Experimental and Computational Insight into Human Mesenchymal Stem Cell Paracrine Signaling and Heterocellular Coupling Effects on Cardiac Contractility and Arrhythmogenicity

Joshua Mayourian\textsuperscript{1}, Timothy J. Cashman\textsuperscript{1}, Delaine K. Ceholski\textsuperscript{1}, Bryce V. Johnson\textsuperscript{2}, David Sachs\textsuperscript{1}, Deepak A. Kaji\textsuperscript{3}, Susmita Sahoo\textsuperscript{1}, Joshua M. Hare\textsuperscript{4}, Roger J. Hajjar\textsuperscript{1}, Eric A. Sobie\textsuperscript{5}, Kevin D. Costa\textsuperscript{1}

\textsuperscript{1}Cardiovascular Research Center, Icahn School of Medicine at Mount Sinai, New York, NY, USA; \textsuperscript{2}Department of Medicine, University of Washington Seattle, Seattle, WA, USA; \textsuperscript{3}Department of Developmental and Regenerative Biology, Icahn School of Medicine at Mount Sinai, New York, NY, USA; \textsuperscript{4}The Interdisciplinary Stem Cell Institute, University of Miami Miller School of Medicine, Miami, FL, USA, and; \textsuperscript{5}Department of Pharmacology and Systems Therapeutics, Icahn School of Medicine at Mount Sinai, New York, NY, USA.

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Address correspondence to:
Dr. Kevin Costa
Cardiovascular Research Center
Icahn School of Medicine at Mount Sinai
1428 Madison Avenue, Room 3-39
New York, NY 10029
Tel: 212-241-7122
kevin.costa@mssm.edu

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ABSTRACT

Rationale: Myocardial delivery of human mesenchymal stem cells (hMSCs) is an emerging therapy for treating the failing heart. However, the relative effects of hMSC-mediated heterocellular coupling (HC) and paracrine signaling (PS) on human cardiac contractility and arrhythmogenicity remain unresolved.

Objective: To better understand hMSC PS and HC effects on human cardiac contractility and arrhythmogenicity by integrating experimental and computational approaches.

Methods and Results: Extending our previous hMSC-cardiomyocyte HC computational model, we incorporated experimentally calibrated hMSC PS effects on cardiomyocyte L-type calcium channel/SERCA activity and cardiac tissue fibrosis. Excitation-contraction simulations of hMSC PS-only and combined HC+PS effects on human cardiomyocytes were representative of human engineered cardiac tissue (hECT) contractile function measurements under matched experimental treatments. Model simulations and hECTs both demonstrated hMSC-mediated effects were most pronounced under PS-only conditions, where developed force increased approximately 4-fold compared to non-hMSC-supplemented controls during physiologic 1-Hz pacing. Simulations predicted contractility of isolated healthy and ischemic adult human cardiomyocytes would be minimally sensitive to hMSC HC, driven primarily by PS. Dominance of hMSC PS was also revealed in simulations of fibrotic cardiac tissue, where hMSC PS protected from potential pro-arrhythmic effects of HC at various levels of engraftment. Finally, to study the nature of the hMSC paracrine effects on contractility, proteomic analysis of hECT/hMSC conditioned media predicted activation of PI3K/Akt signaling, a recognized target of both soluble and exosomal fractions of the hMSC secretome. Treating hECTs with exosomes-enriched, but not exosomes-depleted, fractions of the hMSC secretome recapitulated the effects observed with hMSC conditioned media on hECT developed force and expression of calcium handling genes (e.g., SERCA2a, L-type calcium channel).

Conclusions: Collectively, this integrated experimental and computational study helps unravel relative hMSC PS and HC effects on human cardiac contractility and arrhythmogenicity, and provides novel insight into the role of exosomes in hMSC paracrine-mediated effects on contractility.

Keywords: Electrophysiology, contractility, excitation-contraction coupling, cell therapy, computational biology, exosomes.
Nonstandard Abbreviations and Acronyms:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>APD&lt;sub&gt;90&lt;/sub&gt;</td>
<td>Action potential duration to 90% repolarization</td>
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<tr>
<td>BAX</td>
<td>BCL2-associated X protein</td>
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<tr>
<td>BCL2</td>
<td>B-cell lymphoma 2</td>
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<td>[Ca&lt;sup&gt;2+&lt;/sup&gt;]&lt;sub&gt;peak&lt;/sub&gt;</td>
<td>Diastolic-subtracted calcium transient amplitude</td>
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<td>Casp3</td>
<td>Caspase-3</td>
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<td>Casp9</td>
<td>Caspase-9</td>
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<tr>
<td>CdM</td>
<td>Conditioned media</td>
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<tr>
<td>CSA</td>
<td>Cross-sectional area</td>
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<tr>
<td>cTriT</td>
<td>Cardiac troponin-T</td>
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<tr>
<td>DF</td>
<td>Developed force</td>
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<tr>
<td>+dF/dt</td>
<td>Maximum rate of contraction</td>
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<tr>
<td>-dF/dt</td>
<td>Maximum rate of relaxation</td>
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<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half maximum effective concentration</td>
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<tr>
<td>HC</td>
<td>Heterocellular coupling</td>
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<tr>
<td>hECT</td>
<td>Human engineered cardiac tissue</td>
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<tr>
<td>hiPSC-CM</td>
<td>Human induced pluripotent stem cell-derived cardiomyocyte</td>
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<td>hMSC</td>
<td>Human mesenchymal stem cell</td>
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<td>k</td>
<td>Hill coefficient</td>
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<tr>
<td>LTCC</td>
<td>L-type calcium channel</td>
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<tr>
<td>MHC</td>
<td>Myosin heavy chain</td>
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<td>PC</td>
<td>Principal Component</td>
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<tr>
<td>PS</td>
<td>Paracrine signaling</td>
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<tr>
<td>SERCA</td>
<td>Sarco/endoplasmic reticulum calcium-ATPase</td>
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<td>SFDM</td>
<td>Serum-free defined media</td>
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<tr>
<td>VW</td>
<td>Vulnerable window</td>
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<td>∆G&lt;sub&gt;Lc&lt;/sub&gt;</td>
<td>Maximum saturated effect of hMSC PS on LTCC</td>
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<tr>
<td>∆V&lt;sub&gt;max_up&lt;/sub&gt;</td>
<td>Maximum saturated effect of hMSC PS on SERCA</td>
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<td>τ&lt;sub&gt;Ca&lt;/sub&gt;</td>
<td>Calcium transient duration at 50% decay</td>
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INTRODUCTION

Cardiovascular disease remains a leading cause of morbidity and mortality worldwide. Substantial evidence from in vitro, pre-clinical, and clinical studies supports bone marrow-derived human mesenchymal stem cells (hMSCs) as a promising cardio-reparative approach. However, in several clinical trials, stem cell delivery benefits have been modest and transient, representing an opportunity for improvement. Therefore, to optimize future hMSC-based therapies, it is essential to better understand the underlying hMSC-cardiac interactome.

It has been theorized that hMSCs reduce cardiac fibrosis and enhance angiogenesis largely through paracrine signaling (PS) mechanisms. Moreover, recent studies have demonstrated that hMSC PS can modulate cardiomyocyte ion channel/pump activity. For example, DeSantiago et al. observed that hMSC-conditioned media increased L-type calcium channel (LTCC) current and sarco/endoplasmic reticulum calcium-ATPase (SERCA) activity in mouse ventricular myocytes, yielding an increase in the calcium transient amplitude and an acceleration of the calcium transient decay. Askar et al. later observed that hMSC paracrine conditioned media prolonged action potential duration of neonatal rat cardiomyocytes in a dose-dependent manner.
On the other hand, hMSCs have been shown to form gap junctions with cardiomyocytes, leading to direct heterocellular coupling (HC) and spontaneous fusion that can influence cardiomyocyte electrophysiology. Indeed, our recent computational modeling study demonstrated that if one considers HC mechanisms alone (i.e., neglecting PS), hMSCs would impact cardiac electrophysiology via shortened action potential and decreased conduction velocity, which could potentially increase vulnerability to re-entry.

Taken together, hMSC HC and PS mechanisms each have the potential to modulate single-cell cardiomyocyte action potential and calcium handling waveforms, as well as tissue-level conduction, constituting well-established determinants of excitation-contraction coupling and arrhythmogenicity in myocardium. However, the relative contributions of hMSC-mediated HC and PS mechanisms on these components of human cardiac function have yet to be established, reflecting challenges with isolating these effects in experimental preparations.

In this study, we utilize both mathematical modeling and three-dimensional human engineered cardiac tissues (hECTs) to better understand hMSC PS and HC effects on human cardiac contractility and arrhythmogenicity. First, we build on our previously established hMSC-cardiomyocyte HC computational model to also experimentally calibrate hMSC PS effects on single-cell cardiomyocyte ion channel/pump activity and tissue-level fibrosis. Next, we validate this mathematical model using hECTs under matched experimental hMSC-mediated HC and PS treatments. Finally, we perform proteomic analysis of hECT/hMSC conditioned media and subsequent functional testing/molecular characterization of hECTs treated with exosomes-depleted and exosomes-enriched fractions of the hMSC secretome to provide insight into key hMSC paracrine factors and their potential mechanisms of action.

METHODS
An expanded Methods section describing all computational and experimental procedures and protocols is available in the Online Data Supplement.

RESULTS
Multi-species parameter sensitivity analysis reveals key hMSC PS-modulators of cardiomyocyte action potential and calcium handling metrics.

To build on our previous hMSC-cardiomyocyte HC model by incorporating select hMSC PS effects on single-cell cardiomyocyte ion channel/pump activity, we first performed unbiased hierarchical clustering on a multi-species parameter sensitivity analysis of empirically-determined hMSC PS modulators of action potential and calcium handling behavior—namely the LTCC, SERCA, and transient outward current (coefficients of determination shown in Online Table I).

From this sensitivity analysis and subsequent hierarchical clustering, action potential metrics were most sensitive to LTCC activity, while calcium handling metrics were highly sensitive to SERCA activity (Online Figure I). No metric was most influenced by transient outward current perturbation, the least experimentally studied effect; hMSC PS effects on transient outward current were therefore neglected in subsequent models.
Experimental calibration of the hMSC PS model.

hMSC PS has been reported to affect single-cell ion channel/pump activity,\textsuperscript{19,20} as well as to favorably remodel fibrotic cardiac tissue.\textsuperscript{21,27-33} The pro-arrhythmic effects of increased fibrosis are well recognized,\textsuperscript{34-36} so including anti-fibrotic effects of hMSC PS was a priority in developing a tissue-level model. To develop a model of PS anti-fibrotic effects, we performed an extensive literature search to see if we could uncover a relationship across studies and species between hMSC delivery levels and anti-fibrotic effects.\textsuperscript{21,27-33} To compare between species, hMSC delivery levels in each study were normalized to the approximate number of left ventricular myocytes in that species. Interestingly, when we normalized data across nine studies, we found a nearly linear relationship between anti-fibrotic effects and hMSC delivery within a range of approximately 20-60% hMSCs (Online Figure II). This consistency between studies allowed us to confidently and easily incorporate anti-fibrotic effects into the tissue level model to test for arrhythmogenicity.

Incorporating the effects of hMSC PS on single-cell ion channel/pump activity was not straightforward, as limited dose-response data were available in the literature for characterizing the single-cell responses of LTCC and SERCA activity to varying doses of hMSC paracrine factors. Given this uncertainty, and the fact that dose-response relationships will vary between experiments, we adopted an established method\textsuperscript{25,26} and calibrated a population of models by closely matching simulation outputs to corresponding experimental recordings for different species at three hMSC PS dosages (Figure 1). The resultant population of models would therefore effectively represent hMSC PS effects on cardiomyocyte action potential and calcium transient across a range of hMSC dosages.

We generated a large initial population of 2,500 model variants with randomly chosen parameter sets within physiologically and empirically relevant bounds (Online Table II). In contrast with previous studies,\textsuperscript{25,26} however, the parameters varied were not maximal conductances but rather parameters that controlled the sensitivity of myocytes to PS and the maximal effects caused by saturating PS (see Online Methods). The initial population was then filtered to retain only select models (Figure 1A; blue dots) that were consistent (i.e., within one standard deviation) with all experimentally observed data ranges (Figure 1A; within boxed region) of hMSC PS dose-dependent effects on action potential and calcium transient metrics\textsuperscript{19,20} (Figure 1A). This calibration process reduced the initial population to 100 accepted model parameter sets. The histograms in Figure 1B illustrate the distribution of output simulation metrics resulting from the range of accepted model parameters. Figure 1C shows the distribution of parameters used to model PS effects on LTCC current (left) and SERCA activity (right) for the population of 100 accepted models. In contrast to the latter, the former case is constrained (Figure 1C) with a relationship between the Hill coefficient and the half-maximal dose concentration.

In addition to our previously established model of hMSC-myocyte HC through gap junctions,\textsuperscript{24} this computational model now also includes hMSC PS effects on cardiomyocyte LTCC and SERCA activity, as well as hMSC PS anti-fibrotic effects. To our knowledge, this is the most comprehensive model capable of reproducing a majority of the non-vasculature-related effects of hMSCs on cardiomyocyte action potential, calcium transient, and excitation-contraction metrics, as further examined below.

\textit{hMSC PS and HC effects on action potential and calcium handling behavior.}

First, we simulated the effects of hMSC HC-only, hMSC PS-only, and hMSC HC+PS mechanisms on the cardiomyocyte action potential and calcium transient at 100% hMSC supplementation per myocyte (i.e., 1:1 hMSC-cardiomyocyte ratio) for multiple cardiomyocyte species (Figure 2), representing the high end of hMSC:myocyte ratios used in prior in vitro co-culture studies.\textsuperscript{20} hMSC PS was modeled using the least squares model (Figure 1C and Online Table III). As shown in the Online Figure III, our model can be readily adapted to incorporate time-dependent paracrine effects, such as in vitro data from DeSantiago et
al. Nevertheless, the remainder of this modeling study uses steady-state solutions to examine longer-term PS effects, which is more relevant to our hECT experiments.

Across all cell types, HC tends to decrease action potential duration to 90% repolarization (APD$_{90}$), whereas PS increases APD$_{90}$ and calcium transient amplitude, although the magnitude of the effect is cell-type and hMSC dose-dependent (Figure 2 and Online Figures IV-V). As enforced in our process of calibrating model populations to experimental data (Figure 1), all simulated dose-dependent effects of hMSC PS-only mechanisms of myocyte action potential and calcium transient metrics were within one standard deviation of published data for a range of experimental conditions and cardiomyocyte species. Furthermore, we observed that simulated dose-dependent effects of combined hMSC HC+PS mechanisms on myocyte action potential and calcium transient metrics were also consistent with published data. For example, Askar et al. demonstrated that APD$_{90}$ increases, while Chang et al. reported no significant effect, for rat myocytes co-cultured approximately 4:1 with hMSCs (i.e., ~25% hMSC dose). Our simulations predicted a modest prolongation of APD$_{90}$ under matched conditions (Online Figure IV), intermediate between these findings. Our simulations were also consistent with calcium transient measurements in mouse ventricular cardiomyocytes, where the effects on peak calcium transient and calcium transient decay rate with hMSC HC+PS treatment were not significantly different compared to the hMSC PS-only condition (Figure 2A and Online Figure V).

The consistency of our findings with various experimental trends motivated further validation of simulated hMSC HC and PS effects on cardiac contractile function by comparing to experiments performed with three-dimensional hECTs. Interestingly, the various cardiomyocyte models predicted that human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs)—similar to those used in our hECTs—showed the greatest distinction between hMSC HC+PS and PS-only effects on the action potential and calcium transient (Figure 2C). We therefore hypothesized that hECTs may be well suited to empirically delineate the effects of hMSC HC and PS on contractility.

Counteracting effects of hMSC PS and HC on hECT contractility.

Our custom bioreactor system facilitates simultaneous culture of six hECTs that are each comprised of either unsupplemented (-hMSC) or 10% hMSC-supplemented (+hMSC) cellular composition. hMSCs were employed from lots used in recently published clinical trials. Prior to using the hMSCs, their tri-lineage potential was confirmed, as shown in Online Figure VI.

Using this custom bioreactor system, we studied four different experimental groups as illustrated in Figure 3A: 1) hECTs without co-cultured hMSCs (-hMSC; black), 2) hECTs with co-cultured hMSCs (+hMSC; purple), or 3) and 4) alternating +hMSC and -hMSC hECTs in a shared paracrine media bath (red and blue, respectively). Note that equalizing the total number of hECTs in each bioreactor avoids inequalities in total metabolic demand that could introduce a confounding factor; however, this results in a final sample size for the shared media groups being about half that for the +hMSC and -hMSC groups. These four groups correspond to model conditions of approximately: 1) 0% HC + 0% PS; 2) 10% HC + 10% PS; 3) 10% HC + 5% PS; and 4) 0% HC + 5% PS hMSC treatment interventions, respectively. Images of hECTs on the bioreactor and example twitch force measurements are shown in Figures 3B and 3E, respectively. After functional assessment, selected hECTs were fixed, sectioned, and stained for cardiac-troponin-T, revealing similar myofibrillar structures for both -hMSC (Figure 3F) and +hMSC (Figure 3G) tissues. Based on hECT cross-sectional area (Figure 3C-D), hMSC PS alone caused tissue compaction comparable to having hMSCs co-cultured within the tissue; furthermore, there was no significant difference in resting (diastolic) force between all groups (data not shown), suggesting similar passive tissue stiffness. Finally, hMSCs are known to mechanically couple to myocytes. Altogether, this suggests differences in contractile properties of hECT experimental groups largely reflect underlying cardiomyocyte contractility.
To test the predictive power of our calibrated population of models, we simulated excitation-contraction behavior of hiPSC-CMs subjected to experimentally calibrated hMSC PS (Figure 1) and HC (Online Figure VII) mechanisms, and compared the simulation results to developed force (DF), maximum rate of contraction (+dF/dt), and maximum rate of relaxation (-dF/dt) measurements of hECT contractile function under matched experimental hMSC-mediated treatments (Figure 4).

Simulations of the experimentally calibrated population of PS-only (0% HC + 5% PS) and combined HC+PS (10% HC + 5% PS and 10% HC + 10% PS) effects of hMSCs on hiPSC-CMs were representative of contractile function measurements of hECTs under matched experimental hMSC-mediated treatments (Figure 4). In fact, 63%, 63%, and 61% of simulations fell within one standard deviation of the experimental mean for DF, +dF/dt, and -dF/dt, respectively. The median of each experimental condition was squarely within the range of simulation outputs (Figure 4; right panels). Medians of simulation outputs differed from corresponding experimental medians by a range of -41% to +15%. Coincidentally, the population simulations also recapitulated the variability seen in the experimental measurements; the greatest variability was seen in the 0% HC + 5% PS group, and the least in the 10% HC + 5% PS group.

Both model simulations and hECTs demonstrated that the most pronounced hMSC-mediated effects on contractile function were under PS-only conditions (0% HC + 5% PS), where DF significantly increased by approximately 4-fold on average relative to non-hMSC-supplemented controls during physiologic 1-Hz pacing (Figure 4A). Similarly, ±dF/dt were significantly increased by PS-only conditions, and diminished by HC (Figure 4B-C).

To explore how the apparent counteracting effects of hMSC HC and PS on immature stem cell-derived cardiomyocyte contractility demonstrated both empirically and in silico (Figure 4) translates to mature human cardiomyocytes, we modeled the sensitivity of contractile function of hiPSC-CMs (Figure 5A), healthy human adult cardiomyocytes (Figure 5B), and ischemic human adult cardiomyocytes (Figure 5C) to the least squares calibrated model (Online Table III) with perturbations about a mean hMSC treatment condition of 10% HC and 10% PS. Coefficients of determination can be found in Online Table IV.

Consistent with above findings (Figure 4), these low levels of hMSC PS and HC treatment (relative to Figure 2 conditions) are predicted to have counteracting effects on contractile metrics such as DF and ±dF/dt for hiPSC-CMs (Figure 5A), such that increases in contractile metrics would be most pronounced under hMSC PS-only conditions. The sensitivity analysis in Figure 5 implies there is a positive correlation between hMSC PS dosages and more pronounced effects on DF and ±dF/dt for all cell types. Interestingly, DF sensitivity to HC was minimal in healthy and ischemic human adult cardiomyocytes (Figures 5B-C); this likely reflects the larger size and higher ion channel density of adult cardiomyocytes, which makes them less susceptible to consequences of direct coupling with non-contractile hMSCs, so that PS effects dominate. Simulations using all 100 accepted calibration models under 10% HC + 5% PS and 0% HC + 5% PS interventions confirmed these findings, as pronounced HC effects on the action potential and calcium transient were predicted for immature hiPSC-CMs, but not adult cardiomyocytes (Figures 5D-F). By contrast, hMSC PS was more potent than HC, and was predicted in some cases to restore calcium transients of ischemic myocytes to healthy cell levels (Figure 5F). Taken together, these findings demonstrate the dominant role of hMSC PS mechanisms on adult cardiomyocyte contractile function relative to HC.

**hMSC PS protects from HC effects on arrhythmogenicity.**

Using our models of hMSC PS and HC single-cell and tissue-level effects, we analyzed how various empirically relevant levels of hMSC delivery and engraftment affect the vulnerable window (VW)
for re-entry in moderate and high diffuse fibrotic cardiac tissue (Figure 6). In this analysis, increases and decreases in VW (compared with 0% hMSCs) can be considered pro-arrhythmic and anti-arrhythmic, respectively (see Online Methods for details).

Counteracting PS and HC effects of hMSCs were revealed in the VW analysis of tissue-level arrhythmogenicity in simulated cardiac tissue with moderate (21%) and high (40%) diffuse fibrosis (Figure 6). With increasing levels of hMSC delivery and engraftment, the HC-only conditions led to increases in VW for moderate and high fibrosis compared to control. On the other hand, in all cases, the PS-only conditions decreased VW compared to control, thus minimizing arrhythmogenic risk in an hMSC dose-dependent manner. Spontaneous beat rate variability measurements in hECTs—a proposed surrogate of arrhythmogenicity—40,41—with low-level hMSC HC and PS interventions qualitatively support these trends (Online Figure VIII).

Importantly, by simulating a range of pre-clinically and clinically relevant percentages of hMSC injection and engraftment, our simulations predict that the combined effects of hMSC HC and PS mechanisms (i.e., HC+PS condition) will cause intermediate effects on VW, predominantly leading to beneficial decreases in VW. In only one case (moderate fibrosis at high engraftment) there was a slight increase in the VW compared to control, suggesting that low levels of engraftment commonly found clinically may in fact be advantageous. Overall, our simulated VW analysis may help explain why hMSCs are reported to have either no effect,14 or favorable protective effects,13 on arrhythmogenesis in clinical trials, despite the potential electrophysiological risk of hMSC-cardiomyocyte coupling.

Proteomic analysis of experimental culture media.

To explore the nature of the observed hMSC paracrine-mediated increase of hECT contractility, we first performed a protein microarray on serum-free defined media (SFDM) collected from each experimental condition in Figure 3A, representative of the soluble factors released cumulatively by hECT cellular constituents. As expected, samples collected from each hECT culture media group predominantly up-regulated protein expression levels relative to mean values of serum-free defined media control (Figure 7A). Importantly, the two hECT culture conditions with hMSC intervention clustered together in a principal component (PC) analysis (Figure 7B), supporting a consistent paracrine effect by hMSCs in both groups.

Next, we examined the mean fold-change of soluble proteins in the shared paracrine media bath relative to the hMSC-unsupplemented hECT control media, yielding 39 and 31 factors that were up-regulated and down-regulated at least two-fold, respectively (Figure 7C; black dots with red outline located outside the red lines). Using Ingenuity Pathway Analysis (IPA®) software, functional analyses on the up-regulated proteins significantly modulated cardiac cell-specific pathways (Figure 7D), as well as expected immune-related and pro-angiogenic pathways (Online Table V).

Of the 39 up-regulated factors, 8 are linked to at least one of the cardiac cell-specific pathways (Online Table VI), three of which activate the PI3K/Akt signaling cascade previously hypothesized as the mechanism for hMSC paracrine-mediated effects on contractility (Figure 7E).20

Exosomes play a key role in mesenchymal stem cell-mediated increase of hECT contractile function.

Cardioactive hMSC exosomes have also been shown to modulate myocyte PI3K/Akt signaling,42,43 motivating us to test whether the exosomes-depleted and/or exosomes-enriched fractions of the hMSC secretome may be responsible for the hMSC paracrine-mediated increase of hECT contractility.

First, we confirmed via confocal microscopy that hMSC exosomes are taken up by the cardiomyocytes and fibroblasts that constitute our hECTs (Online Figure IX). Next, we tested the role of
exosomes-depleted and exosomes-enriched fractions (both confirmed via dynamic light scattering analysis; see Online Figure X) of the hMSC secretome on hECT contractile performance by replacing hECT SFDM media with the following treatments following baseline contractile function testing on day 5: 1) SFDM (Control); 2) hMSC conditioned media (hMSC CdM); 3) SFDM enriched with hMSC exosomes (hMSC exo); or 4) hMSC exosomes-depleted conditioned media (hMSC exo-depl). hECTs were cultured an additional 5 days, and then DF was measured post-treatment and was compared to baseline measurements.

As shown in Figure 8A, the Control group DF was unchanged relative to pre-treatment baseline, whereas the hMSC CdM treatment led to statistically significant increases in DF, thus confirming our findings in Figure 4 that hMSC PS was indeed responsible for increasing hECT contractility. Importantly, we further observed that the hMSC exo treatment group yielded a statistically significant increase in hECT DF similar to hMSC CdM, whereas the hMSC exo-depl group was not significantly different from baseline.

These functional findings were corroborated by hECT molecular characterization. Following five days of treatment as above, hECTs were snap-frozen for prospective qRT-PCR of cardiac-specific, calcium handling, and apoptosis genes. Cardiac troponin T, the ratio of α-myosin heavy chain to β-myosin heavy chain, and caspase-3/9 mRNA levels were consistent among all groups; on the other hand, mRNA levels of SERCA2a and LTCC—known to be regulated by PI3K/Akt signaling—significantly increased for hECTs treated with hMSC CdM and hMSC exo (Figure 8B), while the BAX/BCL2 ratio, an apoptosis marker, significantly decreased relative to the hMSC exo-depl group (Figure 8C). Together, these findings indicate a key role of exosomes in the paracrine-mediated effects of hMSCs on cardiac contractility.

DISCUSSION

Understanding the therapeutic influence of hMSC HC and PS mechanisms has important implications for their clinical utility. If HC between hMSCs and myocytes is essential for improving cardiac function, then future work should focus on methods to optimize delivery, retention, and integration with host myocardium. On the other hand, if select PS factors secreted by hMSCs are primarily responsible for desired functional benefits, those factors could be identified, isolated, and implemented as a therapy independent of the hMSCs, potentially circumventing some challenges with delivering live biologics. Alternatively, both effects might be required to optimize the phenotypic and clinical benefits of cellular therapy; our model could facilitate the rational design of cell therapies in the future to achieve desired outcomes.

Herein, we utilized mathematical modeling and three-dimensional hECT experiments to delineate effects of hMSC PS and HC on human cardiac contractility and arrhythmogenicity. This study provides: 1) the first experimental measurements distinguishing the PS and HC effects of hMSC supplementation on contractile function of human stem cell-derived engineered cardiac tissue; 2) a comprehensive mathematical model capable of reproducing a majority of reported effects of hMSCs on cardiomyocyte action potential, calcium transient, and excitation-contraction metrics, allowing for direct comparisons between different species, maturity, and disease conditions; 3) new insight into the discrepancy of why clinical trials of hMSC therapy report either no arrhythmogenic effect or even an anti-arrhythmic effect, whereas hMSCs have been considered pro-arrhythmic in vitro; 4) protein microarray analysis of hECT-conditioned media suggesting PI3K/Akt signaling activation; and 5) functional and molecular data supporting a key role of exosomes in hMSC paracrine-mediated increase of hECT contractility.
Motivation for model development.

In our previous computational modeling work, we predicted that HC of human cardiomyocytes with an hMSC electrophysiological model can lead to action potential shortening at the single-cell level, as well as decreased conduction velocity and increased vulnerability to re-entry in simulated 2-D cardiac monolayer tissue absent of fibroblasts. Such tissue-level simulations reproduced several metrics in empirical monolayer studies of arrhythmogenicity; however, the model could not explain effects of hMSC co-culture on single-cell action potential duration and calcium handling as reported by multiple investigators. Furthermore, our model could not explain pre-clinical and clinical observations, where hMSC delivery to the heart had no significant effect—or even favorable protective benefits—on the arrhythmogenicity of fibrotic cardiac tissue. These discrepancies made it clear that some other mechanism of hMSC interaction with the myocardium, neglected in our original computational model, must be responsible for the inconsistencies with experimental data.

Extensive literature suggests hMSC PS mechanisms can have a profound effect on cardiac single-cell and tissue-level function; we therefore decided to focus our modeling efforts on these PS effects.

Experimental calibration of the hMSC PS model.

Computational studies typically involve model development representing the average behavior of a population. However, important information is lost both experimentally and theoretically when the underlying variability is ignored, ultimately limiting the extrapolation of results at a population level. In this study, we integrate experimental measurements with mathematical modeling to calibrate a population of hMSC PS effects on ion channel/pump activity across various hMSC PS dosages representative of physiological variability and several cardiac cell types commonly used in the literature. We then examine this model in the context of experimentally measured contractility of hECTs following hMSC intervention.

Our approach builds on previous studies that show the importance of modeling intersubject variability in biology. Previous cardiac electrophysiology modeling studies of variability have constructed populations of cardiac cell models by directly adjusting model conductance and kinetic parameters. We expanded on this approach by simulating variability in dose-response curve parameters that effectively describe hMSC PS effects on cardiomyocyte model conductances across a wide range of prescribed treatments.

Model is representative of empirical data.

As enforced in our process of calibrating model populations to experimental data (Figure 1), all simulated dose-dependent effects of hMSC PS-only mechanisms of myocyte action potential and calcium transient metrics were within one standard deviation of published data for a range of experimental conditions and cardiomyocyte species. Importantly, our model was also consistent with published hMSC HC+PS effects on single cells, as well as a wide range of original hECT contractility data that were not used in the model calibration process. Furthermore, our simulations of time-dependent paracrine effects of hMSCs in vitro demonstrate the model’s versatility to incorporate clinically relevant time-course data as it becomes available in the future.

Our model was also consistent with reported hMSC-mediated effects on tissue-level arrhythmogenicity. In VW simulations, the hMSC PS-only conditions were anti-arrhythmic compared to control, consistent with a preclinical study on a rat myocardial infarction model, where hMSC paracrine factors were anti-arrhythmic, suppressed fibrosis, and restored conduction. Furthermore, our model trends are consistent with our original hECT data on spontaneous beat rate variability—a proposed surrogate of...
arrhythmogenicity.40,41 Most importantly, VW analyses predicted that hMSC supplementation (involving hMSC PS+HC mechanisms) did not adversely impact arrhythmogenesis, and may even be anti-arrhythmogenic under some conditions; such findings may help explain why hMSCs are mainly reported to have either no effect,14 or favorable protective effects,13 on arrhythmogenesis in clinical trials.

**Nature of the hMSC paracrine-mediated increase of hECT contractility.**

Previously, Desantiago et al. demonstrated hMSC paracrine-mediated enhancement of mouse ventricular myocyte excitation-contraction coupling via the PI3K/Akt signaling cascade.20 Several soluble factors from our protein microarray are known to modulate this PI3K/Akt signaling cascade; however, cardioactive hMSC exosomes have also been shown to modulate myocyte PI3K/Akt signaling,42,43 motivating us to comprehensively test whether the exosomes-depleted and/or exosomes-enriched fractions of the hMSC secretome are responsible for the hMSC paracrine-mediated increase of hECT contractility.

Our functional testing and molecular characterization of hECTs treated with exosomes-enriched or exosomes-depleted fractions of the hMSC secretome suggest that exosomes play a key role in the hMSC paracrine-mediated increase of contractility. Given the avascular nature of our hECTs, these findings suggest exosomes can augment contractility via mechanisms other than previously established neovascularization.47 Importantly, we show mRNA levels of SERCA2a and LTCC—known to be regulated by PI3K/Akt signaling20—significantly increased for hECTs treated with hMSC CdM and hMSC exo, which: 1) supports inclusion of these factors in our model development; and 2) motivates future work investigating the interplay between hMSC exosomes, their cargo, and this signaling cascade for improving contractility.

**Limitations and future work.**

Several limitations of the study should be noted. First, there was limited experimental data available for modeling hMSC PS ion channel/pump activity, as well as anti-fibrotic dose-response curves. This led us to neglect the least influential current modulated by hMSC PS (i.e. transient outward current), and to predict dose-response values using an established experimental calibration algorithm.25,26 Nevertheless, the ability of the model to reproduce a variety of experimental findings justified utilizing the model to make predictions about hMSC HC and PS effects on cardiac function. The model will be further strengthened as more data become available for experimental calibration.

Second, our VW analyses were performed with 2-D diffuse fibrotic tissue, with active fibroblasts assumed to be the same size as cardiomyocytes, and without the electrical remodeling that typically accompanies diseased conditions. Other types of fibrosis could have also been considered, including patchy, interstitial, and compact—the most representative of diseased infarcted heart in hMSC therapy.48 However, it is believed that ischemic heart disease arrhythmogenicity is not primarily due to macroscopic compact fibrotic scars, but by scars surrounded by a border zone, where diffuse fibrosis coexists with patchy and interstitial fibrosis.48 Similarly, our VW analyses focused on a diffuse random distribution of hMSCs; other localized cell distribution patterns could affect the VW differently. Although more advanced models could hypothetically be developed to incorporate realistic anatomical features and fibrosis/hMSC distribution, our model is computationally efficient, yet still provides qualitative insight into hMSC PS and HC effects on contractility and arrhythmogenicity.

Third, the simplified hECT model system does not fully represent native human myocardium. However, the controlled biocomplexity allows for isolation of hMSC-mediated effects on myocyte contractility independent of immunomodulatory, neurohumoral, neovascular, and other mechanisms that may obscure in vivo findings. This also facilitates comparing tissue-level contractility in stem cell-derived hECTs with in silico single-cell hiPSC-CM excitation-contraction under matched hMSC treatment
conditions, as: 1) hMSC PS alone caused tissue compaction comparable to having hMSCs co-cultured within the tissue; and 2) there was no significant difference in diastolic force between all groups, a surrogate of tissue stiffness, even though hMSCs mechanically couple to myocytes in vitro. To help account for the different output measures, we focused on fold changes in treatment groups relative to control (Figure 4), where the model closely reproduced empirical tissue-level findings.

Based on our findings, crucial future work should include testing translation of hMSC PS effects on contractile function to hECT models of heart disease, and further studying the role of hMSC exosomes and their molecular cargo that may be responsible for increased contractility in vivo. Such research efforts could ultimately lead to improved hMSC-based cardiac therapy.

CONCLUSIONS

In summary, this integrated experimental and computational study provides an improved understanding of the relative effects of hMSC PS and HC on cardiac contractility and arrhythmogenicity. Importantly, we provide new insight into the discrepancy of why recent clinical trials report either no arrhythmogenic risk or even an anti-arrhythmic benefit of hMSC therapy, whereas hMSCs have been shown to be pro-arrhythmic in vitro. Finally, we perform proteomic analysis of hECT/hMSC conditioned media and subsequent functional testing/molecular characterization of hECTs treated with exosomes-depleted and exosomes-enriched fractions of the hMSC secretome, which provides novel insight into the role of exosomes in hMSC paracrine-mediated effects on contractility.

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DISCLOSURES

Dr. Hare discloses a patent for cardiac cell-based therapy. He also holds equity in, board membership, and consulting for Vestion, Inc. Dr. Costa discloses his role as scientific co-founder and Chief Scientific Officer of NovoHeart Ltd. Neither Vestion nor NovoHeart played any role in the design or conduct of this study. The other authors report no conflicts.
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FIGURE LEGENDS

Figure 1: Experimentally Calibrating hMSC PS Effects. hMSC PS effects on LTCC and SERCA activity were experimentally calibrated. (A) Scatter plots of the initial population (white dots) filtered (blue dots) to be within one standard deviation (boxed region) of action potential duration to 90% repolarization (APD\textsubscript{90}), calcium transient duration at 50% decay (\(\tau_{Ca}\)), and diastolic-subtracted calcium transient amplitude ([Ca\textsuperscript{2+}]\textsubscript{peak}) metrics across various experimental species and hMSC dosages (7%, 28%, or saturated (\(\infty\))). (B) Histograms illustrating distributions of the output simulation metrics resulting from the accepted population of calibrated models. (C) Accepted sets of calibrated model parameters of hMSC PS dose response curves (see Online Data Supplement for details). Non-standard abbreviations: \(\Delta G_{LCA}\) and \(\Delta V_{\max,up}\) denote maximum saturated effects of hMSC PS on LTCC and SERCA activity, respectively; \(k_{LCA}\) and \(k_{V}\) denote respective Hill coefficients; \(EC_{50, LCA}\) and \(EC_{50, V}\) denote respective half maximum effective concentrations. Asterisk point in panel C denotes the least squares calibrated model defined in the Online Data Supplement.

Figure 2: hMSC PS and HC Effects on Multi-Species Cardiomyocyte Action Potential and Calcium Transient. The effects of hMSC HC-only (red), hMSC PS-only (blue), and hMSC HC+PS (purple) were simulated on (A) mouse, (B) rat, (C) human induced pluripotent stem cell-derived cardiomyocyte (hiPSC-CM), (D) healthy, and (E) ischemic human adult cardiomyocyte action potential (left) and calcium transient (right) at 100% hMSC supplementation per myocyte, compared to unsupplemented controls (black).

Figure 3: Schematic and Structure of hECTs in the Bioreactor. (A) Schematic of the four experimental hECT groups tested. (B-C) Sample images of hECTs on the bioreactor (scale bars inset). (D) Cross-sectional area (CSA) for each hECT group (n=10-22) normalized to control. (E) Sample force measurements of control and -hMSC (shared bath) intervention conditions normalized to control. Sarcomeric organization and alignment in both (F) -hMSC and (G) +hMSC hECTs stained with cardiac troponin-T (cTnT, green) and DAPI (blue). * p<0.05.

Figure 4: Model Comparison with hECT Measurements Under hMSC HC and PS Interventions. Comparison of control (n=22), 10% HC + 5% PS (n=11), 0% HC + 5% PS (n=10), and 10% HC + 10% PS (n=17) hECT measurements (mean +/- standard deviation; legend with “hECT exp” represents empirical hECT data) to simulations (circles) of all 100 accepted models in the calibration population for (A) DF, \(+dF/dt\), and (C) \(-dF/dt\). All data are normalized to control. **p<0.01 based on ANOVA of hECT experiments. Right panel shows histograms of simulation output distributions for each contractility metric; color-coded vertical lines indicate corresponding experimental median values.

Figure 5: Simulated hMSC HC and PS Effects on Contractile Function of Derived and Adult Human Cardiomyocytes. A contractile function sensitivity analysis was performed for (A) hiPSC-CM, (B) healthy human adult cardiomyocyte, and (C) ischemic human adult cardiomyocyte models, with hMSC HC and PS as inputs, and DF, \(+dF/dt\), and \(-dF/dt\) as outputs. The net sensitivity (HC+PS; purple) to hMSC supplementation is defined as the sum of the sensitivity, B, of hMSC HC (red) and PS (blue) mechanisms individually. Single cell action potential and calcium transient simulations of (D) derived and mature (E) healthy and (F) ischemic human cardiomyocytes across all 100 accepted calibration models under 10% HC + 5% PS (red) and 0% HC + 5% PS (blue) interventions versus untreated controls (black).

Figure 6: Simulated Vulnerable Window Analysis on hMSC-Supplemented Fibrotic Cardiac Tissue. A VW analysis was performed on moderate (21%; left panel) and high (40%; right panel) fibroblast-populated cardiac tissue injected with 0% (control), 20%, 40%, or 60% hMSCs at (A) low (1%), (B) moderate (4.5%), and (C) high (16%) engraftment levels (n=3 randomized cell distributions). In all cases,
the PS-only conditions decreased VW, minimizing arrhythmogenicity. As recommended by White et al., comparative statistics were not implemented, as p-values are dependent on sample size (which can be arbitrarily high in simulations).

**Figure 7.** Proteomic Analysis of hECT Conditioned Media: Visualization of protein microarray data via (A) heatmap and (B) principal component (PC) analysis. Heatmap values (run in duplicate) are relative to mean values of serum-free defined media (SFDM) control. (C) Bland-Altman plot of mean differentially expressed proteins in paracrine media bath relative to hMSC-unsupplemented hECT control media (2-fold threshold). (D) Cardiac-specific pathway functions significantly modulated by up-regulated soluble proteins. (E) Soluble factors involved in modulating cardiac-specific pathways.

**Figure 8.** Effects of hMSC Exosomes on hECT Function and mRNA Levels. (A) Contractility assay shows hECT DF during 0.5-Hz pacing (mean±SEM, n=4-7) at pre-treatment (day 5) and 5-days post-treatment with SFDM, hMSC CdM, hMSC exo, and hMSC exo-depl (see text for details). *p<0.05; p-values from paired t-tests. hECTs from each group (n=3) were snap-frozen for qRT-PCR on day 10, where expression of (B) cardiac-specific, calcium handling, and (C) apoptotic genes were studied. *p<0.05, **p<0.01; p-values from one-way ANOVA with post-hoc Tukey test. Non-standard abbreviations: cardiac troponin T (cTnT); L-type calcium channel (LTCC); sarco/endoplasmic reticulum calcium-ATPase (SERCA2a); myosin heavy chain (MHC), caspase-3 (Casp3); caspase-9 (Casp9); B-cell lymphoma 2 (BCL2); BCL2-associated X protein (BAX).
NOVELTY AND SIGNIFICANCE

What Is Known?

- Delivery of human mesenchymal stem cells (hMSC) offers a promising therapy for heart repair.
- The hMSC-cardiomyocyte interactome involves both paracrine signaling (PS) and heterocellular coupling (HC) mechanisms.
- Understanding the contributions of PS and HC mechanisms to cardiac contractility and arrhythmogenicity would help optimize the therapeutic potential of hMSCs.

What New Information Does This Article Contribute?

- Complementary experimental and computational approaches reveal distinct PS and HC effects of hMSCs on cardiac contractility and arrhythmogenicity.
- Functional and molecular data support a key role of exosomes in hMSC paracrine-mediated increase of cardiac contractility.
- Novel in silico insights help resolve disparate reports of potential pro-arrhythmic risks of hMSCs in vitro versus anti-arrhythmic benefits of hMSCs in vivo.

The exciting promise of emerging hMSC cardiotherapies motivates a systematic investigation into underlying mechanisms of action, aiming to minimize potential risks and maximize therapeutic benefits. In this study, computational and experimental approaches were combined to distinguish the effects of hMSC PS and HC on cardiac contractility and arrhythmogenicity. Based on a newly developed mathematical model and human engineered cardiac tissue (hECT) measurements, hMSC-mediated effects on cardiac contractility were most pronounced under PS-only conditions. Simulations of fibrotic cardiac tissue revealed hMSC PS protected from potentially pro-arrhythmic effects of HC, providing novel insight into the discrepancy between in vitro and pre-clinical/clinical findings. We further show that exosomes likely play a key role in hMSC paracrine-mediated effects on hECT contractility and expression of calcium handling genes. Together, this integrated experimental and computational approach provides an improved understanding of the relative effects of hMSC PS and HC on cardiac contractility and arrhythmogenicity, with evidence supporting the cardioactive potency of hMSC exosomes. Our findings motivate further investigation of how hMSC exosomes and their molecular cargo may yield increased cardiac contractility in vivo, ultimately maximizing the safety and efficacy of hMSC-based cardiac therapies.
Figure 1
Figure 3

A - hMSC Control

+ hMSC Supplementation

Alternating + hMSC (red) and - hMSC (blue) in Shared Bath

B

10 mm

C

Control
+ hMSC
- hMSC
+ hMSC

D

GSA (Normalized to Control)

Control + hMSC - hMSC + hMSC

Shared Bath

E

Developed Force (normalized to Control)

0 0.25 0.5 0.75 1

Time (s)

F

cTnT
DAPI

Scale: 10 μm

G

cTnT
DAPI

Scale: 10 μm
Experimental and Computational Insight into Human Mesenchymal Stem Cell Paracrine Signaling and Heterocellular Coupling Effects on Cardiac Contractility and Arrhythmogenicity
Joshua Mayourian, Timothy J Cashman, Delaine K Ceholski, Bryce V Johnson, David Sachs, Deepak A Kaji, Susmita Sahoo, Joshua M Hare, Roger J Hajjar, Eric A Sobie and Kevin D Costa

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SUPPLEMENTAL MATERIAL

Detailed Methods

Single-Cell Cardiomyocyte Electrophysiology Models

Multiple single-cell endocardial cardiomyocyte electrophysiological models were utilized in this study, including the: Bondarenko et al. mouse ventricular myocyte model; Devenyi et al. rat cardiomyocyte model; Paci et al. human induced pluripotent stem cell-derived ventricular-like cardiomyocyte model; ten Tusscher et al. human healthy cardiomyocyte model; and Weiss et al. human ischemic cardiomyocyte model. All single-cell models were numerically integrated with MATLAB’s (The MathWorks, Natick MA) stiff ordinary differential equation solver (ode15s) until steady state was achieved.

Parameter Sensitivity Analysis

An established multivariable regression analysis was performed; briefly, we randomly varied input parameters of interest in 300 trials by a normally distributed pseudorandom scale factor with a coefficient of variation of 10%. From the changes in the model outputs and input parameters, a linear approximation was made to find the normalized parameter sensitivity vector.

Hierarchical Clustering Analysis

Briefly, hierarchical clustering was performed on our multi-species parameter sensitivity analysis vector elements with a Euclidean distance metric and average linkage.

Modeling hMSC HC and PS Effects on the Single-Cell Cardiomyocyte Action Potential and Calcium Transient

hMSC-cardiomyocyte HC interactions were simulated with our established model, representing an empirically observed mixture of hMSC electrophysiological phenotypes. Effects of hMSC PS were independently simulated using experimentally calibrated dose-response curves to adjust the maximum L-type calcium channel permeability ($G_{\text{L}Ca}$) and SERCA maximum uptake activity ($V_{\text{max}up}$) in the cardiomyocyte models according to the prescribed strength of hMSC treatment, as described next.

Unless noted otherwise, hMSC PS steady-state effects are modeled. Detailed methods for modeling transient effects of hMSC PS can also be found below.

Experimental Calibration of the Steady-State hMSC PS Model

We experimentally calibrated the sigmoidal dose response effects of hMSC PS on $G_{\text{L}Ca}$ and $V_{\text{max}up}$ using published experimental data and a modified version of methods previously described. $G_{\text{L}Ca}$ and $V_{\text{max}up}$ were chosen because they have the strongest influence on action potential and calcium handling metrics, as presented in Online Figure I (i.e., $G_{\text{to}}$, the transient outward conductance, was assumed to be constant). Briefly, we constructed a population of 2,500 models of single-cell myocyte electrophysiology by randomly assigning specific parameter values within physiologically and empirically relevant bounds (Online Table II) to characterize the following sigmoidal dose-response curves:

$$G_{\text{L}Ca}' = G_{\text{L}Ca} (1 + \frac{\Delta G_{\text{L}Ca}}{1 + 10^{k_{\text{G}_{\text{L}Ca}} (EC_{50} - x)}})$$

$$V_{\text{max}up}' = V_{\text{max}up} (1 + \frac{\Delta V_{\text{max}up}}{1 + 10^{k_{\text{V}_{\text{max}up}} (EC_{50} - y)}})$$
where $G'_{LCa}$ and $V'_{maxup}$ represent the fold changes of $G_{LCa}$ and $V_{maxup}$ based on prescribed hMSC PS dosages, respectively; $\Delta G_{LCa}$ and $\Delta V_{maxup}$ are the maximum saturated effects of hMSC PS on $G_{LCa}$ and $V_{maxup}$, respectively; $k_{LCa}$ and $k_{V}$ are the characteristic Hill coefficients for each respective curve; $EC_{50,LCa}$ and $EC_{50,V}$ are the characteristic half maximum effective concentrations for each respective curve; and $x$ is the effective hMSC PS dosage, defined as $\log_{10}$ of the percentage of hMSCs per myocyte. Each parameter set in the experimental calibration had 6 parameters: $\Delta G_{LCa}$, $\Delta V_{maxup}$, $EC_{50,LCa}$, $EC_{50,V}$, $k_{LCa}$, and $k_{V}$.

Our model-calibration algorithm determined whether a given set of model parameters should be added to an “accepted” population based on comparison of output simulated with empirical metrics. This process was constrained by the availability of experimental data at different hMSC dosages. Therefore, we used four dose-dependent output action potential and calcium transient metrics to calibrate the model, specifically: 1) rat myocyte APD 90 at 7% hMSC PS supplementation (APD 90,7%);13 2) rat myocyte APD 90 at 28% hMSC PS supplementation (APD 90,28%);13 3) mouse myocyte $[Ca^{2+}]_{peak}$ at saturated hMSC PS ($[Ca^{2+}]_{peak,\infty}$),14 and 4) mouse myocyte $\tau_{Ca}$ at saturated hMSC PS ($\tau_{Ca,\infty}$).14 Modified from Prinz et al., bounds on allowed output variability were set to one standard deviation around mean values.15 Finally, the set of accepted model parameters that best represented, in a least squares sense, the average of all accepted output metrics was defined as the “least squares” or “best fit” model (Online Table III) and was used for in-depth simulations.

**Modeling hMSC PS Transient Effects**

To model transient effects of hMSC PS, we modified steady-state sigmoidal dose-response curves with best-fit equations to transient experimental data (Online Figure III):

$$G'_{LCa}(t, x) = G_{LCa}(1 + \frac{\Delta G_{LCa}}{1 + 10^{k_{LCa}(EC_{50,LCa}-x)}}(1 - e^{-t/\tau}))$$

$$V'_{maxup}(t, x) = V_{maxup}(1 + \frac{\Delta V_{maxup}}{1 + 10^{k_{V}(EC_{50,V}-x)}}(1 - e^{-t/\tau}))$$

where $G'_{LCa}(t, x)$ and $V'_{maxup}(t, x)$ represent the time- and hMSC dose-dependent fold changes of $G_{LCa}$ and $V_{maxup}$ based on prescribed hMSC PS dosages, respectively; $t$ is time; and $\tau$ is a time constant.

**hECT Tissue Construction**

hECTs were created from differentiated human embryonic stem cells (H7 cell line, NIH Registration #0061) and type-I collagen using methods previously described.16,17

**hECT Functional Test Metrics**

hECT twitch force and dynamics were assessed; hECT developed force (DF) was measured as the difference between maximum systolic and minimum diastolic force, while twitch dynamics were assessed by maximum rates of contraction (+dF/dt) and relaxation (-dF/dt). Beat rate variability, a proposed index of arrhythmogenicity,18,19 was measured from a sequence of contractions in spontaneously beating hECTs using previously established methods.18

**Testing Contributions of HC and PS on hECT Contractile Function**

For unsupplemented control tissues (-hMSC), the cell-matrix solution was directly used for tissue construction in a custom 6-tissue bioreactor. For supplemented tissues (+hMSC), an
additional 10% hMSCs were added to the tissue mix prior to hECT formation. The two tissue types were cultured either separately or in a shared media/paracrine bath. Prior to using the hMSCs, their tri-lineage potential was confirmed (Online Figure VI). The function of both tissue types in each condition was then assessed using established methods.\textsuperscript{16,20} Twitch force measurements were obtained on culture day 7 with hECTs beating during electrical field stimulation at 1 Hz pacing frequency.

**Modeling hMSC HC and PS Effects on Single-Cell Cardiomyocyte Excitation-Contraction**

To compare numerical results to empirical hECT contractile function data, our experimentally calibrated models of hMSC HC and PS effects were applied to Paci and coworker’s single-cell model of human induced pluripotent stem cell-derived ventricular-like cardiomyocytes (hiPSC-CM)\textsuperscript{3}—representing the primary cellular constituent of hECTs—linked to an established excitation-contraction coupling model.\textsuperscript{21} Initial sarcomere length was modified to 1.9 \(\mu\)m to be within the range of published experimental data on such cell types.\textsuperscript{22,23} For each experimental condition, we performed in silico trials using all accepted sets of experimentally calibrated parameters. Twitch developed force (DF), +dF/dt, and –dF/dt were calculated for each simulation.

For contractile function sensitivity analysis, we applied the least squares model parameters to the single-cell Paci et al. hiPSC-CM\textsuperscript{3}, ten Tusscher et al. human endocardial cardiomyocyte\textsuperscript{4}, and Weiss et al. human ischemic\textsuperscript{5} models with the same established excitation-contraction coupling model, with initial sarcomere lengths of 1.9, 2.1, and 2.1 \(\mu\)m, respectively. We then performed a parameter sensitivity analysis (method described above) with output contractility metrics including DF, +dF/dt, and -dF/dt.

**Calibrating HC Gap Junction Conductance to Experimental Conditions**

Valiunas et al. reported a mean HC gap junction conductance between hMSCs and canine ventricular cardiomyocytes on glass coverslips of 1.5 nS, reaching as high as 11 nS with other cell types.\textsuperscript{24} This experimental setup closely represented the conditions of published studies\textsuperscript{13,14} we used to compare to our action potential and calcium handling model simulations. Therefore, a gap junction conductance of 1.5 nS was used to model hMSC HC effects on single-cell cardiomyocyte action potential and calcium handling.

The three-dimensional environment of hECTs formed with human pluripotent stem cell derived-cardiomyocytes differs from monolayer conditions, which may alter effective gap junction conductance between hMSCs and other cell types. Therefore, we measured the excitation-threshold voltage for pacing capture in hECTs with and without hMSC HC interventions, and calibrated the hMSC HC gap junction conductance in the excitation-contraction simulations to match the experimental mean threshold values (Online Figure VII). The resulting 9.1 nS HC gap junction conductance was used for excitation-contraction simulations when comparing to hECT contractile function results.

**In Silico Diffuse Fibrosis Cardiac Tissue Configuration**

A modified version of a monolayer spatial model of two-dimensional cardiac tissue with diffuse fibrosis was used.\textsuperscript{25,26} Specifically, a 5 cm x 5 cm anisotropic cardiac tissue sheet was simulated, with structural remodeling accounted for by adding intercellular uncoupling at randomly inserted fibroblast locations. Moderate and high fibrosis were defined as fibroblasts comprising 21% and 40% of the total cell population, respectively (corresponding to fibrotic areas within experimental ranges\textsuperscript{27}). Using these assumptions, the spatial model can be described as:

\[
\frac{\partial V}{\partial t} = \frac{I_{\text{tot},k}}{C_{m,k}} + I_{\text{stim}} + D_{k,x} \frac{\partial^2 V}{\partial x^2} + D_{k,y} \frac{\partial^2 V}{\partial y^2}
\]
where $V$, $I_{tot}$, $I_{stim}$, $C_m$, $D$, and $k$ are the voltage, total ionic current, stimulus current, cell membrane capacitance, diffusion coefficient, and cell type (e.g., cardiomyocyte or fibroblast) at a given node, respectively.

Cardiomyocyte electrical activity was simulated with the ten Tusscher model of adult human endocardial myocytes, while the electrical activity of fibroblasts was simulated using the MacCannell et al. active formulation. Euclidian geometry was selected, with $\Delta x = \Delta y = 0.01$ cm, and $\Delta t = 0.01$ ms. Neumann-type boundary conditions were implemented to solve the partial differential equations. As performed elsewhere, electrical anisotropy was modeled as $D_{k,x} = 4D_{k,y}$. All relevant model parameters can be found elsewhere.

All tissue simulations were executed in Python.

Modeling hMSC PS and HC Effects on Diffuse Fibrosis Cardiac Tissue

hMSCs were added at 0% (control), 20%, 40%, and 60% of the total cell population in the diffuse fibrosis adult human cardiac tissue simulations described above. To model hMSC HC effects, we assumed the delivered hMSCs engrafted at either low (1%), moderate (4.5%), or high (16%) levels, respectively approximating the minimum, median, and high values of stem cell engraftment reported in various studies. Engraftment was defined as hMSCs that randomly inserted into the monolayer tissue with their own characteristic electrical activity and intercellular coupling, as described elsewhere.

To model hMSC PS tissue-level effects, we developed a relationship (Online Figure II) between hMSC delivery levels (% of total left ventricular cell population) and anti-fibrotic effects ($\alpha$) based on published data. From this relationship, we replace $\alpha$ percent of randomly distributed fibroblast nodes with cardiomyocyte nodes. Based on hMSC delivery levels, hMSC PS also affected L-type calcium channel and SERCA activity at cardiomyocyte nodes using the “best fit” model (methods previously described). All relevant model parameters can be found in Figure 1, Online Figure II, Online Table III, and elsewhere.

Vulnerable Window Analysis

The well-established cross-field stimulation protocol was applied to the 2-D tissue simulations. Briefly, after achieving steady state, two S1 stimuli were applied at the left end of the tissue at a 1000 ms basic cycle length. An S2 stimulation was applied 100–500 ms after the second S1 stimulus—at 1 ms increments—in the bottom left corner of the tissue (1.25 cm wide × 2.5 cm high). For each tissue model, the VW was defined as the range of S1–S2 intervals that led to at least one spiral wave rotation. Mean and standard deviation of VW of three random tissue sheet configurations ($n = 3$) were reported for each condition.

Protein Microarray

To identify soluble factors secreted by hMSCs cultured within the supplemented hECTs, each set of hECTs was switched to serum free defined medium (SFDM) composed of RPMI 1640 (Life Technologies) medium with B27 supplement (Life Technologies), 1% penicillin-streptomycin (Corning) and 0.2% amphotericin B (Sigma-Aldrich) after pacing on day 7. A full change of SFDM was performed 24 hours later. After 48 hours, the entirety of the media was collected and stored at -20°C to await further analysis. The tissues were returned to NBS medium and function re-assessed on day 10 to confirm tissue viability. Media from four conditions from one experiment was collected: cell- and serum-free defined media control, -hMSC hECT controls, +hMSC hECTs, and the paracrine bath shared by -hMSC and +hMSC hECTs (see Figure 3).

Collected medium was thawed; protein concentration was then determined with a bicinchoninic acid (BCA) assay and sent to RayBiotech for analysis. Each of the four samples
was loaded on the human L1000 glass slide antibody microarray (RayBiotech) at equal total protein concentration, each testing for 1000 known proteins in duplicate plus internal controls.

**Computational Analysis of Protein Microarray**

As recommended for RayBioc®, positive control normalization without background data was used for the human L1000 glass slide. Factors with non-negative values across each condition were retained. For hierarchical clustering visualization, the MATLAB clustergram function was used. For the Bland-Altman plot, the MATLAB mairplot function was used to compare average -hMSC hECT control and paracrine bath protein microarray data. The 39 soluble factors up-regulated at least 2-fold were uploaded to Ingenuity Pathway Analysis (IPA®, Qiagen). The biological functions of the set of 39 factors were analyzed and reduced to only those with known relevance to cardiomyocytes. An interaction network between the remaining proteins and PI3K/Akt signaling was subsequently formed. Activation of PI3K/Akt signaling was tested via the Molecule Activity Predictor on the remaining proteins.

**hMSC Conditioned Media and Exosomes Isolation**

Conditioned media was collected from 10 cm dishes of 50% confluent hMSCs (passage less than 5) cultured in SFDM for five days. Exosomes were isolated from hMSC conditioned media via differential centrifugation using previously established methods and characterized via dynamic light scattering using a NanoBrook ZetaPALS (Brookhaven, Holtsville, NY).

**Exosomes Taken Up by Human Stem-Cell Derived Cardiomyocytes and Fibroblasts**

Exosomes from hMSC conditioned media were calcin-stained using methods previously described. Calcin-stained hMSC exosomes were subsequently supplemented to Hoescht stained, fluorescence activated cell sorted, human stem cell-derived SIRPa+ cardiomyocytes and CD90+ fibroblasts for 48 hours in monolayer culture.

**Functional Assessment of hECTs Treated with hMSC Conditioned Media**

Following 48 hours of tissue compaction, hECTs were cultured in SFDM until day 5. Following baseline contractile function testing at 0.5-Hz pacing on day 5, SFDM was replaced with the following treatments: 1) SFDM (Control); 2) fresh hMSC conditioned media (see above; hMSC CdM); 3) SFDM supplemented with exosomes isolated from fresh hMSC conditioned media (hMSC exo); or 4) hMSC exosomes-depleted conditioned media obtained from the supernatant of ultracentrifugation (hMSC exo-depl). hECTs were cultured an additional 5 days, and then DF was measured again.

**qRT-PCR**

Total RNA was extracted from flash-frozen hECTs using the Quick-RNA MiniPrep kit (Zymo Research, Irvine, CA) after lysis using FastPrep Lysis Beads and Matrix Tubes (MP Products, Santa Ana, CA) and quantified using a NanoDrop 2000 (ThermoFisher, Rockville, MD). Reverse transcription was performed using the iScript cDNA synthesis kit (Biorad Laboratories, Hercules, CA) and quantitative PCR (10 ng cDNA/reaction) was performed using a two-step system with SYBR Advantage qPCR Premix (Clontech Laboratories, Mountain View, CA) on the ABI Prism 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA) according to manufacturer recommendations.
All primers used were against human and are shown as 5’ to 3’:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Sequence (5’-3’)</th>
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</thead>
<tbody>
<tr>
<td>β2M</td>
<td>Forward</td>
<td>GTATGCCTGCGCTGTGAAC</td>
</tr>
<tr>
<td>β2M</td>
<td>Reverse</td>
<td>CAAGCAAGCAAGAATTGGAA</td>
</tr>
<tr>
<td>TNNT2</td>
<td>Forward</td>
<td>AAGAGGCAGACTGACGGCCGGAAAA</td>
</tr>
<tr>
<td>TNNT2</td>
<td>Reverse</td>
<td>AGATGCTCTGCCACAGCTTTC</td>
</tr>
<tr>
<td>αMYHC</td>
<td>Forward</td>
<td>GGAAGACAAGGTCAACACGGCCTT</td>
</tr>
<tr>
<td>αMYHC</td>
<td>Reverse</td>
<td>TCCAGTTTCCGCTTGGCTCCT</td>
</tr>
<tr>
<td>βMYHC</td>
<td>Forward</td>
<td>GGAGTTACACGCCTCAAGAGG</td>
</tr>
<tr>
<td>βMYHC</td>
<td>Reverse</td>
<td>TCCTCAGCATCTGCCAGGTGT</td>
</tr>
<tr>
<td>SERCA2a</td>
<td>Forward</td>
<td>CTGTCCATGTCACTCCACTTTCC</td>
</tr>
<tr>
<td>SERCA2a</td>
<td>Reverse</td>
<td>AGCGGTTACTCCAGTATGCGAG</td>
</tr>
<tr>
<td>CASP3</td>
<td>Forward</td>
<td>TTAATAAAGGTATCCATGGAGAAGACT</td>
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<tr>
<td>CASP3</td>
<td>Reverse</td>
<td>TTAGTGATATTTTATAGAGTTCTTTTTGTGAG</td>
</tr>
<tr>
<td>CASP9</td>
<td>Forward</td>
<td>TTCCAGGTTTTGTTTCTCT</td>
</tr>
<tr>
<td>CASP9</td>
<td>Reverse</td>
<td>CCTTTACCGAAACAGCATT</td>
</tr>
<tr>
<td>BCL2</td>
<td>Forward</td>
<td>CTGCACTGTACGCCCTTCC</td>
</tr>
<tr>
<td>BCL2</td>
<td>Reverse</td>
<td>CACATGACCCACCGAATCTGAAGAA</td>
</tr>
<tr>
<td>BAX</td>
<td>Forward</td>
<td>GGACGGAATGGAGACTAAGCTGAG</td>
</tr>
<tr>
<td>BAX</td>
<td>Reverse</td>
<td>GCAAGTAGAAAGGGGCCAACA</td>
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<tr>
<td>LTCC</td>
<td>Forward</td>
<td>TGACTATTTTGCCAAATTGTGTGG</td>
</tr>
<tr>
<td>LTCC</td>
<td>Reverse</td>
<td>CGGGAGGTAGGGCAATTGGG</td>
</tr>
</tbody>
</table>

Fold changes in gene expression were determined using the comparative Ct method (ΔΔCt) with normalization to the housekeeping gene β2M. This experiment was repeated in triplicate.

**Statistical Testing and Analysis**

Unless otherwise specified, results are presented as mean +/- standard deviation (SD) of n independent experiments. Statistical analyses were performed using MATLAB (The MathWorks, Natick MA). One-way analysis of variance (ANOVA), followed by Scheffe’s post-hoc test, was used for multiple pairwise comparisons of unequal group sizes. One-way ANOVA, followed by Tukey’s post-hoc test, was used for multiple pairwise comparisons of equal group sizes. Paired t-tests were used when comparing hECT contractile function pre- and post-treatment. Differences with a p-value less than 0.05 were considered statistically significant.
Online Figure I: Multi-Species Parameter Sensitivity Analysis of hMSC PS-Modulators of Cardiomyocyte Action Potential and Calcium Handling. A multi-species parameter sensitivity analysis of L-type calcium current maximum permeability ($G_{LCa}$), maximum SERCA uptake ($V_{maxup}$), and transient outward current maximum conductance ($G_{to}$) based on metrics of A) action potential and B) calcium handling for mouse ventricular myocyte (Mouse), rat cardiomyocyte (Rat), human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM), adult human cardiomyocytes (Human), and adult ischemic human cardiomyocytes (Ischemic). This method: 1) accounts for the sensitivity of output metrics to model parameters; 2) provides insight into the variability among species and conditions commonly used in studies of hMSC effects; and 3) ranks output metric sensitivity to input parameters in an unbiased manner. Color scale as shown, with + and – designating positive and negative correlations, respectively. Non-standard abbreviations: APD$_{90}$ denotes action potential duration to 90% repolarization; RMP denotes resting membrane potential; $\tau_{Ca}$ denotes calcium transient duration at 50% decay; $[Ca^{2+}]_{peak}$ denotes diastolic-subtracted calcium transient amplitude.
Online Figure II: Data Fitting hMSC PS Anti-Fibrotic Effects. A linear regression with zero-intercept was used to describe the relationship between hMSC injection (as % cells of left ventricular cardiomyocytes) and the anti-fibrotic effect (% decrease in fibrosis). hMSC injection levels were normalized to the approximate number of cells in the left ventricle for a given species. References for each data point are inset, n = 1-3 per data point. Best-fit linear equation and R² values are shown.
Online Figure III: Modeling Transient Effects of hMSC PS. The computational model can be adapted to simulate transient effects of hMSC PS. A) Fitting the asymptotic function $1 - e^{-t/\tau}$ to experimental data from Desantiago et al.\textsuperscript{14} on the percent change of peak $[\text{Ca}^{2+}]$, relative to control ($t=0$ min) and steady-state (assumed to be at $t=180$ min) in mouse ventricular cardiomyocytes treated with hMSC-conditioned media, yielding the best-fit time constant, $\tau = 22.4$ min. B) Validation of this equation by simulating effects on $\tau_{\text{Ca}}$ and comparing to experimental data from Desantiago et al.\textsuperscript{14} In both plots, the mouse cardiomyocyte model was used with the least squares hMSC PS model at a saturated hMSC dose to match the reported experimental conditions.\textsuperscript{14}
Online Figure IV: Effects of hMSC HC and PS on Multi-Species Cardiomyocyte Action Potential Metrics. hMSC HC and PS effects on APD$_{90}$ were simulated for: A) mouse, B) rat, C) human induced pluripotent stem cell-derived, D) adult human, and E) ischemic human cardiomyocytes at different hMSC dosages from 0% to 100% (i.e. 1:1 hMSC:cardiomyocyte ratio).
**Online Figure V: Effects of hMSC HC and PS on Multi-Species Cardiomyocyte Calcium Handling Metrics.** hMSC HC and PS effects on calcium transient duration at 50% decay ($\tau_{Ca}$; left inset), and diastolic-subtracted calcium transient amplitude ($[Ca^{2+}]_{peak}$; right inset) were simulated for: A) mouse, B) rat, C) human induced pluripotent stem cell-derived, D) adult human, and E) ischemic human cardiomyocytes at different dosages.
Online Figure VI: Tri-Lineage Potential of hMSCs. hMSCs display: A) adipogenic, B) osteogenic, and C) chondrogenic potential prior to adding them to hECTs.
Online Figure VII: Calibrating hMSC HC Gap Junction Conductance in 3-D Culture.

hMSC HC gap junction conductance was calibrated to hMSC 10% HC and 5% PS-treated hECT (n = 11) mean excitation threshold relative to hMSC 0% HC and 5% PS-treated hECTs (n = 10). Experimental excitation threshold is defined as the minimum voltage pulse amplitude necessary to capture hECTs at 1 Hz pacing. Simulation excitation threshold was defined as the minimum voltage pulse necessary to excite the human induced pluripotent-stem cell derived cardiomyocyte model at 1 Hz, effectively matching experimental conditions. HC gap junction conductance was varied from 0 nS to 10 nS at 0.1 nS intervals (1 nS intervals shown). The best fit was achieved at 9.1 nS (bold). Data are normalized to control, mean +/- standard error of the mean.
Online Figure VIII: hECT Spontaneous Beat Rate Variability Measurements Under hMSC HC and PS Interventions. Using previously established methods, the spontaneous rate-corrected beat rate variability was measured for -hMSC, +hMSC, and alternating +hMSC and -hMSC hECTs in a shared media bath. A) Plot of collective rate corrected time-to-next and time-to-previous peaks for each group (hECT group shown in top right of plot), where different colors designate different hECT samples. B) Rate-corrected mean distance from centroid for -hMSC (black, n = 8), +hMSC (purple, n = 7), and alternating +hMSC (red, n = 3) and -hMSC (blue, n = 2) in a shared media bath. All data is normalized to control. * p<0.05, mean +/- standard error of mean shown.
Online Figure IX: hMSC Exosomes Taken Up by Cardiomyocytes and Fibroblasts. Calcein-stained hMSC exosomes (green) are taken up by Hoescht stained (blue) human embryonic stem cell-derived SIRPα+ cardiomyocytes (A) and CD90+ fibroblasts (B) within 48 hours of treatment. Scale bar = 20 μm. Maximum intensity projections from confocal microscopy z-stacks shown with orthogonal projections at given locations (yellow lines).
Online Figure X: Dynamic Light Scattering Analysis of Exosomes-Depleted and Exosomes-Enriched Fractions of the hMSC Secretome. Confirmation via dynamic light scattering that the hMSC conditioned media was successfully separated into A) a vesicular exosome-like fraction taken from the ultracentrifugation pellet with a mean diameter of 105.5 nm and B) a non-vesicular soluble fraction taken from the supernatant after ultracentrifugation.
<table>
<thead>
<tr>
<th>Species</th>
<th>$\text{APD}_{90}$</th>
<th>RMP</th>
<th>$\tau_{\text{Ca}}$</th>
<th>$[\text{Ca}^{2+}]_{\text{peak}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>0.83</td>
<td>0.98</td>
<td>0.99</td>
<td>0.99</td>
</tr>
<tr>
<td>Rat</td>
<td>0.99</td>
<td>0.81</td>
<td>0.92</td>
<td>0.99</td>
</tr>
<tr>
<td>hiPSC</td>
<td>0.99</td>
<td>0.73</td>
<td>0.94</td>
<td>0.99</td>
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<tr>
<td>Healthy Human</td>
<td>0.99</td>
<td>0.94</td>
<td>0.99</td>
<td>0.99</td>
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<tr>
<td>Ischemic Human</td>
<td>0.99</td>
<td>0.99</td>
<td>0.98</td>
<td>0.99</td>
</tr>
</tbody>
</table>
### Online Table II: Bounds (Lower, Upper) and Justification for Experimentally-Based Calibration Model

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Bounds</th>
<th>Justification</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta G_{L_{Ca}}$</td>
<td>(0.27,0.62)</td>
<td>Within one standard error of experimental data.\textsuperscript{14}</td>
</tr>
<tr>
<td>$\Delta V_{\text{max,up}}$</td>
<td>(0,1)</td>
<td>0: Based on experimental data,\textsuperscript{14} SERCA activity increases.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1: As performed elsewhere, the conductance is assumed to increase by a maximum of 100%\textsuperscript{11,12}</td>
</tr>
</tbody>
</table>
| $EC_{50,L_{Ca}}$ | $(\log_{10}(28),\log_{10}(200))$ | $\log_{10}(28)$: Experimental data\textsuperscript{13} demonstrates a growth phase when supplementing 7\% and 28\% hMSC PS. Therefore, the half maximum effective concentration is assumed to be greater than $\log_{10}(28)$.  
$\log_{10}(200)$: We assume the half maximum effective concentration is achieved prior to a 2:1 hMSC:myocyte ratio. |
| $EC_{50,v}$ | $(0,\log_{10}(200))$ | 0: Due to limited experimental data, our lower bound is the minimum feasible value.  
$\log_{10}(200)$: As above, we assume the half maximum effective concentration is achieved prior to a 2:1 hMSC:myocyte ratio. |
| $k_{L_{Ca}}$ | (0.6,5)           | 0.6: The APD\textsubscript{90} slope between 7\% and 28\% hMSC PS was 0.45\textsuperscript{13}, corresponding to a $G_{L_{Ca}}$ slope of 0.6 (sensitivity coefficient of 0.75). Therefore, we assume $k_{L_{Ca}}$ must be greater than 0.6.  
5: Given our lower bound on the half maximum effective concentration, a k value of 5 will lead to no change in $G_{L_{Ca}}$ at 7\% hMSC dosage. This would lead to no effect on APD\textsubscript{90} at this level, conflicting with experimental data.\textsuperscript{13} |
| $k_v$    | (0,5)             | 0: Due to limited experimental data, our lower bound is the minimum feasible value.  
5: Assumed to be the same upper limit as $k_{L_{Ca}}$. |
Online Table III: Parameters for Least Squares “Best” Accepted Model

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta G_{Ca}$</td>
<td>0.61</td>
</tr>
<tr>
<td>$\Delta V_{max,up}$</td>
<td>0.017</td>
</tr>
<tr>
<td>EC$_{50, Ca}$</td>
<td>Log$_{10}(38.4)$</td>
</tr>
<tr>
<td>EC$_{50, V}$</td>
<td>Log$_{10}(45.9)$</td>
</tr>
<tr>
<td>$k_{Ca}$</td>
<td>1.77</td>
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<tr>
<td>$k_V$</td>
<td>3.51</td>
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</table>
## Online Table IV: Coefficients of Determination for Contractile Function Sensitivity Analysis

<table>
<thead>
<tr>
<th>Species</th>
<th>Force</th>
<th>+dF/dt</th>
<th>-dF/dt</th>
</tr>
</thead>
<tbody>
<tr>
<td>hiPSC</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
</tr>
<tr>
<td>Mature hCM</td>
<td>0.99</td>
<td>0.99</td>
<td>0.99</td>
</tr>
<tr>
<td>Ischemic Mature hCM</td>
<td>0.98</td>
<td>0.98</td>
<td>0.98</td>
</tr>
</tbody>
</table>
Online Table V: Immunogenic and Angiogenic Pathways Significantly Modulated by Up-Regulated Factors in Paracrine Bath

<table>
<thead>
<tr>
<th>Category</th>
<th>Function</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune</td>
<td>Immune Cell Trafficking</td>
<td>1.96E-9 to 2.35E-6</td>
</tr>
<tr>
<td></td>
<td>Hematological System Development and Function</td>
<td>7.85E-7 to 9.38E-2</td>
</tr>
<tr>
<td></td>
<td>Inflammatory Response</td>
<td>6.89-13 to 6.61E-2</td>
</tr>
<tr>
<td></td>
<td>Cellular Movement</td>
<td>1.45E-15 to 3.98E-2</td>
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<tr>
<td></td>
<td>Inflammatory Disease</td>
<td>2.86E-13 to 6.61E-2</td>
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<tr>
<td></td>
<td>Lymphoid Tissue Structure and Development</td>
<td>1.19E-12 to 2.39E-6</td>
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<tr>
<td></td>
<td>Immunological Disease</td>
<td>1.97E-10 to 4.29E-2</td>
</tr>
<tr>
<td></td>
<td>Cell-mediated Immune Response</td>
<td>1.47E-8 to 1.83E-7</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>Angiogenesis</td>
<td>3.94E-13</td>
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<tr>
<td></td>
<td>Vascularogenesis</td>
<td>8.82E-11</td>
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<tr>
<td></td>
<td>Neovascularization</td>
<td>5.54E-07</td>
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<tr>
<td></td>
<td>Branching</td>
<td>4.71E-07</td>
</tr>
<tr>
<td></td>
<td>Tubulation</td>
<td>1.72E-06</td>
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<tr>
<td>Cardiac</td>
<td>Hypertrophy of Heart Cells</td>
<td>2.29E-01</td>
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<tr>
<td></td>
<td>Hypertrophy of Ventricular Myocytes</td>
<td>3.78E-02</td>
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<td></td>
<td>Proliferation of Cardiomyocytes</td>
<td>1.53E-04</td>
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<tr>
<td></td>
<td>Proliferation of Ventricular Myocytes</td>
<td>1.32E-04</td>
</tr>
<tr>
<td></td>
<td>Enlargement of Heart Cells</td>
<td>2.83E-02</td>
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<tr>
<td></td>
<td>Enlargement of Cardiomyocytes</td>
<td>2.25E-02</td>
</tr>
<tr>
<td></td>
<td>Survival of Ventricular Myocytes</td>
<td>1.06E-02</td>
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<tr>
<td></td>
<td>Apoptosis of Cardiomyocytes</td>
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Online Table VI: Up-Regulated Factors Linked to Cardiac Cell-Specific Pathways from Proteomic Analysis of hECT Conditioned Media

<table>
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<tr>
<th>Factor</th>
<th>Fold Change</th>
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<td>FADD</td>
<td>5.3</td>
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<tr>
<td>Survivin</td>
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</tr>
<tr>
<td>IGF-1</td>
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</tr>
<tr>
<td>FoxO1</td>
<td>2.5</td>
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<tr>
<td>IL-1 R9</td>
<td>2.3</td>
</tr>
<tr>
<td>IGF-II</td>
<td>2.2</td>
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<tr>
<td>Caspase-8</td>
<td>2.2</td>
</tr>
<tr>
<td>KLF4</td>
<td>2.1</td>
</tr>
</tbody>
</table>
Supporting References:


44. Thery C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol.* 2006;Chapter 3:Unit 3 22

