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Characterizing functional stem cell–cardiomyocyte interactions

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Abstract

Despite the progress in traditional pharmacological and organ transplantation therapies, heart failure still afflicts 5.3 million Americans. Since June 2000, stem cell-based approaches for the prevention and treatment of heart failure have been pursued in clinics with great excitement; however, the exact mechanisms of how transplanted cells improve heart function remain elusive. One of the main difficulties in answering these questions is the limited ability to directly access and study interactions between implanted cells and host cardiomyocytes *in situ*. With the growing number of candidate cell types for potential clinical use, it is becoming increasingly more important to establish standardized, well-controlled *in vitro* and *in situ* assays to compare the efficacy and safety of different stem cells in cardiac repair. This article describes recent innovative methodologies to characterize direct functional interactions between stem cells and cardiomyocytes, aimed to facilitate the rational design of future cell-based therapies for heart disease.

Keywords

arrhythmia; cellular cardiomyoplasty; co-culture; contractile force; micropatterning; stem cells

With over 1 million new and recurring myocardial infarctions per year and more than 5 million people diagnosed with heart failure in the USA alone [1], cardiovascular disease remains a pervasive and significant clinical problem. Over 15 years ago, the transplantation of exogenous stem cells in the heart was proposed as a strategy to decrease or restore the loss of myocytes that occurs with infarction and other cardiac pathologies, thereby preventing or reversing heart failure [2,3]. The severity and prevalence of the disease, along with exciting preclinical results, have prompted a number of clinical trials with mixed but encouraging results. A few different stem cell types (skeletal myoblasts [4,5], bone marrow-derived cells [6,7], peripheral blood-derived cells [8,9], cardiosphere-derived cells [10] and c-kit-positive cardiac stem cells [11]) have already been or are about to be used in clinical trials, and several others are under consideration for future studies. However, our understanding of the potential mechanisms of stem cell-based cardiac repair, and specifically the structural and functional interactions between implanted cells and host cardiomyocytes, remains limited.

The current view is that implanted stem cells secrete multiple paracrine factors that not only promote the vascularization and survival of infarcted myocardium, but also potentially attenuate inflammation, improve the remodeling, and aid in the recruitment and differentiation of endogenous stem cells [12–14]. However, as the implanted cells are short-lived and poorly retained in the heart, the resulting functional benefits are mainly transient in nature (for several

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months) rather than being sustained long term. Furthermore, the cells currently used in clinics have a limited capacity to attain cardiac fate and permanently contribute to new heart muscle, thus making significant and long-lasting recovery of heart contractile function less likely [15]. As a result, recent efforts in the field have been directed towards the discovery and use of novel cardiogenic stem cells [10,11,16,17], which are expected to provide large numbers of functional cardiomyocytes after implantation, and exert safe and efficient repair by strongly coupling with host myocytes. For these stem cells, direct, contact-mediated interactions with host cells (along with potential paracrine actions) are expected to be the main 'mode of action' upon implantation.

In general, it is well recognized that both contact-mediated and local paracrine interactions between implanted stem cells and host cardiac tissue can influence the survival and function of host cardiomyocytes and nonmyocytes, as well as the integration, proliferation, differentiation and survival of implanted stem cells. These complex bilateral interactions can assume different spatiotemporal patterns after implantation (depending on the cell type and mode of delivery) and in turn, determine both the benefits and potential risks of cell therapy. For example, cell–cell contact-mediated activation of the Notch signaling pathway has been shown to enhance cardiogenic differentiation of mesenchymal stem cells [18] and resident cardiac progenitors [19]. Electrical coupling between stem cells and cardiomyocytes via gap junctions has been correlated with arrhythmogenic risks postimplantation [20,21]. Secretion of different growth factors and cytokines by bone marrow-derived, circulating, tissue-resident or embryonic stem cells has been shown to profoundly influence the functional outcome of cell therapy [13,22].

Despite their importance, systematic studies of paracrine and contact-mediated heterocellular interactions *in vivo* are hampered by a number of confounding factors, including low spatial resolution of functional measurements in the beating heart, the lack of direct experimental access to implanted cells, and the presence of endogenous paracrine and neurohumoral factors. Recent review articles [12,13] have discussed several paracrine actions and related mediators by which implanted stem cells can affect survival and function of host cardiomyocytes and vascular cells. This article will therefore focus on direct interactions between stem cells and cardiomyocytes, and specifically on the existing *in vitro* and *in situ* methodologies to systematically study the functional consequences of stem cell implantation. As the number of potential donor cell sources and approaches for cardiac cell therapy is increasing, so is the importance of understanding heterocellular interactions in a cardiac milieu. The ability to systematically modify individual components of these interactions (e.g., cell type, geometry, distribution and density, cell–cell contact length, coupling strength and paracrine actions) is expected to improve our ability to interpret and understand the results of clinical cell therapy studies. We believe that through the use of various donor cell types, as well as genetic and pharmacological applications, standardized heterocellular assays will be instrumental in guiding the rational design of novel cell-based therapies for safe and efficient treatment of cardiac infarction and arrhythmias.

***In situ* characterization of functional stem cell–cardiomyocyte interactions**

The ability of implanted stem cells to electrically couple with surrounding cardiomyocytes and locally alter (augment or deteriorate) cardiac electrical activity and contractile function should be ideally assessed in a noninvasive fashion, with high spatial and temporal resolution, and over a significant volume of the heart. Such assessment would allow longitudinal tracking of stem cell fate and function in the same individual (a clear advantage regarding the large variability between individuals and implantation procedures) and enable understanding of the true functional impact of all, or most, of the implanted cells in different regions of the heart. Direct labeling of cells with genetic reporters, magnetic nanoparticles or radioisotopes

combined with bioluminescent imaging, MRI, PET or single photon-emission computed tomography [23–27] is being pursued to allow short- or long-term noninvasive tracking of the survival, migration and differentiation of implanted cells within treated animal and human hearts. For clinical applications, current research in this field is aimed at identifying suitable intracellular labels that are safe for cells even when accumulated at high concentrations (for comprehensive reviews on this topic see [28,29]). Besides cell tracking, several nondestructive methods are used to assess the global functional consequences of stem cell implantation *in vivo*, including echocardiography, standard ECG and ECG imaging, intracardiac mapping (e.g., Ensite®, CARTO®), pressure catheterization and cardiac magnetic resonance tagging [30–32]. The restricted spatial resolution of these techniques, however, precludes an accurate description of how stem cell–cardiomyocyte interactions affect cardiac function *in vivo*.

Therefore, the next-best option to assess these interactions in the treated heart is to perform histological assessments [33] and/or high resolution recordings of intracellular Ca^{2+} transients [34–37]. These *in situ* techniques, while significantly limited by small sample size, invasiveness and terminal nature, currently represent the gold standard in assessing heterocellular interactions within intact cardiac tissue. Usually, implanted cells expressing a fluorescent reporter are: immunohistologically assessed for the presence of coupling proteins (connexins, cadherins) at their borders with host cells or other implanted cells; or stained with the rest of the heart with a cell-permeable Ca^{2+} -sensitive dye (Rhod-2, Fluo-2, Fluo-4), mounted onto a two-photon laser-scanning microscope, and assessed during pacing or sinus rhythm for their ability to generate intracellular Ca^{2+} transients in synchrony with surrounding cardiomyocytes. Excitation–contraction decouplers (cytochalasin-D [36], blebbistatin [38]) are added to the heart perfusate during these recordings to prevent the occurrence of motion artifacts.

Using two-photon imaging of Rhod-2-stained mouse hearts, Field and coworkers were the first to demonstrate functional coupling (i.e., synchronous Ca^{2+} transients) between host cardiomyocytes and implanted green fluorescent protein-labeled mouse fetal cardiomyocytes or fused myoblast–cardiomyocytes [34,39]. Similarly, by two-photon imaging mouse hearts, Anversa and coworkers demonstrated functional coupling between the host cardiomyocytes and cardiomyocytes differentiated from implanted mouse bone marrow cells [35] or human cardiac stem cells [11]. The use of two-photon excitation for Ca^{2+} imaging offers several advantages over traditional single-photon confocal imaging, including:

- No need for a pinhole in the emission path;
- Less photobleaching and photodamage;
- Higher signal–noise ratio;
- Larger penetration depth (up to a few hundred microns below the heart surface) [40].

Nevertheless, the methods based on traditional confocal imaging, recently described by Fujiwara *et al.* [37], may also enable simultaneous high-resolution recording of action potentials (using voltage sensitive dyes such as Di-4 ANEPPS or RH237) and Ca^{2+} transients in heterocellular cardiac milieu few cell layers below the heart surface. Using these methods, complete and partial conduction blocks and potentially arrhythmogenic behaviors of implanted cells could be studied at subcellular and cellular spatial scales [37,41].

Importantly, to ensure that no signal crosstalk occurs from the surrounding cardiomyocytes, the two-photon excitation volume needs to be confined within the cytoplasm of implanted cells, thus requiring the use of objectives with a high numerical aperture (and magnification) [40]. As a result, the corresponding field of view has to be relatively small (usually $<0.1 \text{ mm}^2$ in area), encompassing only a few implanted and host cells. In this setting, the synchrony of

Ca²⁺ transients in implanted and host cells does not guarantee that the implanted cells can actively generate and propagate action potentials, fire mature Ca²⁺ transients (by release of Ca²⁺ from sarcoplasmic reticulum) or contribute to contraction. Rather, it only demonstrates their ability to passively (electrotonically) bridge short conduction distances in cardiac tissue through functional coupling with host cells. In other words, unexcitable nonmuscle cells, either alone or in small clusters, could also display similar Ca²⁺ transients when strongly coupled with host myocytes. The signals in this case could be generated by passive Ca²⁺ inflow through:

- Gap junctions with excited myocytes;
- Existing voltage-sensitive Ca²⁺ channels activated by passive membrane depolarization due to coupling with myocytes;
- Existing Na⁺/Ca²⁺ exchangers in response to the influx of Na⁺ through gap junctions.

To definitely establish that implanted fluorescently labeled cells can actively generate action potentials and Ca²⁺ transients as well as contract upon stimulation, these cells need to be enzymatically isolated from the heart and studied using conventional patch clamp and edge detection techniques, as demonstrated by Rota *et al.* [35].

To assess the ability of implanted cells to actively propagate action potentials, electrophysiological recordings need to be performed over distances spanning at least several electrotonic space constants (>2–3 mm) [42]. Furthermore, the mechanistic studies of the potential reentrant or focal arrhythmias caused by implanted cells require an even larger field of view (>tens of mm²). These propagation studies are usually performed *ex vivo* using macroscopic optical mapping techniques that employ voltage- and Ca²⁺-sensitive dyes in conjunction with high numerical aperture photographic lenses and fast cameras (CCD, CMOS) or photodiode arrays [43,44]. For example, Mills *et al.* optically mapped action potentials in an 8.3 × 8.3 mm² infarct area in Langendorff-perfused rat hearts to study the arrhythmic potential of implanted skeletal myoblasts or bone marrow-derived mesenchymal stem cells (MSCs) [21]. These mappings successfully revealed conduction slowing, block or arrhythmia induction in the implant area. However, for macroscopic optical mapping, all cells in the heart are nonselectively stained by dye indicators and recordings are performed with low spatial resolution (>100 μm) in relatively thick optical sections [43]. As a result, recorded signals can not be used to reliably differentiate activity in implanted cells from surrounding cardiomyocytes. Therefore, even if implanted cells are unexcitable or unable to couple to host cells, the mapping in the implant area may still display relatively uniform action potential propagation that proceeds through adjacent cardiomyocytes. Similarly, recorded signals can not be used to reliably derive action potential shape or coupling strength of the implanted cells.

Finally, all available voltage- and Ca²⁺-sensitive dyes are cytotoxic, which precludes repeated or continuous recordings over long periods.

In contrast to fluorescent dyes, novel genetically encoded Ca²⁺ indicators [45] can be utilized for long-term *in situ* recordings and targeted only to implanted cells (or their progeny). gCaMP2 is one such indicator designed as a fusion protein between green fluorescent protein and calmodulin that rapidly changes fluorescence upon binding to Ca²⁺ ions [46]. The kinetics of binding and unbinding are slower than that of the dye indicators; however, the resulting fluorescence signals still allow reliable recordings of intracellular Ca²⁺ transients in various cell types, including neurons [47], smooth [48], cardiac [49] and skeletal muscle [20], and endothelial cells [50]. If the gCaMP2 gene is driven by a constitutive, inducible or tissue-specific promoter and transfected or transduced into donor cells prior to implantation, Ca²⁺ transients can be selectively recorded in implanted cells without any need for staining with indicator dyes. For example, Roell *et al.* have applied gCaMP2 labeling to embryonic cardiomyocytes and connexin-43-expressing skeletal myoblasts and used optical mapping to

unambiguously demonstrate their electrical coupling with host cardiomyocytes *in vivo* [20]. These cells supported action potential propagation in the infarct region, thereby reducing the incidence of postinfarction arrhythmias. One drawback of gCaMP2 is its relatively low baseline fluorescence [46], which may hamper identification of quiescent gCaMP2-labeled cells in the heart. A possible strategy to overcome this limitation is to use a bicistronic plasmid containing both gCaMP2 and a strong fluorescent reporter such as mCherry. We constructed this plasmid and applied it to transfect cultured neonatal rat ventricular myocytes (NRVMs). The quiescent transfected cells were first identified by strong mCherry fluorescence, and gCaMP2 fluorescence was subsequently recorded during electrical field stimulation using a fast EMCCD camera (Figure 1).

The described techniques for studies of heterocellular interactions *in situ* have led to much progress in our understanding of the fate and function of stem cells delivered to the myocardium. While *in situ* functional studies are most relevant for testing the potential clinical consequences of stem cell implantation, they suffer from inherent limitations, including:

- Large variability in the injury site, transplantation procedure and 3D heart tissue microstructure and function among test subjects;
- Difficulty in identifying and accessing implanted stem cells more than a few hundred microns below the heart surface;
- Random spatial and geometrical relationships between implanted cells and host cardiomyocytes and nonmyocytes.

Because of these limitations, *in situ* functional studies can, in general, address only a small number of heterocellular interactions in non-reproducible conditions. Thus, these studies are often descriptive in nature rather than mechanistic or quantitative. For example, when assessing a heterogeneous stem cell population for its ability to structurally or functionally couple with cardiomyocytes, it would be ideal to quantify the frequency and spatial distribution of this coupling in the heart, rather than only specify if some of the implanted cells can couple or not.

A number of new donor cells including embryonic [16,51] and induced pluripotent stem cells [52,53], as well as stem cells derived from epicardium [54], pericardium [55], adipose tissue [56,57], liver [58], umbilical cord blood [59] and amniotic fluid [60,61] are being contemplated for future clinical treatments of heart disease. Systematic and quantitative comparison of these cells for their intrinsic ability to electromechanically interact with host cardiomyocytes and affect their function requires a biochemically and geometrically reproducible setting that is unattainable in intact hearts. Similarly, mechanistic studies of the potential of different molecules (cytokines, genes, drugs) to promote cardiomyocyte–stem cell interactions and safely and efficiently improve cardiac function are difficult to perform *in situ*. For these reasons, well-defined and reproducible *in vitro* assays to systematically study how complex heterocellular interactions affect cardiac electrical and mechanical function at different spatial scales would be invaluable to the progress of the field. These assays are described in more detail in the following text.

***In vitro* characterization of functional stem cell–cardiomyocyte interactions**

Standardized *in vitro* assays for well-controlled studies of functional stem cell–cardiomyocyte interactions can promote our understanding of the potential arrhythmogenic risks and functional benefits associated with the implantation of different stem cells in the heart. Monitoring how this risk/benefit ratio changes with time (i.e., with the differentiation of stem cells, establishment of cell contacts and electromechanical interactions), and in the presence of specific genetic, biophysical and biochemical modulators of stem cell fate, function and integration is eventually expected to facilitate the rational design of heart cell therapies. The

development of these cell co-culture assays is becoming increasingly more important as the field is turning towards the use of cardiogenic stem cells that can actively change pacemaking, electrical and contractile cardiac function by directly coupling to host cardiac cells. Since the late 1950s, different types of cardiac cell cultures have been used as simplified model systems for well-controlled studies of cardiac function at the cellular and molecular scale. Their main advantages over intact tissue and organ preparations include:

- Simplified structure with a relatively small number of studied cells (up to few hundred thousand);
- The ability to reproducibly define cellular composition and biochemical microenvironment;
- Direct experimental access to all cells;
- The absence of capillaries, neurohumoral and other confounding factors present *in vivo*.

In addition, direct access to cultured cells allows controlled conditioning and manipulation by soluble factors, gene vectors, and electrical and mechanical stimulation.

In the last two decades, different micropatterning and microfluidic techniques [62] have been increasingly utilized in cardiac research *in vitro* because they allow precise control of single cell and cell network geometry through selective deposition of extracellular matrix proteins to predetermined regions on the substrate. Since they are based on computer-assisted design, these techniques are inherently reproducible and can even be combined with high-resolution imaging modalities (e.g., diffusion tensor MRI) to create cell cultures that replicate anatomically accurate myocardial structure [63]. Importantly, the ability to control geometric and spatial relationships within small groups of different cells, along with the 2D nature of the preparation, allow functional measurements to be directly related to the underlying cellular structure and composition at both microscopic and macroscopic spatial scales. Previously, micropatterned cardiac cell cultures have been used in conjunction with electrical stimulation and optical mapping of action potential and Ca^{2+} transients to study the roles of tissue microstructure in electrical propagation, propensity to conduction block and arrhythmias, and outcomes of electrical shock [64–68]. Importantly, since electrical propagation is studied in a single cell layer, any potential interference due to nonselective recordings from deeper tissue layers (as present *in situ*) is eliminated.

With the current advancements in cell culture technologies at the micro- and nano-scale, *in vitro* assays for comparative and quantitative studies of functional stem cell–cardiomyocyte interactions should become a standard complementary tool to clinical and various *in vivo* and *in situ* animal studies. Ideally, these assays are expected to be designed as specialized 2D and 3D co-culture systems where the cell type of interest (e.g., stem cell and primary nonmyocyte) can be simply ‘plugged’ into a geometrically and biochemically reproducible cardiomimetic environment and then studied in a systematic and relatively high-throughput fashion. Ideally, these co-culture systems are expected to facilitate:

- Studies of the basic mechanisms behind the observed functional outcomes in clinics, including occurrence of arrhythmias;
- Meaningful and quantitative comparisons of different stem cells for their potential to improve cardiac electrical and mechanical function;
- A predictive and high-throughput prescreening of novel cell types, pharmaceuticals, genetic manipulations and combinations thereof, designed to advance existing therapies.

To increase the efficacy and accuracy of the functional studies in stem cell–cardiomyocyte co-cultures, stem cells can be genetically labeled with fluorescent proteins and identified during live imaging. Furthermore, micropatterning and microfluidic techniques can be utilized to design the structurally simplest and most controlled heterocellular setting that still permits a desired functional test to be performed in a reproducible and systematic fashion. For example, the ability of different stem cells to structurally and functionally couple with cardiomyocytes and change their electrical properties can be studied in a number of different cell co-cultures with varying degrees of structural complexity. Nevertheless, the simplest well-controlled setting to perform comparative studies is a micropatterned cell pair as described in the text below.

In Figure 2, different co-culture settings are listed from top to bottom in order of increasing structural complexity and qualified with regard to their suitability for different types of functional studies. Besides using the same co-culture setting to functionally compare different stem cell types, each stem cell type can be additionally characterized relative to two control groups: pure cardiomyocyte cultures representing healthy tissue; and cardiomyocyte–fibroblast co-cultures representing fibrotic or ‘diseased’ tissue. Alternatively, comparative functional tests can be performed after adding different stem cell types (or their paracrine factors) to cardiomyocyte–fibroblast co-cultures.

Micropatterned cell pairs for studies of cell–cell coupling

Cell–cell coupling studies in traditional co-cultures involve random cell shapes and length of cell–cell contacts, as well as the presence of multiple homo- and hetero-typic contacts between interacting cells. These factors make it difficult to interpret and quantify the obtained results and perform comparative studies among various cell types. To systematically study heterocellular coupling between cardiomyocytes and stem cells at a single-cell level while overcoming the inconsistencies of traditional co-culture settings, we fabricated a large number of individual micropatterned cell pairs with reproducible shape, size and region of cell–cell contact (Figure 3A) [69]. The culture parameters in this system were optimized to maximize the formation of heterotypic cell pairs made of NRVMs and different noncardiomyocytes relevant for cardiac disease and cell therapies, including cardiac fibroblasts and two clinically relevant stem cells types: skeletal myoblasts and bone marrow-derived MSCs.

The structural and biochemical reproducibility of this assay enables both quantitative and comparative studies of the probability of expression and the spatial distributions of the main electrical (connexin-43, Figure 3A) and mechanical (N-cadherin, not shown) coupling proteins. Using high-throughput studies of more than 5000 geometrically identical cell pairs, we found that:

- Only a fraction of noncardiomyocytes (maximum of 40% for MSCs and minimum of 9% for fibroblasts) formed connexin-43 and N-cadherin junctions with the contacting NRVMs for cell–cell contact lengths of up to 100 μm ;
- Noncardiomyocytes caused internalization of junctions in NRVMs;
- Abutting NRVMs enhanced the formation of junctions in noncardiomyocytes;
- The incidence of junctional formation increased with increase in the cell–cell contact length.

The differences in frequency of junctional formation in different stem cells as well as in stem cell–stem cell versus stem cell–cardiomyocyte contacts will have direct consequences on the ability of implanted clusters of stem cells to propagate or initiate electrical activity as well as synchronize or initiate contractions in surrounding host cardiomyocytes.

In addition to structural immunostaining studies, the micropatterned cell pairs can be used for the assessment of homo- and heterocellular functional coupling, albeit not in a high-throughput fashion. Nevertheless, reproducible cell area and contact length in this setting significantly reduce the variability of the results and the number of samples required. Fluorescence recovery after photobleaching [70,71], a technique in which fluorescence in a photobleached cell is recovered by dye passage from the adjacent cell through functional gap junctions, is one method to assess functional coupling (Figure 3B). For geometrical parameters defined by micropatterning, the analysis of the time course of fluorescence recovery (e.g., the exponential time constant) enables quantitative comparisons between different cell types or environmental conditions (i.e., soluble factors added to the culture medium).

Although fluorescence recovery after photobleaching can provide a relative measure of the strength of coupling, it cannot quantify the macroscopic electrical conductance between the coupled cells. Dual whole-cell patch clamping [72,73] is the ultimate quantitative method to directly measure the intercellular conductance between two cells (Figure 3C). Usually, pulled glass electrodes with tip resistances of 2–5 M Ω when filled with a conductive internal solution are giga-sealed to each cell. Upon establishing whole cell access, both cells are clamped at the common holding potential (e.g., -40 mV). As one cell is stepped to a depolarized voltage (e.g., +20 mV), and the other is held at common potential, the resulting transjunctional voltage (e.g., 60 mV) will elicit junctional current equal in magnitude (and opposite in sign) to the current measured by the electrode in the nonstimulated cell (Figure 3C). The macroscopic conductance between the two cells is then calculated from the known transjunctional voltage and the measured junctional current after accounting for series resistances from the two patch electrodes [74]. Analysis of junctional currents elicited by a range of voltage steps (e.g., for transjunctional voltages from -100 to +100 mV) is used to evaluate junctional current–voltage (I–V) relationships and thus assess the behavior of gap junctions at different voltage gradients between the two cells. This generally nonlinear behavior may be particularly relevant if stem cell–cardiomyocyte coupling is weak, when a large delay between action potential initiations in neighboring cells can create significant transjunctional voltage gradients.

Micropatterned cell strands for studies of electrical propagation

While individual micropatterned cell pairs are well suited for systematic studies of isolated heterocellular interactions, understanding the functional consequences of stem cell presence within the cardiac milieu requires the use of multicellular preparations. Specifically, the geometrically simplest, well-controlled *in vitro* setting to study the effects of stem cell implantation on cardiac electrical propagation is a relatively narrow (50–100 μ m) and long (~1 cm) micropatterned cell ‘strand’ (Figures 2 & 4). Since the width of the strand is smaller than the cardiac electrotonic space constant [42], electrical propagation along the strand is pseudo-1D. This allows microscopic tracking of electrical propagation along a distinct linear path (a few cells wide) without any ambiguity about the exact pattern of propagation. By fluorescently labeling stem cells, their sites of contact with cardiomyocytes can be readily identified during live imaging. Electrical activity can then be mapped in the regions of heterocellular contacts using optical recordings of membrane potentials or intracellular Ca²⁺ concentration with fast photodetectors [65,75], and extracellular recordings of field potentials with microelectrode arrays [76,77]. The advantage of the first mapping method is the ability to assess the shapes of action potentials and Ca²⁺ transients with subcellular resolution, while the latter method, despite low spatial resolution (200–500 μ m), can allow long-term recordings without concerns about phototoxicity or photo bleaching. In addition, action potentials in selected cells can be recorded by a sharp microelectrode during propagation [78] or by a patch clamp electrode after pharmacological decoupling of cells [79].

Two basic settings for studies of heterocellular electrical propagation include:

- ‘Mixed strands’, where stem cells and cardiomyocytes are interspersed randomly at a specified mixture ratio (Figure 4A & 4B);
- ‘Strands with inserts’ where stem cells form a distinct cellular bridge (insert) of a defined length within a cardiomyocyte strand (Figures 4C & 4D).

In the mixed setting, the ability of a single, or only a few, stem cells to bridge conduction between neighboring cardiomyocytes can be studied, while in the insert setting, stem cells are tested for their ability to bridge electrical conduction over lengths between one and several hundred micrometers. Because undifferentiated stem cells are relatively depolarized and electrically unexcitable, their action potential propagation will be passive and highly dependent on their size and orientation (i.e., number of gap junctions per length of the insert [79]) and gap junctional conductance. In general, this passive (detrimental) propagation in pure stem cell inserts will be limited to maximum distances of several hundred microns [65,75,80]. As the velocity of detrimental conduction nonlinearly decays with distance, apparent propagation velocities within the insert can only be meaningfully compared for inserts of identical length. Adding a small percentage of cardiomyocytes as ‘active conduction boosters’ in the insert (i.e., creating mixed inserts) may significantly increase the apparent propagation velocity and maximum propagation distance.

To be able to compare different stem cells for their ability to conduct electrical activity within a cardiac strand, the length of the insert needs to be precisely varied. We have recently described a method combining the use of cell micropatterning and elastomeric stencils (thin membranes made of polydimethylsiloxane [PDMS] with holes of defined shape and size [81]) to create inserts with defined length [75]. Specifically, patterned fibronectin lines are covered by stencils containing a narrow spacer to selectively block cardiomyocyte attachment and growth in the insert area. After the stencil is removed, empty inserts are carefully seeded with various cells. The cardiac strand is electrically paced a few millimeters away from the insert to allow studies of propagation free of influence from the stimulus. The advantages of this approach are high reproducibility and the potential to perform relatively high-throughput studies by simultaneously pacing and mapping multiple, closely spaced strands. These strands can be specifically designed to quantify either the probability of successful conduction through an insert of a given length or the maximum insert length that allows successful propagation. In addition to the use of stencils, two alternative methods to create empty inserts of microscopic size employ a narrow adhesive tape [65] or a high-power laser beam [79] to remove a portion of an already confluent cardiomyocyte sheet; however, this approach may introduce injury in the surrounding cells.

Using micropatterning/stencil technology and optical mapping of intracellular Ca^{2+} transients, we studied propagation through 200–500 μm inserts made of different cells, including rat skeletal myoblasts (not shown), cardiac fibroblasts, MSCs and mouse embryonic stem cell-derived cardiomyocytes (mESC-cardiomyocytes) selected using resistance to the antibiotic puromycin driven by an α -myosin heavy chain promoter (Figure 4D). After 2 days of co-culture, we found that cells exhibiting negligible amounts of connexin-43 (fibroblasts, myoblasts) failed to conduct electrical impulses over distances above 200 μm . By contrast, cells able to couple to cardiomyocytes by connexin-43 junctions passively (MSCs) or actively (mESC-cardiomyocytes) conducted electrical activity between proximal and distal portions of the strand [75]. From all studied cells, MSCs showed the most efficient short range (<500 μm) conduction, likely due to their relatively large cell size [65]. The early-stage mESC-cardiomyocytes, although capable of active Ca^{2+} transient generation, conducted significantly slower, likely due to their small cell size, low connexin-43 expression and/or immature ion channel properties.

Cell monolayers for studies of cardiac arrhythmias

While the pseudo-1D setting of a micropatterned strand enables well-defined studies of impulse conduction at a microscopic spatial scale, larger, centimeter-sized *in vitro* preparations are required to systematically study macroscopic cardiac electrical conduction and arrhythmia generation. Specifically, heterocellular monolayer sheets (Figures 2 & 5) represent the geometrically simplest (2D) *in vitro* setting to perform macroscopic, tissue-level studies of the effect of stem cell implantation on cardiac electrical and mechanical function. Usually, for arrhythmia studies, optical mapping of action potentials or Ca^{2+} transients is performed with a sufficient spatial (200–500 μm) and temporal (>500 Hz) resolution to track wave propagation in a monolayer of a few cm^2 area [82–84]. Programmed pacing regimes similar to standard clinical tests of arrhythmia vulnerability [85] (e.g., burst or premature pacing) are applied to study the occurrence of wavebreaks and the induction and dynamics of reentrant activity [67, 84]. The spatial distribution and proportion of stem cells and cardiomyocytes can be assessed by live fluorescence imaging or immunostaining immediately after functional tests to allow one-to-one correlation between the monolayer composition and the corresponding functional outcomes. Similar to *in situ* studies, stem cells can be transfected or transduced with the gCaMP2 indicator to unambiguously demonstrate their functional coupling with cardiomyocytes. In addition, as the progression of cardiac differentiation is associated with an increase in intracellular Ca^{2+} transient amplitude (due to the switch from sarcolemmal influx- to sarcoplasmic reticulum release-based Ca^{2+} transient [86,87]), relative gCaMP2 signal amplitudes can be used as a live indicator of cardiogenic differentiation.

The most basic monolayer setting for studies of stem cell–cardiomyocyte interactions is a confluent co-culture of randomly oriented and distributed stem cells and NRVMs. This basic isotropic setting has been previously used in conjunction with mapping of action potential propagation to demonstrate that diffusely distributed cardiac myofibroblasts [88], MSCs [89] or skeletal myoblasts [90] (i.e., electrically unexcitable or uncoupled cells) can be arrhythmogenic when present in a sufficient number within the cardiac milieu. To create heterocellular monolayers with a more physiological, anisotropic architecture, the culture substrate can be modified by the use of microfabrication and micropatterning techniques to create parallel microgrooves or narrow lines of extracellular matrix protein. These topographical and biochemical cues can successfully guide uniform alignment of cardiomyocytes and stem cells (Figure 5A & 5B). In the anisotropic setting [91], differences in electrical conduction along versus across aligned cells may amplify stem cell-induced conduction heterogeneities and precipitate conduction blocks and reentrant arrhythmias under conditions that would appear nonarrhythmogenic in the isotropic cultures.

Furthermore, instead of the uniform dispersion of stem cells and NRVMs throughout the entire monolayer area, a more realistic setting of a localized stem cell implant can be produced *in vitro* using a combination of micropatterning and stencil techniques. This approach enables the creation of distinct cellular ‘islands’ with a defined shape and size surrounded by NRVMs. The islands are made either of pure fibroblasts or stem cells (mimicking a discrete scar or implant, Figure 5C) or a defined mixture of NRVMs with fibroblasts or stem cells (mimicking a diffuse scar or implant, or multiple small implants, Figure 5D). In conjunction with optical mapping of electrical propagation, these settings can be utilized to evaluate the stem cell type-specific conditions that lead to successful electrical conduction, cause partial and complete conduction block or promote arrhythmic activity (Figure 4E). In our preliminary studies with 3 mm circular islands made of MSCs or fibroblasts mixed with NRVMs, the incidence of the conduction block in the island increased with increasing fraction of nonmyocytes, reaching its maximum in the pure nonmyocyte islands; however, the incidence of reentrant arrhythmias during rapid pacing (Figure 5F) was highest for specific mixture ratios that yielded relatively continuous conduction at lower pacing rates, and significant conduction slowing and eventually

block at high pacing rates. In addition, pure or mixed stem cell islands can be designed with defined perimeter:area ratios to systematically study the geometrical source–sink relationships that permit generation and exit of the action potential from a stem cell area into surrounding myocytes. These and similar studies could aid the potential development of stem cell-based biological pacemakers [92–94].

Cell bilayers for studies of electrical loading

Upon implantation, stem cells in the heart have been identified as: single cells or small cell clusters [34] incorporated in the cardiac syncytium, thus potentially contacting both the sides and ends of the surrounding myocytes; and compact multilayer grafts bordering the myocardium over a relatively large area, thus mainly contacting the sides but not the ends of the abutting myocytes [95]. In the first scenario, during electrical propagation, stem cells can act as conduction obstacles if they are not coupled to cardiomyocytes, or as electrical loads and short- or long-range conductors [96], depending on their excitability and ability to form functional gap junctions. In the second scenario, stem cell implants can either cause no direct effect on cardiac conduction if they are not coupled to bordering myocardial layers, or exert active or passive electrical loading of the myocardium via functional gap junctions.

While the first scenario can be studied *in vitro* using heterocellular monolayers, the ability to systematically study how electrical loading by different stem cells affects cardiac conduction and arrhythmogenicity requires the use of a heterocellular bilayer setting. In this setting, stem cells are cultured on top of confluent cardiomyocyte monolayers or strands, either in a diffuse or localized fashion (Figures 2 & 6). The regions of contact between the two cell layers can be precisely assessed and their area quantified [97] using live imaging of fluorescently labeled stem cells. This structural assessment can be further correlated with microscopic and macroscopic functional studies in the same culture. In addition, the bilayer setting provides stem cells with an underlying cardiac layer as a naturally soft substrate for growth, in contrast to the rigid substrate present in the monolayer setting. This softer growth substrate is expected to better mimic the mechanical environment experienced by implanted cells, and possibly promote cardiac differentiation of cultured stem cells. On the other hand, stem cell alignment and the shape and size of the localized stem cell island are significantly harder to control in the bilayer compared with the monolayer setting.

The heterocellular bilayer setting has been previously used to demonstrate that myofibroblast loading can reduce the velocity of conduction and action potential upstroke [78], as well as increase the incidence of ectopic activity [98], in micropatterned NRVM strands. Furthermore, electrical loading of isotropic NRVM monolayers with NIH/3T3 fibroblasts modified to express the potassium channel Kv1.3 was shown to cause conduction slowing and block at sites of fibroblast loading [99]. In addition, isotropic NRVM and HL-1 (a murine cardiomyocyte cell line) monolayers locally loaded with mouse or human ESC-cardiomyocytes have been utilized for *in vitro* studies of stem cell pacemaking [93,100]. We have recently used the bilayer setting to examine how diffuse electrical loading with unexcitable cells through different types of gap junctions affects macroscopic cardiac conduction [97]. Specifically, confluent anisotropic NRVM monolayers were covered at varying densities with fibroblasts or with fluorescently labeled human embryonic kidney-293 cells engineered to express connexin-43 or -45. We found that reduced loading via connexin-45 junctions caused only a weak effect on cardiac impulse conduction. Loading through connexin-43 junctions with coupling strengths comparable to that of NRVMs yielded significant conduction slowing and action potential prolongation in NRVM monolayers while causing no change in conduction velocity anisotropy ratio or the incidence of spontaneous activity. Finally, a related loading setting was recently applied to study functional integration of mouse or human ESC-cardiomyocytes cultured on top of a thin ventricular slice (Figure 2)

from embryonic mice [101,102] or neonatal rats [103]. The main advantage of this preparation is the potential to perform long-term monitoring of stem cell–cardiac tissue integration in a setting of realistic tissue architecture. In addition, the ventricular slices can be obtained from both untreated and stem cell-treated ischemic or infarcted hearts [103] thus allowing functional studies of heterocellular interactions under pathological conditions.

Tissue bundles for studies of contractile force generation

While fast electrical propagation within the stem cell-treated cardiac milieu is a prerequisite for safe, nonarrhythmogenic cardiac repair, the potential of implanted cells to improve the pumping function of the heart will also depend on their ability to form electromechanical junctions with host myocytes and augment or maintain their contractile function, by either: electrically synchronizing contractions of unconnected myocytes; or undergoing cardiogenic differentiation with the development of mature excitation–contraction coupling. To systematically study the ability of different stem cells to affect cardiac contractile function *in vitro*, stem cells and cardiomyocytes need to be co-cultured within a reproducible, mechanically compliant 2D or 3D setting that can support macroscopic tissue contractions and allow mechanical tests using standard force transducers. The described strand, monolayer and bilayer settings utilize rigid substrates for cell growth, and although well suited for studies of electrical propagation, they prevent any macroscopic contractions and thus the ability to measure generated contractile forces. On the other hand, cardiomyocyte or skeletal myoblast monolayers cultured on elastic 2D substrates such as thin microgrooved PDMS films [104] or aligned electrospun polyurethane fibers [105] have been successfully used for measurements of isometric contractile force. While the main advantage of these settings is direct experimental and diffusional access to cultured cells, the measured forces [104] are an order of magnitude smaller than in thicker 3D cardiac multilayers [106–108] and the uniaxial measurements of active and passive force can be harder to interpret due to the 2D nature of the preparation and the presence of the underlying adhesive growth substrate.

3D stem cell–cardiomyocyte assays based on the use of natural or synthetic hydrogels, although in general more complicated to develop, offer several advantages compared with heterocellular monolayers. First, cells in a soft, 3D tissue culture environment attain more physiologically relevant shapes, cell–cell and cell–matrix interactions compared with cells in a 2D environment [109]. As a result, micro- and macro-scopic electrical and mechanical function, including the mechanical stresses and electrical load experienced by cardiomyocytes [42], are distinctly different in 2D and 3D settings. Furthermore, compared with the large extracellular bath that surrounds monolayer cultures, the confined extracellular space in dense 3D cultures can amplify local autocrine and paracrine actions of cells [110] and directly affect the cardiac electrical propagation by modifying local circuit currents [111] and extracellular ion concentrations [112]. In addition, 3D cultures can be maintained intact over a relatively long period of time, in the order of several weeks or months, as opposed to monolayers that typically last between 1 and 2 weeks. This longer culture time under more physiological conditions may influence the proliferation, differentiation and function of stem cells to more closely mimic an *in vivo* state. Finally, the presence of multiple cell layers and no adhesive substrate in 3D cultures allows relatively robust and straightforward force measurements.

The geometrically simplest 3D setting to systematically study the effects of stem cell implantation on cardiac mechanical properties is a relatively thin (100–500 μm in diameter) and long ($\sim 1\text{--}3\text{ cm}$) tissue cylinder in the form of a heterocellular bundle (Figures 2 & 7) or a ring. This pseudo-1D tissue configuration is created by culturing a mixture of cells and fibrin or collagen gel within cable- [113] or ring-shaped molds [108,114]. Through the process of cell-mediated gel compaction and under the passive tension resulting from imposed geometrical constraints (e.g., two fixed Velcro® felts at the ends of the hydrogel, Figure 7A),

the NRVMs in the gel spread, align and interconnect to form functional 3D cardiac tissue bundles (Figure 7B). Fibroblasts or various stem cell types (including nonadherent cells) can be incorporated into the NRVM bundles either diffusely (Figure 7C) or as a defined central bridge (Figure 7D). The resulting unidirectional cell alignment is ideally suited for well-controlled uniaxial viscoelastic tests and isometric contractile force measurements (Figure 7E–G). For these measurements, tissue bundles are usually clamped via the two Velcro felts, while tissue rings are threaded onto two small metal hooks [114]. The mounted tissues are stimulated either by a strong field shock to measure the maximum contractile force of all cells regardless of their connectivity, or by near-threshold point pacing to measure the force physiologically generated secondary to electrical propagation. The difference between the two measured forces along with mapping of electrical propagation can reveal the degree of functional connectivity between the cells. For 3-week-old NRVM bundles, the generated contractile forces and velocities of electrical propagation are respectively a few hundred μN (Figure 7E) and 30 cm/s. In addition, standard force-frequency (Figure 7F), force-length (Figure 7G) and different pharmacological tests can be used to further characterize the functional consequences of stem cell ‘implantation’ in this system.

In addition to macroscopic tissue bundle or ring settings, novel miniature tissue fabrication approaches have been recently described [115,116]. In these methods, a large number of few millimeter- or sub-millimeter-sized tissue bundles were created by molding a cell/collagen gel mixture within individual tissue wells containing two thin PDMS pillars (Figure 2). The two pillars served to anchor the spontaneously formed tissue rings and by acting as calibrated cantilevers, enabled force measurements based on the linear bending theory. The small dimensions of the rings allowed for rapid penetration of soluble agents into the tissue. If proven to be sufficiently sensitive, these technically delicate but promising approaches have a potential to be developed into:

- Relatively high-throughput, low volume screening applications to test different soluble factors for their ability to improve direct stem cell–cardiomyocyte interactions and generated contractile forces;
- Platforms to noninvasively monitor, in the same tissue ring, the stem cell differentiation-induced changes in cardiac force generation as a function of culture time.

Future perspective

Owing to its potential impact on the lives of millions of people worldwide, the exciting field of cell-based cardiac therapies is rapidly moving forward under extremely high expectations. Although some stem cell therapies for postinfarction heart disease have already become a clinical reality, there exist several fundamental questions that if answered, would allow significant progress in the field. These include:

- To what extent do stem cells and host cardiomyocytes experience cell contact-mediated interactions?
- Can these interactions be manipulated through the use of soluble or genetic factors to improve electrical propagation and force generation in the treated host tissue?
- Which stem cell type(s), mode(s) of cell implantation and level(s) of cell maturity will offer the most functional benefit with the least arrhythmogenic risk?

These questions are rapidly gaining in importance as new stem cells capable of generating large numbers of functional cardiomyocytes with the ability to strongly couple with host tissue are actively being considered for clinical trials.

With the advancements in our knowledge of basic stem cell biology and increased feedback from clinical trials, the number of potential therapeutic combinations involving different cell types and approaches is becoming intractably large. A rational and systematic approach to the design of novel stem cell therapies represents the only viable path forward. Although the techniques to label, track and functionally examine implanted stem cells in the intact myocardium have improved, the development of standardized high-fidelity *in vitro* and *in situ* platforms that can help bridge the knowledge gap between clinical outcomes and basic functional studies of heterocellular interactions will be vital for ensuring accelerated progress in the field. In particular, new high-resolution noninvasive imaging modalities, nontoxic genetic indicators of cellular function and high-throughput micropatterning and microfluidic techniques are just some of the promising approaches to allow more focused and unifying studies in the field. The main short-term goals of these methodological developments will be to enable: systematic screening and mechanistic studies of different chemical, genetic and biophysical factors that can modulate functional stem cell–cardiomyocyte interactions; as well as quantitative comparisons of different stem cell types in well-defined and reproducible conditions for their ability to improve cardiac function while exerting minimal arrhythmogenic risks.

The ability to precisely control spatial distribution of stem cells within different co-culture assays will also lead to a better understanding of how specific modes of cell delivery (e.g., large bolus injection vs several small injections vs dispersed delivery through coronaries vs tissue patch) affect the functional results of cell therapies.

In summary, this article has described some of the recent innovative methodologies to characterize direct functional interactions between stem cells and cardiomyocytes. While conventional immunohistology and transmission electron microscopy can reveal structural cell interactions *in situ*, developing versatile methods to dynamically study the real-time functional interactions between stem cells and cardiomyocytes *in vivo*, *in situ* and *in vitro* at the cellular and tissue scales would allow us to dissect and manipulate the active roles that implanted cells may have upon the electrophysiological or contractile function of the host tissue. In order to accelerate progress in this field, the development of new theories regarding the fate and function of various implanted stem cells must be accompanied by new techniques that can rigorously test these theories. A successful ‘functional interaction’ between bioengineering and biology will be one of the keys to achieving this goal.

Executive summary

In situ assays for functional stem cell–cardiomyocyte characterization

- Immunohistochemistry is used for the assessment of structural stem cell–cardiomyocyte interactions.
- Two-photon microscopy allows high spatial resolution, small field-of-view studies of functional stem cell–cardiomyocyte coupling, up to a few hundred microns below the heart surface.
- Macroscopic optical mapping allows heart surface studies of electrical propagation and arrhythmia generation over a few cm² area with a few hundred micron resolution.
- Genetic indicators of intracellular Ca²⁺ concentration such as gCaMP2 allow cell-targeted, long-term *in situ* and *in vivo* studies of functional coupling and electrical propagation.
- Current *in situ* functional studies of stem cell–cardiomyocyte interactions suffer from low reproducibility, limited sample size and no direct access to studied cells.

***In vitro* assays for functional stem cell–cardiomyocyte characterization**

- Standardized *in vitro* heterocellular assays offer several levels of structural complexity, high biochemical and geometrical reproducibility, and direct access to studied cells.
- These assays are specialized for a particular functional test and have a ‘plug-in’ nature to allow systematic quantitative comparisons among different types of stem cells.
- Micropatterned cell-pair assays provide control over cell size, shape and length of cell–cell contact to enable high-throughput reproducible studies of structural and electrical heterocellular coupling.
- Micropatterned cardiomyocyte strands with interspersed or defined inserts of stem cells permit pseudo-1D studies of heterocellular electrical conduction at a microscopic scale.
- Heterocellular monolayer and bilayer cultures enable studies of 2D electrical propagation, loading and arrhythmogenesis in defined geometries that mimic stem cell implantation scenarios.
- 3D tissue bundles with defined densities or distinct regions of embedded stem cells present a more native *in vivo*-like cellular environment and permit the measurements of contractile force generation.
- 3D miniature tissue bundles with defined densities of stem cells allow contractile force measurements combined with high-throughput, low-volume screening applications.
- Thin ventricular slices enable long-term integration studies in the setting of realistic cardiac architecture and permit the measurement of both electrical conduction and contractile force.

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Figure 1. Optical recording of calcium transients in gCaMP2-expressing neonatal rat ventricular myocytes

(A) Cultured myocytes were transfected to express the mCherry fluorescent protein (B) as well as gCaMP2 (D & E). (C) During application of a 4-V/cm² Hz field stimulus train, mCherry signal exhibited only bleaching and no fluctuations, while (F) gCaMP2 fluorescence levels significantly varied with changes in intracellular Ca²⁺ concentration. (G) Spatial distribution of color-coded gCaMP2 fluorescence intensity in a different cell at five time points during the Ca²⁺ transient. Fluorescent signals were recorded at 20× magnification using an Andor IxonEM +860 EMCCD camera and a Nikon TE2000U microscope.

Stem cell-Cardiomyocyte Co-cultures	Functional Study				Main Advantages	Main Disadvantages	Ref.
	Cell-cell Coupling	Electrical Conduction	Arrhythmias	Contractile Force			
Cell Pairs	+++				Defined cell shape and cell-cell contact length, high throughput	Relatively short-term studies	[69]
Cell Strands	+	+++	+		Well-defined path of electrical propagation, relatively high throughput, microscale studies	Difficult to prevent cell overlap, no curvature during propagation	[65,75]
Cell Monolayers		+	+++	+	Control of proportion and spatial distribution of stem cells, control of structural anisotropy, similar optical access to all cells	Difficult to prevent cell overlap, rigid substrate, absence of 3D matrix and conduction paths	[76,79,80, 88-90]
Cell Bilayers		++	+++	+	Can dissect role of stem cells as an electrotonic load vs. a short-range conductor, easy access to cells	Not true 3D environment, diluted paracrine action, difficult to assess stem cell-cardiomyocyte coupling	[79,92-94,97-100]
Tissue Bundles		++	+	+++	Native-like 3D environment and paracrine actions, 3D electrical loading, soft contractile substrate, uniaxial force generation	Limited thickness and access to cells, pseudo 1D conduction	[107,108, 113,114]
Miniature Tissue Bundles				++	Small volume, high throughput, fast diffusion access to cells	Low contractile forces, no studies of impulse propagation, technically challenging to manufacture	[115,116]
Ventricular Slices		++	++	+	Realistic healthy or pathological tissue architecture, long-term studies, easy access to stem cells	Low reproducibility, potential damage during sectioning, measurements of non-uniaxial force and conduction hard to interpret	[101-103]

Figure 2. *In vitro* settings for functional studies of stem cell–cardiomyocyte interactions

Co-culture settings in different rows increase in structural complexity from top to bottom. The number of + signs describes relative suitability of different settings for a specific type of functional study.

(c) denotes control settings.

Figure 3. Heterocellular micropatterned cell pairs for studies of cell coupling

(A) Immunostaining examples for the expression of Cx43 gap junctions formed at short end-to-end contacts in homocellular neonatal rat ventricular myocytes (NRVMs) (1) and heterocellular NRVM–mesenchymal stem cell (MSC) (2) cell pairs, as well as at long side-to-side contacts of homocellular MSC–MSC pairs (3). No Cx43 junctions were formed in some homocellular MSC (4) and heterocellular (5) pairs despite the existing cell contact, while in other heterocellular pairs Cx43 expression was diffuse and intracellular (6). (B) Fluorescence recovery after photobleaching assay in micropatterned cell pairs. Left: calcein fluorescence (green) in a heterocellular NRVM–MSC pair immediately before bleaching, immediately after bleaching (a), 1 min after bleaching (b) and 4 min after bleaching (c). Right: time course of fluorescence recovery with the points corresponding to the images shown left. (C) Dual whole cell patch clamp in micropatterned cell pairs. A cell pair made of two square-shaped cells was analyzed by clamping both cells to the same holding potential. A voltage stimulus was applied to one cell (V_1 or V_2) to elicit a current response from the other cell (I_2 or I_1) equal to the

junctional current. These experiments enable the quantification of macroscopic gap junctional conductance (120 nS in this NRVM–NRVM pair).

Dashed line denotes 0 mV or 0 nA.

Cx43: Connexin-43; WGA: Wheat germ agglutinin.

Figure 4. Heterocellular micropatterned strands for studies of electrical conduction

(A & B) Examples of 'mixed' strands, where neonatal rat ventricular myocytes (red) are co-cultured with (A) genetically or (B) chemically labeled rMSCs (green). (C & D) Examples of cardiac strands with defined inserts made of different cell types. These strands were paced 5 mm left of the insert and intracellular Ca^{2+} transients were recorded with 37 μm resolution. Traces at recording sites 1–3 demonstrate that rat CFs, rMSCs or mCherry-labeled mESC-derived cardiomyocytes respectively caused conduction block, conducted passively without calcium transients or conducted actively with calcium transients.

CF: Cardiac fibroblast; CM: Cardiac myocyte; cTnT: Cardiac troponin-T; GFP: Green fluorescent protein; mESC: Mouse embryonic stem cell; rMSC: Rat bone marrow-derived mesenchymal stem cell.

Figure 5. Heterocellular monolayers for studies of cardiac arrhythmias

(A & B) Examples of 'mixed' aligned (anisotropic) monolayers where neonatal rat ventricular myocytes (NRVMs; F-actin or sarcomeric α -actinin) are interspersed with (A) cardiac fibroblasts (vimentin) or (B) mesenchymal stem cells (MSCs; unstained, shown by arrowheads). (C & D) Example of NRVM monolayers with central islands made of (C) pure GFP-labeled mouse embryonic stem cell-derived cardiomyocytes or (D) mixed NRVMs and fibroblasts (unstained, shown by arrowheads). Note well-defined and confluent contact between the cells in island and surrounding NRVMs. (E & F) Mapping of action potential propagation in the NRVM monolayer from (D) during (E) 3-Hz steady pacing and (F) a 6-Hz pacing burst. Successive isovoltage frames with corresponding times given at their top right corners are shown from left to right and top to bottom. Blue and red denote rest and peak of the action potential. Arrows show the direction of propagation. Pulse signs denote pacing sites. Small black circles are the 504 recording sites. Central circle is the border of the central island. Note slowing of impulse propagation through the island at 3-Hz pacing rate (E). During 6-Hz pacing burst (F), the propagating action potential wave breaks in the island area and a self-sustained reentrant wave forms in the cardiac-only area (shown at 195 ms). With time, the reentrant wave drifts, and after 1.5 s anchors at a location within the island (last three frames). Stars denote the tip of the reentrant wave. GFP: Green fluorescent protein.

Figure 6. Heterocellular bilayers for studies of electrical loading

(A) After 5 days of culture, confluent aligned NRVMs (green) are covered on top with loading cells (red) at a desired density. (B) Example of a 2 day co-culture with cardiac fibroblasts as the loading cells. (C) Action potential propagation is optically mapped during electrical stimulation from the center of the monolayer (pulse sign). Circles denote recording sites. Activation isochrones are elliptical as a consequence of the NRVM alignment. NRVM: Neonatal rat ventricular myocyte.

Figure 7. Heterocellular tissue bundles for studies of force generation

(A) Example of a cardiac bundle attached to Velcro® felts and mounted within the force measurement setup. (B) Pure (control) cardiac bundles after 2 weeks of culture contain aligned, cross-striated and coupled neonatal rat ventricular myocytes (NRVMs). (C) An example of a 2-week-old 'mixed' bundle containing co-aligned NRVMs (green) and mCherry-labeled mESC-CMs (red). (D) Example of a cardiac bundle with central bridge made of mESC-CMs, cultured inside a silicone 'boat'. Live fluorescence overlaid with phase contrast demonstrates the location of the mESC-CM bridge. (E) Traces of isometric contractile force in cardiac bundles elicited at different frequencies by a field electrode. (F) Negative force-frequency relationship characteristic of mouse and rat hearts. (G) Force-length relationship exhibits physiological shape.

CM: Cardiac myocyte; mESC: Mouse embryonic stem cell.