitrogen) and rabbit anti-α-actinin (Sigma) and then stained with Alexa 488 and Alexa 546 conjugated secondary antibodies (Invitrogen).

For functional measurements, scaffolds were washed and equilibrated at room temperature and stained with Fluo-4 AM (Invitrogen). A 2-dimensional optical mapping system was used to measure impulse propagation at room temperature.

**Canine studies**

cMSCs were prepared as above and used in passages 2 to 4. All batches used had consistently high SkM1 sodium current expression in green fluorescent protein–expressing cells. At the time of in vivo experimentation, cells were thawed, and trypan blue exclusion was used to obtain the percentage and total number of viable cells. 1×10⁶ viable cells were suspended in 0.75 mL PBS. The percentage of viable cells was 70% to 90%.

Adult male mongrel dogs (22–25 kg; Chestnut Ridge Kennels, Shippensburg, PA) were anesthetized with thiopental (17 mg/kg IV) and mechanically ventilated. Anesthesia was maintained with isoflurane (1.5%–3.0%). A left thoracotomy was
Figure 1. Skeletal muscle Na⁺ channel (SkM1) and SCN5A expression in canine mesenchymal stem cells (cMSCs). A, SkM1 and SCN5A activation in cMSCs held at −100 mV and then pulsed to test potentials from −80 mV to +40 mV, per Methods. B, Inactivation of SkM1 and SCN5A currents in cMSCs held at potentials from −100 mV to 0 mV, with 5-mV increments. C, Current-voltage relationship of SkM1 (n=8) and SCN5A (n=8) in cMSCs, normalized to maximum peak current. D, Inactivation curve (the h∞ curve) of SkM1 (n=8) and SCN5A (n=8). Data are normalized to the maximum peak current and fit to the Boltzmann equation \( f = \frac{1}{1 + \exp \left(\frac{E_m - V_h}{K}\right)} \), where \( V_h \) is the midpoint membrane potential and \( K \) is the slope factor.
performed by sterile techniques, and coronary artery ligation was performed as previously described. 13 1×10⁶ cMSCs were injected using a 23-gauge needle into 3 sites in the epicardial border zone (EBZ). The injection protocol was similar to that of adenoviral delivery. 4 The chest was closed, and lidocaine (50 µg·kg⁻¹·min⁻¹) was infused during surgery and for 24 to 48 hours postoperatively. Seven days later, dogs were anesthetized, the heart exposed, and ECGs and electrograms (EGs) acquired, digitized, and stored on a personal computer (EMKA Technologies, Falls Church, VA).

EG recordings, induction of VT, microelectrode studies, infarct sizing, and immunohistochemistry were all performed as previously reported  4 and are detailed in the Data Supplement.

**Statistical analysis**

Data are expressed as mean±SEM. For in vitro studies, t tests were used to compare between 2 groups, and Kruskal-Wallis 1-way ANOVA followed by the Dunn multiple comparison test was used to analyze conduction velocity. Arrhythmia incidence in sham and cMSCs or cMSC/SkM1-treated animals was analyzed by Fisher exact test. ECG parameters, EG width recordings, and microelectrode data were analyzed using 1-way ANOVA followed by Bonferroni posttests. During PES at different cycle lengths, QRS duration was analyzed using 2-way ANOVA for repeated measurements. P<0.05 was considered significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the article as written.

**Table 1.** ECG and Local EG Measurements During Sinus Rhythm

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Sham</th>
<th>cMSC</th>
<th>cMSC/SkM1</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECG parameters, ms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycle length</td>
<td>567±37.2</td>
<td>562±30.1</td>
<td>560±33.6</td>
</tr>
<tr>
<td>PR</td>
<td>96±4.4</td>
<td>98±4.3</td>
<td>100±3.5</td>
</tr>
<tr>
<td>QRS</td>
<td>56±1.4</td>
<td>47±2.4 *</td>
<td>44±1.2 *</td>
</tr>
<tr>
<td>QT</td>
<td>220±5.6</td>
<td>207±5.5</td>
<td>210±7.9</td>
</tr>
<tr>
<td>QTc</td>
<td>294±4.8</td>
<td>277±6.5</td>
<td>282±5.7</td>
</tr>
<tr>
<td>EG duration, ms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LV basal (PS)</td>
<td>21±0.8</td>
<td>22±1.3</td>
<td>21±2.1</td>
</tr>
<tr>
<td>LV anterior (EBZ)</td>
<td>32±1.9</td>
<td>26±1.4 *</td>
<td>21±1.6 †</td>
</tr>
<tr>
<td>RV anterior</td>
<td>22±1.1</td>
<td>20±0.4</td>
<td>21±0.8</td>
</tr>
</tbody>
</table>

EG indicates electrogram; cMSC, canine mesenchymal stem cells; SkM1, skeletal muscle Na⁺ channel; QTc, corrected QT; LV, left ventricular; PS, paraseptal site; EBZ, epicardial border zone; RV, right ventricular. Note that only the local EG duration in anterior wall of the LV, the injected EBZ, differs among groups. *: P<0.05 vs sham. †: P<0.05 vs cMSC and sham.
Figure 2. Effect of canine mesenchymal stem cells (cMSCs)/skeletal muscle Na⁺ channel (SkM1) on conduction velocity (CV) in in vitro cardiac syncytium. A and B, According to previously described methods, cMSCs were loaded with quantum dots (QDs; red) before being cocultured with myocytes. Cultures were fixed and stained 4 days after initiation of coculture. Nuclei were counterstained using 4’,6-diamidino-2-phenylindole (DAPI; blue). A, Low-amplification micrographs showing the distribution of QDs loaded with cMSCs (red) in relation to the cultured myocytes stained for connexin 43 (Cx43; green); scale bar, 40 µm. B, High-amplification micrographs showing Cx43 expression (green) at the interface (yellow arrows) between myocytes (stained orange for α-actinin) and cMSCs loaded with QDs (red); scale bar, 10 µm. C, Comparison of CV in myocyte-only (n=33), myocyte-cMSC (n=17), and myocyte-cMSC/SkM1 (n=28) cultures in normal and high K Tyrode (*: P<0.05).
RESULTS

Biophysical comparison of SkM1 and SCN5A in cMSCs

To study voltage-dependent activation of SkM1 and SCN5A currents in green fluorescent protein–positive cMSCs (n=8 per group), cells were held at −100 mV to prevent inactivation and then pulsed to test potentials from −80 to +40 mV, with 5-mV increments (Figure 1A). SkM1 current started activating at −40 mV, was half maximal at −30 mV, and peaked at −20 mV. Comparable SCN5A current measurements were −50 mV, −40 mV, and −25 mV, respectively (Figure 1C). This suggests a 5- to 10-mV shift between activation of SkM1 and SCN5A channels. Reversal potentials were at 21.69±1.87 mV and 21.09±3.66 mV, respectively, both close to the Nernst potential for Na⁺ (+23.31 mV at 22°C). Peak current density and peak conductance density were 38.52±4.74 pA/pF and 0.95±0.14 nS/pF for SkM1, respectively, and 55.09±10.60 pA/pF and 1.27±0.27 nS/pF for SCN5A, respectively. There were no significant differences in peak current density or peak conductance density between groups, suggesting comparable expression levels of both genes in cMSCs.

To characterize steady-state SkM1 and SCN5A inactivation, cMSCs were prepulsed for 500 ms to holding potentials from −100 mV to 0 mV, with 5 mV increments, and then stepped to 0 mV (Figure 1B). Normalized currents were fitted with the Boltzmann equation. SkM1 channel inactivation had a midpoint of −58.6±0.4 mV and a slope factor of 6.0±0.2 mV. SCN5A had a midpoint of −73.9±0.1 mV and a slope factor of 5.9±0.1 mV (Figure 1D). Thus, SCN5A inactivation was ≈15 mV negative to SkM1 (P<0.05). These data confirmed the relatively positive position of SkM1 inactivation voltage dependence, suggesting cMSC/SkM1 may function better to deliver Na⁺ current than cMSC/SCN5A in depolarized cells.

Similar to our human embryonic kidney 239 cell results,8 time constants for recovery from inactivation of SkM1 in cMSCs are smaller than those for SCN5A at all holding potentials (Data Supplement Figure IV), especially at more depolarized potentials, suggesting much faster recovery of SkM1 in cMSCs.

cMSCs and cMSC/SkM1 effects on in vitro impulse propagation

To confirm electrical coupling between cMSCs and cardiac myocytes, we tested SkM1 effects on conduction velocity, with cMSCs as the delivery system in the coculture of myocytes and cMSCs expressing SkM1 on polydimethylsiloxane scaffolds (Figure 2A–2B). A linear platinum electrode was placed at 1 edge of the scaffold to pace at 1 Hz. Macroscopic optical mapping was carried out at room temperature to record propagation in 2 dimensions. Comparison of conduction velocities among myocyte-only, myocyte-cMSC, and myocyte-cMSC/SkM1 cocultures showed significantly higher conduction velocities in SkM1 cocultures in normal and high K⁺ solutions (Figure 2C).

Studies in the canine model

Fourteen dogs were injected with cMSCs, 10 with cMSC/SkM1, and 12 were not injected (sham). One cMSC animal died of VT 2 hours after surgery. Two sham animals
died of arrhythmias, 1 during surgery and 1 after 2 days. All cMSC/SkM1 animals survived. Animals that died in the first 2 days were excluded from further analysis. We performed terminal experiments at 7 days. During sinus rhythm, ECG cycle length, PR, QT, and QTc did not differ among groups (Table 1). However, QRS duration in cMSC and cMSC/SkM1 dogs was shorter than sham (P<0.05). EBZ showed broad, fragmented EGs in sham, narrower EGs in cMSC, and narrow and unfragmented EGs in cMSC/SkM1 dogs (Table 1; Figure 3).

**Effective refractory period, QRS duration, and arrhythmia incidence during PES**

Effective refractory period did not differ among groups (Table 2). During paraseptal site stimulation, QRS duration in cMSC and cMSC/SkM1 dogs was shorter than sham (P<0.05); during EBZ site stimulation, QRS duration was shorter in cMSC/SkM1 than cMSC or sham dogs (P<0.05; Figure 4). Despite the potentially therapeutic actions of cMSC/SkM1 on conduction, sustained VT/VF was induced in 7 of 10 cMSC/SkM1-injected dogs versus 5 of 13 cMSC-injected dogs and 7 of 10 shams (P>0.05).

**Microelectrode studies**

After the in situ protocol, tissue slabs of injected regions were used to study the impact of cMSCs and cMSC/SkM1 on EBZ cellular electrophysiology. Resting membrane potential and action potential duration did not differ among groups (P>0.05; Table 3). However, $V_{\text{max}}$ in cMSC/SkM1-injected preparations was faster than sham and cMSCs (P<0.05; Table 3).

| Table 2. ERP Recorded During PES From the PS and From the EBZ/Injection Region |
|-----------------------------|---------|----------|-------------|
| Measurement                 | Sham    | cMSC     | cMSC-SkM1   |
| ERP (PS), ms                | 168±2.8 | 157±4.4  | 163±5.3     |
| ERP (EBZ), ms               | 167±4.2 | 158±4.8  | 153±4.0     |

ERP indicates effective refractory period; PES, programmed electrical stimulation; PS, paraseptal site; EBZ, epicardial border zone; cMSC, canine mesenchymal stem cells; SkM1, skeletal muscle Na$^+$ channel.

| Table 3. In Vitro Electrophysiological Parameters Recorded From EBZ Tissues |
|-----------------------------|---------|----------|-------------|
| Measurement                 | Sham    | cMSC     | cMSC-SkM1   |
| MDP, -mV                    | 78.2±1.6| 77.9±1.1 | 78.5±1.6    |
| $V_{\text{max}}, V/S$       | 157.8±10.4| 148.0±8.0| 201.0±13.9 †|
| APD30, ms                   | 38.7±7.4 | 43.9±4.8 | 49.6±8.1    |
| APD50, ms                   | 54.9±8.6 | 70.0±7.3 | 74.8±11.5   |
| APD90, ms                   | 87.3±9.1 | 109.9±8.6| 113.2±12.9  |

†: P<0.05 vs. cMSCs and Sham. Sham n=10, cSMC n=13, cMSC/SkM1 n=10.
To provide further insight into the relationship between $V_{\text{max}}$ and membrane potential, we plotted them against one another for all groups (Figure 5). This demonstrated that cMSC/SkM1 preparations have faster $V_{\text{max}}$ over the full membrane potential range.

**Infarct size, Western blotting, and histology**

No differences in infarct size were seen among sham, cMSC, and cMSC/SkM1 does (29±2.4, 28±2.1, and 28±3.3%, respectively; $P>0.05$). Western blotting indicated persistent presence of SkM1 protein in the injection site of cMSC/SkM1-injected animals, whereas the noninjected site in these animals and all tested sites in cMSC or sham-treated animals persistently showed absence of SkM1 protein (Figure 6A). Immunohistochemistry of cMSC/SkM1-injected regions demonstrated anti-Cx43
**Figure 4.** QRS duration during normal and premature stimulation. QRS duration during stimulation from the paraseptal site (PS; left) and epicardial border zone (EBZ)/injection (right) regions. Note shorter QRS duration in canine mesenchymal stem cell (cMSC)/SkM1-injected animals (*: $P<0.05$). Electrical stimulation applied per Methods. Some animals could not be included in this analysis because they fibrillated before completing the protocol (PS: sham, $n=7$; cMSC, $n=12$; cMSC/SkM1, $n=9$; EBZ: sham, $n=5$; cMSC, $n=9$; cMSC/SkM1, $n=8$). SkM1, skeletal muscle Na⁺ channel.

**Figure 5.** Action potentials have higher membrane responsiveness ($V_{max}$ vs MP) curves in cMSC/SkM1-injected preparations vs sham and cMSC (*$P<0.05$). MP indicates membrane potential; cMSCs, canine mesenchymal stem cells; SkM1, skeletal muscle Na⁺ channel.
staining at the cMSC and adjacent myocardium interface (Figure 6B). SkM1/green fluorescent protein–positive cMSCs were not found in sham EBZ (Figure 6B).

DISCUSSION

The present study demonstrates the following: 1) SkM1 biophysical properties in cMSCs are more favorable than SCN5A in restoring fast conduction in depolarized tissue, 2) cMSC/SkM1 but not unloaded cMSCs maintain relatively fast conduction in depolarized tissue, 3) cMSC/SkM1-injected animals show prominent restoration of fast impulse propagation (narrow EGs, narrow prematurely stimulated QRS complexes, and high $V_{\text{max}}$ in excised tissue), 4) despite the potentially therapeutic actions of cellular SkM1 delivery there was no antiarrhythmic effect, contrasting with our previous work using viral delivery, and 5) whereas prior literature suggests MSC may be proarrhythmic, we found that MSC delivery to a healing canine infarct does not increase VT/VF incidence.

Biophysics of speeding conduction using cMSC-SkM1

Early work on circus movement arrhythmias predicted cessation of reentry if conduction accelerated, such that the activation wave front encountered its own refractory tail. Yet, initial pharmacological strategies to speed conduction (eg, neurohormones) were hampered by proarrhythmia and limited success. Novel drugs (rotigaptide and analogs) and gene therapies provided experimental means to speed conduction by enhancing gap junctional function, but their efficacy is still being debated, and there are concerns that maintaining or increasing gap junctional function during acute infarction will increase infarct size.

Cell and gene therapies are being explored as means to prolong effective refractory period and prevent reentry. Cell-based strategies can induce postrepolarization refractoriness, but their antiarrhythmic efficacy is still under investigation. Prolonging repolarization and refractoriness are effective in monomorphic VT, and tachy-pacing induced atrial fibrillation; efficacy against polymorphic VT/VF is still unknown. Despite concerns regarding proarrhythmia accompanying local prolongation of repolarization, no proarrhythmia occurred in proof-of-concept studies.

Ischemic tissue is often depolarized, contributing to reduced Na-channel availability, slow conduction, and reentry. Cardiac Na+ channel inactivation is pivotal here and motivated our gene transfer of SkM1 channels whose inactivation kinetics favor current flow in depolarized tissue. These studies showed efficient restoration of conduction and protection against PES- or ischemia/reperfusion-induced arrhythmias.

Concerns regarding use of viral vectors led us to explore alternatives to viral gene transfer. Investigation of biophysical differences between SkM1 and the native cardiac Na-channel SCN5A in cMSCs showed that the midpoint of Na-channel inactivation shifted positively by 15 mV in SkM1-expressing cMSCs compared with cMSC-expressing SCN5A—an outcome similar to results in human embryonic kidney cells and neonatal rat ventricular myocytes.
Approximately 60% of SkM1 channels are available at −60 mV, contrasting with <10% of SCN5A channels. Other important predictors of Na⁺ channel availability include fast inactivation and recovery from fast inactivation: both were accelerated in SkM1 versus SCN5A. Together, these results suggest that cellular SkM1 delivery should efficiently restore the pool of available Na⁺ channels in a fashion superior to cellular SCN5A delivery and natively available Na⁺ channels.

Efficient coupling of delivery cells (here, cMSCs) to cardiac myocytes via gap junctions is central to ion current delivery. To this end, we previously reported that human MSCs express Cx40 and Cx43, allowing efficient electrical coupling, delivery of overexpressed HCN2 current, and introduction of HCN2-based spontaneous activity in adjacent myocytes.6,30,31 In those studies, HCN2 current and spontaneous activities were blocked by carbonoxalone, highlighting the criticality of gap junctional coupling. To investigate the capability of cMSC/SkM1 to couple to myocytes and speed conduction, we cocultured them with neonatal rat ventricular myocytes. Cx43 was expressed at myocyte/cMSC interfaces, and conduction velocities were increased in normal and depolarized conditions compared with myocyte-only and myocyte/cMSC cell strands (Figure 2). This outcome encouraged our in vivo experiments.

Efficient and specific restoration of fast conduction in canine EBZ

One concern about the cMSC-based approach was the reduced pH of ischemic tissue, which results in closure of Cx43 gap junctions.32,33 This might limit the efficiency of SkM1 current delivery. Despite the potential for suboptimal coupling between myocytes and cMSCs, cMSC-SkM1 efficiently restored fast conduction in EBZ, as evidenced by the following: 1) local EGs in EBZ were broad and fragmented in sham, narrow and less fragmented in cMSC, and narrow and unfragmented in cMSC-SkM1 dogs (Figure 3), 2) QRS duration after application of PES in the EBZ was narrow in cMSC/SkM1-injected dogs, comparable with that of uninfarcted dogs, 3) QRS duration was shorter at normal and short coupling intervals in cMSC/SkM1 dogs than in shams or those receiving cMSCs (Figure 4), and 4) $V_{\text{max}}$ in isolated EBZ tissues of cMSC/SkM1 was significantly faster than in noninjected or cMSC-injected tissues (Figure 5). These results reflect restoration of fast inward Na⁺ current and speeding of conduction in EBZ by cMSC/SkM1, with efficiency comparable with that of viral SkM1.

Also similar to viral SkM1 delivery was the lack of proarrhythmia in hearts receiving cMSC/SkM1. Note as well that QT and corrected QT (Table 1), effective refractory period (Table 2), and action potential duration (Table 3) were similar across groups, further illustrating that introducing SkM1 primarily impacts conduction without affecting repolarization. This suggests the outcomes of SkM1-based interventions arise from an effect on conduction.

Absence of protection against inducible VT/VF

Despite the efficacy of cMSC/SkM1 in restoring fast conduction in EBZ, protection against PES-induced VT/VF was not achieved. Several considerations might explain the absence of an antiarrhythmic effect.
Figure 6. Western blotting and immunohistochemistry of epicardial border zones (EBZs). A, Western blotting of injection site samples from canine mesenchymal stem cell (cMSC)/skeletal muscle Na⁺ channel (SkM1)–treated animals showed a specific positive band at 250 kDa comparable to the signal obtained from virally transduced (using an SkM1 adenovirus [Ad-SkM1]) neonatal rat ventricular myocytes (NRVMs) that were used here as a positive control. This 250-kDa SkM1-specific signal was not obtained in tissue from noninjected EBZ of cMSC/SkM1-treated animals nor was it obtained from sham- (not shown) or cMSC-treated animals. GAPDH was used as a loading control. B, In cMSC/SkM1-injected tissue, coimmunohistochemical experiments showed connexin 43 (Cx43; green) on the interface (yellow arrows) between myocardium and SkM1-positive cells. Green fluorescent protein (GFP) is visualized via direct fluorescence (green) and not optometrically separated from the Cx43 signal. SkM1-positive cells were not detected in sham. Nuclei were counter stained (blue) using 4',6-diamidino-2-phenylindole (DAPI). Scale bar, 25 µm.
First, the extent of speeding of conduction might be insufficient to prevent PES-induced VT/VF. This seems unlikely, as we previously demonstrated significant reduction in the incidence of PES-induced VT/VF with a similar acceleration of conduction based on SkM1 gene transfer. We also showed SkM1 gene therapy speeds longitudinal conduction and prevents ischemia/reperfusion-induced arrhythmias, further supporting the notion that the extent of conduction speeding (similar to viral SkM1) should be antiarrhythmic.

Second, cMSCs could negatively impact conduction by acting as a current sink, thereby slowing conduction and compensating the SkM1 effects. This is unlikely because unloaded cMSCs did not slow conduction. In light of potential current sink effects, it should be noted that we use a small number of cells (≈1 million) because we previously established this dose to generate significant ion channel–based biological function. This dose is much lower than that typically used in studies of cardiac regeneration (≈200 million cells).

Third, prior research has shown that MSCs form low-resistance junctions but not intercalated disks with myocytes. Therefore, although conduction is sped by cMSC/SkM1, the organization needed for an antiarrhythmic effect may not be achieved. In addition, it is possible that absence of intercalated disks in the cMSC-SkM1/myocyte unit supports formation and maintenance of reentrant pathways that otherwise would have remained incomplete.

Safety concerns of MSC-based therapies
When considering gene-modified or unmodified MSC transplantation as therapeutic approaches in cardiac disease, 2 concerns have been extensively discussed: 1) the potential of MSCs to be proarrhythmic, and 2) the risk for neoplasia. The proarrhythmia concern is based on studies illustrating slowing of conduction and reentrant arrhythmias in vitro and effective refractory period shortening in vivo after MSC transplantation. However, other in vivo studies report absent or protective effects with regard to ventricular arrhythmias. Our study supplements these findings by showing that transplanting a low dose of allogeneic MSCs into the EBZ is safe and that it improves the conduction properties of the myocardium (Table 1; Figure 4).

Concern for potential tumorogenesis resulting from MSC transplantation has primarily arisen because even early passage MSCs can manifest chromosomal aberrations. A murine model of MSC transplantation confirmed this concern. Although a large body of literature suggests that the use of human MSCs is safe, recent reports of tumor formation in rodents clearly warrant extensive safety analysis of MSC-based therapies.

Study Limitations
In the present study, we asked whether cellular delivery of SkM1 protects against inducible arrhythmias 7 days after myocardial infarction. We worked with a fixed end point 1) because previous research indicated this provides a stable substrate for induction of reentrant arrhythmias, and 2) to allow direct comparison between
cellular delivery of SkM1 and previously reported viral delivery. Furthermore, we tested only 1 dose of cells, which had been highly effective in delivering electrical signals locally. Other experiments had indicated that 7 days is sufficient for MSCs to form gap junctions with myocytes and deliver ionic currents. We cannot exclude that a protocol using higher doses or later time points might have had a different outcome. Yet the outcome with the cellular approach, highly efficient normalization of conduction, suggests that sufficient dose and time were available for cellular delivery of SkM1. The lack of specific antiarrhythmic effects of the cMSC/SkM1 intervention suggests that the cell delivery approach was complicated by the mechanistic problems discussed above, rather than resulting from insufficient dose or time.

CONCLUSIONS

Using in vitro and in vivo approaches, we have shown that cMSCs provide an efficient platform to control ion channel function in the vicinity of myocardial infarcts. We also have shown that the effectiveness of SkM1-based antiarrhythmic therapy critically depends on the delivery vehicle, with viral gene delivery seeming to be superior. Further attempts to modify conduction in infarcted tissue may, therefore, be better focused on viral delivery of Na⁺ channels, while cells might be reserved for myocardial repair.

SOURCES OF FUNDING

This work was supported by the National Heart, Lung and Blood Institute, HL094410, and New York State Department of Health, Stem Cell Contract CO24344. Gerard Boink received support from the Netherlands Heart Foundation, the Netherlands Foundation for Cardiovascular Excellence, the Dr Saal van Zwanenberg foundation, and the Interuniversity Cardiology Institute of the Netherlands.

REFERENCES

7. Plotnikov AN, Shlapakova I, Szabolcs MJ, et al. Xenografted adult human mesenchymal stem cells provide a


CLINICAL PERSPECTIVE

In patients with a reduced ejection fraction, the annual risk for sudden cardiac death in the healing phase after myocardial infarction is estimated to be as high as 10%. Ischemic heart disease is typically complicated by areas of membrane depolarization, which inactivate cardiac sodium channels (encoded by SCN5A) and generate areas of slow conduction predisposing to reentrant arrhythmias. At present, available therapies for these arrhythmias are both limited and frequently ineffective. As a potential novel gene therapy, we recently reported overexpression of the skeletal muscle Na⁺ channel (SkM1). SkM1 channels are relatively resistant to inactivation by membrane depolarization and when overexpressed in the infarct epicardial border zone they locally restored the speed of conduction and significantly reduced the incidence of induced ventricular tachycardia/fibrillation. As an alternative to the viral approach, the present study investigated the delivery of SkM1 channels via mesenchymal stem cells. We found that mesenchymal stem cells couple to myocardium and can be safely administered to the depolarized epicardial border zone. We also found that SkM1-loaded mesenchymal stem cells efficiently deliver sodium current and restore the speed of impulse propagation. Yet despite these potential therapeutic outcomes and in contrast to the viral approach, cellular delivery of SkM1 seemed not to be antiarrhythmic, thus indicating the criticality of the delivery platform in obtaining the antiarrhythmic effect. No proarrhythmia occurred. Future SkM1-based antiarrhythmic approaches should, therefore, focus on viral delivery.
SUPPLEMENTARY MATERIAL – CHAPTER 12

EXPANDED MATERIALS AND METHODS

Plasmid Construction
The rat SkM1 insert was released from pl-2-SkM1, kindly provided by Dr. Gail Mandel (SUNY, Stony Brook, NY) and subcloned into a mammalian expression vector pIRES2-EGFP (BD Bioscience Clontech, Mountain View, CA) at the EcoR I site. Human SCN5A-pcDNA3.1 was a kind gift from Dr. Robert Kass (Columbia University, New York, NY). The insert was excised using Hind III and Xba I restriction enzymes, blunt ended, and subcloned into the Sma I site of pIRES2-EGFP.

Cell Isolation and Culture
Canine mesenchymal stem cells were isolated by Ficoll-Paque Plus density gradient centrifugation from aspirated bone marrow (Figure I). Primary cultures of cMSC were maintained at 37°C in 5% CO₂/95% air with an initial medium for 48h. Medium was then changed every 3-4d. Cell colonies with spindle-like morphology were transferred 7d after initial plating. After confluence cells were harvested with 0.25% trypsin-EDTA, and replated. Isolated cells were characterized at passages 2-4 by flow cytometric analysis of specific surface antigens with fluorescein isothiocyanate- (FITC) conjugated rat anti-canine CD44, FITC-conjugated rat anti- canine CD45 unconjugated rat anti-canine CD90, and phycoerythrin-(PE) conjugated mouse anti-canine CD34. A large majority of the cells were CD44 (99.61%) and CD90 (93%) positive – and 98% were CD34 and CD45 negative; suggesting a significant majority were MSCs (Figure II).

To further validate the MSC properties of the isolated cells we subjected subsets of the cells to osteogenic, adipogenic and chondrogenic differentiation protocols. For adipogenic and osteogenic differentiation, the cells were plated in 6-well or 12-well plates. Adipogenic and osteogenic induction was initiated using designated kits from Lonza. For adipogenesis, three to five cycles of the following media changes were performed: 2-3 days of exposure to adipogenic induction medium followed by 2-3 days of exposure to maintenance medium. Osteogenic induction was carried out by feeding the cells with osteogenic induction medium every 3-4 days for 2-3 weeks. Chondrogenic induction was performed by pelleting 2.5×10⁵ cells in chondrogenic induction medium containing TGF-β3. Complete media changes were performed every 2-3 days for 3-4 weeks. At the end of the induction protocols, the cells were rinsed with PBS and fixed with 10% formalin. Adipogenesis was assayed using Oil Red O staining. Osteogenesis was assayed by staining for calcium deposition using Alizarin Red staining. Chondrogenic pellets were embedded in cryogenic cutting medium, sectioned for histology, and glycosaminoglycans were stained using Safranin O. Figure III illustrates the osteogenic, adipogenic and chondrogenic potential of the isolated cells.
Transfection
cMSCs were transfected with pIREs2-EGFP-SkM1 or -SCN5A construct by electroporation using Nucleofector technology (Amava Lonza, Gaithersburg, MD) as directed and were incubated at normal culture conditions. Expression of EGFP and Na⁺ currents was examined 24-48h after transfection. 30-45% of the cells were GFP positive. Na⁺ currents were measured in GFP-positive cells perfused with 15 mM Na⁺ Tyrode’s solution. cMSC/SkM1 and cMSC/SCN5A cell capacitances were 57.63±6.74 pF and 56.38±6.93 pF, respectively (n=8/group).

Patch clamp studies
Whole cell patch clamp with a signal amplifier (Model Axopatch-1B, Axon Instruments Inc.) was used to measure single cell membrane current. Voltage and current signals were digitized (Model DIGIDATA 1320A, Axon Instruments) and transferred to a personal computer. Data acquisition and analysis were performed using CLAMPAC 9.2 and CLAMMFIT 9.2 software (Axon instruments), respectively. Normal Tyrode’s solution contained (mM): NaCl 137.7, KCl 5.4, NaOH 2.3, CaCl₂ 1.8, MgCl₂ 1, Glucose 10, and HEPES 10 (pH adjusted to 7.4 with NaOH). Low Na⁺ Tyrode’s solution contained (mM): NaCl 15, TEACl 122.7, KCl 5.4, NaOH 2.3, CaCl₂ 1.8, MgCl₂ 1, Glucose 10, and HEPES 10 (pH adjusted to 7.4 with NaOH). Electrodes with resistances=3-4 MΩ were made from capillaries with a P-87 Flaming/Brown micropipette puller (Sutter Instrument Company, Novato, CA) and filled with (mM): KCl 50, K-aspartic acid 80, MgCl₂ 1, EGTA 10, HEPES 10 and Na₂-ATP (pH adjusted to 7.2 with KOH). The liquid junction potential (~8 mV between bath and electrode solutions) was not corrected because exchange between pipette and cell are never complete.¹

Generation of a cardiac Syncytium
Neonatal Sprague-Dawley rats were sacrificed and ventricular myocytes were isolated by an approved Stony Brook University IACUC protocol as previously described.² Ventricles were excised and washed free of blood, tissue cut into small pieces and enzymatically digested with trypsin at 4°C (1mg/ml, USB, Cleveland, OH), and, the next morning, with collagenase at 37°C (1mg/ml, Worthington, Lakewood, NJ). Cardiac fibroblasts were removed by 90 min preplating. Isolated ventricular myocytes were re-plated at 4×10⁵ cells/cm² for the control group and 3.5×10⁵ cells/cm² for the coculture groups at a 20:1 ratio with cMSC onto grooved fibronectin-coated polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, Midland, MI) scaffolds in M199 medium (GIBCO Invitrogen) supplemented with 10% fetal bovine serum (GIBCO Invitrogen) for 2d and then reduced to 2%. Cultures were maintained in an incubator at 37°C with 5% CO₂ for 4-5d before functional measurements.

Microscopic Dynamic Functional Measurements and Analysis
All scaffolds were washed and equilibrated at room temperature in normal Tyrode’s solution. Samples were then stained with Fluo-4 AM (Invitrogen, Carlsbad, CA) for 20 min for tracking Ca²⁺ waves. After washing with Tyrode’s, scaffolds were removed
Figure I. Canine mesenchymal stem cell (cMSC) isolated by Ficoll-Paque Plus density gradients centrifugation. A, Cells isolated from the density interface of 1.073g/ml attached and grew as symmetric spindle-like morphology cell colonies in about 7-10 days after initial planting. B-C, Cells obtained from densities higher than 1.073g/ml showed different cell morphology and did not develop into colonies over 7-10 days.

Figure II. Flow cytometric identification of Ficoll-Paque Plus density gradient isolated cMSC. A Typical flow cytometry histograms indicating nearly all cells are negative for CD34 (2.01%; upper panel) and CD45 (2.18%; lower panel). B, Typical flow cytometry histograms indicating a majority of cells are positive for CD90 (93.45%; upper panel) and nearly all cells are positive for CD44 (99.61%; lower panel).
Figure III. Canine MSCs differentiation. A-B, Osteogenic differentiation. B, Red-brown staining indicates Ca$^{2+}$ deposition based on alkaline phosphatase - suggesting osteogenic differentiation in induced cultures. A, Non-induced cultures did not show Ca$^{2+}$ depositions. C-D, Adipogenic differentiation. D, Positive Oil red-O staining indicates adipogenic differentiation in induced cells. C, Non induced cells did not show positive staining for Oil red-O. E-F, Chondrogenic differentiation. F, Cell pellets of induced cells showed positive safranin O staining – indicative for presence of proteoglycans and therefor suggesting cartilage formation. E, Pellets of non-induced cells do not show safranin O positivity. Cytoplasm stains green and nuclei stain black.
Figure IV. Recovery of SkM1 current (A) and SCN5A current (B) in cMSCs. Cells were held over a range of holding potentials and double-pulsed to 0 mV. Each pulse had a 10 msec duration, with an increasing time interval between the two pulses. Data were normalized to the current amplitude of the first pulse (n=8 for each holding potential) and curve fit the equation $f=1- \exp (-t/\tau)$. C, Comparison of recovery time constant $\tau$ between SkM1 and SCN5A at different holding potentials.
from the surface so that only the cells in the grooves remained, forming 5 linear cultures. A 2-D optical mapping system\textsuperscript{3} as in the companion paper (Chapter 11)\textsuperscript{4} was used to measure impulse propagation. To vary the degree of membrane depolarization, we used 5.4 and 10.4 mM K\textsuperscript{+} Tyrode's solutions.

**Induction of VT/VF**

Pacing threshold was determined by incrementally increasing the current until capture. Ventricular pacing was performed at 2X pacing threshold. Extrastimulus pacing with a programmable stimulator (Bloom Associates, Reading, Pa) was performed sequentially in the high paraseptal region (PS), infarct lateral EBZ/injection region and within the infarct. Pacing trains began with 10 stimuli at a cycle length of 350-400 ms. S2 was initiated at 250 ms, and S1-S2 was decreased in 10-ms steps until loss of capture. For S3 pacing, S1-S2 was set at the shortest interval with reliable S2 capture (equivalent to the effective refractory period; ERP). S3 was initiated at a coupling interval of 100 ms and increased in 10-ms steps until S3 capture occurred. If VT was \( \geq 60 \) seconds or when the protocol was finished, the heart was removed and prepared for microelectrode study, histology, and infarct sizing.

**Microelectrode Methods**

Hearts were removed and immersed in Tyrode's solution\textsuperscript{5} equilibrated with 95% O\textsubscript{2}/5% CO\textsubscript{2}. Epicardial strips (\( \approx 10 \times 5 \times 0.5-1 \) mm) were filleted parallel to the left ventricular free wall surface from non-injected EBZ sites and EBZ sites injected with blank cMSC or cMSC/SkM1. Preparations were pinned epicardial surface up to the bottom of a 4-mL tissue bath and superfused (36°C, pH 7.35±0.05) at 12 mL/min. Superfusate [K\textsuperscript{+}] was varied from 4-7 mmol/L to permit \( V_{\text{max}} \) measurement at various membrane potentials. Preparations were paced at a cycle length of 500 ms, and AP recorded at 30–50 sites/preparation after 3h equilibration to reach steady state.\textsuperscript{5}

**Infarct Sizing**

After tissues were removed for microelectrode study and histology, the heart was cooled to 4°C and cut into 1-cm-thick transverse slices from apex to base. Slices were incubated x20 min in 1% tetrazolium red (pH, 7.4 buffer at 37°C), immersed in 10% formalin x15 min, and pressed between 2 glass plates to obtain uniform 1-cm thickness. Apical sides of slices were photographed, and a digital image planimetered (Image J Analysis 1.40g, National Institutes of Health, Bethesda) to determine overall infarct size. Volume of infarcted myocardium was calculated by multiplying planimetered areas by slice thickness and expressed as % total left ventricular volume.

**Histology and Immunohistochemistry**

Tissue blocks were snap-frozen in liquid nitrogen; 5 \( \mu \)m serial sections were cut with a cryostat (Microm HM505E) and air-dried. Sections were washed in PBS, blocked x20 min with 10% goat serum, and incubated overnight at 4°C with anti-SkM1 antibody (1:200, Sigma-Aldrich, St Louis, Mo) alone or together with Cx43 antibody.
(1:500, Invitrogen, Breda, The Netherlands). Antibody bound to target antigen was detected by incubating sections x2h with goat anti-mouse IgG labeled with Cy3 (red fluorescence for SkM1) and goat anti-rabbit IgG labeled with Alexa 488 (green fluorescence for Cx43), together with detection of GFP with a Nikon E800 fluorescence microscope.

**Western Blotting**

Tissue samples were sonicated in lysate buffer, which contains 1X PBS, 1% Triton X-100, 0.5% NaDoc, 0.1% Tween-20 and protease inhibitor tablet (Roche), for 45 second and incubated on ice for 30 min. After centrifugation at 3000 rpm for 10 minutes to remove connective tissue and unbroken cells, the supernatant were used as the whole cell lysate. Samples were separated on a 4-20% tris-glycine gradient gel (invitrogen) and transferred to PVDF membrane (Biorad). After blocking with 5% milk for an hour at room temperature membranes were incubated with anti-Nav1.4 mouse monoclonal antibody (Sigma-Aldrich, 1:500) or anti-GAPDH rabbit polyclonal antibody (Fitzgerald Industries International, 1:5000) in 5% milk overnight at 4°C. After washing, membranes were incubated with secondary antibody for an hour at room temperature (GE Healthcare), followed by enhanced chemiluminescence processing (Amersham Pharmacia Biotech). In Figure 6B, the sample neonatal rat ventricular myocytes (NRVMs) transduced with a SkM1 overexpressing adenovirus (Ad-SkM1) was used as a positive control – loaded to the gel with 1 µg/lane (as compared to 75 µg/lane for the other samples) – and is not included for quantitative comparison. The results for the cMSC/SkM1-treated animal is typical for a total of 4 animals tested.

**REFERENCES**