Cellular Biology

Spiral Waves and Reentry Dynamics in an In Vitro Model of the Healed Infarct Border Zone

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Rationale: Reentry underlies most ventricular tachycardias (VTs) seen postmyocardial infarction (MI). Mapping studies reveal that the majority of VTs late post-MI arise from the infarct border zone (IBZ).

Objective: To investigate reentry dynamics and the role of individual ion channels on reentry in in vitro models of the “healed” IBZ.

Methods and Results: We designed in vitro models of the healed IBZ by coculturing skeletal myotubes with neonatal rat ventricular myocytes and performed optical mapping at high temporal and spatial resolution. In culture, neonatal rat ventricular myocytes mature to form striated myocytes and electrically uncoupled skeletal myotubes simulate fibrosis seen in the healed IBZ. High resolution mapping revealed that skeletal myotubes produced localized slowing of conduction velocity (CV), increased dispersion of CV and directional-dependence of activation delay without affecting myocyte excitability. Reentry was easily induced by rapid pacing in cocultures; treatment with lidocaine, a Na⁺ channel blocker, significantly decreased reentry rate and CV, increased reentry path length and terminated 30% of reentrant arrhythmias (n=18). In contrast, nitrendipine, an L-type Ca²⁺ channel blocker terminated 100% of reentry episodes while increasing reentry cycle length and path length and decreasing reentry CV (n=16). K⁺ channel blockers increased reentry action potential duration but infrequently terminated reentry (n=12).

Conclusions: Cocultures reproduce several architectural and electrophysiological features of the healed IBZ. Reentry termination by L-type Ca²⁺ channel, but not Na⁺ channel, blockers suggests a greater Ca²⁺-dependence of propagation. These results may help explain the low efficacy of pure Na⁺ channel blockers in preventing and terminating clinical VTs late after MI. (Circ Res. 2009;105:1062-1071.)

Key Words: arrhythmia ■ Ca²⁺ channels ■ cardiac electrophysiology ■ electrophysiology ■ mapping ■ Na⁺ current ■ optical mapping

In the United States, myocardial infarction (MI) affects more than 7.1 million people, with 865,000 new cases each year.¹ Late after an MI, surviving myocyte bundles in the infarct, and the infarct border zone (IBZ) have been implicated in the genesis of post-MI reentrant ventricular tachyarrhythmias² and sudden cardiac death. Currently, the treatment options for post-MI arrhythmias include defibrillator implantation combined with antiarrhythmic drug therapy and/or ablation. Unfortunately, effective drug therapy for post-MI ventricular arrhythmias has been discouraging because they are proarrhythmic,³ lack efficacy,⁴-⁷ and cause toxicity.⁸,⁹ Understanding the role of ion channels on arrhythmia dynamics is crucial to developing tailored, new drug and gene-based therapies for ventricular tachy-arrhythmias. Currently, technical obstacles preclude 3D mapping in whole heart studies, making 2D and theoretical models attractive options for studying the biophysics of reentrant arrhythmias.

Mapping studies have revealed that the majority of VTs originate from the IBZ, a substrate that is characterized by nonuniform anisotropic architecture resulting from fibrosis that separates myocyte bundles and gap junction remodeling of surviving myocytes.¹⁰ Despite extensive ultrastructural and electrophysiological characterization of the “healed” IBZ, very little information exists regarding reentry dynamics and the role of individual ion channels on reentry. In this study, we adopted a reductionist, tissue engineering approach and created a new 2D in vitro model of the healed IBZ. We used optical mapping to characterize the substrate and study the...
effects of Na\(^+\), Ca\(^{2+}\), and K\(^+\) channel blockers on reentrant arrhythmia dynamics. We found that this 2D, coculture model resembled the healed IBZ in several architectural and electrophysiological respects. An increased contribution of the L-type Ca\(^{2+}\) current to impulse propagation was observed in cocultures (consisting of electrically uncoupled myotubes mixed with electrically coupled myocytes\(^{11}\)) but not in myocyte-only controls. Low doses of nifedipine (5 \(\mu\)mol/L), an L-type Ca\(^{2+}\) channel (LTCC) blocker, terminated 100% of reentrant arrhythmias, but high doses of lidocaine (200 \(\mu\)mol/L) only terminated 30% of reentry episodes in cocultures.\(^{12}\) These results may help explain the low efficacy of pure Na\(^+\) channel blockers in terminating and preventing ventricular tachycardias that occur late after MI.

### Methods

We investigated impulse propagation and arrhythmias by performing optical mapping of novel in vitro models of the healed epicardial\(^{10}\) and lateral\(^{14,15}\) IBZs generated by coculturing human skeletal myotubes (SkMs) with neonatal rat ventricular myocytes (NRVMs). We used SkMs to simulate fibrosis seen in the healed IBZ because they: (1) lack gap junctions unlike myofibroblasts that express connexin Cx43\(^{14,15}\); (2) assume a linear morphology that resembles fibrosis unlike myofibroblasts that express connexin Cx45\(^{14,15}\); and (3) orient neighboring myocytes into bundles, resulting in a nonuniform anisotropic architecture.\(^{10,17}\) A cardinal feature of the healed IBZ.

The healed “epicardial” IBZ was simulated by plating a mixture of 20% SkMs with 80% NRVMs on 21-mm fibronectin-coated plastic coverslips (Figure 1B and 1C), whereas a model of the healed “lateral” IBZ was created by micropatterning a sector (\(\theta=120^\circ\), 115 mm\(^2\); Figure 5A) composed of a coculture of (20% to 30%) SkMs and (70% to 80%) NRVMs adjacent to an NRVM-only region on fibronectin-coated polydimethylsiloxane-treated glass coverslips. Controls for this study consisted of NRVM-only monolayers. Optical mapping (i.e., micromapping (20-\(\mu\)m spatial and 125-\(\mu\)s temporal resolution) and macromapping (1 mm spatial and 1-ms temporal resolution) were performed after 9 to 11 days in culture. Reentry was induced by rapid pacing; Na\(^+\), Ca\(^{2+}\), or K\(^+\) channel blockers were added to stable reentry (5 minutes after reentry initiation), and reentry dynamics were analyzed using custom software written in MATLAB. An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

### Results

#### Architectural Characterization

We studied NRVM-only controls (n=50) and cocultures (n=50). Figure 1A shows a representative control, NRVM-only monolayer and Figure 1B and 1C shows a representative transmitted light and corresponding fluorescent microscopy image of a coculture where myotubes are labeled with green fluorescent protein. In culture, NRVMs mature to form striated myocytes (Figure 1D), whereas myoblasts mature to form electrically uncoupled myotubes (100 \(\mu\)m to 2 mm in length) that resemble ingrowths of fibrous tissue in the healed IBZ (Online Figure I).\(^{10,17}\) Furthermore, the myotubes orient bundles of myocytes in several directions (Figure 1B and 1C and Online Figure II), resulting in a nonuniform anisotropic structure, which has been observed in the IBZ because of disarray of the usually parallel-oriented fiber bundles by fibrosis and lateralization of gap junctions.\(^{10,17}\) Figure 1E shows Cx43 immunostaining of a coculture that reveals lateralization of gap junctions in myocytes and lack of gap junctions in myotubes (arrows), both of which are normal features for these cell types. Additionally, impulse conduction in the epicardial border zone is often confined to a 2D plane.\(^{18}\)

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** A, Transmitted light image of an NRVM-only control monolayer. B and C, Transmitted light and corresponding overlaid fluorescent microscopy image of a coculture where myotubes are labeled with green fluorescent protein. The myotubes are heterogeneously distributed; their orientation promotes neighboring NRVM alignment in several directions. D, Fluorescent image of a day 10 NRVM-only monolayer stained for \(\alpha\)-actinin (white) reveal striations resembling those observed in adult myocytes. E, Immunostaining for Cx43 (white) and nuclear labeling with Hoechst (blue) in a coculture. Arrows point to areas of myotubes that have Hoechst-positive nuclei but lack Cx43.
Electrophysiological Characterization

Electrophysiological studies in transplanted and intact human hearts as well as experimental models of healed MIs reveal that the healed IBZ is characterized by decreased conduction velocity (CV), increased dispersion of CV, and easily inducible, stable reentrant arrhythmias, all of which are reproduced in this in vitro coculture model.

Key electrophysiological features of the healed IBZ that are reproduced in this model are (1) reduction in CV at a macroscopic scale (Figure 2A) but normal myocyte excitability at a microscopic scale (Figure 2D, 2F, and 2G) but negligible directional differences in impulse propagation at a macroscopic scale (Figure 2H); (2) susceptibility to functional block (Figure 2I) and reentrant arrhythmias (Figure 2J and 2K) following rapid pacing. Remarkably, these features were observed in all cocultures that were mapped (n=50 monolayers) but not in controls (Figure 2E).

Optical mapping revealed that CV was decreased by 77% (Figure 2A; 5.7 ± 1.1 versus 24.4 ± 4.9 m/sec, P<0.001) and action potential duration (APD) was prolonged by 38% (Figure 2L; 143 ± 29 versus 197 ± 39 ms, P<0.04) in cocultures compared to NRVM-only controls. This decrease in CV was primarily attributable to the underlying architecture and not decreased excitability as demonstrated by similar upstroke velocities measured by micromapping (dF/dt max = 0.33 ± 0.02 versus 0.34 ± 0.02 ms⁻¹, P=0.33, n=7 each, controls and cocultures, respectively; Figure 2B and 2C) and similar resting membrane potential (~77 mV in cocultures versus ~74 mV in controls) by patch clamp in cocultures and NRVM-only controls.
Myotubes oriented the NRVMs into bundles that resulted in rapid conduction when stimulated along the long axis of the bundles (Online Figure III, A) but slower conduction when stimulated in the transverse direction (Online Figure III, B). Additionally, presence of electrically uncoupled myotubes interposed between myocytes produced slowing of CV but not complete block because of overlap between myotubes and myocytes. Figure 2D shows areas of widely spaced isochrones interspersed with significant slowing, evidenced by crowding of isochrones in a region containing myotubes (labeled with green fluorescent protein), as well as circumvention of wave fronts around myotubes. In contrast, isochrones are uniformly spaced in all areas of control (NRVM-only) monolayers as seen in Figure 2E and Online Figure IV, which show propagation along the transverse and longitudinal axis, respectively. Another interesting feature that has been reported in the healed IBZ is directional differences in activation delay, resulting from variable amounts and architecture of fibrosis that predispose to unidirectional block and reentry. Figure 2F illustrates this point: here, stimulation from the right resulted in a 20-ms activation delay, whereas stimulation from the bottom resulted in an increase in the activation delay to 80 ms attributable to large numbers of myotubes between the stimulation and recording sites. Paradoxically, propagation in cocultures was uniform on a macroscopic scale following point stimulation at the center of the monolayer (Figure 2H), suggesting that, as in the case of the healed IBZ, directional differences in propagation induced by myocyte bundles oriented in several directions on a “microscopic” scale canceled out.

Functional block and reentry can be readily induced in hearts with healed infarcts and in this in vitro model. In fact, reentrant arrhythmias could be initiated in all cocultures by rapid pacing, and 90% of the reentrant arrhythmias sustained for >5 minutes, making them amenable to pharmacological manipulation. Reentry initiation is illustrated in Figure 2I and 2J; here, uniform impulse propagation was observed at 3-Hz pacing (Figure 2H), but localized conduction block was observed at 4-Hz pacing (Figure 2I), culminating in sustained reentry (Figure 2J); pseudo-ECGs of reentry resembled monomorphic VT (Figure 2K).

Effects of Sodium and Calcium Channel Blockade on Spiral Wave Dynamics
Na⁺ channel blockers like lidocaine and agents like amiodarone that block Na⁺, Ca²⁺, and K⁺ channels are commonly used for the treatment and/or prevention of sustained ventricular tachycardia in patients with prior MI. We were interested in dissecting out the role of individual ion channels on spiral wave dynamics, so we used specific Na⁺, Ca²⁺, and K⁺ channel blockers, rather than amiodarone. In normal myocardium, lidocaine, exhibits use dependent Na⁺ channel blockade, slows CV, and flattens CV restitution without affecting APD restitution, whereas nitrendipine, which produces use-dependent LTCC blockade, should flatten APD restitution but not significantly affect CV restitution.

In this study, reentry cycle length (Figure 3A) was significantly increased by 79±38% (263±25 versus 446±29 ms, \( P<0.001 \), n=18 monolayers) and 30±14% (242±25 versus 309±35 ms, \( P=0.03 \), n=16 monolayers) after perfusion with lidocaine (200 μmol/L) and nitrendipine (5 μmol/L), respectively. This increase in reentry cycle length was attributable to a decrease in reentry CV (−28±23%, 6.2±0.4 versus 4.4±0.5 ms/sec, \( P<0.005 \), n=11; and −13±11%, 8.0±0.7 versus 6.8±0.6 ms/sec, n=8, \( P=0.01 \); Figure 3B) and an increase in spiral core size, reflected by increase in the spiral tip path length by 207±160% (16.9±2.7 versus 42.6±5.0 mm/cycle, \( P<0.001 \), n=13) and 71±42% (16.5±3.9 versus 27.4±5.5 mm/cycle, \( P<0.001 \), n=10) for lidocaine and nitrendipine, respectively (Figure 3C and Online Figure V). Additionally, lidocaine increased reentry APD₅₀ by 28±8% (82±2 versus 106±4 ms, \( P=0.02 \), n=15), whereas nitrendipine, produced a small decrease in APD₅₀ (2%±3%, 79±2 versus 77±2 ms, \( P=0.02 \), n=12; Figure 3D). The underlying mechanism of the observed changes in reentry APD is probably APD restitution, where APD prolongs in response to an increase in cycle length. A modest prolongation of APD would be expected under lidocaine superfusion but not significantly increase the APD restitution relationship and (2) a significant decrease in reentry rate. On the other hand, the small decrease in reentry APD with nitrendipine can be explained by a decrease in Ca²⁺ influx during the plateau phase of the APD attributable to LTCC blockade that is opposed (to a lesser extent) by the increase in reentry cycle length that tends to prolong APD.

An unexpected finding was that reentry terminated after nitrendipine superfusion for 4 to 5 minutes, but only 30% of reentry episodes were terminated by lidocaine despite super-
fusion for >10 minutes. Nitrendipine (Figure 4A), but not lidocaine (Figure 4B) induced wave breaks before reentry termination. These results were confirmed by power spectrum analysis, which revealed an additional peak following nitrendipine (Figure 4D and 4E), suggesting wave break, whereas a single dominant frequency was seen during lidocaine superfusion (Figure 4C). Also, optical recordings revealed 2:1 block (resembling graded response) at the recording site corresponding to the wave break (Figure 4F), whereas recording sites distant to the wave break demonstrated 1:1 capture (Figure 4G).

**Potassium Channel Blockade Does Not Terminate Reentry**

Potassium channel blockers like sotalol are often used to prevent ventricular tachyarrhythmias in conjunction with ICD therapy. We evaluated d-sotalol (300 μmol/L), an IKr blocker, and tetraethylammonium (TEA) (20 mmol/L), a nonspecific K⁺ channel blocker. These agents prolonged reentry APD (Online Figure VI, A; P<0.005) by 7.6±1.9% during d-sotalol and 3.8±0.8% during TEA superfusion but did not affect reentry CL or induce wave breaks (Online Figure VI, B); d-sotalol did not terminate reentry (n=6), whereas TEA terminated 1/6 reentry, an effect probably stemming from insufficient reentry APD prolongation.

**Calcium-Dependent Propagation**

Next, we investigated impulse propagation and arrhythmias in the sector model that simulates the lateral IBZ (n=7 monolayers; Figure 5A). The coculture region demonstrated slow propagation (CV was decreased by 54±7% [P<0.01]) relative to the control region during normal tyrode perfusion, and conduction block developed in the coculture region within 5 minutes of nitrendipine superfusion (Figure 5B and 5C).

**Spiral Waves With Transient Concave Wavefront**

A novel finding observed in these patterned cultures was the induction of stable, spiral waves with transient concave wave fronts, following rapid pacing. This is illustrated in Figure 6B and the Online Movie, where the spiral wave propagates in the counterclockwise direction. Figure 6A (1 through 5) shows the voltage maps following the 5-point stimuli delivered just before induction of these spiral waves.
In this study, we designed an in vitro model that reproduces several architectural and electrophysiological features of the healed IBZ (Table). We demonstrated that this model exhibits readily inducible reentrant arrhythmias that are consistently terminated by nitrendipine, an LTCC blocker, but not high doses of lidocaine, a Na\(^+\) channel blocker or K\(^+\) channel blockers, suggesting increased contribution of the L-type Ca\(^{2+}\) current to impulse propagation attributable to decreased cell-cell coupling. These results may help explain the low efficacy of Na\(^+\) channel blockers like lidocaine in terminating sustained ventricular tachycardia in patients with old myocardial infarcts; significantly, these clinical arrhythmias are often terminated by intravenous amiodarone, which when administered acutely, produces use-dependent Na\(^+\) and Ca\(^{2+}\) channel blockade, without an appreciable effect on cardiac repolarization.\(^{30}\)

**Effects of LTCC Blockade in Cocultures**

Impulse propagation in the heart is dependent on active membrane properties determined by the ion channel compo-

![Figure 5. Sector (lateral) IBZ model exhibits Ca\(^{2+}\)-dependent propagation. A, Schematic of the sector model. B and C, Tableau and voltage maps, respectively, during 2-Hz stimulation (from the right) during normal tyrode (left) and nitrendipine (right) superfusion. During normal tyrode superfusion, all recording channels exhibit action potentials and voltage maps reveal slow conduction in the coculture region. Nitrendipine superfusion produces conduction block of a planar wavefront at the interface of the coculture and NRVM-only region.](image)

![Figure 6. Spiral waves with transient concave wavefront in the sector model were initiated by 3-Hz pacing. A (1 through 5), Voltage maps following the first through fifth delivered point stimuli, respectively, before induction of spiral waves with transient concave wavefront. A (1), Propagation through the entire monolayer. A (2 and 3), Conduction block at 7 o’clock. A (4), Conduction block at the 7 and 11 o’clock with excitation extinguishing in the center. A (5). Conduction block at 7 o’clock, whereas the upper arm at 11 o’clock continued to propagate, facilitating the initiation of a spiral waves with transient concave wavefront. B, Spiral wave with transient concave wavefront rotating in the counterclockwise direction.](image)
Reentry Initiation With Rapid Pacing

Clinical and experimental data support reentry as the most common cause of ventricular arrhythmias in healed infarcts. Rentry can be classified into anatomic or functional; mitral isthmus-dependent VT after inferior MI is an example of anatomic reentry, here, the impulse circulates around an anatomic obstacle, the scar.

Functional reentry on the other hand, can be induced even in homogeneous tissues, take the form of spirals and has also been observed in ex vivo whole heart preparations. Functional block is essential for reentry initiation; a short wavelength (product of CV and refractory period) favors maintenance of both anatomic and functional reentry by decreasing the size of tissue needed to sustain reentry. Decreased cell–cell coupling combined with nonuniform anisotropic architecture significantly decreases CV, thereby decreasing wavelength and thus increasing the likelihood that a reentry circuit or spiral wave would be contained within the IBZ of healed infarcts or a 21 mm coverslip. Rapid pacing or premature stimuli would amplify dispersions in CV and wavelengths.

### Table. Architectural and Electrophysiological Similarities Between SkM-NRVM Cocultures and the Healed IBZ

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<th>Characteristics of the Healed Infarct</th>
<th>SkM-NRVM Co-culture IBZs</th>
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<td>Border Zone</td>
<td>Inexcitable and electrically uncoupled</td>
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<td>SkMs represent 10–20% of the 2D monolayer (Figure 1B, 1C, and 1E and Online Figure II)</td>
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<td>Resting membrane potentials (coculture vs controls: −77 vs −74 mV; n=1 control and 2 cocultures) and upstroke velocities by micromapping were similar in cocultures and controls (cocultures vs controls: 0.33 ± 0.02 vs 0.34 ± 0.02 ms−1; P=0.33, n=7; Figure 2B and 2C)</td>
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<td>Decreased conduction velocity (CV) and dispersion of CV at a microscopic scale (12, 52)</td>
<td>Significant reduction of macroscopic CV (Figure 2A) in cocultures and marked dispersion of CV on a microscopic scale (Figure 2D) when compared with control NRVM-only monolayers (Figure 2E)</td>
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<td>Prolongation of APD in cells from the border-zone4,10</td>
<td>APD in cocultures are prolonged compared with controls (Figure 2L; 143 ± 29 vs 197 ± 39 ms, P&lt;0.04)</td>
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<td>Negligible directional differences in impulse propagation at a microscopic scale14,18</td>
<td>Negligible directional differences in impulse propagation at a macroscopic scale (Figure 2G and 2H) despite significant dispersion of CV on a microscopic scale in cocultures</td>
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<td>Highly susceptible to reentrant arrhythmias and functional block60–24</td>
<td>Functional block at high pacing rates culminating in stable reentrant arrhythmias (Figure 2I and 2J); the in vitro equivalent of monomorphic VT (Figure 2K) was seen only in cocultures</td>
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<tr>
<td>Direction-dependent propagation delay on a microscopic scale37,57</td>
<td>Micromapping revealed direction dependent propagation delay (Figure 2F) in cocultures but not controls</td>
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activation times, predisposing to functional block and reentrant arrhythmias. Additionally, heterogeneities would also promote anchoring of spiral waves and an ECG pattern of monomorphic VT (Figure 2K). Our experiments reveal that functional block produces wave breaks that precede reentry initiation (Figure 2I and 2J). We hypothesize that electrically uncoupled myotubes or fibrous tissue ingrowths combined with nonuniform anisotropic architecture predispose to areas of source-load mismatch that block at high pacing rates or following premature beats, resulting in reentry initiation. Slow CV caused by decreased cell–cell coupling also increases the safety factor of propagation, which would promote sustained reentrant arrhythmias.

Mechanisms Underlying Reentry Termination
Reentry termination can occur because of detachment of the wave from an obstacle, increase in reentry APD that promotes head–tail collision, or interruption of the reentry circuit. The limited length of optical recordings (2 to 4 seconds) did not allow us to capture the termination of reentry. We hypothesize that the mechanism of reentry termination with nitrendipine, an LTCC blocker was decreased safety factor for propagation and conduction block in multiple areas of the monolayer; these conduction blocks would decrease the size of excitatory medium available for reentry and consequently terminate reentry. This hypothesis was bolstered by our observation of conduction block in large areas of the monolayer after the termination of reentry. Similar results are illustrated in Figure 5C which demonstrates conduction block only in the coculture region of the sector following nitrendipine superfusion.

We speculate that LTCC-mediated conduction block in reentry circuits may help explain clinical studies where Ca2+ channel blockers have been observed to reduce mortality in patients without significant left ventricular dysfunction, conditions where the negative inotropic effects of Ca2+ blockers may not be detrimental. Furthermore, our findings may also explain our clinical experience where patients with sustained VT and old infarcts often respond to intravenous amiodarone (which mainly inhibits the depolarization phase of action potentials by use-dependent Ca2+ and Na+ channel blockade) but not lidocaine a relatively pure Na+ channel blocker. In the 3D heart, where an increased number of shunt pathways are available for impulse propagation, pure Ca2+ channel blockers may not be effective, and a combination of LTCC and Na+ channel blockade may be necessary to terminate or prevent reentrant arrhythmias late after MI.

Mechanism Underlying Spiral Waves With Transient Concave Wavefront
We have demonstrated for the first time the presence of spiral waves with transient concave wavefront in biological tissue. This phenomenon has been observed in experimental and computational studies of chemical reactions as well as in the FitzHugh-Nagumo computational model by imposing inhomoogeneous excitability with specific geometric features.

Limitations
The present study has several limitations. NRVMs used in the study are electrophysiologically different from adult cardiac myocytes. Nevertheless, previous studies using patch clamp have demonstrated that the biophysical properties of LTCCs in neonatal and adult rat ventricular myocytes are similar, although Na+ channel blockade by lidocaine was more pronounced in NRVMs when compared to adult myocytes. Hypertrophy and ion channel remodeling that occurs in the IBZ is not completely replicated in this in vitro model; despite this drawback, we were still able to reproduce important structural and electrophysiological features of the healed IBZ. Photobleaching of di-4-ANEPPS limited our ability to obtain long-term voltage recordings, resulting in our inability to capture termination of reentry during nitrendipine treatment; hence, we had to rely on the last recording (2 minutes) before reentry termination for spiral wave analysis. Consequently, the actual changes in specific reentry parameters under maximum Ca2+ channel blockade could be larger than those presented in this study. The macromapping system used in this study is a contact fluorescence imaging system that precludes assessment of architecture concurrently with optical mapping. Lastly, we minimized but were unable to fully eliminate fibroblasts in our cultures by 2 preplating steps. Although fibroblasts may affect the electrophysiological properties of cultures, we assume that they are present to a similar extent in cocultures and controls and hence would not affect the validity of our results.

Conclusion
Our results indicate that a mixture of SkMs and NRVMs forms a substrate that reproduces several architectural and electrophysiological features of the healed IBZ. All reentrant arrhythmias in this model were terminated by LTCC blockade, whereas only 30% were terminated by Na+ channel blockade. This study highlights the differential effects of Na+ and Ca++ channel blockers in an in vitro model of the healed IBZ and may help explain the low efficacy of lidocaine in the treatment of incessant VT episodes in patients with healed infarcts. Furthermore, we have shown for the first time the presence of spiral waves with transient concave wavefront in cardiac tissue, suggesting that this novel arrhythmia phenotype may exist in the in vivo setting.

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Disclosures
None.

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Spiral Waves and Reentry Dynamics in an In Vitro Model of the Healed Infarct Border Zone

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Supplemental Methods

All protocols used conform to standard set forth by the National Institutes of Health in the *Guide for the Care and Use of Animals* (NIH publication No. 85-23, Revised 1996).

**Human Skeletal Myoblast Cell Culture** As previously described\(^1,2\), human skeletal myoblasts were obtained from Cambrex (Walkersville, Md), and grown at 37°C and 5% CO\(_2\) in myoblast basal grown medium (SkBM, Clonetics) containing 10% fetal bovine serum, recombinant human epidermal factor (10 ng/mL), dexamethasone (3 µg/mL), L-glutamine, gentamicin, and amphotericin–B. In order to minimize the fusing of skeletal myoblasts to form myotubes (SkM), myoblasts were plated at 3500 cells/cm\(^2\) and maintained at cell densities of 60 to 70% and amplified up to 6 passages in culture. For co-cultures used for micro-mapping and microscopy experiments, the cells were transduced with a 3\(^{rd}\) generation lentivirus expressing GFP (LV-GFP)\(^1\) at a multiplicity of infection of 20 during their second passage to enable cell tracking.

**Cardiac Myocytes Isolation.** Neonatal rat ventricular myocytes (NRVMs) were dissociated from ventricles of 2-day-old neonatal Sprague-Dawley rats (Harlan, Indianapolis, Ind), using trypsin (US Biochemicals) and collagenase (Worthington) and re-suspended in M199 culture medium (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), as previously described\(^1\). The cells were then counted and plated on 21 mm plastic coverslips to generate control NRVM-only monolayers or mixed with SkMs to form co-cultures.

**Co-culture model.** We created 2 co-culture models: 1) mixtures of SkMs and NRVMs to generate a random dispersion of SkMs among myocytes to resemble the *healed epicardial* infarct border-zone and 2) using patterning techniques, a sector (approx \(\theta=120^\circ\), 115mm\(^2\)) consisting of SkM-NRVM mixtures surrounded by NRVMs (on one side and the edge of the coverslip on the other side) to resemble the *healed lateral* border-zone that abuts scar on one side and normal myocardium on the other (Fig 5A).

SkMs were mixed thoroughly with NRVMs in M199 culture medium containing 10% serum, in a proportion of 20% and then plated on 21-mm plastic cover slips coated with human fibronectin (25 µg /mL) or plated as sectors on PDMS-coated glass coverslips. For the patterning
experiments, polydimethyl siloxane (PDMS) “stamps” were created with dimensions corresponding to the myocyte region, coated with fibronectin and engaged to the surface of the coverslips. Subsequently, SkMs were plated, stamps were removed and NRVMs were added. SkMs were transduced with Lv-GFP to track them during micro-mapping and microscopy experiments. Following 24 hours of plating, the cultures were washed twice with PBS to remove any dead cells. On day 2, the serum was reduced to 2% to minimize fibroblast and SkM proliferation. By day 5 of culture, monolayers were confluent and myocytes showed striations; optical mapping was performed after 9-14 days in culture to allow maturation of Ca\(^{2+}\) handling in myocytes. Transmitted light and fluorescent microscopy images were obtained using an Eclipse TE-2000 inverted fluorescence microscope (Nikon, Melville, NY) with a cooled CCD camera attached (Micro Max, Roper Scientific) using WinView32 acquisition software (Roper Scientific). GFP was imaged with 465 to 495 nm fluorescence excitation and 515 to 555 nm emission.

**Immunostaining.** Cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature and then permeabilized with 0.075% saponin. α-Actinin was detected using a monoclonal Anti-α-actinin (sarcomeric) antibody produced in mouse (Sigma, St. Louis, MO) at a dilution of 1:200 and an Alexa Fluor 594-conjugated secondary antibody (Molecular Probes, Carlsbad, CA) at a dilution of 1:333. The nuclei were labeled with bisbenzimide (Hoechst) at a concentration of 1:500. Images were recorded using an Eclipse TE2000-U (Nikon, Melville, NY) inverted fluorescence microscope with a cooled CCD camera attachment and WinView32 acquisition software (Roper Scientific). α-Actinin was imaged with 528 to 553 nm excitation and 578 to 633 nm emission.

**Optical Mapping**

1. **Macromapping**

Transmembrane potentials were recorded using a contact fluorescence imaging system\(^3\) with 253 recording sites arranged in a 17-mm diameter hexagonal array\(^4\) to investigate (1) impulse propagation at a macroscopic scale (spatial resolution of 1mm); and (2) reentry dynamics; the temporal resolution of this system is 1 ms.
Monolayers were visually inspected under the microscope, and non-beating or non-confluent monolayers were rejected. The coverslips were placed in a custom-designed chamber, stained with 5 µmol/L di-4-ANEPPS (Molecular Probes) for 5 minutes, and then continuously superfused with warm (36.5°C) oxygenated Tyrode solution containing (in mM) 135 NaCl, 5.4 KCl, 1.8 CaCl\(_2\), 1 MgCl\(_2\), 0.33 NaH\(_2\)PO\(_4\), 5 HEPES, and 5 glucose. Unipolar point stimulation was applied using a platinum electrode placed directly above the monolayer with 2 platinum line electrodes at the sides of the chamber acting as the return. An LED array with an interference filter (530 +/- 25 nm) was used to excite the voltage sensitive dye that emitted light at an intensity proportional to the transmembrane potential. This light was filtered through an emission filter (long pass cutoff of 590nm) that consisted of a No.1 circular glass coverslip, spin-coated with 3 layers of red photoresist (Brewer Science, Rolla, MO), residing at the bottom of the chamber upon which the monolayer rested. The hexagonal mapping array was positioned directly below the filter; optical signals were low-pass filtered at 500 Hz and amplified with 8 custom-designed 32-channel printed circuit boards. Signals were sampled at 1 kHz and digitized with 4, 64 channel 16 bit analog-to-digital boards (Sheldon Instruments). The optical recordings were normalized to compensate for non-uniformity in dye staining. Data were stored, displayed, and analyzed using software written in Visual C++ (Microsoft) and LabVIEW (National Instruments).

2. **Micromapping**

Micromapping experiments were performed to investigate the effects of architecture on propagation and excitability at a single cell scale. The spatial resolution of this system is 20µm and the temporal resolution is 125µsecs. The micromapping setup used in this study has been previously described in detail\(^5\). The fluorescence signal was focused onto a bundle of 57 fibers arranged in 5 rows (consisting of 11, 12, 11, 12, 11 fibers, respectively). A 60X oil immersion objective lens was used, giving each recording site a diameter of 20µm in the specimen plane, with a total mapping region spanning ~220µm x 90 µm. The optical signals were acquired at a sampling rate of 8 kHz per channel, and the time delays of all of the channels were corrected to be within 20 µs of each other. The data acquisition program was written in LabView. A CCD camera (Model 4910, Cohu Inc., San Diego, CA, USA) mounted on the camera port of the microscope was used to record bright-field and fluorescent images of the co-cultures.
Experimental Protocol

Macromapping

An initial one-second recording was made to check for spontaneous activity. For all experiments, trains of monophasic pulses at 1.5X diastolic threshold and 10ms duration were used. The only exception was for calculation of restitution after lidocaine superfusion, where pulses at 3-4 X the diastolic threshold (pre-drug) had to be used due to Na⁺ channel blockade and consequent decreased excitability. Recordings of impulse propagation at 2Hz were acquired for both controls and co-cultures to analyze for differences in action potential duration (APD) and CV, and to visualize any macroscopic directional differences in CV.

To examine the effect of ion channel blockade on spiral waves, rapid pacing was employed to initiate reentry: stimulation was begun at 1Hz, and increased by 1Hz increments until reentry initiation. Once reentry was sustained for >5 minutes, a recording long enough to obtain 6 cycles of reentry was obtained before superfusing with 200µM lidocaine, 5µM nitrendipine, 300µM d-sotalol, or tetraethylammonium (TEA (20mM). We used modest doses of nitrendipine in order to prevent concomitant Na⁺ channel blockade that has been reported with di-hydropyridines; patch clamp studies reveal that 10µM nitrendipine blocked 86+/-8% of I(CaL) in control myocytes⁶, but also blocked Na⁺ current⁷. We used a very high dose (200µM) of lidocaine in order to produce as complete a block of Na⁺ channels as possible. Katsube et al. demonstrated that lidocaine (30µM) produced a 23.2 +/- 7% reduction in Na⁺ current amplitude and 22.5 +/- 5.6% use-dependent block in neonatal rat ventricular myocytes (using patch clamp⁸).

Recordings were taken two minutes after superfusion of drug, and every minute thereafter, until reentry termination or until reentry had sustained for >8 minutes. A high dose of sotalol (300µM), an IKr blocker⁹, was used because previous data showing a significant decrease in channel open probability (-50+/-6%) and increase in $\tau_{\text{closed}}$ (3.37+/-0.6ms to 7.74+/-0.7ms) at a moderate dose of 100µM in NRVMs¹⁰. A high dose of TEA (20mM) was used based on previous reports of substantial action potential prolongation in NRVMs¹¹.

Data Analysis: All data was analyzed in MATLAB (The MathWorks Inc., Natick, MA, USA) using custom-written scripts. The baseline drift due to photobleaching of the potentiometric dye
was reduced by subtracting a third order polynomial best fit line of the optical signals. To reduce the noise of the optical signals, a seventh-order median filter was applied to the detrended data.

**Micromapping**

The experimental conditions used during micromapping experiments were the same as those described above for macromapping experiments. Cells were stimulated using a rectangular pulse (10ms duration) at 1.5X diastolic threshold, from several sites 2mm away from the central recording channel to assess local dispersion in CV within the field of view and directional differences in activation delay. To reduce phototoxic cell damage generated by the excitation light, two exposures, each 300ms in duration were obtained per recording site. Field stimulation was applied at 1.5X diastolic threshold in order to obtain the action potential upstroke velocity. Bright field and fluorescent images to localize the GFP-labeled myotubes were obtained after stimulation in order to minimize phototoxicity.

**Macromapping**

Animations of electrical propagation were generated from signals that filtered using a 100 Hz low pass filter. The activation time was defined as the instant of maximum positive slope. The relative activation times at each recording point of the hexagonal array were used to calculate CV. To compare CVs among different episodes in the same monolayer, conduction velocity was calculated along the same path and averaged over different stimulus responses. Paths were chosen to be sufficiently far away from the stimulus site so that latency delays associated with excitation could be neglected.

Phase mapping analysis was performed to study the organizing center of the spiral waves. Phase plane plots (Supplemental Fig 8B) were generated using time-embedded analysis by plotting the fluorescent signal (Supplemental Fig 8A) against the same fluorescent signal with a time delay of 10ms. The phase (\( \phi \)) is computed by using the mean value of the phase plane plot (as indicated by the red dot in the phase plot) as the origin. The phase and voltage maps are shown in Supplemental Fig 8C Phase singularities are easily identified as sites where all phases converge and were used to track the spiral tip trajectories (Supplemental Fig 8D) In addition to tracking
the spiral wave tip, phase singularities were used to indicate sites of wave breaks as indicated by the white arrow in Fig 4A.

Reentry CV and wavelength 3mm away from the tip were computed using a method adapted from that outlined in Kay and Gray\textsuperscript{12}. Statistical analysis of the effects of channel blockers was performed using a paired t-test.

*Power spectrum analysis*

Power spectrum analysis was performed by taking the square modulus of the discrete-time fourier transform of data obtained from individual channel traces. The mean value across a channel trace was subtracted in order to reduce a zero-frequency baseline artifact, and then a 4N-point DFT was taken of N-point channel traces to obtain more distinct peaks.

*Tip pathlength calculations*

Reentry wavetips were manually tracked as previously described. The tip pathlength was determined as the total distance the wavetip moved over each frame (at 5 ms intervals) used for tip tracking.

*pEKG*

Pseudo EKGs were computed using the concept of the lead field. The potential difference was determined between two virtual electrodes positioned 5mm above diametrically opposite points on the monolayer, and located parallel to the horizontal edge of the recording hexagonal area\textsuperscript{13}.

*Micromapping*

Dispersion in local CV was assessed by comparing the variance of the local CV values computed using the activation times within the field of view. Upstroke velocities were compared using the maximal derivative of the normalized recordings. Directional delays in activation times were assessed by comparing differences in activation times of the FOV relative to the stimulation time.
Statistics

Data are means ± SE and were analyzed using a paired Student's t-test. P values <0.05 were considered to be significant.

References


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Supplemental Figure Legends

**Supplemental Figure 1.** *In vivo* healed infarct border zone. Low (left) and high (right) power image of Masson’s trichome stained sections showing the infarct border zone and the infarct area of 12 week old male SCID mice that had undergone permanent ligation of LAD and injection of PBS. Red represents viable tissue; and blue represents interstitial fibrosis that has similar structural characteristics to the thin and elongated GFP-labeled myotubes in the co-culture model.

**Supplemental Figure 2.** Panels A and B shows a transmitted light and corresponding fluorescent microscopy image (overlaid on transmitted light image) of a co-culture, respectively, where myotubes are labeled with GFP (green) and myocytes with a membrane dye, Di-I (red) after 6 days in culture. The myotubes are heterogeneously distributed; their orientation promotes neighboring NRVM alignment in several directions.

**Supplemental Figure 3.** Isochrones (0.3ms apart) reveal significantly faster CV when bundles of myocytes in co-cultures are stimulated along the longitudinal axis (Panel A) compared to transverse axis (Panel B).

**Supplemental Figure 4.** Propagation along the longitudinal axis in control (myocyte-only) culture. Widely spaced isochrones (0.2ms apart) reveals rapid conduction (~40cm/s).

**Supplemental Figure 5.** Spiral tip trajectories before and after lidocaine (Panel A) and nitrendipine (Panel B) superfusion reveal that Na$^+$ and Ca$^{2+}$ channel blockade increases size of the spiral core.
**Supplemental Figure 6.** Panel A shows $7.6 \pm 1.9\%$ APD prolongation ($p<0.005$) during d-Sotalol and $3.8 \pm 0.83\%$ APD prolongation during TEA superfusion. Panel B shows the isochrone maps and power spectrum prior to and after superfusion of $K^+$ channel blockers d-sotalol (left) and TEA (right). All power spectrums depict a single dominant frequency indicating the absence of spiral wave breakup.

**Supplemental Figure 7.** Micromapping reveals conduction slowing in some regions containing myotubes in co-cultures.

**Supplemental Figure 8.** Phase mapping analysis was performed to study the organizing center of the spiral waves. Phase plane plots (Panel B) were generated using time-embedded analysis by plotting the fluorescent signal (Panel A) against the same fluorescent signal with an embedded time delay of 10ms. The phase ($\phi$) is computed by using the mean value of the phase plane plot as indicated by the red dot in the phase plot as the reference point. The phase and voltage maps are shown in Panel C. Phase singularities are easily identified as sites where all phases converge and were used to track the spiral tip trajectories (Panel D). In addition to tracking the spiral wave tip, phase singularities were used to indicate regions of wave breaks (white arrow in Fig 4A).

**Supplemental Movie.** Spiral wave with transient concave wavefront observed in sector model that simulates the lateral IBZ.
Supplemental Figures

Figure 1
Figure 2

A

B
Figure 3
Figure 4
Figure 5

A

Pre-Lidocaine

Post-Lidocaine

B

Pre-Nitrendipine

Post-Nitrendipine
Figure 6

A

Reentry APD

% Change

- d-Sotalol
- TEA

B

Isochrone Maps

Pre-Sotalol  Post-Sotalol  Pre-TEA  Post-TEA

Power Spectrums
Figure 7
Figure 8

A. Fluorescent Signal

B. Phase Plane Plot

C. Voltage Map
   Phase Map

D. Tip Trajectory
   2x enlargement