Interrogating functional integration between injected pluripotent stem cell-derived cells and surrogate cardiac tissue

Hannah Song[a], Charles Yoon[a], Steven J. Kattman[b], Jana Dengler[a,c], Stéphane Massé[d], Thushaanthini Thavaratnam[e], Mena Gewarges[f], Kumaraswamy Nanthakumar[d], Michael Rubart[g], Gordon M. Keller[b], Milica Radisic[a,c,h,1], and Peter W. Zandstra[a,b,c,h,1]

[a]Institute of Biomaterials and Biomedical Engineering, Departments of [c]Chemical Engineering and Applied Chemistry, [e]Cell and Molecular Biology, and [f]Human Biology, University of Toronto, Toronto, ON, Canada M5S 1A1; [b]McEwen Centre for Regenerative Medicine, University Health Network, Toronto, ON, Canada M5G 1L7; [d]The Toby Hull Cardiac Fibrillation Management Laboratory, Toronto General Hospital, Toronto, ON, Canada M5G 2C4; [g]Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN 46202; and [h]The Heart and Stroke/Richard Lewar Centre of Excellence, Toronto, ON, Canada M5S 3E2

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Myocardial infarction resulting in irreversible loss of cardiomyocytes (CMs) remains a leading cause of heart failure. Although cell transplantation has modestly improved cardiac function, major challenges including increasing cell survival, engraftment, and functional integration with host tissue, remain. Embryonic stem cells (ESCs), which can be differentiated into cardiac progenitors (CPs) and CMs, represent a candidate cell source for cardiac cell therapy. However, it is not known what specific cell type or condition is the most appropriate for transplantation. This problem is exasperated by the lack of efficient and predictive strategies to screen the large numbers of parameters that may impact cell transplantation. We used a cardiac tissue model, engineered heart tissue (EHT), and quantitative molecular and electrophysiological analyses, to test transplantation conditions and specific cell populations for their potential to functionally integrate with the host tissue. In this study, we validated our analytical platform using contractile mouse neonatal CMs (nCMs) and noncontractile cardiac fibroblasts (cFBs), and screened for the integration potential of ESC-derived CMs and CPs (ESC-CMs and -CPs). Consistent with previous in vivo studies, cFB injection interfered with electrical signal propagation, whereas injected nCMs improved tissue function. Purified bioreactor-generated ESC-CMs exhibited a diminished capacity for electrophysiological integration; a result correlated with lower (compared with nCMs) connexin 43 expression. ESC-CPs, however, appeared able to appropriately mature and integrate into EHT, enhancing the amplitude of tissue contraction. Our results support the use of EHT as a model system to accelerate development of cardiac cell therapy strategies.

cardiac progenitor | engineered heart tissue | regenerative medicine | myocardial infarction | cell therapy

Heart failure due to myocardial infarction (MI) is the main cause of death in the world (1). Myocardial ischemia due to MI results in the irreversible loss of cardiomyocytes (CMs), which are typically replaced by noncontractile fibrotic scars. Recent advances in stem cell biology have raised considerable hopes for the development of therapeutic approaches for replacing cardiac tissue through cell transplantation (2). However, despite this enthusiasm, most clinical cell transplantation studies have yielded modest results, motivating the need for fundamental studies into the barriers limiting cell-based regeneration of the injured heart.

Uniquely, pluripotent cells such as embryonic stem cells (ESCs) hold the potential to generate bona fide CMs and other derivatives (3–5). Using cardiac promoter specific selection strategies and bioreactor technologies, large numbers of ESC-derived CMs (ESC-CMs) can be produced (6, 7) and appear capable of forming grafts in the adult mice heart (8). Additionally, it now appears possible to identify and isolate specific ESC-derived cardiac progenitors (ESC-CPs) (9–11), opening the possibility of using progenitor cells for cardiac cell therapy, as has been explored using pancreatic endoderm progenitors for the generation of insulin responsive islet-like cells (12, 13). Importantly, although multiple CMs and CPs can be generated from pluripotent cells, it is not clear which specific cell types will be the most effective in treating cardiac disease, or what additional factor(s) may be important for functional cell integration. Furthermore, the large experimental matrix generated by testing different cell types cultured with different survival and integration molecules, creates a barrier to the rapid development of candidate therapies. It is with this limitation in mind that we, herein, explore the utility of an engineered heart tissue (EHT) as a predictive test-bed for functional cell integration. EHT, generated from rat neonatal CMs and collagen scaffolds, and conditioned under "biomimetic" environments, exhibits many of the properties of native heart tissue (14).

We hypothesized that analysis of the changes in the molecular and electrophysiological properties of EHT upon test cell injection could provide insight into the potential (or limitations) of different test cell populations and conditions for cardiac cell therapy. Consistent with previous in vivo studies, injection of mouse cardiac fibroblasts (cFBs) interfered with electrical signal propagation, whereas injected mouse neonatal CMs (nCMs) improved tissue function. Purified bioreactor-generated ESC-CMs exhibited a diminished capacity for electrophysiological integration, a result correlated with lower connexin 43 (Cx43) expression. ESC-CPs, however, appeared able to appropriately mature and integrate into EHT, significantly enhancing the amplitude of tissue contraction. Our results support the use of EHT as a model system to accelerate the development of cardiac cell therapy strategies.

Results

Cell Injection Impacts EHT Function: The Effect of Neonatal Cardiomyocytes and Cardiac Fibroblasts. We have used an electrical stimulation chamber to cultivate EHT under conditions that mimic the native heart. Fig. 1*A* shows a schematic diagram of the experimental and analytical set-up, including yellow fluorescent protein (YFP) cells injected into EHT. To generate EHT, isolated rat nCMs were seeded onto collagen scaffolds, and a biphasic

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[1]To whom correspondence may be addressed. E-mail: m.radisic@utoronto.ca or peter.zandstra@utoronto.ca.

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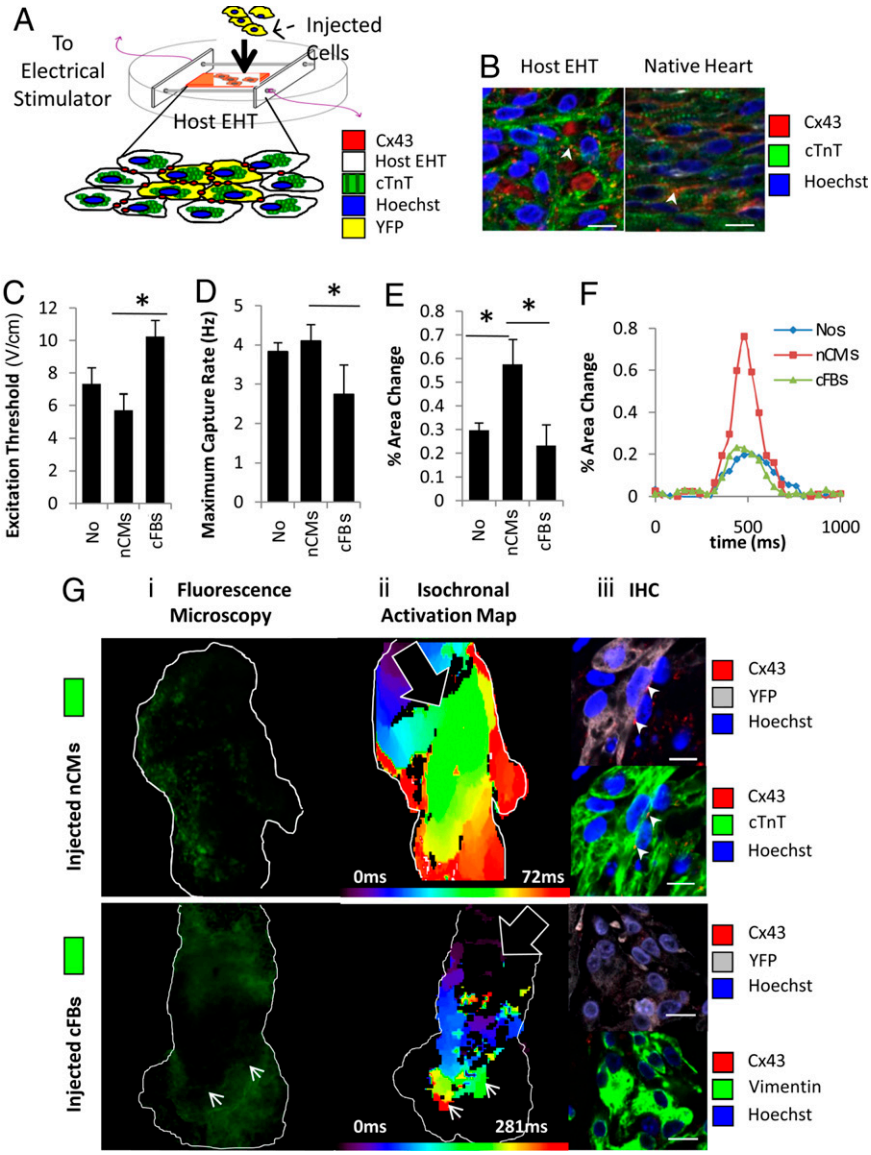

Fig. 1. Validation of EHT as host model heart tissue for in vitro injection analysis. (*A*) Experimental setup of the EHT and schematic diagram of integration of injected and host cells. (*B*) IHC staining of the host EHT and the native heart sections: Cx43 (red), cTnT (green), and Hoechst (blue) (Arrowheads denote Cx43). (*C*) ET, (*D*) MCR, and (*E*) AC analysis on day 13 shows that nCM injection improved the electrical properties of the EHT compared with mouse cardiac fibroblast (cFB) injection. (*F*) Representative contraction cycle with no injection control, nCM and cFB injection. (*Gi*) Fluorescence microscopy of EHT shows the location of YFP+ (green) injected cells. (*ii*) Isochronal activation maps represent the electrical impulse propagation through the EHT, black arrows indicate the electrode, and white arrow heads mark the end point of the impulse. (*iii*) Representative IHC staining denotes the presence of Cx43 junctions (arrow heads) between injected YFP cells and host CMs. Cx43 (red), YFP (gray), cTnT/vimentin (green), and nucleus (blue). $n = 4$–6 samples per group. (Scale bar, 10 μm.)

electrical field was applied starting at day 3 and continued throughout cultivation. As expected (14, 15), cells in EHT aligned and elongated in the direction of the electrical field, and formed Cx43 junctions (Fig. 1*B*).

To evaluate functional integration between test injected cells and host EHT, we analyzed the excitation threshold (ET), the maximum capture rate (MCR), and the amplitude of contraction (AC). ET is the minimum electrical field voltage required to induce synchronous contractions of the EHT (14), and represents the electrical excitability of the construct. MCR is the maximum beating frequency attainable while maintaining synchronous contraction, indicative of the degree of integration and beating capacity (14). Finally, AC is a parameter that indirectly correlates with the force of contraction. In addition to the electrophysiological parameters, we also used optical mapping to measure electrical impulse propagation between injected cells and the host EHT along with immunohistochemical evaluation of Cx43 [a major player in the connectivity between injected fetal and host adult CMs in vivo (16)] expression.

To determine whether the electrophysiology of EHT could be manipulated by test cell populations, we first injected nCMs or cFBs isolated from YFP transgenic mice. Upon cFB injection (Fig. 1*C*), the ET increased significantly ($P = 0.031$), suggesting that these cells require a higher excitation potential to initiate contraction. In marked contrast, nCM injection lowered the ET (Fig. 1*C*), suggesting that EHT function could be increased by the injection of an appropriate cell type. Consistent with these results, cFB injection significantly ($P = 0.048$) decreased MCR (Fig. 1*D*), a parameter not significantly influenced by nCMs. Finally, as expected, both the average (Fig. 1*E*) and time-dependent (Fig. 1*F*) changes in AC across the entire EHT were quantitatively inhibited by the injection of noncontractile cFBs ($P = 0.018$).

To monitor impulse propagation through injected cells within the host EHT, membrane depolarization was measured using optical mapping. With YFP+ nCM injection (Fig. 1*Gi*), the electrical signal propagated uniformly through EHT, as shown by the corresponding isochronal activation map (Fig. 1*Gii*). Immunohistochemical (IHC) analysis supports the optical mapping results showing the presence of Cx43 between cardiac troponin T (cTnT+) injected (YFP+) cells and host (YFP−) cells (Fig. 1*Giii*), suggesting intercellular connections. In contrast, the impulse propagation (Fig. 1*Gii*) was blocked by the injection of nonintegrating cFBs (Vimentin+, Fig. 1*Giii*).

Together, these data support a correlation between the tissue level electrophysiological properties of EHT, and molecular connections between the injected and host cells. cFB injection blocked

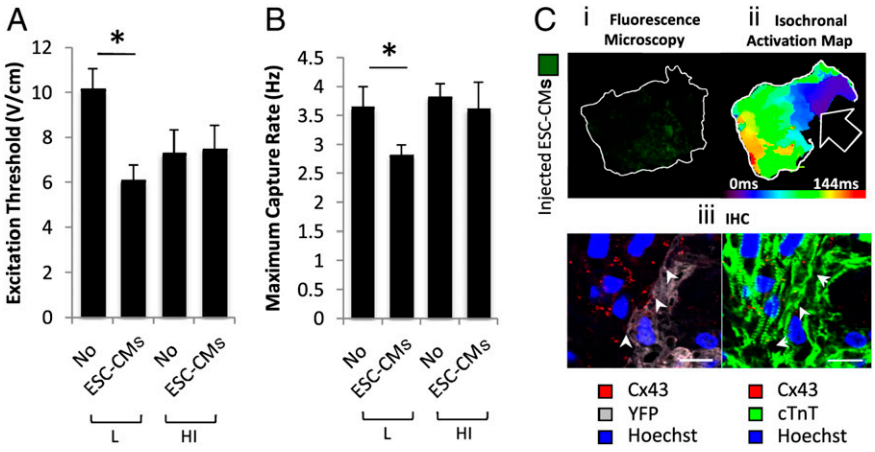

Fig. 2. Injection of genetically selected ESC-derived CMs (ESC-CMs) into the model host tissue. ESC-CMs were injected into EHT in low glucose (L), or high glucose and insulin (HI). (*A*) ET and (*B*) MCR were measured on day 13. (*C*) Representative images of (*i*) YFP-injected cells, (*ii*) activation map of impulse propagation, (*iii*) IHC for Cx43 (red), YFP (gray), cTnT (green), and Hoechst (blue). White arrowheads show presence of Cx43 in gap junctions between ESC-CMs and host CMs. *, $P < 0.05$ by Tukey's post hoc test with one-way ANOVA; $n = 3$–7 samples per group. (Scale bar, 10 μm.)

electrical impulse propagation whereas nCMs improved or maintained the electrical function of the EHT. In vivo studies have similarly reported that while fetal CMs improved cardiac function (17), noncontractile cells such as fibroblasts, or mesenchymal stem cells did not (18). As a next step, we asked whether ESC-CMs, which have been shown to exhibit some but not all of the properties of nCMs (19), would appropriately integrate into the EHT.

Biochemical Factors Modulate Integration of the Injected ESC-CMs with the Host EHT. ESC-CMs represent an important candidate cell population for cardiac cell therapy as clinically relevant cell numbers can be generated in bioreactors (6, 20). In addition to investigating the integration of ESC-CMs into EHT, we also screened the role of the integration environment on the functional properties of the cell injected EHT. We specifically sought to determine if the in vivo and in vitro observations that insulin improves cardiac contractile function (21, 22) in a glucose-dependent manner (23, 24) could be recapitulated in our cell-injection system.

Using genetically engineered YFP ESCs containing a plasmid carrying an α-myosin heavy chain promoter driving neomycin resistance (8), we generated up to 20 million ESC-CMs (>90% cTnT+) after 14 days of culture in 250-mL flasks (Fig. S1). One hundred thousand cTnT+ ESC-CMs were injected into the EHT exposed to low glucose (L), or high glucose and insulin (HI). ET and MCR significantly decreased with L; in contrast there was no significant difference with HI relative to their respective no injection controls (Fig. 2 *A* and *B*). In the L condition, the decrease in the ET suggests that the EHT became more excitable upon the contractile ESC-CM injection; however, the decrease in MCR is indicative of a lack of functional coupling between the host and injected cells (IHC analysis failed to detect connectivity between these two). In the HI condition, the ET and MCR were maintained, and associated IHC demonstrated Cx43 connectivity between injected cells (cTnT+/YFP+) and host (cTnT+/YFP−) tissue (Fig. 2*Ciii*). Electrical impulse propagation was continuous throughout the construct, as manifested via optical mapping (Fig. 2*C i* and *ii*).

These results indicate that the injected ESC-CMs do not interfere with EHT electrophysiological properties, and appear capable, when exposed to an appropriate microenvironment, of integrating with the host tissue. These results also demonstrate that EHT function is sensitive to introduced molecules, supporting the use of this system to screen for cardiac tissue function modifiers. We next asked whether the differential output from the EHT, with different cell-type injections, could be correlated with the molecular properties of individual cells.

ESC-CMs Express Fewer Cx43 Molecules Compared with nCMs Injected into the EHT. The injection of nCMs improved electrical function of the host EHT ($P < 0.05$) (Fig. 1 *C–E*). However, ESC-CM injection showed no change in function compared with the no-injection EHT control (Fig. 2 *A* and *B*, HI conditions; the same condition for Fig. 1 *C–E*), despite the apparent presence of Cx43 junctions between injected and host cells (Fig. 2*C*). Because optical mapping can concurrently detect impulse propagation signals throughout the EHT, the local conduction velocity (CV) through the injected YFP cell area and host EHT area was measured as described in Fig. 3*Ai*. As shown in the activation map (Fig. 1*Gii*), the CV of the cFBs was zero in all constructs, and the CV of the host EHT was 2.5 ± 0.6 cm/s. However, nCM injection increased the CV significantly to 7.4 ± 1.5 cm/s ($P = 0.006$) (Fig. 3*A*) in the host EHT compared with the cFB injection. The CV from nCMs was not significantly different from the host EHT. In contrast, the CV of the ESC-CM injection area was significantly decreased compared with the host EHT ($P = 0.013$) (Fig. 3*Aii*). These data indicate that even though both nCMs and ESC-CMs appeared capable of integrating into host EHT, the characteristics of the injected cells differentially impact the resultant host EHT function.

We hypothesized that the differences between nCMs and ESC-CMs may be attributable to their expression of functional markers associated with CM identity or maturation. A quantitative comparison of Cx43 expression per cell injected was performed. Fig. 3*B* shows that nCMs expressed 1.6 times more Cx43 compared with ESC-CMs ($P < 0.05$). To investigate additional correlations between marker expression and CV, qRT-PCR was used to analyze a time course of ESC-CM differentiation; these data are compared with nCMs (Fig. 3*C*). Both Cx43 and Cx45 mRNA expression in ESC-CMs was lower than in nCMs ($P = 0.013$ and $P = 0.090$, respectively, between nCMs and ESC-CMs) whereas other contractile mRNA expression levels, including cTnT, myosin heavy chain α and β (MHC-α and -β), and myosin light chain v (MLCv), were comparable. Interestingly, the expression of atria-associated myosin light chain α (MLCα) was significantly higher in ESC-CMs ($P = 0.036$). This observation is consistent with the slower CV of atrial CMs relative to ventricular CMs in vivo (25).

The results thus far suggest that the maturation and electrical properties of the injected cells may be important parameters for integration between host and injected cells. We next asked whether using cardiac progenitor cells, which may be capable of responding to local differentiation signals in situ, could functionally integrate into EHT.

Injection of ESC-Derived CPs Improves the Electrical Function of EHT. A number of ESC-CPs have been identified and characterized (10, 26, 27). These cells have been isolated at different efficiencies using transcription factor-driven promoters, or using cell surface expressed antigens. Although these strategies are able to significantly enrich for cells with cardiogenic potential, cell populations are generally heterogeneous and likely contain progenitors and mature

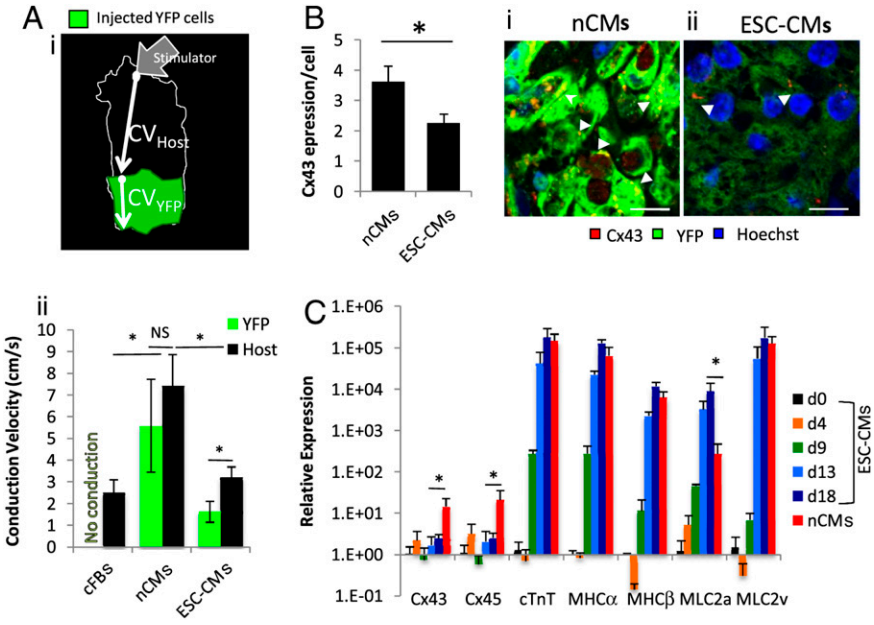

Fig. 3. Characterization of neonatal CMs and ESC-derived CMs in model host tissue. (*Ai*) Schematic diagram to show the calculated local conduction velocity (CV) within the injected YFP+ cells and the host EHT, (*ii*) Quantification demonstrates that injected cell characteristics differentially impact overall EHT function. *, $P < 0.05$ by Tukey's post hoc test with one-way ANOVA, $n = 4$–8 samples per group. (*B*) Cx43 expression per YFP+ cell indicated that (*i*) nCMs expressed significantly more Cx43 than (*ii*) ESC-CMs. *, $P < 0.05$ by *t* test, Cx43 (red) and YFP (green). (*C*) qRTPCR differentiation time course analysis was performed on ESCs during ESC-CM production (see Fig. S1) from days 0–18 and compared with nCMs. Expression was normalized to the average day zero ESCs.

cells from a number of different mesoderm and endoderm lineages. For the purposes of this report, we chose to focus on a cell population defined based on the cell surface expression of the fetal liver kinase 1 (Flk1) and platelet-derived growth factor receptor α (PDGFRα) antigens. This population is enriched for CPs (26) and can be generated using a serum-free directed differentiation protocol (9).

ESC-CPs were enriched to >90% purity using FACS from day 4 cultures and injected into the host EHT for functional and phenotypic analyses. ET and MCR were measured after 1, 4, and 8 days of cocultivation. IHC staining from nCM injection shows that by day 4, cells start to elongate and conjugate with the EHT (Fig. S2). Parallel analysis of ESC-CP maturation independent of EHT shows similar maturation kinetics (Fig. S3). The ET transiently increased with both injection of nCMs and ESC-CPs after 1 day, but markedly decreased in a time dependent manner by day 8 (Fig. 4*A*); concomitantly MCR values increased, suggesting a higher or maintained connectivity with the host EHT (Fig. 4*B*). Injection of ESC-CPs also increased the AC as measured by the percentage area change (Movies S1–S3). We also measured host cell and injected cell numbers over 8 days of cultivation after digesting the construct, and showed that the injection of ESC-CPs has a survival effect on the EHT (Fig. 4*C*). The continuous electrical impulse propagation between injected cells and the host EHT, as determined by optical mapping (Fig. 4*D i* and *ii*), suggests functional integration between ESC-CPs and CMs. Further, IHC for Cx43 shows junction formation between injected cells (YFP+/cTnT+) and the host EHT (YFP−/cTnT+) (Fig. 4*Diii Left*). IHC analysis for cTnT on large areas of the ESC-CPs demonstrates that they can mature in situ, evidenced by striation (Fig. 4*Diii Right*). However, the amount of Cx43 expression in ESC-CPs (2.0 ± 0.5/cell) was comparable to ESC-CMs. Therefore, to further evaluate coupling between ESC-CPs and EHT, we examined intracellular calcium transients using laser scanning confocal microscopy in combination with the calcium-sensitive fluorophore rhod-2. Calcium transients in YFP-expressing nCMs (Fig. 4*Ei*) or ESC-CPs (Fig. 4*Eii*) occurred synchronously with those in neighboring non-YFP-expressing host CMs, suggesting functional integration of injected cells. Together, these results suggest that sorted ESC-CPs can differentiate into cardiac cells in model cardiac tissue and that conditions could be defined for the use of ESC-CPs as an appropriate test cell type for in vivo transplantation studies.

Discussion

Cardiac cell transplantation is an area of intense research due to excitement around the potential of cell therapy to the heart. Several groups have investigated restoration of cardiac cell function in vivo using a number of different cell types, including fetal CMs (16), adult bone marrow-derived cells (28), hematopoietic stem cells (29), ESC-CMs from mouse and human (8, 30, 31), and human fetal cardiac Sca1+ progenitors (32). It is not clear, however, what cell type will be best suited for cardiac cell therapy, or under what conditions cells should be transplanted. Furthermore, in vivo studies designed to test multiple variables and combinations can be experimentally daunting. Recently, other groups have published studies using murine ventricular slices to test cell injection ex vivo (33, 34). This approach, although powerful, does not allow for independent control of tissue properties such as CM content and sample heterogeneity, or the design and synthesis of cardiac tissue using human pluripotent cells. Therefore, we have developed an in vitro injection system that should serve as a powerful test-bed to screen multiple conditions for future in vivo cell transplantation studies.

In this study, we tested the ability of nCMs, cFBs, ESC-CMs, and ESC-CPs to integrate into model cardiac tissue. Functional and molecular analyses of the model tissue-injected cell construct provided quantitative insights into cell injection parameters; analyses that have proved very difficult in vivo. Using optical mapping (35), we obtained an electrical impulse propagation map, and used this output to examine spatial integration between injected cells and the host EHT. Our results intriguingly suggest a reciprocal relationship between injected cell and host cell electrical properties; in particular, the CV of the host tissue appeared to be responsive to the CV of the injected cells.

Importantly, our results demonstrate that injected cells can influence the electrical properties of model host tissue. Based on electrical performance measured by ET, MCR, and AC, the EHT system could distinguish between different injected cell types. Injected cFBs blocked electrical impulse propagation completely and aggravated the electrical properties of the EHT, whereas nCM injection improved these parameters. EHT should thus be a useful platform to enable functional screening for integration analysis before in vivo experiments.

By screening for injection conditions to improve the electrical function and integration, we showed that the functional properties

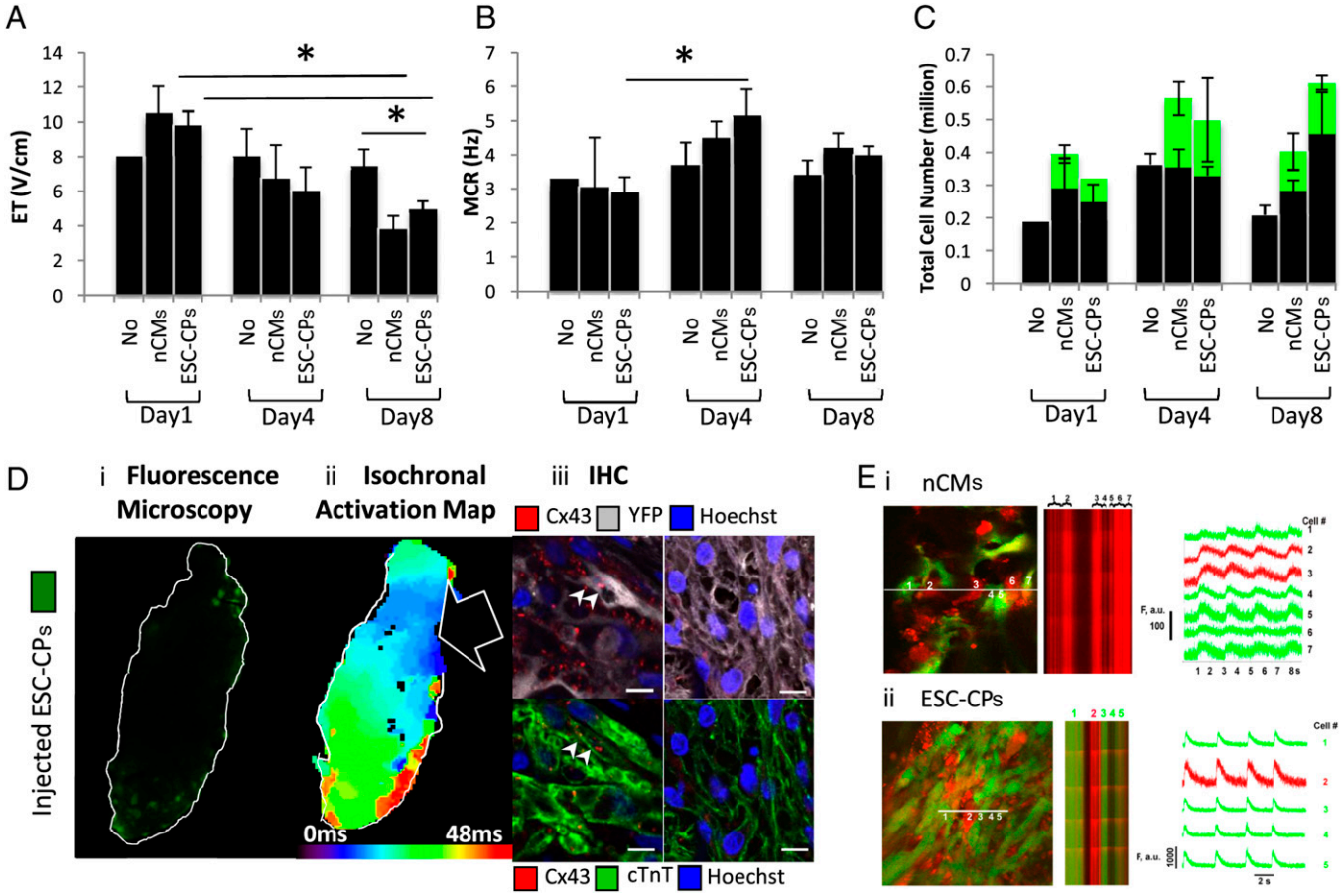

Fig. 4. Functional analysis injection of ESC-derived Flk1+/PDGFRα+. Time course of (*A*) ET, (*B*) MCR, and (*C*) injected and host cell numbers show that nCMs and ESC-CPs injected into EHT improved model host tissue function over time. *, $P < 0.05$ by *t* test, $n = 3$–6 samples per group. (*Di*) Injected YFP+ CP cells and (*ii*) the corresponding activation map indicate successful integration and electrical propagation through both the injected CPs and host EHT, further supported by IHC (*iii*) of Cx43 junctions (*Left*). For maturation, IHC (*Right*) shows cTnT+ ESC-CPs. (*E*) Entrainment of action potential-induced changes in intracellular calcium in YFP-expressing and nonexpressing cardiomyoytes within EHTs. (*i*) Frame (*Left*) and line-scan mode image (*Middle*) taken from an EHT injected with YFP-expressing nCMs. The line-scan image was obtained by repeatedly scanning along the white line in *Left* and stacking the lines vertically. *Right* shows the spatially averaged changes in rhod-2 fluorescence as function of time for each cell along the white scan line. Changes in rhod-2 fluorescence occur synchronously in YFP+ (green) and YFP− myocytes (red), indicating that both cell types are functionally coupled. (*ii*) Identical results were obtained when images were taken during spontaneous activity of an EHT injected with ESC-CPs.

of the test cell injected EHT could be modulated by the cell-injection environment. Whereas, under baseline conditions, injected ESC-CMs showed a reduced potential to integrate with the host tissue (compared with nCMs), ESC-CM injection in the presence of insulin, likely via modulation of PI3 kinase/Akt signaling (36), improved injected cell-EHT connectivity. These results are consistent with observations in that the use of "survival cocktails" may be useful adjutants for cardiac cell therapy (37).

Finally, ESC-CPs were tested for functional integration. We found that ESC-CPs can survive and integrate into the EHT, and appear to improve electrical function to a greater extent than that observed using ESC-CMs (via an as yet undefined mechanism). Importantly, we extended our tissue level analysis to the single cell level and confirmed the functional coupling between individual injected cells and host EHT using confocal microscopy and simultaneously tracking reporter protein expression and calcium-dependent fluorescence. As additional early cardiogenic markers are identified and characterized, this cardiac model tissue-cell injection system is well poised to provide rapid quantitative insight into the cardiac therapy-related potential of these cells.

Motivated by these promising results, further improvements to the EHT-cell injection system should be considered. Sekine et al. (38) generated a cardiac patch and investigated the connection between the host and graft with or without a cFB layer, showing that a single cFB layer inhibits the CMs bridging between the host and graft. Miragoli et al. (39) also showed that cFBs have a negative impact on the overall propagation velocity as the ratio between cFBs and CMs increases. Pillekamp et al. (40, 41) have generated a heart explant model from normal heart and ischemically damaged tissue using glucose/oxygen deprivation. Using similar approaches, EHT could be engineered with an artificial scar tissue layer using cFBs deposited within the EHT to create an MI model. Subsequent screening studies for the effect of matrix proteolytic enzymes to break down scar tissue before the injection of candidate cardiac cells, or other strategies to maximize the integration between injected and host CMs, should prove informative.

The EHT system may also prove useful in screening for adjuvants to cardiac regeneration. We and others have demonstrated that adding survival factors including insulin or IGF-1 improves cell injection outcomes (42). We further speculate that maturation cues may be an important component of progenitor cell–transplantation strategies. These issues highlight the utility of the EHT platform to systematically monitor multiple parameters for screening cell survival and integration in the presence of supportive biomaterials, inflammatory cytokines or cells, and small molecules. Finally, because of the large number of conditions and small molecules that

may affect cardiac tissue function and survival that could be tested, scaling EHT into a functional tissue array platform (reviewed in ref. 43) for high-throughput screening should be considered as a valuable next step.

One of the main advantages of the EHT system lies in its customizability. The EHT can be created using human ESC- or human-induced pluripotent stem cell-derived CMs (iPS-CMs). This strategy is particularly advantageous in terms of the human cardiac studies where EHT made from iPS-CMs can be used as the host tissue, and targeted population of human cells can be injected. Not only could this system enable highly relevant human injection studies, it could also enable patient specific EHT for individualized drug and cell injection screens.

In summary, the present study provides a platform for in vitro functional screens of multiple different cardiac cell types and conditions. This platform should not only contribute to a large reduction in experiment time and effort needed to identify candidate strategies for in vivo analysis, it should also provide mechanistic insights into strategies to overcome the host tissue-injected cell integration barrier.

Methods

Generation of the Host EHT and Injected nCMs, cFBs, ESC-CMs, and -CPs. Neonatal rat (Sprague–Dawley) and mouse [YFP transgenic, 129-Tg(CAG-EYFP)7AC5 Nagy/J; Jackson Laboratory] hearts were harvested and enzymatically digested using trypsin (6120 U/mL, 4 °C, 5–8 h), followed by series of collagenase digestion steps. CMs were seeded onto the collagen scaffold and cultivated in the environmental chamber with electrical stimulation until nCMs, ESC-CMs, or ESC-CPs were injected. For differentiation protocols of ESCs, refer to *SI Text*.

Functional Analysis. ET, MCR, and AC were measured as described in ref. 14. Optical mapping was performed using 5 mM Di-4-ANEPPS (Invitrogen), a voltage-sensitive dye, and dye fluorescence was recorded using a microscope mapping system (Ultima Scimedia).

Full methods are available in *SI Text*.

Statistical Analysis. Results are expressed as mean ± SEM. Statistical significance was assessed using Tukey's test followed by one-way ANOVA or Dunn's test for more than three groups (SigmaStat), or *t* test for between two groups, and denoted with * when the *P* value for at least three independent experiments was <0.05.

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