Physiologic, Pathologic, and Therapeutic Paracrine Modulation of Cardiac Excitation-Contraction Coupling

Joshua Mayourian, Delaine K. Ceholski, David M. Gonzalez, Timothy J. Cashman, Susmita Sahoo, Roger J. Hajjar, Kevin D. Costa

Abstract: Cardiac excitation–contraction coupling (ECC) is the orchestrated process of initial myocyte electrical excitation, which leads to calcium entry, intracellular trafficking, and subsequent sarcomere shortening and myofibrillar contraction. Neurohumoral β-adrenergic signaling is a well-established mediator of ECC; other signaling mechanisms, such as paracrine signaling, have also demonstrated significant impact on ECC but are less well understood. For example, resident heart endothelial cells are well-known physiological paracrine modulators of cardiac myocyte ECC mainly via NO and endothelin-1. Moreover, recent studies have demonstrated other resident noncardiomyocyte heart cells (eg, physiological fibroblasts and pathological myofibroblasts), and even experimental cardiotherapeutic cells (eg, mesenchymal stem cells) are also capable of altering cardiomyocyte ECC through paracrine mechanisms. In this review, we first focus on the paracrine-mediated effects of resident and therapeutic noncardiomyocytes on cardiomyocyte hyper trophy, electrophysiology, and calcium handling, each of which can modulate ECC, and then discuss the current knowledge about key paracrine factors and their underlying mechanisms of action. Next, we provide a case example demonstrating the promise of tissue-engineering approaches to study paracrine effects on tissue-level contractility. More specifically, we present new functional and molecular data on the effects of human adult cardiac fibroblast conditioned media on human engineered cardiac tissue contractility and ion channel gene expression that generally agrees with previous murine studies but also suggests possible species-specific differences. By contrast, paracrine secretions by human dermal fibroblasts had no discernible effect on human engineered cardiac tissue contractile function and gene expression. Finally, we discuss systems biology approaches to help identify key stem cell paracrine mediators of ECC and their associated mechanistic pathways. Such integration of tissue-engineering and systems biology methods shows promise to reveal novel insights into paracrine mediators of ECC and their underlying mechanisms of action, ultimately leading to improved cell-based therapies for patients with heart disease. (Circ Res. 2018;122:167-183. DOI: 10.1161/CIRCRESAHA.117.311589.)

Key Words: cardiomyocytes ■ endothelial cells ■ fibroblasts ■ stem cells ■ systems biology ■ tissue engineering

Cardiomyocyte excitation–contraction coupling (ECC) is the crucial process that links calcium (Ca²⁺) trafficking to active force generation and subsequent relaxation of the cardiomyocyte. After suprathreshold myocyte excitation, Ca²⁺ rapidly influxes into the myocyte cytosol through depolarization-activated inward Ca²⁺ channels. This Ca²⁺ influx induces Ca²⁺ release from the sarcoplasmic reticulum; together, these mechanisms raise free cytosolic Ca²⁺ concentration, increasing the probability of Ca²⁺–troponin C binding and thus activating the contractile machinery. In normal physiology, a fine-tuned percentage of cytosolic Ca²⁺ is then pumped back into the sarcoplasmic reticulum reservoir for future calcium-induced calcium release. A simplified overview of this highly orchestrated ECC process is shown in Figure 1.

Sympathetic β-adrenergic signaling is a well-established rapid inotropic and lusitropic modifier of ECC. Briefly, β-adrenergic receptor stimulation of Gs type G-protein–coupled receptors activates adenylyl cyclase to form cAMP. cAMP subsequently activates protein kinases that phosphorylate various proteins essential to electrophysiology, calcium handling, and, thus, ECC, including the L-type calcium channel, phospholamban, ryanodine receptor, troponin I, and myosin-binding protein C (Figure 1). A comprehensive review of sympathetic activation effects on ECC is available elsewhere.

Cardiomyocyte ECC and associated β-adrenergic signaling can be further modulated by factors secreted by both distant and nearby cells. For example, chemokine CXCL12, also known as stromal cell–derived factor, can activate its receptor CXCR4 to attenuate β-adrenergic–mediated increases in adult...
cardiomyocyte calcium handling and fractional shortening. In addition, it is well established that cardiac endothelial cells secrete certain paracrine factors (eg, NO) that can act as physiological signaling mediators of myocyte ECC and compete with β-adrenergic signaling pathways. Recent work has demonstrated that several other neighboring physiological and pathological noncardiomyocytes in the heart, as well as experimental cardiotherapeutic cells, are capable of modulating cardiomyocyte ECC by paracrine signaling mechanisms. Importantly, these effects are relatively slower than previously described β-adrenergic signaling, likely reflecting underlying cardiomyocyte remodeling. Note that although cardiomyocytes are also known to release cardioactive paracrine factors, their mechanisms of action are largely independent of direct ECC modulation and are thus beyond the scope of this review; for more details, see elsewhere.

Paracrine Effects of Cardiac Endothelial Cells on Cardiomyocyte ECC

Cardiac endothelial cells, including but not limited to endocardial and intramyocardial capillary endothelial cells, regulate cardiac function and are well-established paracrine mediators of myocyte ECC; they release a vast array of cardioactive factors, including NO, endothelin-1, prostaglandins, natriuretic peptides, angiotensin II, kinins, reactive oxygen species, adenylnucleotides, neuregulin-1, parathyroid hormone–related peptide, and others. Two major ECC factors, NO and endothelin-1, are discussed below. A schematic summarizing these mediators and their effects on cardiomyocyte ECC is shown in Figure 2.

Endothelial NO

NO is reported to have biphasic inotropic effects on basal contractility, where only low doses increase basal contractility. For example, various ex vivo studies on isolated perfused rat hearts and cardiac myocytes demonstrated that low levels of NO modestly increase basal myocardial contractility, while higher doses inhibit it. Therefore, the balance between NO production and degradation by NO synthases (NOS) determines the overall cardiac response to NO. Prolonged exposure to high levels of NO can lead to apoptosis and necrosis of cardiomyocytes, whereas moderate levels of NO are necessary for normal cardiac function.

In this review, we will first discuss both established and unresolved key ECC-related paracrine factors secreted by endothelial cells, fibroblasts, myofibroblasts, cardiac progenitor cells (CPCs), and mesenchymal stem cells (MSCs), together representing neighboring physiological, pathological, and therapeutic paracrine mediators of cardiomyocyte ECC (for a summary of neighboring noncardiomyocyte paracrine effects on cardiomyocyte ECC, see Table). Next, we provide a case example to demonstrate the promise of tissue-engineering approaches to study noncardiomyocyte paracrine effects on contractile function and associated gene expression. Finally, we discuss systems biology approaches to help identify key cardiomyocyte ECC paracrine mediators of cardiotherapeutic cells, the least studied cell types in this context.

Figure 1. β-Adrenergic signaling and cardiomyocyte excitation–contraction coupling. β-Adrenergic receptor (β-AR) activation by binding of epinephrine/norepinephrine leads to the following signaling cascade: (1) activation of the GTP-binding protein α, (2) stimulation of adenylyl cyclase (AC), and (3) an increase in cAMP. Increase in intracellular cAMP leads to protein kinase A (PKA) activation, which subsequently phosphorylates and thus increases ion flux through the L-type Ca²⁺ channel and ryanodine receptor (RyR). Additionally, PKA relieves phospholamban (PLB) inhibition on sarcoplasmic reticulum Ca²⁺-ATPase (SERCA), allowing for an increase in Ca²⁺ storage within the sarcoplasmic reticulum (SR). In normal physiology, SERCA removes ≈70% of the activator cytosolic calcium (Ca²⁺) during diastole. Finally, PKA phosphorylates troponin I, leading to positive lusitropic effects via myofilament Ca²⁺ desensitization. Blue and red arrows denote calcium flow during systole and diastole, respectively.

Table 1. Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>APD</td>
<td>action potential duration</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium</td>
</tr>
<tr>
<td>CPC</td>
<td>cardiac progenitor cell</td>
</tr>
<tr>
<td>DF</td>
<td>developed force</td>
</tr>
<tr>
<td>ECC</td>
<td>excitation–contraction coupling</td>
</tr>
<tr>
<td>hACF CdM</td>
<td>human adult cardiac fibroblast conditioned media</td>
</tr>
<tr>
<td>hECT</td>
<td>human engineered cardiac tissue</td>
</tr>
<tr>
<td>HFF CdM</td>
<td>human foreskin fibroblast conditioned media</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin-like growth factor 1</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal stem cell</td>
</tr>
<tr>
<td>PLSR</td>
<td>partial least squares regression</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarcoplasmic reticulum Ca²⁺-ATPase</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor β</td>
</tr>
</tbody>
</table>

Note: The table lists key abbreviations and acronyms used throughout the text.
Studies have shown that the positive inotropic effect of endothelin receptors expressed on cardiomyocytes is predominantly attributed to enhanced Ca$^{2+}$ entry, increased myofilament sensitivity to cytosolic Ca$^{2+}$, and enhanced calcium-induced calcium release. Positive inotropic effects across various species via activation of endothelin receptors expressed on cardiomyocytes. Studies have shown that the positive inotropic effect of endothelin-1 is predominantly attributed to enhanced Ca$^{2+}$ entry, increased myofilament sensitivity to cytosolic Ca$^{2+}$, and enhanced calcium-induced calcium release.

Mechanistically, endothelin-1–mediated enhancement of Ca$^{2+}$ entry results from (1) activation of Na$^+$/Ca$^{2+}$ exchanger and Ca$^{2+}$-induced calcium release. 

**Table. Brief Summary of Noncardiomyocyte Paracrine Effects on Cardiomyocyte ECC**

<table>
<thead>
<tr>
<th>Source</th>
<th>Likely Paracrine Mediator(s)</th>
<th>Paracrine-Mediated Effect on ECC</th>
<th>Potential Mechanisms of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelial cell</td>
<td>NO</td>
<td>↑ basal myocardial contractility</td>
<td>LTCC$^{17}$ and RyR2 nitrosylation.$^{11,18}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Attenuate β-adrenergic inotropic effects</td>
<td>cGMP-dependent degradation of cAMP via PDEII$^{19,20}$; PKG-mediated decrease of LTCC activity.$^{21,22}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Positive lusitropy</td>
<td>cGMP-dependent PKG phosphorylation of troponin I.$^{23-24}$</td>
</tr>
<tr>
<td>Endothelin-1</td>
<td></td>
<td>↑ Ca$^{2+}$ entry</td>
<td>PKC-mediated activation of NCX forward/reverse modes$^{25-29}$; PKG-mediated activation of LTCC.$^{30-35}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alter myofilament Ca$^{2+}$ sensitivity</td>
<td>PKC/D-dependent phosphorylation of troponin I and myosin-binding protein C.$^{36-40}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ calcium-induced calcium release</td>
<td>IP$_3$-induced calcium release from the SR via IP$_3$R, which subsequently sensitizes RyR2.$^{41,44}$</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>TGF-β</td>
<td>Hyper trophy$^{44,45}$; ↑ resting membrane potential$^{46}$; ↓ conduction/upstroke velocity$^{46}$; ↑ calcium transient amplitude$^{46}$; ↑ contractility (see Figure 4 and elsewhere$^{46}$)</td>
<td>Noncanonical, non-Smad TGF-β MEK/ERK signaling pathway$^{46}$; Na$^+$ and K$^+$ ion channel remodeling (see Figure 5 and elsewhere$^{46}$)</td>
</tr>
<tr>
<td></td>
<td>Exosomal miRNA-21-3p</td>
<td>Hyper trophy$^{44}$</td>
<td>Targeting the mediators sorbin and SH3 domain-containing protein 2, as well as PDZ and LIM domain 5$^{46}$</td>
</tr>
<tr>
<td>Myofibroblast</td>
<td>TGF-β</td>
<td>Hyper trophy$^{44}$; ↓ calcium transient amplitude$^{47}$</td>
<td>TGF-β signaling$^{47}$</td>
</tr>
<tr>
<td>CPC</td>
<td>Unresolved</td>
<td>↑ contractility$^{40}$</td>
<td>↑ SERCA/NCX ratio$^{40}$</td>
</tr>
<tr>
<td>MSC</td>
<td>Unresolved</td>
<td>↑ action potential duration$^{51}$; ↑ calcium transient amplitude$^{25,51}$; ↑ contractility$^{25,54}$; ↓ calcium transient alternans$^{25,54}$; predominantly anti-arrhythmic effects$^{25,54,57,58}$</td>
<td>PI3K/Akt signaling$^{25,54}$; increase in SERCA and LTCC activity/gene expression$^{25,54}$</td>
</tr>
</tbody>
</table>

IP$_3$ indicates inositol trisphosphate; IP$_3$R, inositol trisphosphate receptor; LTCC, L-type calcium channel; NCX, sodium–calcium exchanger; PDEII, phosphodiesterase II; PK, protein kinase; RyR2, ryanodine receptor; SERCA, sarco/endoplasmic reticulum Ca$^{2+}$-ATPase; SR, sarcoplasmic reticulum; and TGF-β, transforming growth factor β.

**Endothelial Endothelin-1**

Endothelin-1 is a peptide that is a potent vasoconstrictor. In addition to its vascular effects, endothelin-1 has demonstrated positive inotropic effects across various species via activation of endothelin receptors expressed on cardiomyocytes. Studies have shown that the positive inotropic effect of endothelin-1 is predominantly attributed to enhanced Ca$^{2+}$ entry, increased myofilament sensitivity to cytosolic Ca$^{2+}$, and enhanced calcium-induced calcium release.

Mechanistically, endothelin-1–mediated enhancement of Ca$^{2+}$ entry results from (1) activation of Na$^+$/Ca$^{2+}$ exchanger forward and reverse modes via protein kinase C downstream of the endothelin A receptor$^{25-28}$ and (2) indirect activation of the Na$^+$/Ca$^{2+}$ exchanger reverse mode by protein kinase C–dependent increase of Na$^+$–H$^+$ exchanger activity, effectively increasing intracellular Na$^+$ and thus driving the Na$^+$/Ca$^{2+}$ exchanger to increase intracellular calcium.$^{25,30}$ Other mechanisms, such as endothelin-1 activation of L-type calcium channel current, remain controversial$^{11-34}$ but are also likely mediated via protein kinase C downstream of the endothelin receptor.$^{35}$

The increased extrusion of H$^+$ via the Na$^+$–H$^+$ exchanger was originally hypothesized to affect myofilament calcium sensitivity via cytosolic alkalization.$^{76}$ However, this was later debunked because the bicarbonate anion transporter largely compensates for changes in cytosolic H$^+$ concentration.$^{76}$ Instead, endothelin-1 likely alters myofilament sensitivity by protein kinase C/D–dependent phosphorylation of troponin I and myosin-binding protein C.$^{36-40}$

Finally, endothelin-1 can stimulate inositol trisphosphate–induced calcium release from the sarcoplasmic reticulum of ventricular myocytes via inositol trisphosphate receptors, which can subsequently sensitize ryanodine receptors.$^{41-43}$ However, these mechanisms are not considered large contributors to endothelin-1 inotropic effects on ventricular myocytes, which are known to have low abundance of inositol trisphosphate receptors.$^{76,77}$

**Paracrine Effects of Cardiac Fibroblasts on Myocyte ECC**

Conventionally, healthy cardiac tissue is believed to have a cellular composition that is up to two thirds nonmyocytes, the
majority of which are cardiac fibroblasts. Interestingly, Pinto et al. recently demonstrated that the number of fibroblasts might not be so high—potentially even less than myocytes—leaving this issue unresolved. Nevertheless, although under normal physiological conditions fibroblasts are key to maintaining homeostasis of myocardial structure and function, they are also activated in pathological wound healing, leading to tissue remodeling and fibrosis. The maladaptive remodeling of fibrotic scar formations then forms obstacles at the tissue level that interfere with normal electric propagation and is considered proarrhythmic. Such pathophysiological responses have motivated genetic modification of unexcitable primary human fibroblasts to create engineered cells that can conduct action potentials and rescue conduction slowing in a cell culture model of fibrosis. In general, cardiac fibroblasts are capable of expressing gap junctions and coupling to cardiomyocytes (see elsewhere for a comprehensive review) and communicating via paracrine signaling.

As described below, physiological fibroblasts and pathological myofibroblasts have been found to alter myocyte size, electrophysiology, and calcium handling, thus influencing ECC at both cellular and tissue levels via paracrine signaling mechanisms.

Figure 2. Endothelial–cardiomyocyte interplay through paracrine factors. Endothelial cell NO increases basal contractility via nitrosylation of L-type Ca²⁺ channel (LTCC) and ryanodine receptor (RyR2). NO attenuates β-adrenergic effects on cardiomyocyte excitation–contraction coupling via cGMP-dependent degradation of cAMP and protein kinase G (PKG)-mediated decrease of LTCC activity. PKG also phosphorylates troponin I, leading to myofilament calcium desensitization and thus increased lusitropy. Endothelin-1, which mainly acts through the endothelin A (ETₐ) receptor in ventricular cardiomyocytes, may increase calcium entry via protein kinase C (PKC)-mediated (1) increase of LTCC activity, (2) indirect activation of sodium–calcium exchanger (NCX) reverse mode by increasing Na⁺⁻H⁺ exchanger activity, and (3) direct activation of NCX reverse (shown) and forward (not shown) mode. Endothelin-1 alters myofilament Ca²⁺ sensitivity via protein kinase C/D (PKC/D) phosphorylation of troponin I and myosin-binding protein C. Finally, endothelin-1 may increase calcium-induced calcium release via inositol trisphosphate (IP₃) activation of inositol trisphosphate receptor (IP₃R), which sensitizes RyR2 on the sarcoplasmic reticulum (SR). Green and red arrows denote activation and inhibition, respectively. PLB indicates phospholamban; and SERCA, sarcoendoplasmic reticulum Ca²⁺-ATPase.
**Paracrine Effects on Cardiomyocyte EC Coupling**

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**Fibroblast-Derived Soluble Factors Can Mediate Cardiomyocyte Hypertrophy**

In addition to their role in fibrosis, cardiac fibroblasts may influence the cardiac hypertrophic response. Indeed, a hypertrophic non–myocyte-derived growth factor released by fibroblasts was hypothesized by Long et al over 20 years ago. Since then, various soluble factors—such as angiotensin II, TGF-β (transforming growth factor β), endothelin-1, IGF-1 (insulin-like growth factor 1), and interleukin 6—have been suggested as fibroblast paracrine mediators of hypertrophy.

Angiotensin II was one of the initial fibroblast paracrine factors hypothesized to induce hypertrophy in myocytes. Later work then demonstrated that fibroblasts are required to be present for angiotensin II–dependent hypertrophic effects, motivating investigators to study downstream paracrine mediators released by angiotensin II–stimulated fibroblasts to induce myocyte hypertrophy.

For example, independent of its profibrotic effects, TGF-β has been demonstrated to induce cardiac hypertrophy both in vitro and in vivo. Rosenkranz et al demonstrated that overexpression of TGF-β in transgenic mice led to increased hypertrophy. Importantly, others showed stretched or angiotensin II–stimulated fibroblast conditioned media neutralized of TGF-β circumvents the cultured myocyte hypertrophic responses, suggesting that TGF-β is required for fibroblast paracrine-mediated effects on myocyte hypertrophy. Using a similar approach, fibroblast-released endothelin-1 has also been suggested to induce hypertrophy. Conditioned media from fibroblasts stimulated with cyclic stretching or angiotensin II did not induce hypertrophy in myocytes treated with endothelin receptor antagonists.

Other paracrine factors have also been suggested to stimulate cardiac myocyte hypertrophy; neutralizing cardiortropin-1—a member of the interleukin 6 family—or IGF-1 also attenuated hypertrophic effects of fibroblast conditioned media. Takeda et al further demonstrated that IGF-1 acts downstream of Krüppel-like factor 5 to induce such hypertrophic responses, suggesting that TGF-β is required for fibroblast paracrine-mediated effects on myocyte hypertrophy. Using a similar approach, fibroblast-released endothelin-1 has also been suggested to induce hypertrophy. Conditioned media from fibroblasts stimulated with cyclic stretching or angiotensin II did not induce hypertrophy in myocytes treated with endothelin receptor antagonists.

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**Fibroblast-Derived Insoluble Extracellular Vesicles Mediate Cardiomyocyte Hypertrophy**

Recently, studies have shown that cell–cell communication can also take place via secreted exosomes, which are bilayer membrane-bound nanovesicles packaging cargoes of select lipids, proteins, and RNAs. In the context of cardiovascular disease, various resident heart cells and therapeutic stem cells have been shown to communicate with myocytes and myocardial tissue via exosomes, predominately involved in cardioprotective, proangiogenic, and antifibrotic effects. However, recently, it was shown that exosomes are also potential mediators of hypertrophy. Bang et al observed that cardiac fibroblasts release exosomal miRNA passenger strand miRNA-21-3p to induce cardiomyocyte hypertrophy in vitro. Mechanistically, they identified sorbin and SH3 domain-containing protein 2, as well as PDZ and LIM domain 5 as the main targets and mediators of miRNA-21-3p–induced hypertrophy. Their findings translated in vivo, whereby microinjection of antagoniRNA-21-3p was found to attenuate angiotensin II–induced hypertrophy in mice. Thus, it is possible that beyond secreted proteins, cardiac fibroblast–derived exosomes may also be involved in the cardiac response to stress.

**Fibroblast Paracrine Factor Effects on Electrophysiology**

An established electrophysiological phenotype of cardiac hypertrophy is prolonged action potential duration (APD) via reduction of voltage-gated potassium channel currents. Thus, it is plausible that hypertrophy–inducing fibroblast conditioned media could also alter myocyte electrophysiology. Indeed, Guo et al first demonstrated with neonatal rat cardiac myocytes in vitro that 72-hour treatment with fibroblast conditioned media not only induced hypertrophy but also prolonged APD at 25% repolarization and decreased transient outward current density. Nearly a decade later, electrophysiological remodeling effects of fibroblast conditioned media were further elucidated. Pedrotty et al demonstrated various consistent single-cell and tissue-level pathological, dose-dependent, electrophysiological effects of cardiac fibroblasts on neonatal rat cardiomyocytes, including (1) decreased monolayer conduction velocity; (2) prolongation of APD; (3) increased resting membrane potential; and (4) decreased upstroke velocity. These effects were likely caused by ion channel remodeling because inward rectifying potassium, sodium, and transient outward potassium channel genes were downregulated while there were no significant changes in fibroblast proliferation, cardiomyocyte apoptosis, or connexin–43 function. Interestingly, these effects were not only dose dependent but also microenvironment dependent because they were not reproduced when the treatment used conditioned media from cardiac myocyte–fibroblast cocultures.

Such observations of cardiac fibroblast conditioned media on the electrophysiological properties of cardiomyocytes are not limited to neonatal rat cardiac myocytes; indeed, they have been reported consistently across several myocyte sources. With adult rat cardiomyocytes, fibroblast conditioned media was shown to decrease peak transient outward current, lengthen APD, and decrease sodium channel current. Adult mouse cardiac fibroblast conditioned media was found to prolong APD to 50% repolarization, increase peak notch action potential voltage (a possible surrogate of transient outward current activity), and decrease beat rate of mouse embryonic stem cell–derived cardiomyocytes.

The fibroblast–released factors mediating such electrical remodeling remain unresolved. Guo et al demonstrated that fibroblast–released IGF-1 may play a role in hypertrophy but not ion channel remodeling. They observed that anti–IGF-1 antibody–treated fibroblast conditioned media partially inhibited myocyte hypertrophy, whereas transient outward ion channel activity was unaffected relative to the fibroblast conditioned media group. Although TGF-β has been shown to induce hypertrophy (as discussed above), it is also a valid...
candidate as a mediator of the electrophysiological effects of cardiac fibroblast conditioned media, as Kaur et al. showed that TGF-β was responsible for ion channel remodeling in adult rat cardiomyocytes. Such effects could potentially contribute to the electrical remodeling that typically accompanies myocardial injury. Additionally, the findings of Kaur et al. were consistent with other studies examining exogenous TGF-β, which has been found to (1) reduce sodium, inward rectifier potassium, and outward sustained potassium currents in neonatal rat atrial cardiomyocytes, (2) increase and decrease sodium and transient outward potassium currents, respectively, in adult rat cardiomyocytes, and (3) prolong APD in adult rat cardiomyocytes. One important area for future work involves testing the extension of these findings in large animal and human cardiomyocytes, as we begin to investigate below.

**Fibroblast-Derived Paracrine Mediators of ECC and Contractility**

Recently, fibroblast paracrine factors have also been shown to also alter calcium handling—a direct determinant of ECC—and cardiac tissue contractility. Motivated by the hypertrophy and electrophysiology studies described above, Cartledge et al. sought to further elucidate the role of physiological fibroblasts and pathological myofibroblasts on adult rat myocyte viability, size, and calcium transients. Using Transwells to prevent direct cell–cell contact, they showed that physiological cardiac fibroblasts and pathological cardiac myofibroblasts both secrete paracrine factors that increase myocyte size, accelerate calcium transient decay, and decrease myocyte viability. However, effects on myocyte calcium transients were fibroblast phenotype-dependent: physiological fibroblast paracrine factors increased the myocyte calcium transient amplitude, whereas pathological myofibroblast paracrine factors decreased the calcium transient amplitude. Interestingly, Cartledge et al. observed that TGF-β levels increased in Transwells containing myocytes with either fibroblasts or myofibroblasts relative to myocyte-only cultures.

To test the role of TGF-β in fibroblast paracrine-mediated effects on myocyte calcium handling, viability, and hypertrophy, they used SB431542 to block TGF-β type 1 receptors. Blockage of the TGF-β type 1 receptor prevented the previously described effects on cardiomyocyte viability, size, and calcium transient amplitude.

Liau et al. recently extended this work to investigate developmental stage-dependent effects of cardiac fibroblast conditioned media on contraction. Specifically, mouse embryonic stem cell-derived–cardiomyocyte miniature tissue micropatches were supplemented with conditioned media from adult or fetal cardiac fibroblasts. Adult cardiac fibroblast conditioned media significantly increased contraction of the mouse micropatches; these effects were amplified with fetal cardiac fibroblast conditioned media. Using pathway inhibitor experiments and Western blot analysis, they concluded these effects to be mediated via MEK-ERK signaling, which is a non-Smad pathway in TGF-β signaling. Altogether, these findings suggest that TGF-β is a likely mediator of cardiac fibroblast paracrine-mediating effects on contraction via ion channel remodeling and hypertrophy.

**Stem Cell Secretomes Can Impact Cardiomyocyte ECC**

Resident adult cardiac stem cells—which include CPCs, endothelial progenitor cells, and MSCs—also have the potential to increase cardiomyocyte ECC through paracrine mechanisms. For example, Tang et al. demonstrated that cell-mimicking synthetic microparticles carrying the human cardiac stem cell secretome promoted neonatal rat cardiomyocyte contractility in vitro. Delivery of CPCs and bone marrow–derived MSCs are emerging approaches to treat heart disease. Like several other stem cell sources (eg, embryonic and induced pluripotent stem cells, CD34+ stem cells, etc), CPC and MSC paracrine factors have been shown to enhance angiogenesis, decrease fibrosis, and promote other cardiac repair mechanisms (comprehensive reviews on this topic are available elsewhere), which can indirectly modulate cardiomyocyte contractility. Moreover, CPC and MSC paracrine factors have each shown the potential to more directly alter myocyte electrophysiology and calcium handling and, thus, ECC.

**Figure 3.** Human engineered cardiac tissue (hECT) contractility assay. A, hECTs are created, cultured, and tested in a custom bioreactor with integrated force-sensing end-posts; as the tissue beats, deflections of the end-posts are tracked. Output contractile metrics include, but are not limited to, developed force (DF), maximum rates of contraction and relaxation (+/− df/dt, respectively), and beat rate. B, Confocal microscopy of hECTs labeled with cardiac troponin I (green) and DAPI (4',6-diamidino-2-phenylindole; blue) displays cardiomyocytes with striated sarcomeres and regions of aligned myofilibrils. Inset shows magnified view of registered sarcomeres. C, hECT labeled with sarcoplasmonic reticulum Ca²⁺-ATPase 2 (red) and DAPI (blue) shows sarcoplasmic reticulum structures distributed throughout the tissue. Bar = 40 μm.
As depicted in the Table, CPC and MSC paracrine effects on cardiomyocyte ECC are primarily associated with increasing expression of calcium-handling genes. In comparison to nearly instantaneous β-adrenergic signaling mechanisms, paracrine mechanisms are expected to be slower and longer-lasting. Indeed, DeSantiago et al demonstrated that MSC paracrine effects on ECC take at least 5 minutes in isolated ventricular myocytes, and in our recent work, such in vitro effects of MSCs were evident at least 5 days post-treatment.

Clearly, a better understanding of the kinetics of stem cell paracrine effects is warranted to optimize future stem cell cardiotherapies for both ischemic and nonischemic cardiomyopathies. To do so, further insight is required into CPC and

Figure 4. Effects of human adult cardiac fibroblast (hACF) conditioned media (CdM) on human engineered cardiac tissue (hECT) contractile function. A, Contractility assay shows hECT developed force during 0.5-Hz pacing (mean±SEM, n=4–6) at pretreatment (day 5) and 5 d post-treatment with serum-free defined media (SFDM, control), hACF CdM, and HFF CdM. Daily measurements of (B) developed force and (C) beat rate during spontaneous contractions for each group (mean±SEM, n=3–4). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; comparison of time point relative to pretreatment in same group; †P<0.05, ††P<0.01, comparison of given group relative to human foreskin fibroblast (HFF) CdM at same time point; †††P<0.05, ††††P<0.01, comparison of same group at 2 different time points. P values from repeated measures obtained using ANOVA followed by Bonferroni multiple comparisons test.

Figure 5. Molecular characterization of human engineered cardiac tissues (hECTs). hECTs from each group (n=3–4, mean±SEM) were snap-frozen for real-time quantitative polymerase chain reaction on day 10, where expression of (A) cardiac-specific/calcium handling, (B) apoptotic, and (C) potassium/sodium channel genes were quantified. *P<0.05, **P<0.01; P values from 1-way ANOVA with Scheffe post hoc test. ANF indicates atrial natriuretic peptide; BAX, BCL2-associated X protein; BCL2, B-cell lymphoma 2; Casp3, caspase-3; Casp9, caspase-9; cTnT, cardiac troponin T; Cx43, connexin-43; Kir2.1, inward rectifier potassium current; Kv4.2, transient outward potassium current; Kv11.1, delayed rectifier potassium current; LTCC, L-type calcium channel; MHC, myosin heavy chain; Nav1.5, sodium current; and SERCA2a, sarco/endoplasmic reticulum calcium-ATPase.

**Kinetics of Stem Cell Paracrine Effects on ECC**

As depicted in the Table, CPC and MSC paracrine effects on cardiomyocyte ECC are primarily associated with increasing expression of calcium-handling genes. In comparison to nearly instantaneous β-adrenergic signaling mechanisms, paracrine mechanisms are expected to be slower and longer-lasting. Indeed, DeSantiago et al demonstrated that MSC paracrine effects on ECC take at least 5 minutes in isolated ventricular myocytes, and in our recent work, such in vitro effects of MSCs were evident at least 5 days post-treatment.

Clearly, a better understanding of the kinetics of stem cell paracrine effects is warranted to optimize future stem cell cardiotherapies for both ischemic and nonischemic cardiomyopathies. To do so, further insight is required into CPC and
MSC paracrine effects on ECC; current knowledge on each of these respective fields is discussed below.

CPC Paracrine Effects on Cardiomyocyte ECC
Few studies have investigated CPC paracrine effects on cardiomyocyte ECC. Maxeiner et al. observed that human and rat CPC conditioned media increased fractional shortening in isolated rat adult cardiomyocytes in a concentration-dependent manner relative to pure media control treatment, largely independent of CPC donor age. In addition, conditioned media derived from several human adult and juvenile CPC lines led to an increase in the ratio of sarcoendoplasmic reticulum Ca²⁺-ATPase (SERCA)/sodium–calcium exchanger gene expression. Interestingly, increases in contractile function were most prominent when the conditioned media was highly diluted, with further investigation required to understand these phenomena.

Several preliminary attempts have been made to identify which CPC-secreted factors may impact myocyte ECC. For example, in the same study, Maxeiner et al. performed cytokine arrays on released soluble factors by rat and human CPCs; the interleukin family, vascular endothelial growth factor, and tissue inhibitors of metalloproteinases-1 were consistently up-regulated in both juvenile and adult rat and human CPCs. Considering other components of the CPC secretome, a series of studies by Gray et al. and Agarwal et al. observed that the insoluble exosomal fraction of CPC conditioned media led to no significant effect on rat ventricular myocyte calcium transient amplitude and sarcomere shortening at 12 hours post-treatment. It remains undetermined whether there may be a time or species dependence to these observations. Clearly, further investigation is necessary to better understand mechanisms and cargo involved in CPC paracrine-mediated effects on cardiomyocyte ECC. As discussed below, paracrine effects of MSCs on ECC have been more extensively studied.

MSC Paracrine Effects on Electrophysiology
The electrophysiological effects of MSC paracrine factors seem to vary across species and experimental conditions. Mureli et al. first showed that mouse MSC conditioned media has a positive dromotropic effect on healthy atrial-like murine HL-1 monolayers by increasing connexin-43 mRNA expression and protein level. Hwang et al. found increased total connexin-43 protein levels significantly increased in hypoxic rat myocytes exposed to hypoxic human MSC (hMSC) conditioned media (exposure to normoxic hMSC conditioned media trended toward increased levels, with no statistical significance). However, these findings were not consistent with healthy ventricular myocytes using hMSC conditioned media, suggesting the effects of hMSC conditioned media may depend on cell phenotype and environment. Askar et al. showed with Transwell experiments that hMSC conditioned media prolonged healthy neonatal rat cardiomyocyte APD in a dose-dependent manner but did not affect conduction velocity in myocyte monolayers. Comparable to the 5% to 10% hMSC supplementation (percent per number of cardiomyocytes and fibroblasts in engineered cardiac tissue) used in our recent experiments, Askar et al. observed that dosages of 7% hMSC conditioned media (percent per number of myocytes in 2-dimensional substrate) led to significant effects on cardiomyocyte electrophysiology. Dose-dependent prolongation of APD led to increased APD dispersion and vulnerability of reentry in the setting of healthy neonatal rat cardiomyocyte monolayers. On the contrary, in hydrogen peroxide–treated human induced pluripotent stem cell–derived cardiac myocyte monolayers, hMSC conditioned media significantly decreased action potential alternans relative to control.

In a recent study, silico findings from mathematical models provide novel insights that help resolve disparate reports of potential proarrhythmic risks of hMSCs in vitro that appear contradictory to in vivo reports of hMSCs having no effect, or favorable cardioprotective effects, on arrhythmogenesis in preclinical animal studies and clinical trials. In simulations representing healthy myocyte monolayers (ie, similar to experimental conditions from Askar et al.), both hMSC paracrine signaling and heterocellular coupling have the potential to increase arrhythmogenicity (Online Figure IA; see Mayourian et al. for details). However, in vulnerable window simulations of fibrotic cardiac tissue (ie, similar to preclinical/clinical conditions for hMSC intervention), hMSC paracrine signaling-only conditions were antiarrhythmic compared with control (Online Figure IB). Consistent with a preclinical study in a rat myocardial infarction model, where hMSC paracrine factors were antiarrhythmic, suppressed fibrosis, and restored conduction. Most importantly, in silico vulnerable window analyses predicted that hMSC supplementation (involving both hMSC paracrine signaling and heterocellular coupling mechanisms) did not adversely impact fibrotic cardiac tissue arrhythmogenesis and may even be antiarrhythmic (Online Figure IB). These simulations may help explain why hMSCs are mainly reported to be safe or even antiarrhythmic in the clinical trial setting where paracrine effects can be present despite low cell engraftment efficiency.

Although our simulations may suggest maximizing hMSC engraftment increases arrhythmogenic risk, it is important to note that several in vitro studies demonstrate that hMSCs have the potential to repair conduction block in neonatal rat cardiomyocyte cultures by filling conduction gaps via connexin-mediated heterocellular coupling. To our knowledge, this experimental preparation has yet to be simulated; the potentially important implications in post–myocardial infarction tissue warrant further investigation.

MSC Paracrine Effects on Calcium Handling
The effects of MSC paracrine factors on calcium handling are observed across several studies that look at healthy and injured myocytes. DeSantiago et al. first showed that hMSC conditioned Tyrode-treated healthy mouse cardiomyocytes had increased calcium transient amplitude and accelerated calcium transient decay compared with control. Beneficial effects of hMSC paracrine factors on calcium handling were found in injured myocytes, where both normoxic and hypoxic hMSC conditioned media suppressed effects of hypoxia on neonatal rat ventricular myocyte cytosolic free calcium levels. Sattayapraser et al. further showed that hMSC conditioned media reduces calcium transient alternans and shortens calcium transient duration in hydrogen peroxide–treated human
induced pluripotent stem cell–derived cardiac myocyte monolayers. Similarly, mouse MSC conditioned media attenuated the occurrence of arrhythmic calcium transients of ischemia/reperfusion-injured isolated ventricular myocytes.56 Thus, across species, hMSC conditioned media seems to have favorable effects on calcium handling in cultured cardiomyocytes.

**MSC Paracrine-Mediated Effects of Cardiac Contractile Function**

In our most recent work, we demonstrated that the effects of hMSC paracrine factors on calcium handling translated to increased contractility in both computer model simulations and our custom human engineered cardiac tissue (hECT) system.54 We simulated ECC of human induced pluripotent stem cell–derived cardiomyocytes—representative of hECT cellular constituents—subjected to experimentally calibrated hMSC heterocellular coupling128 and paracrine signaling effects.54 We then compared the in silico results to hECT contractility measurements under matched hMSC treatments. The in silico and hECT data together corroborate the paracrine-mediated effects of hMSCs on cardiomyocyte contractility.54 Interestingly, when hMSCs were cocultured within hETCs, the hMSC paracrine-mediated increase on contractility was attenuated41; our simulations predicted that underlying myocyte–hMSC heterocellular coupling may act as a sink and reduce calcium transient amplitude and thus decrease contraction.54 A similar phenomenon was recently reported by Aratyn-Schaus et al129 as neonatal cardiomyocytes coupled with stem cell–derived cardiomyocytes led to calcium transients and fractional shortening intermediate between the 2 cell types. Clearly, further investigation is needed to better understand direct cell–cell contact effects on cardiomyocyte ECC.

The previously described findings of MSC paracrine effects on contractility were consistent with a pair of studies by DeSantiago et al,52,56 where MSC-conditioned Tyrodes solution increased cell shortening in healthy and injured mouse myocytes. Furthermore, they complement the findings by Luo et al,130 where hMSC-secreted factors packaged into poly(lactic-co-glycolic acid) microparticles coated with the hMSC membrane promoted neonatal rat cardiomyocyte contraction in vitro. These positive inotropic effects of the hMSC secretome motivate efforts to (1) investigate the underlying mechanisms and (2) identify the responsible cardioactive factors.

**Underlying Mechanisms of MSC Paracrine Effects on ECC**

Substantial progress has been made toward elucidating the underlying mechanisms responsible for MSC paracrine effects on electrophysiology and calcium handling. Hwang et al53 observed that hypoxic rat myocytes treated with normoxic hMSC conditioned media trended toward increased mRNA levels of L-type calcium channel and SERCA2a relative to control, with statistical significance achieved when hMSCs were cultured under hypoxic conditions. We recently demonstrated that treating hETCs with hMSC conditioned media also led to increased mRNA levels of L-type calcium channel and SERCA2a relative to untreated control54; additionally, DeSantiago et al52 found that the activity of L-type calcium channel and SERCA2a activity increases in healthy mouse myocytes exposed to hMSC paracrine factors relative to control.

DeSantiago et al52 were one of the first groups to find that the PI3K/Akt signaling pathway was activated in mouse ventricular myocytes by MSC conditioned media. Through proteomic analysis, we also showed recently that PI3K/Akt signaling is predicted to be activated in hECT/hMSC conditioned media.54 This signaling pathway was also implicated in MSC paracrine-mediated cardioprotection from ischemia/reperfusion injury in vitro, where hMSC conditioned Tyrode solution attenuated the hyperpolarization of the mitochondrial membrane potential via PI3K/Akt signaling,56 prolonging cell survival and reducing arrhythmic early afterdepolarizations. The role of the PI3K/Akt signaling pathway in hMSC conditioned media–mediated ECC enhancement was further defined in human induced pluripotent stem cell–derived cardiomyocyte calcium handling by Sattayaprasert et al,59 where inhibitors of PI3K and endothelial NO synthase (a downstream mediator of PI3K) attenuated the benefits of hMSC paracrine media on calcium transient alternans and duration, whereas S-nitrosoglutathione, an NO donor, had opposite effects. Taken together, these findings confirm that the PI3K/Akt pathway is a critical mediator of the effects of hMSC paracrine factors on cardiomyocyte ECC.

**Proposed MSC Factors Responsible for Enhancing ECC**

Numerous studies have investigated the composition of the hMSC secretome120,131–136; however, although many secreted factors have been associated with proangiogenic120,137–139 and antifibrotic120,139–141 effects, little is known about the hMSC paracrine factors responsible for the effects on electrophysiology, calcium handling, and contractility described above.

Cardioactive hMSC exosomes have been shown to modulate myocyte PI3K/Akt signaling,100,142 a crucial pathway involved in hMSC paracrine effects on cardiomyocyte ECC (Table). Indeed, we found that treating hETCs with the exosome-depleted fraction of the hMSC secretome recapitulated the effects observed with complete hMSC conditioned media on hECT contractile force and expression of calcium-handling genes (eg, SERCA2a and L-type calcium channel), whereas treatment with the exosome-depleted fraction was similar to unsupplemented controls.54 These findings motivate further investigation into the role of hMSC exosomes, their membrane (although certain exosomal membranes appear necessary for proangiogenic and antifibrotic cardioactivity,101 in general, little is known about their role on cardiomyocyte ECC), and their cargo responsible for increased cardiomyocyte contractility.

hMSC exosomal cargo has been implicated in multiple studies related to cardiac repair99,100,143–145; however, little is known about the specific effects of hMSC exosomes and their cargo on contractility. On the basis of previous literature, exosomal miRNA-21-5p may be one possible candidate responsible for increasing ECC in cardiomyocytes. miRNA-21-5p is the only miRNA consistently in the top 5 most abundantly found in hMSC exosomes across at least 3 independent studies.131,135,146 Interestingly, CPC exosomal miRNA-21-5p has been shown
to prevent cardiomyocyte apoptosis by targeting programmed cell death 4, known to be downstream of the PI3K/Akt signaling pathway.147 This is consistent with the findings by Wei et al148 and Cheng et al,149 where miRNA-21-5p also regulated cardiomyocyte apoptosis by targeting programmed cell death 4. miRNA-21-5p has also been shown to bind strongly to phospholamban150 and to activate endothelial NO synthase,151 both of which increase SERCA2a activity.52,150 Clearly, further work is necessary to elucidate the cardiotoxic role of miRNA-21-5p and other key constituents of the hMSC secretome.

A major challenge in identifying key CPC and hMSC paracrine factors is that the composition of their secretomes strongly depends on the microenvironment. For example, Gray et al151 and Agarwal et al152 showed that the cardioactivity of CPC exosomes increases under hypoxia. Similarly, Hwang et al153 showed that hypoxic conditioning of hMSCs leads to more potent paracrine effects on calcium-handling enhancement, fibrosis reduction, and cardioprotection. Kinnaird et al154 demonstrated that 72 hours of hypoxic preconditioning of hMSCs led to a significant increase in the secretion of proangiogenic factors relative to normoxic control conditions. Furthermore, genetic manipulation of hMSCs155 or molecular preconditioning of hMSCs156 can also increase the potency of their paracrine effects. A systematic investigation using a reliable contractility assay is warranted to optimize these environmental variables and to help identify key cardioactive hMSC paracrine factors.

Tissue-Engineering Approaches to Study Noncardiomyocyte Paracrine Effects on Cardiac Contraction

Human Engineered Cardiac Tissues as an In Vitro Contractility Assay

A major advancement in the cardiac research field has been the growing sophistication of in vitro models of functional human myocardium by combining tissue-engineering technology with human stem cell biology,155–157 as recently reviewed.158–160 In our laboratory, hECTs are created, cultured, and tested using a custom bioreactor161 with integrated force-sensing end-posts. When the tissue beats (either spontaneously or by electrical pacing), deflections of the end-posts are tracked in real time by high-speed video to noninvasively monitor cardiac contractile performance throughout a sequence of twitch cycles (Figure 3A). From the twitch tracings, extracted contractile metrics include, but are not limited to, developed force (DF), maximum rates of contraction and relaxation, and beat rate (Figure 3A, bottom).

These hECTs have been designed to ensure cell viability throughout the full tissue thickness,162,163 with cardiomyocytes that exhibit regions of aligned myofibrils and registered sarcomeres (Figure 3B). With SERCA2 found throughout the tissue (Figure 3C), hECTs recapitulate key aspects of native cardiac muscle physiology including ECC, the Frank–Starling mechanism, and pharmacological responses.162 Such hECTs can be maintained for 4 weeks or longer and are particularly suited for longitudinal in vitro investigation of healthy and diseased human cardiac contractile function and effects of therapeutic intervention. To this end, in the cardiac tissue-engineering field, we are one of the first groups to model familial dilated cardiomyopathy164 and to test stem cell therapies.54,161,165 In the context of this review, our hECT system is advantageous for uniquely providing a configuration of cocultured tissues in a shared media bath to investigate paracrine signaling effects on cardiac contractility. The ability to noninvasively measure contractile function facilitates the collection of multiday longitudinal data on the effects of paracrine factors on contractile function. Altogether, hECTs offer a system that helps bridge a gap in previous experimental models of the heart and provides new opportunities for advancing our understanding of stem cell paracrine signaling and exosome biology.

Indeed, our recent work on hMSC paracrine-mediated effects on hECT contractile function and gene expression recapitulated many findings from murine studies using alternative experimental setups54 but also reveal some interesting species-specific and platform-specific differences. To further demonstrate the use of hECTs for testing paracrine-mediated effects on contractility, we provide new data examining how the effects of the fibroblast secretome on cardiomyocyte contractility and ECC-relevant gene expression translates to human cell sources (for detailed methods, see the Online Data Supplement). After baseline contractile function testing on day 5 using our custom 3-D hECT contractility assay system (Figure 3A), we treated hECTs with (1) serum-free defined media control, (2) fresh human foreskin fibroblast conditioned media (HFF CdM), or (3) fresh human adult cardiac fibroblast conditioned media (hACF CdM). hECTs were cultured an additional 5 days in their respective treatments, after which contractile function was measured and compared with baseline measurements. Spontaneous beat rate and DF were also measured on a daily basis, providing a simple method of monitoring longitudinal contractile function without having to replace culture media (as is normally done after electric pacing to eliminate electrolysis byproducts).

As shown in Figure 4A, the hACF CdM treatment led to a statistically significant increase in DF relative to pretreatment at 0.5-Hz pacing, whereas the HFF CdM and untreated control groups were not significantly different from pretreatment. Furthermore, hACF CdM led to a statistically significant increase in hECT DF relative to HFF CdM at 0.5-Hz pacing 5 days post-treatment (Figure 4A).

To understand the time dependence of treatments, we looked at spontaneous beat rate and DF. As shown in Figure 4B, hACF CdM treatment led to statistically significant increases in DF days 2 to 5 post-treatment relative to day 0 pretreatment, even though beat rate was not significantly different over time in this group (Figure 4C). In the HFF CdM and control groups, beat rate and DF tended to be relatively stable over time (the control group showed a significant decrease in beat rate on day 5 post-treatment, which coincided with a small but significant decrease in DF at this time point). These results complement murine studies on cardiac fibroblast paracrine-mediated contractility effects, as previously described.47,48

These hECT functional measures were corroborated by molecular characterization. After 5 days of treatment and testing as above, hECTs from each group were snap-frozen for prospective real-time quantitative polymerase chain reaction
of cardiac-specific, calcium-handling, apoptosis, and potassium/sodium ion channel (ie, repolarization/depolarization, respectively) genes. In general, calcium-handling and cardiосpecific genes were not significantly different between groups (Figure 5A). Furthermore, apoptosis genes were not significantly different between groups (Figure 5B). Interestingly, this hACF CdM–mediated gene profile is distinctly different from that of hMSC-treated hECTs (noted above and elsewhere54) despite similarities in the increase of hECT contractile function by the 2 cell types. Like others have suggested,46 this implies that the effects of hACF CdM must be mediated through alternative mechanisms.

In particular, expression of the Kir2.1 inward rectifier and Kv11.1 delayed rectifier potassium channel genes significantly decreased in the hACF CdM intervention group compared with both HFF CdM and control groups (Figure 5C). These findings are also consistent with others.46 Surprisingly, there was a statistically significant increase in Kv4.2 transient outward potassium channel gene expression in the hACF CdM intervention group compared with both HFF CdM and control groups (opposite of previous reports in rodent cells46), whereas there was no significant difference in the Nav1.5 sodium channel gene between groups (which was found to decrease in rodent studies46). This may partly reflect species-dependent variations in ion channel distributions; for example, Kv4.2 is highly expressed in rat myocytes but not in human myocytes.183 Accordingly, out of all genes analyzed, Kv4.2 had the highest threshold cycle (ie, lowest expression) in control hECTs. Furthermore, transient outward activity plays a much less influential role in the human stem cell–derived myocyte action potential waveform relative to murine action potential (see multispecies sensitivity analysis in our previous work54 for justification). This suggests that the increase in Kv4.2 is likely not physiologically significant, even though it is statistically significant. Altogether, these studies further support the use of hECT functional and molecular characterization for testing noncardiomyocyte paracrine effects on cardiac ECC.

On the basis of these findings and the previous studies described, we hypothesize that reduction of outward potassium currents by hACF CdM leads to prolongation of the APD and, therefore, increased calcium handling with a concomitant increase in contractile force. The proposed mechanism is similar to the described inotropic effects of class III antiarrhythmic drugs.166–168 APD is an established correlate of contractile force169–171, therefore, by effectively prolonging the plateau period, calcium availability is increased, augmenting calcium-induced calcium release, elevating the calcium transient, and increasing ECC.

**Limitations of Human Engineered Cardiac Tissues**

Despite the advantages of hECT technology, one recognized limitation is the relatively immature cardiac phenotype that mimics newborn human heart muscle,186 reflecting an ongoing scientific challenge with cardiomyocytes derived from pluripotent stem cells.172 Although perhaps not ideal for cardiac repair applications,173 immaturity may be less problematic for in vitro screening of factors that can modulate contraction given that aspects of the fetal gene program are reactivated as a stress-protective mechanism in failing adult myocardium.174 Also, hMSC therapy has recently been proposed to treat pediatric dilated cardiomyopathy.175 Thus, immature hECTs may adequately represent some aspects of the heart failure disease state and, as such, offer a unique contractility assay for studying cell-based treatment mechanisms and developing future cardiac therapies.

Nevertheless, like others in the field, we continue to develop strategies to promote phenotypic maturation of human pluripotent stem cell–derived cardiomyocytes and hECTs including improved electrophysiology and contractility,176 structurally advanced sarcomeres and evidence of T-tubule formation,177 and the ability to model various forms of hypertrophic and dilated cardiomyopathies.164,178 Generating physiological levels of force may be a prerequisite for surgical implantation applications of engineered cardiac tissues157; however, for in vitro studies, the current subphysiological but cardiomimetic performance of our hECTs appears suitable for therapeutic screening applications.

**Systems Biology Approaches to Study Stem Cell Paracrine Effects on ECC**

As previously reviewed,120,134 bioinformatics methods have proven valuable for investigating paracrine mechanisms of stem cell therapy, such as angiogenesis, fibrosis, and immune modulation. However, in the specific context of paracrine effects of stem cells on cardiac ECC, the application of computational and systems biology approaches is a relatively nascent field. As discussed below, recent systems biology approaches have shown promise for (1) complementing experimental data to better understand noncardiomyocyte paracrine effects on contractile function and (2) identifying key paracrine mediators responsible for increases in contractility.

In our previous work,54 complementary experimental and computational methods were used to study hMSC paracrine-mediated effects on contractility and arrhythmogenicity. The mathematical models could simulate how paracrine signaling affects cardiac cell and tissue physiology (eg, action potentials and calcium transients) based on calibrating the models with measured effects on cellular physiology and tissue-level fibrosis. One benefit of this approach is that the relative importance of specific effects can be independently controlled and readily delineated in the simulations, which is often challenging in an experimental setting. A second benefit is that because the model is calibrated using data covering a range of hMSC concentrations, the effects of intermediate concentrations can also be simulated. Our model was successful in reproducing paracrine-mediated effects on hECT contraction that were not included in the model calibration process, demonstrating the predictive power of the model. Together, these 2 approaches, which provide complementary information, can yield greater insights than either strategy used in isolation.

Nevertheless, such phenomenologic simulations cannot be used to infer which components of the secretome are crucial in altering myocyte physiology. Recent advancements in systems biology have enabled linking complex networks of cues (eg, hMSC microenvironment, signals (eg, hMSC factors), and responses (eg, phenotypes of interest). Janes et al179 first demonstrated that partial least squares regression (PLSR) analysis is useful for forming relationships between signaling molecules and cell decisions. The PLSR method has been
applied to a range of bioanalytics problems\textsuperscript{180–183}; recently, Gray et al\textsuperscript{184} and Agarwal et al\textsuperscript{112} used PLSR in a pair of studies to form relationships between CPC exosomal miRNA effectors and proangiogenic, antifibrotic, and antihypertrophic responses. This method was successful in predicting various key exosomal miRNAs, although it has yet to be applied in the context of cardiomyocyte ECC.

### Integrating Tissue-Engineering and Systems Biology Approaches to Identify Novel Cardioactive Factors

Ranganath et al\textsuperscript{120} first proposed a systematic approach to optimize and harness the hMSC secretion profile by conditioning hMSCs with an array of small molecules and subsequently characterizing the customized secretomes via high-throughput human cytokine antibody array. In the context of identifying key components of the hMSC secretome responsible for increasing contractility, this method could address the issue of hMSC secretome dependence on microenvironment/preconditioning, but it would not reveal (1) the key factors responsible for a given contractile phenotype nor (2) the role of insoluble factors, including exosomes and their cargo, which have been demonstrated to influence contractile function.\textsuperscript{55}

We propose using the PLSR method to help identify key hMSC factors responsible for increasing contractility. By collecting sets of quantitative measurements of input signaling molecules and output metrics of interest across an array of culturing conditions, PLSR can be used to predict relationships between the cues, signals, and responses (details of this method are available elsewhere\textsuperscript{101,112}). This method is an extension of that proposed by Ranganath et al\textsuperscript{120}; in addition to signals and cues, it would also require responses of interest. Given our stated focus on ECC, we propose using hECTs as an in vitro contractility assay to generate physiologically relevant response data for the PLSR analysis. By testing the contractile function of hECTs supplemented with hMSC conditioned media from an array of preconditioning environments, PLSR can be applied to systematically reveal relationships between factors identified via protein- and miRNA-profiling techniques and functional contractile outputs of hECTs. Such methodology could equally be applied to identify key CPC-secreted ECC-modulating factors. Examples of conditioning environments for these cell types that may alter their secretome composition and potency include normoxia versus hypoxia,\textsuperscript{101,112} 2-dimensional versus 3-dimensional substrates,\textsuperscript{54} soft versus hard substrate stiffness,\textsuperscript{154,158} and homocellular versus heterocellular culture.\textsuperscript{54,166}

### Conclusions

Recent studies have demonstrated that in addition to classical endothelial cell paracrine modulation of ECC, other resident noncardiomyocyte heart cells (eg, physiological fibroblasts and pathological myofibroblasts) or even experimental cardiotherapeutic cells (eg, MSCs and CPCs), are also capable of altering cardiac ECC through indirect paracrine mechanisms. On the basis of our recent work and the new experimental data herein, we demonstrate that hECTs are a promising contractility assay to further study noncardiomyocyte paracrine effects on tissue-level contractility. Systems biology approaches can provide complementary information to identify the key stem cell paracrine mediators of ECC and their mechanisms of action. Altogether, integrating tissue engineering and systems biology approaches shows exciting potential for providing novel insight into paracrine mediators of ECC and their underlying mechanisms of action, ultimately leading to improved cell-based therapies for patients with heart disease.

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### Disclosures

K.D. Costa discloses his role as scientific cofounder and Chief Scientific Officer of NovoHeart Ltd. The other authors report no conflicts.

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Physiologic, Pathologic, and Therapeutic Paracrine Modulation of Cardiac Excitation-Contraction Coupling

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SUPPLEMENTAL MATERIAL

Methods

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.¹,²

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, using beam bending theory as described elsewhere.³ Beat rate was measured from a sequence of contractions in spontaneously beating hECTs using previously established methods.³

hACF/HFF Conditioned Media
Conditioned media was collected from 10 cm dishes of 50% confluent hACFs or HFFs cultured in serum-free defined media (SFDM) for five days and used within 3 hours of collection.

Functional Assessment of hECTs Treated with hACF/HFF Conditioned Media
Following 48 hours of tissue compaction, hECTs were cultured in SFDM until day 5. Following spontaneous and 0.5 Hz pacing baseline contractile function testing on day 5, SFDM was replaced with the following treatments: 1) SFDM (Control); 2) fresh hACF conditioned media; or 3) fresh HFF conditioned media. hECTs were cultured an additional 5 days in their respective treatments, after which contractile function was measured and compared to baseline measurements. Spontaneous beat rate and DF were also measured on a daily basis.

hECT Immunofluorescence:
hECTs were fixed in 4% paraformaldehyde, and were later frozen and embedded in Tissue-Tek optimal cutting temperature compound (Sakura, Torrance, CA, USA). 10 µm sections were stained using either anti-cardiac troponin I (H-170, 1:100; Santa Cruz Biotechnology, Dallas, TX, USA) or anti-sarcoendoplasmic reticulum Ca²⁺-ATPase 2 (MA3-919, 1:100; Invitrogen, Carlsbad, CA, USA), followed by Alexa Fluor 488 secondary antibody (A-11034, 1:200; Invitrogen) or Alexa Fluor 594 secondary antibody (A-11032, 1:200; Invitrogen), respectively. All sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI); images were obtained using a laser-scanning confocal microscope (Leica TCS SP5 DMI; Leica Microsystems, Buffalo Grove, IL, USA) using a 63x oil immersion objective.

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 human-specific and are shown in the 5’ to 3’ direction:

<table>
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<th>Gene</th>
<th>Direction</th>
<th>Sequence (5’→3’)</th>
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<tr>
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<td>GGAAGACAAGGTCACAGCCTG</td>
</tr>
<tr>
<td>αMYHC</td>
<td>Reverse</td>
<td>TCCAGTTCCTGGTCGTGGG</td>
</tr>
<tr>
<td>βMYHC</td>
<td>Forward</td>
<td>GGAAGTTCAGCAGCCTGAAGAG</td>
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<tr>
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<td>Reverse</td>
<td>TCCTCAGCATCCTGCCAGGTGT</td>
</tr>
<tr>
<td>SERCA2a</td>
<td>Forward</td>
<td>CAGCTCATGCTCGAGCTCC</td>
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<tr>
<td>SERCA2a</td>
<td>Reverse</td>
<td>AGCGGTACCCTCATGATTGCAG</td>
</tr>
<tr>
<td>CASP3</td>
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<td>TTAGTGAAAAAATAGAGTCTTCTTTTGAG</td>
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<td>Forward</td>
<td>TCCCAGGTTTTTGTTTCTG</td>
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<td>Reverse</td>
<td>CTTTCACCGAAGACGGATT</td>
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<tr>
<td>BCL2</td>
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<td>CTGCACCTGAGCGCCCTTCACC</td>
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<td>CACATGACCCCCAAGCAGTCGGAAGA</td>
</tr>
<tr>
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<td>Forward</td>
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<tr>
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<td>Reverse</td>
<td>GCAAAATGAGAAAAGGCAGCAAC</td>
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<tr>
<td>LTCC</td>
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</tr>
<tr>
<td>Cx43</td>
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<td>GGTTAAAAGGAAAGGAGAGCACC</td>
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<td>Reverse</td>
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</tr>
<tr>
<td>Kv4.2</td>
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<td>CCAACTTCAGTCGTACCCTACC</td>
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<tr>
<td>Kv4.2</td>
<td>Reverse</td>
<td>GCTCTGCGATGATGCGCCCTTC</td>
</tr>
<tr>
<td>ANF</td>
<td>Forward</td>
<td>ACAATTGCGGTGTTCAAGCAGA</td>
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<tr>
<td>ANF</td>
<td>Reverse</td>
<td>CTTCATTGCTGCTCAGGCAC</td>
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<tr>
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<td>Forward</td>
<td>AACAGTGAGGAGCCGCTTGT</td>
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<tr>
<td>Kir2.1</td>
<td>Reverse</td>
<td>AAGACAGAAGGCAGCCAGAAGA</td>
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<tr>
<td>Kv11.1</td>
<td>Forward</td>
<td>CATCTGCTGCTACGCTGTC</td>
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<td>Reverse</td>
<td>TCTCGTGAGACGGAATAGGA</td>
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<tr>
<td>Nav1.5</td>
<td>Forward</td>
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<tr>
<td>Nav1.5</td>
<td>Reverse</td>
<td>GTCGACGATCTCAAGCAGA</td>
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</table>

Fold changes in gene expression were determined using the comparative threshold cycle method (ΔΔCt) with normalization to the housekeeping gene beta-2-microglobulin (β2M). This experiment was repeated in triplicate.

**Statistical Testing and Analysis**

The descriptive statistics used to present results are specified within each figure legend. Statistical analyses were performed using Prism 6. Repeated measures analysis of variance (ANOVA), followed by Bonferroni’s multiple comparisons test, was used for Figure 4. One-way ANOVA, followed by Scheffé’s post-hoc test, was used for multiple pairwise comparisons of unequal group sizes in Figure 5. Differences with a p-value less than 0.05 were considered statistically significant.
Supporting References:


2. Cashman TJ, Josowitz R, Gelb BD, Li RA, Dubois NC, Costa KD. Construction of defined human engineered cardiac tissues to study mechanisms of cardiac cell therapy. *J Vis Exp.* 2016:e53447


**Online Figure I: Simulated Vulnerable Window (VW) Analysis on hMSC-Supplemented Fibrotic Cardiac Tissue.** A VW analysis was performed on low (4%; left panel) and high (40%; right panel) fibroblast-populated cardiac tissue injected with 0% (control), 20%, 40%, or 60% hMSCs at high (16%) engraftment levels (n=3 randomized cell distributions). In low fibrosis (i.e., more representative of myocyte monolayers in vitro), both paracrine signaling (PS) and heterocellular coupling (HC) increases the VW, while in high fibrosis (i.e., more similar to preclinical/clinical conditions for hMSC intervention), PS decreases the VW and overcomes potentially pro-arrhythmic HC. Panel B adapted from Mayourian et al.,⁵ which also provides detailed methods for these in silico simulations. As recommended by White et al.⁶, comparative statistics were not implemented, as p-values are dependent on sample size (which can be arbitrarily high in simulations).