Cardiac Progenitor Cells Improve Function in Human Engineered Cardiac Tissues

Jack Murphy
Mentored by Dr. Irene Turnbull and Dr. Kevin Costa
Cardiovascular Research Center
Icahn School of Medicine at Mount Sinai
High School for Math, Science, and Engineering @ CCNY

Abstract

Heart failure (HF) is a condition whereby the heart is not pumping blood effectively, depriving the body of blood and oxygen it needs to properly function.

Stem cell therapies offer an attractive alternative because they avoid many of the risks associated with traditional HF treatment methods while being able to actively improve heart function.

To identify the mechanisms by which human cardiac progenitor cells (hCPC) improve cardiomyocyte (hCM) contractile function, we studied three groups of human engineered cardiac tissues (hECTs) in custom multi-tissue bioreactors.

1.Contol (-hCPC): No hCPC supplementation.
2.p.+hCPC: With hCPC supplementation (10% of total cells); in shared media bath with p.-hCPC.
3.p.-hCPC: Without supplementation; in shared media bath with p.+hCPC.

hECTs were tested with and without electrical stimulation on Days 6, 8, and 10 post-fabrication.

p.+hCPC showed increased twitch force vs. control and p.-hCPC ($p<0.05$), implicating direct coupling of hCPC and hCM as an important mechanism.

Introduction

One major problem in developing novel HF treatments is the lack of a suitable model for the human heart.

Most laboratory based models of human myocardium have important limitations.

- Animal models are often inaccurate due to species-specific differences.
- Traditional 2-D cell culture models lack bio-complexity needed to represent the human heart.
- Limited access to cardiomyocytes from patients.

hECTs overcome these limitations by being made from human induced-Pluripotent Stem Cell-derived cardiomyocytes (CM), which help solve the lack of cardiomyocytes and ensures human specificity. The tissues are also 3-D, more closely resembling native human heart muscle.

It is generally accepted that there must be some exchange of materials or information between the transplanted stem cells and the host cardio-myocytes. Two possible mechanisms are:

- **Paracrine Signaling:** Indirect communication via secreted molecules.
- **Cell-Cell Coupling:** Direct physical contact is needed for communication.

Hypothesis and Research Design

- Previous research in the Costa Lab showed that paracrine signaling is primarily responsible for pro-contractile effects of human Mesenchymal Stem Cells (hMSC).
- I hypothesized that paracrine factors are also mainly responsible for pro-contractile benefits of hCPC.

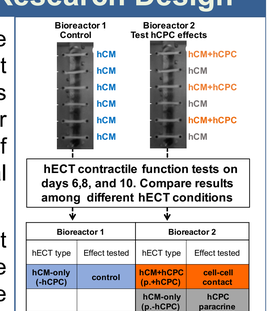


Fig. 1: Research design diagram.

Methods

Fig. 2: Schematic of hECT fabrication using custom bioreactor designed by the Costa Lab.

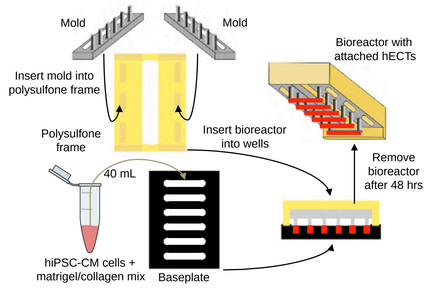


Fig. 3: Adding collagen-1/Matrigel + hiPSC-CM mix into bioreactor resulting in 6 hECTs on pairs of end-posts.

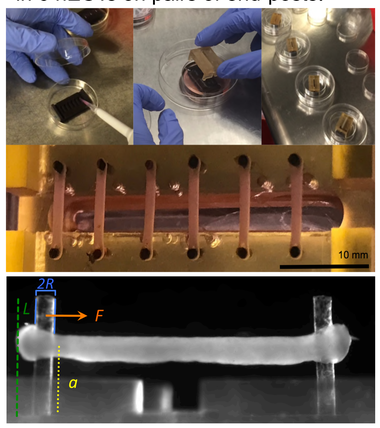
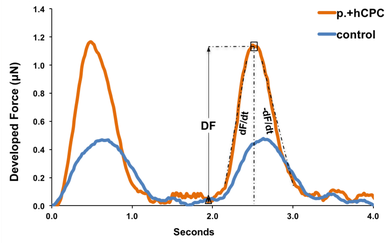


Fig. 4: Side view of hECT with end-posts, showing variables used in the twitch force equation.

$$F = \left\lfloor \frac{3\pi E R^4}{2a^2(3L-a)} \right\rfloor \delta$$

Fig. 5: Twitch force tracings at 0.5Hz stimulation, comparing a control tissue (blue) with a supplemented tissue (orange), showing contractility parameters of developed force (DF) and maximum rates of contraction (+dF/dt) and relaxation (-dF/dt).



Results

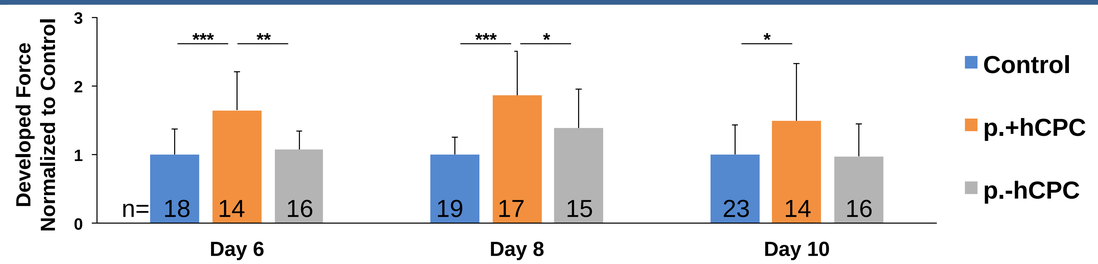


Fig. 6: Developed force under electrical stimulation (0.5 Hz)

- hCPC in direct contact with hCMs (**p.+hCPC**) increased hECT developed force approximately 1.5-fold compared to unsupplemented hECT controls. Statistical significance determined using ANOVA with Scheffe's post-hoc pairwise comparisons, error bars represent standard deviation, *p<0.5, **p<0.01, ***p≤0.001. Sample size, n, as shown.
- hCPC supplemented tissues (**p.+hCPC**) were also stronger than hCPC paracrine-only tissues (**p.-hCPC**).

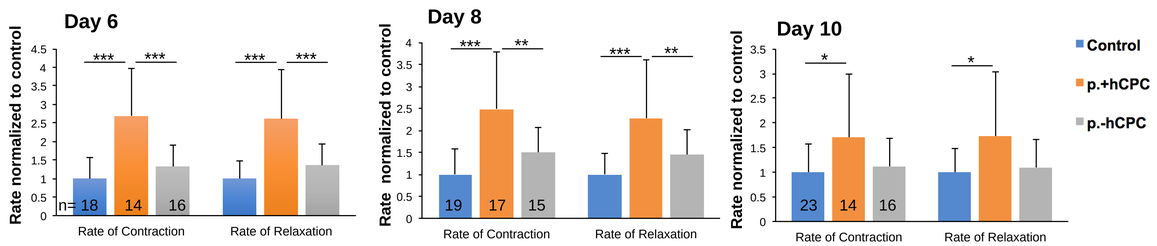


Fig. 7: Rate of contraction under electrical stimulation (0.5 Hz)

- Despite no significant differences in twitch duration parameters (not shown), the p.+hCPC tissues had significantly higher maximum rates of contraction (+dF/dt) and relaxation (-dF/dt) versus unsupplemented hECT controls for all time points tested. Additionally, ±dF/dt were much higher for p.+hCPC than paracrine-only p.-hCPC tissues.

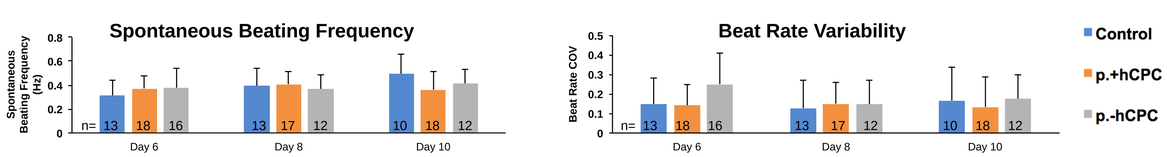


Fig. 8: Spontaneous Beating Frequency and Beat Rate Variability

- There were no observable differences in the spontaneous beating frequency.
- Beat rate variability was similar among all groups. Beat rate coefficient of variation (COV) was calculated by (beat rate std dev)/(average beat rate) with a higher COV indicating less consistency and increased chance of arrhythmia.

Discussion

- Previous studies showing that hCPCs have the ability to stimulate cardiac function in vivo (Bolli et al., 2011) are consistent with our finding that the hCPC supplemented tissues (p.+hCPC) were the strongest at all time points.
- The benefit of hCPC-supplemented tissues declined slightly by day 10. Similarly, clinical trials using MSCs showed that while treatment enhanced heart function, the benefit declined over time (Meyer et al., 2009); mechanistic understanding could lead to improved efficacy.
- hCPC-supplement tissues did not display a pro-arrhythmic phenotype, unlike their hMSC supplemented counterparts (Chang, 2006).
- Recent studies have seen that as CPCs age they become decreasingly effective (Castaldi et al., 2017; Xiao & Thum, 2017), but in this study using adult hCPCs has proven extremely effective.

Conclusions

- hCPC supplemented tissues provided the largest enhancement to contractile force (approximately 1.5 fold increase vs. unsupplemented controls).
- Contrary to my hypothesis, this implies direct cell to cell coupling, not paracrine signaling, is primarily responsible for functional benefits of hCPC on cardiac contractility.
- Unlike MSCs, CPC do not express a pro-arrhythmic phenotype.

Future Directions

- Work to improve retention and engraftment of hCPC implanted in the heart.
- Evaluate if combined MSC + CPC therapies can provide a synergistic benefit.

Acknowledgements

This work was supported by the Center for Excellence in Youth Education (CEYE) Program at the Icahn School of Medicine at Mount Sinai (Jack Murphy), NIH/NHLBI K01-HL133424 (Irene C. Turnbull), and NIH/NHLBI R01-HL132226 (Kevin D. Costa). Adult hCPCs were generously provided by Dr. Mark Sussman at SDSU.

References

1. Bolli R, et al. Cardiac stem cells in patients with ischaemic cardiomyopathy (SCIPIO): initial results of a randomised phase 1 trial. Lancet. 2011;378(9806):1847–57
2. Cai, C., and Molkentin, J. 2017. "The Elusive Progenitor Cell in Cardiac Regeneration." *Circ. Res.* 120(2): 400–406.
3. Castaldi, A. et al. 2017. "Decline in Cellular Function of Aged Mouse C-Kit+ Cardiac Progenitor Cells." *J. Physiol.* 595(19): 6249–6262.
4. Chang, M.G. 2006. "Proarrhythmic Potential of Mesenchymal Stem Cell Transplantation Revealed in an in Vitro Coculture Model." *Circulation* 113(15): 1832–1841.
5. Meyer, GP et al. 2009. "Intracoronary Bone Marrow Cell Transfer After Myocardial Infarction: 5-Year Follow-Up From the Randomized-Controlled BOOST Trial." *Eur. Heart J.* 30(24):2978–2984.
6. Turnbull, I.C. et al. 2014. "Advancing Functional Engineered Cardiac Tissues Toward a Preclinical Model of Human Myocardium." *FASEB J.* 28(2): 644–654.
7. Turnbull, I.C. et al. "Cardiac Tissue Engineering Models of Inherited and Acquired Cardiomyopathies." *Methods Mol. Biol.* (in press).
8. Xiao, K. and Thum, T. 2017. "Exosomal MicroRNAs Released by Pediatric Cardiac Progenitor Cells." *Circ. Res.* 120(4): 607–609.

Cardiac Progenitor Cells Improve Function in Human Engineered Cardiac Tissues

Jack Murphy

Center for Excellence in Youth Education
at the Icahn School of Medicine at Mount Sinai

Jack Murphy

NYCSEF

Title: Cardiac Progenitor Cells Improve Function in Human Engineered Cardiac Tissues

Abstract

Heart failure is a condition whereby the heart is not pumping blood as effectively as it should, depriving the body of blood and oxygen it needs to properly function. Current treatments for heart failure are limited and have major drawbacks; options for treatment include drugs, surgery or medical device implantation. Drugs only provide short-term benefits, often need to be administered daily, and are mostly focused on lowering blood pressure which doesn't help restore strength to the heart. Surgery to help open blocked arteries or repair damaged valves can be dangerous to the patient. Medical devices such as pacemakers and left ventricular assist devices (LVAD) have risks associated with their implantation and can have dangerous side effects or impede everyday life. Stem cells are an attractive new possible treatment because they avoid many of these risks. However, results in clinical trials so far have shown the treatments only provide moderate benefits in the short run and lack long-term significance. If the underlying mechanisms behind the stem cell interactions are better understood, treatments could be modified to have a more favorable clinical impact.

To better understand these mechanisms, specifically between cardiac progenitor cells (CPC) and cardiomyocytes (CM), I generated 3-D human engineered cardiac tissues (hECT) to model the human myocardium. By using human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) I was able to create spontaneously beating tissues. These tissues were fabricated in two different multi-tissue bioreactors to test three different conditions. One bioreactor contained CM-only hECT and acted as the control. The other bioreactor shared media between CPC supplemented hECTs (p. +hCPC), which experience the effects of CPCs from a combination of direct cell-cell contact and indirect paracrine signaling, together with CM-only hECTs (p. -hCPC), which are only exposed to the paracrine effects from CPCs in the neighboring p.+hCPC tissues. With these hECTs suspended between two flexible posts, the deflection of the posts due to hECT contraction was recorded and translated into a developed force. This developed force was calculated while the hECTs were being electrically stimulated to control beat rate, and was used to compare the strength of hECTs among groups.

When comparing CM-only (control) and CM+CPC (p. +hCPC) the CPC supplemented hECTs had a significantly higher developed force when normalized to control (1.65 ± 0.56, 1.86 ± 0.65, 1.49 ± 0.82; on days 6, 8, and 10 respectively; $p<0.05$) whereas the CPC paracrine group was similar to control (CM-only) ($1.06 \pm .23$,$1.38 \pm .57$, 0.97 ± 0.47; p= NS at matched time points). Additionally, the spontaneous beat rate variability, presented as beat rate coefficient of variation (COV) was found to be similar among all three groups; this lack of difference indicates that CPC treatment does not appear to increase the arrhythmogenic risk.

These results were confirmed in four separate iterations of the experiment and suggest that direct cell to cell contact is the most predominant mechanism for CPCs to influence the function of cardiac cells. This suggests that cardiac treatments involving CPCs should focus on improving delivery methods for cell survival and engraftment into the patient's heart in order to maximize clinical outcomes.

Introduction

Heart disease is a leading cause of death both in the United States and abroad, meaning there is an urgent need to find new, reliable treatments (Huffman et al., 2016). One major problem in developing novel treatments is the lack of a suitable model for the human heart. This means that before beginning a clinical trial, which is expensive and risky, there is very limited knowledge as to whether the treatment will be successful or if it will harm the patient. Most lab based models of the human myocardium have severe limitations. Animal models are often inaccurate due to species-specific differences, while traditional 2-D cell culture models lack the complexity needed to represent the human heart. Another limitation in creating a suitable model is access to human cardiomyocytes. This lack of cardiomyocyte availability can be overcome by access to cardiomyocytes through the directed differentiation of human induced pluripotent stem cells (hiPSC). With the development of 3-D human engineered cardiac tissues (hECTs) the Costa Lab has come closer than ever before to creating a realistic model of the human myocardium *in vitro* (Turnbull et al., 2014), and has been able to grow hECTs entirely from hiPSC-CMs (Ceholski et al., 2017; Stillitano et al., 2016). With 3-D hECTs the twitch force of the tissues, the frequency of the contractions, as well as the tissue response to electrical stimulation or certain drugs or compounds can be recorded. These measurements would not be available in any other model of study and can help to better predict how the human heart will respond to a certain treatment *in vivo.*

The myocardium, which is primarily composed of cardiomyocytes, is the muscular tissue that gives the heart its specialized ability to contract and pump blood throughout the body. Myocardial infarction, commonly known as a heart attack, results in local myocardial necrosis after the delivery of oxygen to the tissue is compromised, often due to a blocked coronary artery. Although myocardial infarction survival rates are increasing, this leaves more people at risk for developing heart failure (Velagaleti et al., 2008). In short, current treatments are able to keep the patient alive after a heart attack but are not advanced enough to prevent the devastating complications that arise later. This calls for a treatment that will not only ensure survival but is able to actually repair the damaged tissue and restore function to avoid heart failure related complications in the future. Previous studies have shown that certain types of stem cells have this very effect (Karantalis et al., 2014). Human mesenchymal stem cells (hMSCs) are multipotent stem cells that have the ability to differentiate into bone cells, cartilage cells, fat cells, and most importantly related to the myocardium, muscle cells. Human cardiac progenitor cells (hCPCs) are more specialized stem cells that naturally reside in the heart, but in insufficient numbers to repair the billions of myocytes typically lost during a heart attack. Both of these cells are of particular interest because their surgical delivery to the heart has been shown to stimulate a restoration of cardiac function post myocardial infarction (Bolli et al., 2011; Chugh et al., 2012; Meyer et al., 2009). However, stem cells therapies have often been associated with arrhythmia because of poor integration with the host myocardium. These benefits and drawbacks are not fully understood and are what this project is focused on investigating.

It is generally accepted that there must be some exchange of materials or information between the transplanted stem cells and the host cardiomyocytes, but it is not known how exactly the cells exchange this information. Four of the possible mechanisms for this transfer are reprogramming the cardiomyocytes into a cardiac progenitor-like state, trans-differentiation of the stem cells into cardiomyocytes, paracrine signaling via secreted factors, and direct hetero-cellular electrophysiological coupling (Cashman, Gouon-Evans, & Costa, 2013). If

cardiomyocytes were being reprogrammed into a cardiac progenitor-like state then they could proliferate and re-differentiate back into cardiomyocytes and replace the dead tissue (Acquistapace et al., 2011). Similar to this is the idea of trans-differentiation, whereby a cell progressing towards one cell lineage changes into another cell type via genetic programming (Song, Song, & Tuan, 2004). This would again provide a way of replenishing the dead cells through a form of differentiation. One way to influence the electrical potential post myocardial infarction is through electrophysiological coupling. This method would directly couple stem cells, for example, MSCs with CMs, although it is somewhat controversial whether this would improve or interfere with cardiac function (Mills et al., 2007; Mayourian, Savizky, Sobie, & Costa, 2016). Paracrine signaling, the last proposed method, would mean that certain soluble factors or extracellular vesicles are being excreted from the stem cells and impacting the surrounding cardiomyocytes (Mirotsou, Jayawardena, Schmeckpeper, Gnecchi, & Dzau, 2011).

If paracrine effects are primarily responsible for the restoration of cardiac function in the context of stem cell therapy post-MI, it could obviate the need for direct cell-cell contact that the other mechanisms require. This is important because it could fundamentally alter clinical treatment strategies by circumventing the need to deliver living cells to the patient; they could instead be treated using extracellular vesicles harvested from hCPC or hMSC. This could greatly decrease the chances of harmful side effects associated with injecting living stem cells. This would also open new avenues of research into exactly what is contained in these extracellular vesicles as well as how one can stimulate the cells into producing more of the desired molecules. On the other hand, if direct cell-cell contact is found to be necessary for therapeutic efficacy, then future research would have to focus more on methods for efficient delivery and integration of hCPC and hMSC and lowering the risk of rejection.

Hypothesis/Research Question/Expected Results

It has been shown that hCPCs have the ability to stimulate cardiac tissue function after a necrotic event. I hypothesized that this effect is primarily due to the paracrine signaling between cells and not the result of direct cell-cell contact. This was tested through measurement of the twitch force, the frequency of contractions, and arrhythmic potential of hECTs on multi-tissue bioreactors (**Fig. 1**). If paracrine effects are responsible for the increased tissue response, then the CM-only (CPC paracrine effect, p.-hCPC) tissues in the same bioreactor as the CM+CPC (p. +hCPC), would show the strongest twitch force. If, however, direct cell-cell contact is primarily responsible, then the CPC supplemented tissues (p. +hCPC) will exhibit the strongest twitch force while the CM-only in the same bioreactor (p. -hCPC) will have very little change compared to untreated controls. I also expected

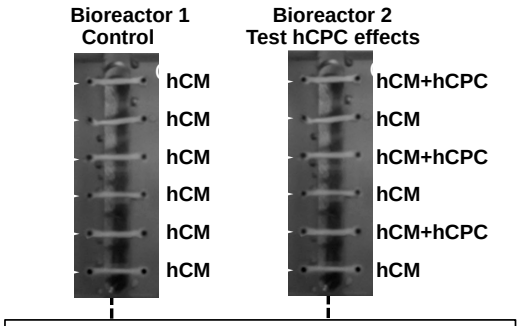


Figure 1. Schematic of experimental design.

that the CM-only tissues (p. –hCPC) in the bioreactor with shared media baths will have a lower arrhythmic potential and be most similar to native human myocardium. This will further support the idea that paracrine factors are responsible for the benefits observed after the introduction of CPCs and can reduce the risk of stem cell therapies by using secreted factors instead of whole cells.

Materials and Methods

Cardiomyocyte Differentiation

Human cardiomyocytes are needed in order to create functional hECTs. These cardiomyocytes were derived from human induced pluripotent stem cells (hiPSC) (healthy cell line 33.1 generously provided by the Hajjar laboratory) (Karakikes et al., 2013)) following a differentiation protocol resulting in a beating monolayer within 10 days. The hiPSCs were maintained in a low oxygen incubator (5% CO_2, 5% O_2) in 6-well plates with 2ml of mTeSR™ media changed daily. After the hiPSC reach confluency of 80-90%, they were either passaged for maintenance or the differentiation protocol was begun. For the first 7 days, the -I media (RPMI 1640 + B-27 Supplement Minus Insulin (50X) + 1% penicillin-streptomycin) was aspirated and each well was washed with 1ml of DMEM/F12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) to make sure no small molecules were left. After the wash, new media was added, with or without small molecules as shown in **Table 1**. On Day 7 the media was changed to +I media (RPMI 1640 + B-27 Supplement (50X) + 1% penicillin-streptomycin) and the plate was transferred from the low oxygen incubator to an incubator with 5% CO_2. On subsequent days, the cells received new +I media daily until they were ready to be harvested for tissue fabrication.

Table 1: hiPSC Differentiation Protocol D0-D7.

Day	Media Change (2ml/well)	Small Molecule (/well)
D0	-I	CHIR99021 (0.67 µl; 30mM stock)
D1	-I	--
D2	--	--
D3	-I	IWR-1 (1 µl; 10mM stock)
D4	-I	IWR-1 (1 µl; 10mM stock)
D5	-I	--
D6	-I	--
D7	+I	--

Cardiomyocyte Isolation

Ranging from Day 18 to Day 28 of differentiation the hiPSC-CM were harvested for tissue fabrication. First, the media was removed from the wells and was given a phosphate-buffered saline (PBS) wash. After PBS was removed it was replaced with 0.025% Trypsin/ Ethylenediaminetetraacetic acid (EDTA) for 5 minutes. After 5 minutes, using a 5ml pipette and

the trypsin in the wells, the cells were dissociated from the plate and collected in a 15ml conical tube. A 1:1 ratio of Trypsin Neutralizing Solution (TNS) was added to the tube to stop the Trypsin reaction. These cells (hiPSC-CM) were then centrifuged at 300g for 5 mins and the supernatant was removed. They were then re-suspended in +I media and a cell count was taken. Depending on the cell count, the number of tissues that could be created was estimated (~1 million cells per tissue for the CM-only condition, and ~0.9 million for co-culture, that is the tissues supplemented with CPC). After this calculation, the total number of CPC needed could be determined (~100,000 per supplemented tissue).

CPC Maintenance and Isolation

The hCPCs, isolated from human heart tissue and generously provided by the Sussman Laboratory at San Diego State University (Monsanto et al., 2017), were maintained in the incubator (37 °C, 5%CO_2) in 100mm dishes with CPC media (HAM'S F12, 10% Embryonic Fetal Bovine Serum, 0.2mM L-Glutathione, 5mU/ml human Erythropoietin, 10ng/ml basic FGF, 1% Penicillin-Streptomycin-Glutamine), with exchange every other day. For passaging the hCPCs, the hCPC dishes were washed 3 times with sterile PBS. Then 1.5 ml of a 1ml:1ml mix of Cellstripper and TripLE was dispensed drop by drop over the 100mm dish. The cells were then incubated for 5 mins in the incubator (37 °C, 5%CO_2). After 5 mins, 1.5 ml of CPC media (described above) was added to the dish and using a pipette the cells were washed off the plates and collected into a 15ml conical tube. The cells were then spun down at 300g for 5 mins, the supernatant discarded, and then re-suspended in CPC media and counted. If no tissues were being fabricated with these cells then they were re-plated at a density of ~ 200,000-250,000 cells per dish. If tissues were being fabricated then, after the cells that were needed were collected, the remaining cells were re-plated.

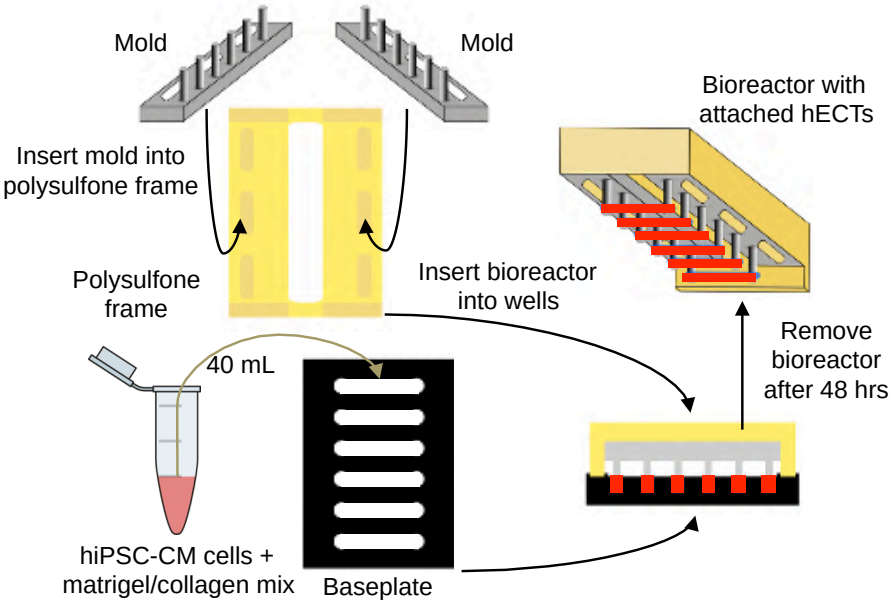


Figure 2. Custom multi-tissue bioreactor designed by the Costa Lab. **1.** The Polydimethylsiloxane (PDMS) master molds are prepared by marking the posts with an ethanol resistant lab marker and being placed on the Polysulfone frame. **2.** The cells are mixed with collagen/Matrigel in an Eppendorf tube. **3.** 40µl of the mix is dispensed into each well on the baseplate. **4.** The Polysulfone frame with the molds are fitted tightly into the baseplate. **5.** After 48 hrs the baseplate is removed and the tissues remain suspended between the posts. Adapted from Turnbull et. al. Methods in Molecular Biology: Cardiac Tissue Engineering Models of Inherited and Acquired Cardiomyopathies (*In Press).*

Tissue Fabrication

After the cell counts described above, the cells were distributed as follows, ~1,000,000 hiPSC-CM per tissue that will not be in direct contact with CPC in one Eppendorf tube and ~900,000 hiPSC-CM plus ~100,000 CPC per CPC supplemented tissue in another Eppendorf tube; spun at 300g for 5 mins and the supernatant was removed keeping only the cell pellets. A mixture of ice cold Collagen Type 1 and Matrigel was added to each of the Eppendorf tubes and mixed with the respective cell pellets (CM-only or, CM+hCPC). This mixture of cells, collagen, and Matrigel was then added to the wells of the baseplate of the multi-tissue bioreactor, using 40 µl per channel (**Fig. 2**). After incubating for 2 hours (37 °C, 5% CO_2), +I media was added and put back in the incubator. After 48 hours, the base plate was removed and the tissues were left on the PDMS molds.

There were two different bioreactor setups resulting in three separate groups of hECT (**Fig. 1)**. The first bioreactor consisted of hECT fabricated with hiPSC-CM (called CM-only) and was used as the control. The second type of bioreactor condition consisted of hECT fabricated with 90% hiPSC-CM supplemented with 10% hCPC (called CPC-Supplemented, p.+hCPC) alternating with hECT fabricated with hiPSC-CM (like the CM-only controls) but under the paracrine effect of hCPCs from the CPC-supplemented tissues (called CPC-paracrine, p.-hCPC); both CPC-supplemented and CPC-paracrine share the same media bath in this bioreactor setup.

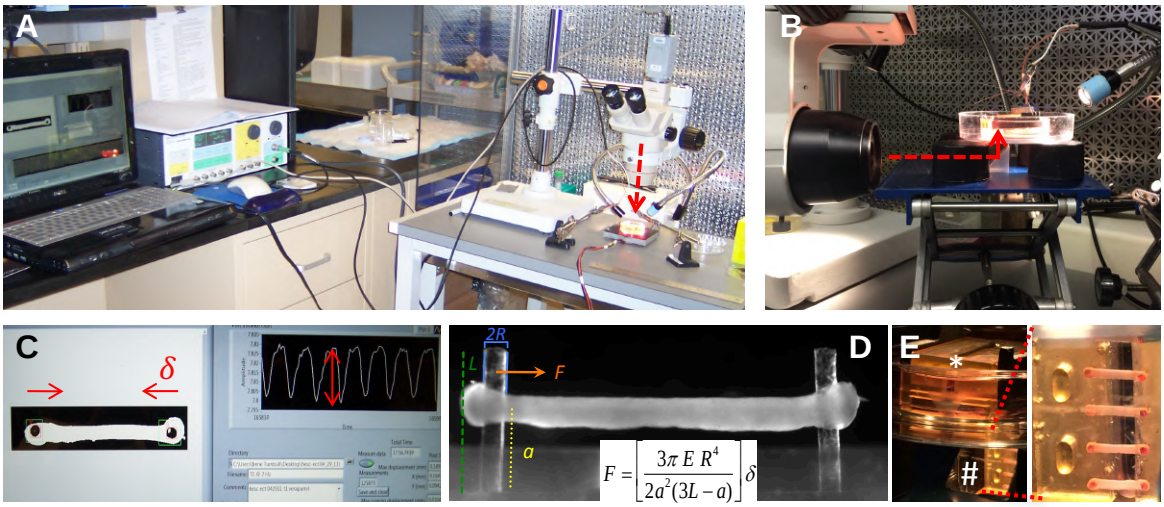


Figure 3. Summary of Methods for Data Collection **A.** Shows the setup for the single tissue bioreactor with the high-speed camera looking directly down at the tissue. **B.** With the use of the 6-tissue bioreactor the setup had to be modified because the bioreactor was inverted. The solution for this was to have the camera aiming at a right-angle mirror placed under the bioreactor so that the camera could detect the top of the post as shown in (C) **C.** The left shows the tissue from the top and the arrows indicate the direction of the contractions. The right shows how LabVIEW graphs the displacements. **D.** Side view of a hECT on flexible posts. The force equation and relevant variables are shown. **E.** This shows how the tissues in the 6-well bioreactor (*) look from the side and from the bottom through the mirror (#) (left) and with zoom in on the image reflected on the mirror (right). Each tissue is suspended between two flexible posts 6mm apart. Adapted from Turnbull et al. Methods in Molecular Biology: Cardiac Tissue Engineering Models of Inherited and Acquired Cardiomyopathies. (*In Press).*

hECT Contractile Function Measurements

On days 6,8, and 10 post-fabrication the hECT twitch force amplitude and beating frequency were recorded both during spontaneous conditions as well as under electrical stimulation. This was carried out using a testing apparatus (**Fig. 3**) developed in the Costa Laboratory (Serrao et al., 2012; Turnbull et al., 2014). The contractile function of the different hECT culture conditions was tested and recorded using LabVIEW and analyzed using MATLAB software to produce a twitch force graph with the amplitude being the developed force (**Fig. 4**). These developed forces were then compared amongst groups to determine the effects of hCPCs. The developed force is calculated from the difference between the maximum and minimum force, with the maximum being at the peaks when the tissue is actively contracted and the minimum being when the tissue is relaxed.

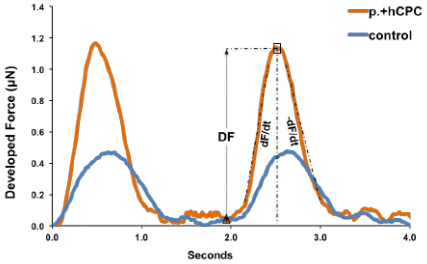


Figure 4. Twitch force tracings at 0.5Hz stimulation, comparing a control tissue (blue) with a hCPC supplemented tissue (orange), showing contractility parameters of developed force (DF) and maximum rates of contraction (+dF/dt) and relaxation (-dF/dt).

Analysis of Combined hCPC+hMSC Therapy

hECT were also fabricated and their contractile function measured to evaluate the benefits provided by a combined hCPC+hMSC therapy. We analyzed the following conditions from three separate bioreactors: 1) Unsupplemented hiPSC-CM-only (Control), 2) 90% hiPSC-CM supplemented with 5% hCPC and 5% hMSC (+hCPC+hMSC), 3) 90% hiPSC-CM supplemented with 10% hMSC (+hMSC).

Statistics

Descriptive statistics are reported as mean ± SD. Analysis of variance (ANOVA) with Scheffe's post hoc test was used for pairwise comparisons among the three groups. Statistical significance was accepted for $p < 0.05$.

Results

hiPSC were successfully differentiated into a beating monolayer. Often times the differentiation protocol can result in many isolated beating clusters. While these can be viable it is more effective when the entire well beats together as a monolayer as shown in **Fig. 5A**. After the beating monolayer was achieved these cells were able to be adapted into fully functioning hECTs with or without the addition of hCPC.

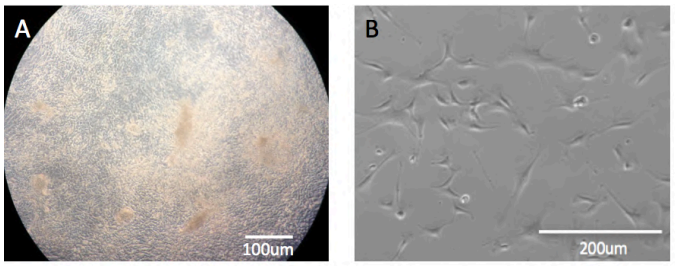


Figure 5. A. hiPSC derived-cardiomyocyte beating monolayer. **B.** Cultured human cardiac progenitor cells.

hCPCs were successfully maintained and expanded in vitro. As shown in **Fig. 5B**, hCPCs are characterized by a high aspect ratio meaning they appear long and thin. These cells were collected and added to a mix of hiPSC-CM + collagen and Matrigel, resulting in fully functioning hECTs.

On days 6, 8 and 10 after hECT fabrication, tissues electrically stimulated at 0.5 Hz with hCPC in direct contact (CPC-supplemented, p. +hCPC) had approximately 1.5-fold the developed force (1.65 ± 0.56, 1.86 ± 0.65, 1.49 ± 0.82; on days 6, 8, and 10 respectively; $p<0.05$) than that of the hECT control (**Fig. 6**). CPC-paracrine (p. –hCPC), which were CM tissues not in direct contact with CPC but in a shared media with the CPC-supplemented, lacked any statistically significant differences when compared to the control CM-only tissues (n=18,14,16; n=19,17,15; n=23,14,16 for each of the three groups respectively on days 6, 8, and 10). Additionally, despite no significant differences in twitch duration parameters (not shown), the p.+hCPC tissues had significantly higher maximum rates of contraction (+dF/dt) and relaxation (-dF/dt) versus hECT controls for all time points tested (**Fig. 7**) (n=18,14,16; n=19,17,15 and n= 23, 14, 16 for day 6, 8 and 10 respectively).

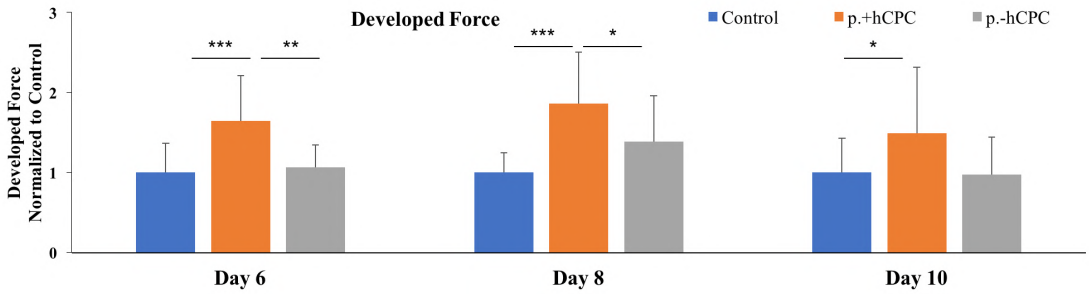


Figure 6. Average developed force for tissues under electrical stimulation (0.5Hz). Shows that CPC supplemented tissues are stronger than both the control as well as the CPC paracrine tissues ($p<0.05$). CPC tissues on day 10 are still significantly stronger than either control or paracrine tissues, however, less so than on days 6 and 8. Bar graphs represent mean ± SD, *$p<0.5$, **$p<0.01$, ***$p\leq0.001$.

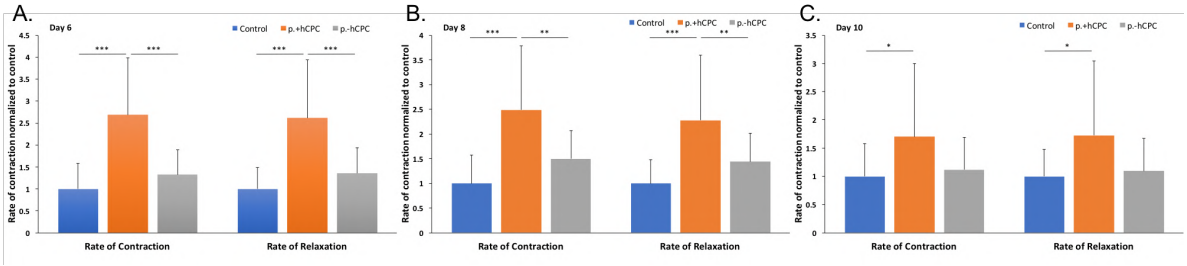


Figure 7. Rate of contraction and relaxation under electrical stimulation (0.5 hz) **A.** Rate of contraction and relaxation for the supplemented tissues (p. +hCPC) was significantly faster than either the paracrine (p. –hCPC) or control tissues (***$p\leq0.001$). This trend continued through days 8 and 10, however, declined slightly by day 10 as shown in **B** and **C** respectively. Bar graphs represent mean ± SD, *$p<0.5$, **$p<0.01$, ***$p\leq0.001$.

To investigate the beat rate variability in each of the groups, twitch force data of the spontaneously beating tissues was analyzed, and it was found that although the CPC paracrine tissues had a slightly higher COV (i.e. more beat-to-beat variability) than either the control or CPC supplemented tissues, the differences were not statistically significant (**Fig. 8**) (n=13,18,16; n=13,17,12 and n= 10, 18, 12 for day 6, 8 and 10 respectively).

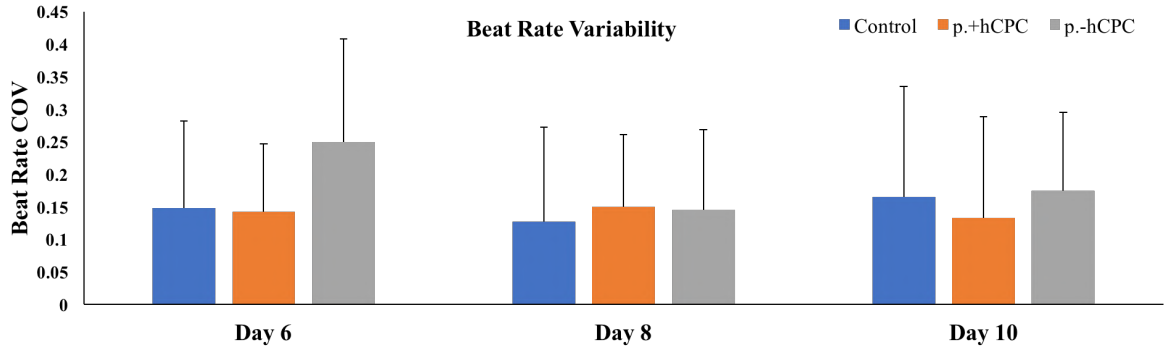


Figure 8. Beat rate variability under spontaneous conditions represented by the coefficient of variance (COV). Beat Rate COV was calculated using the equation $\frac{Beat\ rate\ standard\ deviation}{Average\ beat\ rate}$.

Effectiveness of a combined hCPC+hMSC therapy was analyzed on days 6, 8 and 10 after hECT fabrication. Tissues were electrically stimulated at 0.5 Hz, and those with hCPC+hMSC in direct contact (+hCPC+hMSC) generated approximately a 4-fold increase in the developed force (5.20 ± 3.60, 4.55 ± 3.13, 3.45 ± 2.00; on days 6, 8, and 10 respectively) compared to unsupplemented control hECTs (**Fig. 9**). hMSC supplemented (+hMSC) tissues had nearly 1.75-fold the developed force (1.81 ± 1.21, 1.76 ± 0.63, 1.78 ± 0.66) of the control tissues (n=4,3,3; n=2,3,3; n=3,3,5 for each of the three groups respectively on days 6, 8, and 10). Despite these clear trends, due to the small sample size of this exploratory study, the statistical power was not sufficient to detect significant differences between these groups.

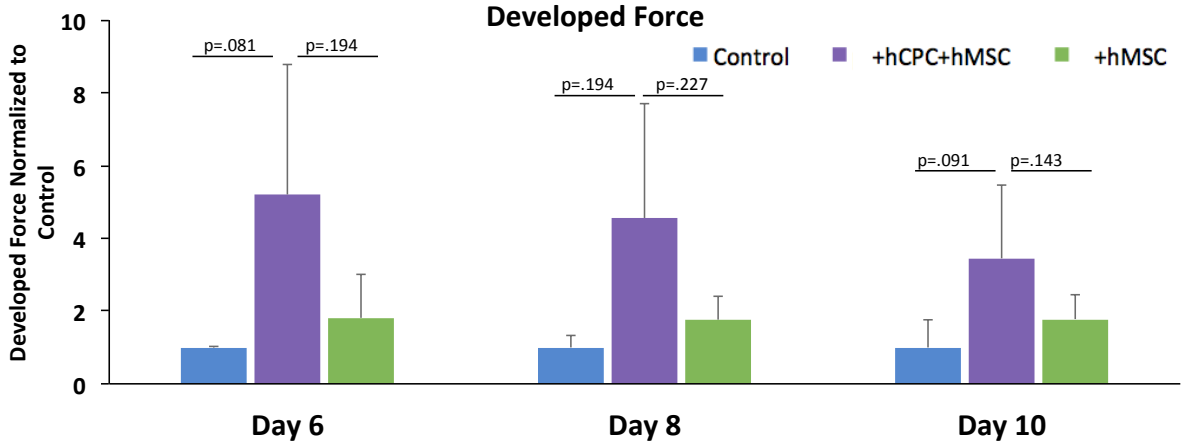


Figure 9. Average developed force for combined hCPC+hMSC tissues under electrical stimulation. Shows that hCPC+hMSC supplemented tissues are stronger than both the control as well as the paracrine only tissues. Although not statistically significant, showing a clear trend of enhancement of contractile force in the presence of combined hCPC+hMSC therapy.

Discussion

The objective of this research was to study the underlying mechanisms behind CPC - cardiomyocyte communication and determine the primary underlying mechanism as well as the CPCs effect on contractility strength and beat rate variability. The CPC supplemented tissues were found to be the strongest of the three conditions compared at all time points tested. This result is supported by previous studies reporting that CPCs have the ability to stimulate function of cardiomyocytes in the heart (van Berlo et al., 2014). Although the mechanisms and significance are debated, there is growing evidence to support an enhancement of contractile function (Cai & Molkentin, 2017) . Although the CPC supplemented tissues remained nearly double the strength of the control and CPC-paracrine hECTs, their strength declined slightly by day 10. This decline in force over-time can be supported by the findings in previous clinical trials relating to other stem cell therapies that indicated that while MSCs did provide a benefit it was transient and declined over time (Meyer et al., 2009). Seeing that both MSC and CPC are able to provide benefits but that the enhanced function only lasts short term implies that something other than the delivery methods needs to be altered to increase long term impact of the treatment.

CPC-supplement tissues did not display a pro-arrhythmic phenotype, unlike their MSC supplemented counterparts (Chang, 2006). Although these results suggest that in order for CPCs to be an effective therapeutic tool the entire cell would need to be transplanted into the damaged heart, which carries the risk of rejection, it also shows that if delivery methods were effective enough to bring the CPCs in direct contact with the damaged or weakened myocardium and the cells were not rejected, they would be able to restore the cardiac muscle strength without increasing the risk of arrhythmia. This presents a tradeoff for future treatment options.

MSCs have been shown to primarily use paracrine signaling, which means that for an effective treatment, the secretome or certain isolated molecules would need to be administered (Mayourian et al., 2017). While this greatly decreases the risk, MSC have been shown to potentially induce the dangerous side effects of arrhythmia. Both stem cell treatments need to be further investigated to determine which one provides the greatest benefits with the lowest risk, or whether there may be synergistic benefits of combining both MSC and CPC treatments (Williams et al., 2013).

One favorable characteristic of the CPC used in this study is that their origin is from heart tissue that is normally discarded after the installment of a LVAD, but that was instead used to isolate CPCs as well as MSC and even endothelial progenitor cells (EPC). Interestingly, in this experiment, the CPC cells seem to be impactful even though recent literature would suggest otherwise. Recent studies have seen that as CPCs age they become decreasingly effective (Castaldi et al., 2017; Xiao & Thum, 2017), but in this study using adult CPCs has proven extremely effective. This makes studying these cells worthwhile as there is still much ambiguity as to how they are performing their function. Furthermore, if these cells were found to be effective, then one way of avoiding the rejection risk would be by taking a biopsy from someone and using the cells isolated to individualize their treatment (autologous transplant). This could not only be effective for CPC isolation but for MSC as well, and if both types of cells could be obtained from a single biopsy, that would help to make it less difficult to offer a possible co-treatment, since there are studies describing that using combined MSC+CPC prove to be even more effective than a single cell type therapy (Quijada et al., 2015; Williams et al., 2013). Obvious limitations include the dangers associated with open heart surgery to simply take a

small piece of the heart as well as how little is understood about how to best implement these treatments, one possible solution would be to differentiate iPSCs into CPC to avoid taking a heart biopsy.

My research shows that CPCs need direct cell contact with cardiomyocytes to exert a beneficial effect on contractility, but the best way to achieve this in a patient setting remains unclear. If cells are injected directly into the myocardium then the cells may not spread to help the entire muscle and may only provide a limited benefit to a localized target region. With this limitation, however, there may still be therapeutic uses, for example with a localized infarct (Williams et al., 2011).

Interestingly, we observed an almost 4-fold increase in developed force when using a combined hCPC+hMSC therapy compared to unsupplemented controls, the greatest benefit so far. Although not achieving statistical significance, likely due to a limited sample size, there is a clear trend showing an increase in the contractile force enhancement provided by the combined therapy versus the benefits provided by either the CPC or the MSC therapies on their own. Further experiments to verify this trend and understand the underlying mechanisms are ongoing.

Limitations and Future Work

Even though 3-D hECTs create an attractive model of the native human myocardium, there are noteworthy limitations in the application of the results from this experiment. One reason is that the hECTs only model the myocardium, the middle layer of the heart, and omit the other two layers (i.e., the epicardium and endocardium), meaning they may not reveal complications that do not directly impact the myocardium. This limitation, however, presents interesting new avenues of research. For example, is it possible to make a 3-D hECT that could emulate the structural organization of the three layers (epicardium, myocardium and endocardium)? If so, would it better model the human heart as a whole, making it a more effective tool in the search for novel treatments? Also, these 3-D hECTs lack blood vessels which would be further advantageous in studying the conditions of ischemia. Lastly, in some respects the hECT structural and contractile features resemble the immature human myocardium more closely than the mature myocardium (Turnbull et al., 2014); enhancing the maturity of the hECT contractile features could advance the field by providing a more precise model of the healthy adult human myocardium.

Conclusions

When comparing the hCPC supplementation to hCPC paracrine effect in hECT, the hCPC supplemented tissues provided the largest enhancement to contractile force (approximately 1.5-fold) while the hCPC paracrine group remained similar to the unsupplemented control. This implies that direct cell coupling is a primary mechanism contributing to therapeutic capabilities of the hCPC. Additionally, hCPC paracrine effects had minimal effect on contractility, and overall hCPC treatments were not shown to heighten the risk of arrhythmia. Therefore, future strategies to optimize hCPC therapy for cardiac repair should focus on improving the retention and engraftment of implanted cells within the host tissue. Finally, combined hCPC + hMSC therapy seems promising, with preliminary results showing the enhancement of contractile force being nearly double that of either hCPC or hMSC cell therapy on its own. This study suggests the hECT model system may be a powerful in vitro assay for investigating and optimizing future stem cell therapies for treating the human heart.

Acknowledgments

This work is supported by Center for Excellence in Youth Education Program (CEYE) at the Icahn School of Medicine at Mount Sinai (Jack Murphy), NIH 1K01HL133424-01 (Irene C. Turnbull), and NIH/NHLBI R01HL132226 (Kevin D. Costa).

References

Acquistapace, A., Bru, T., Lesault, P. F., Figeac, F., Coudert, A. E., Le Coz, O., et al. (2011). Human mesenchymal stem cells reprogram adult cardiomyocytes toward a progenitor-like state through partial cell fusion and mitochondria transfer. *Stem Cells*, *29*(5), 812–824. http://doi.org/10.1002/stem.632

Bolli, R., Chugh, A. R., D'Amario, D., Loughran, J. H., Stoddard, M. F., Ikram, S., et al. (2011). Effect of Cardiac Stem Cells in Patients with Ischemic Cardiomyopathy: Initial Results of the SCIPIO Trial. *Lancet (London, England)*, *378*(9806), 1847–1857.

Cai, C.-L., & Molkentin, J. D. (2017). The Elusive Progenitor Cell in Cardiac Regeneration. *Circulation Research*, *120*(2), 400–406. http://doi.org/10.1161/CIRCRESAHA.116.309710

Cashman, T. J., Gouon-Evans, V., & Costa, K. D. (2013). Mesenchymal stem cells for cardiac therapy: practical challenges and potential mechanisms. *Stem Cell Reviews*, *9*(3), 254–265. http://doi.org/10.1007/s12015-012-9375-6

Castaldi, A., Dodia, R. M., Orogo, A. M., Zambrano, C. M., Najor, R. H., Gustafsson, S. B., et al. (2017). Decline in cellular function of aged mouse c-kit+ cardiac progenitor cells. *J Physiol*, *595*(19), 6249–6262. http://doi.org/10.1113/jp274775

Ceholski, D. K., Turnbull, I. C., Pothula, V., Lecce, L., Jarrah, A. A., Kho, C., et al. (2017). CXCR4 and CXCR7 play distinct roles in cardiac lineage specification and pharmacologic β-adrenergic response. *Stem Cell Research*, *23*(Supplement C), 77–86.

Chang, M. G. (2006). Proarrhythmic Potential of Mesenchymal Stem Cell Transplantation Revealed in an In Vitro Coculture Model. *Circulation*, *113*(15), 1832–1841. http://doi.org/10.1161/CIRCULATIONAHA.105.593038

Chugh, A. R., Beache, G. M., Loughran, J. H., Mewton, N., Elmore, J. B., Kajstura, J., et al. (2012). Administration of Cardiac Stem Cells in Patients With Ischemic Cardiomyopathy: The SCIPIO Trial: Surgical Aspects and Interim Analysis of Myocardial Function and Viability by Magnetic Resonance. *Circulation*, *126*(11_suppl_1), S54–S64. http://doi.org/10.1161/CIRCULATIONAHA.112.092627

Huffman, M. D., Lichtman, J. H., Woo, D., Judd, S. E., Writing Group Members, Nichol, G., et al. (2016). Heart Disease and Stroke Statistics-2016 Update: A Report From the American Heart Association. *Circulation*, *133*(4), e38–360. http://doi.org/10.1161/CIR.0000000000000350

Karakikes, I., Senyei, G. D., Hansen, J., Kong, C.-W., Azeloglu, E. U., Stillitano, F., et al. (2013). Small Molecule-Mediated Directed Differentiation of Human Embryonic Stem Cells Toward Ventricular Cardiomyocytes. *STEM CELLS Translational Medicine*, *3*(1), 18–31. http://doi.org/10.5966/sctm.2013-0110

Karantalis, V., Difede, D. L., Gerstenblith, G., Pham, S., Symes, J., Zambrano, J. P., et al. (2014). Autologous mesenchymal stem cells produce concordant improvements in regional function, tissue perfusion, and fibrotic burden when administered to patients undergoing coronary artery bypass grafting: The Prospective Randomized Study of Mesenchymal Stem Cell Therapy in Patients Undergoing Cardiac Surgery (PROMETHEUS) trial. *Circulation Research*, *114*(8), 1302–1310. http://doi.org/10.1161/CIRCRESAHA.114.303180

Mayourian, J., Hare, J. M., Cashman, T. J., Ceholski, D. K., Johnson, B. V., Sachs, D., et al. (2017). Experimental and Computational Insight Into Human Mesenchymal Stem Cell Paracrine Signaling and Heterocellular Coupling Effects on Cardiac Contractility and ArrhythmogenicityNovelty and Significance. *Circulation Research*, *121*(4), 411–423. http://doi.org/10.1161/CIRCRESAHA.117.310796

Mayourian, J., Savizky, R. M., Sobie, E. A., & Costa, K. D. (2016). Modeling Electrophysiological Coupling and Fusion between Human Mesenchymal Stem Cells and Cardiomyocytes. *PLoS Computational Biology*, *12*(7), e1005014–29. http://doi.org/10.1371/journal.pcbi.1005014

Meyer, G. P., Wollert, K. C., Lotz, J., Pirr, J., Rager, U., Lippolt, P., et al. (2009). Intracoronary bone marrow cell transfer after myocardial infarction: 5-year follow-up from the randomized-controlled BOOST trial. *European Heart Journal*, *30*(24), 2978–2984. http://doi.org/10.1093/eurheartj/ehp374

Mills, W. R., Mal, N., Kiedrowski, M. J., Unger, R., Forudi, F., Popovic, Z. B., et al. (2007). Stem cell therapy enhances electrical viability in myocardial infarction. *Journal of Molecular and Cellular Cardiology*, *42*(2), 304–314. http://doi.org/10.1016/j.yjmcc.2006.09.011

Mirotsou, M., Jayawardena, T. M., Schmeckpeper, J., Gnecchi, M., & Dzau, V. J. (2011). Paracrine mechanisms of stem cell reparative and regenerative actions in the heart. *Journal of Molecular and Cellular Cardiology*, *50*(2), 280–289. http://doi.org/10.1016/j.yjmcc.2010.08.005

Monsanto, M. M., White, K. S., Kim, T., Wang, B. J., Fisher, K., Ilves, K., et al. (2017). Concurrent Isolation of 3 Distinct Cardiac Stem Cell Populations From a Single Human Heart Biopsy. *Circulation Research*, *121*(2), 113–124. http://doi.org/10.1161/CIRCRESAHA.116.310494

Quijada, P., Salunga, H. T., Hariharan, N., Cubillo, J. D., El-Sayed, F. G., Moshref, M., et al. (2015). Cardiac Stem Cell Hybrids Enhance Myocardial RepairNovelty and Significance. *Circulation Research*, *117*(8), 695–706. http://doi.org/10.1161/CIRCRESAHA.115.306838

Serrao, G. W., Turnbull, I. C., Ancukiewicz, D., Kim, D. E., Kao, E., Cashman, T. J., et al. (2012). Myocyte-Depleted Engineered Cardiac Tissues Support Therapeutic Potential of Mesenchymal Stem Cells. *Tissue Engineering Part A*, *18*(13-14), 1322–1333. http://doi.org/10.1089/ten.tea.2011.0278

Song, L., Song, L., & Tuan, R. S. (2004). Transdifferentiation potential of human mesenchymal stem cells derived from bone marrow. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *18*(9), 980–982. http://doi.org/10.1096/fj.03-1100fje

Stillitano, F., Turnbull, I. C., Karakikes, I., Nonnenmacher, M., Backeris, P., Hulot, J. S., et al. (2016). Genomic correction of familial cardiomyopathy in human engineered cardiac tissues. *European Heart Journal*, *37*(43), 3282–3284. http://doi.org/10.1093/eurheartj/ehw307

Turnbull, I. C., Karakikes, I., Serrao, G. W., Backeris, P., Lee, J. J., Xie, C., et al. (2014). Advancing functional engineered cardiac tissues toward a preclinical model of human myocardium. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *28*(2), 644–654. http://doi.org/10.1096/fj.13-228007

Turnbull, I. C., Mayourian, J., Murphy, J. F., Stillitano, F., Ceholski, D. K., & Costa, K. D. (In Press). Cardiac Tissue Engineering Models of Inherited and Acquired Cardiomyopathies. Methods in Molecular Biology.

van Berlo, J. H., Kanisicak, O., Maillet, M., Vagnozzi, R. J., Karch, J., Lin, S.-C. J., et al. (2014). c-kit(+) Cells Minimally Contribute Cardiomyocytes to the Heart. *Nature*, *509*(7500), 337–341.

Velagaleti, R. S., Pencina, M. J., Murabito, J. M., Wang, T. J., Parikh, N. I., D'Agostino, R. B., et al. (2008). Long-term trends in the incidence of heart failure after myocardial infarction.

Circulation, *118*(20), 2057–2062. http://doi.org/10.1161/CIRCULATIONAHA.108.784215
Williams, A. R., Hatzistergos, K. E., Addicott, B., McCall, F., Carvalho, D., Suncion, V., et al. (2013). Enhanced Effect of Human Cardiac Stem Cells and Bone Marrow Mesenchymal Stem Cells to Reduce Infarct Size and Restore Cardiac Function after Myocardial Infarction. *Circulation*, *127*(2), 213–223.
Williams, A. R., Trachtenberg, B., Velazquez, D. L., McNiece, I., Altman, P., Rouy, D., et al. (2011). Intramyocardial stem cell injection in patients with ischemic cardiomyopathy: functional recovery and reverse remodeling. *Circulation Research*, *108*(7), 792–796. http://doi.org/10.1161/CIRCRESAHA.111.242610
Xiao, K., & Thum, T. (2017). Exosomal MicroRNAs Released by Pediatric Cardiac Progenitor Cells. *Circulation Research*, *120*(4), 607–609. http://doi.org/10.1161/CIRCRESAHA.117.310443