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Spatiotemporal Tracking of Cells in Tissue Engineered Cardiac Organoids

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Abstract

Cardiac tissue engineering aims to create myocardial patches for repair of defective or damaged native heart muscle. The inclusion of non-myocytes in engineered cardiac tissues has been shown to improve the properties of cardiac tissue compared to tissues engineered from enriched populations of myocytes alone. While attempts to mix non-myocytes (fibroblasts, endothelial cells) with cardiomyocytes have been made, very little is understood about how the tissue properties are affected by varying the respective ratios of the three cell types and how these cells assemble into functional tissues with time. The goal of this study was to investigate the effects of modulating the ratios of the three cell types as well as to spatially and temporally track cardiac tri-cultures of cells. Primary neonatal cardiac fibroblasts and D4T endothelial cells were incubated in 5 μ M of CellTracker™ Green dye and CellTracker™ Red dye respectively while neonatal cardiomyocytes were labeled with 20 μ g/mL of DAPI. The non-myocytes were seeded either sequentially (Pre-culture) or simultaneously (Tri-culture) in Matrigel-coated microchannels and allowed to form organoids, as in our previous studies. We also varied the seeding percentage of cardiomyocytes while keeping the total cell number constant in an attempt to improve the functional properties of the organoids. Organoids were imaged on days 1 and 4. Endothelial cells were seen to aggregate into clusters when Simultaneously Tri-cultured with myocytes and fibroblasts, while Pre-cultures contained elongated cells. Functional properties of organoids were improved by increasing the seeding percentage of enriched cardiomyocytes from 40% to 80%.

Keywords

cell tracking; cardiac myocytes; microfabrication; tissue engineering; fibroblasts; endothelial cells; PEG

1. Introduction

Cardiac tissue engineering aims to create myocardial tissue constructs *in vitro* that can be used *in vivo* as functional replacements for damaged tissue or as *in vitro* models for pathophysiological testing. In this effort, it is necessary to engineer tissues which recapitulate, as closely as possible, the structure and function of the native myocardium, which comprises multiple cell types working in concert as a single, cohesive tissue unit. It

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has been estimated that one third of the cells in native myocardium are cardiomyocytes (CM) (Nag, 1980, Banerjee, et al., 2006), which are highly specialized to generate contractile force and occupy 80–90% of the total volume due to their large size (Nag, 1980). The remaining two-thirds are non-myocytes (Nag, 1980, Banerjee, et al., 2006) and include endothelial cells (EC) which line the dense network of coronary vasculature (Seghezzi, et al., 1998) and fibroblasts (FB) which are crucial in secreting and degrading extracellular matrix (ECM) proteins to maintain tissue homeostasis (Banerjee, et al., 2006, Kuzuya and Kinsella, 1994). A smaller percentage of other non-myocyte cell types such as, smooth muscle cells, and pericytes are also found in the myocardium (Nag, 1980, Banerjee, et al., 2006). While early attempts at cardiac tissue engineering were focused on removing the non-myocytes from primary heart cell isolates (Bursac, et al., 1999), recent studies have shown that including the non-myocytes may actually enhance cardiac function in engineered tissues (Naito, et al., 2006, Caspi, et al., 2007, Levenberg, et al., 2005).

Through a rapid screening approach, we previously showed that pre-culturing defined ratios of EC and FB in microchannels for two days prior to seeding CM (termed “Pre-culture”) resulted in dense, viable cardiac organoids resembling native myofibres (Iyer, et al., 2008).

The Pre-cultured tissues maintained cardiac function that was comparable to organoids engineered from myocytes alone, even though fewer myocytes were present in these organoids. Pre-cultured organoids contained more elongated and densely arranged cells compared to organoids engineered from cell suspensions which were enriched for cardiomyocytes. Pre-cultured organoids also displayed more marked evidence of cell-cell communication through gap junctions (determined by immunofluorescence microscopy for connexin-43, a key cardiac gap junction marker) (Iyer, et al., 2008). Interestingly, the same three ratios of cell types (CM, FB, and EC) seeded simultaneously rather than pre-culturing (termed “Simultaneous Tri-culture”) resulted in non-functional organoids displaying a two-dimensional morphology with clusters of endothelial cells. The organoids were also completely devoid of gap junctions.

However, in our previous study, we investigated only a single ratio of the three cell types, we used a fibroblast cell line instead of primary cardiac fibroblasts and the organoids were not analyzed for relative position of the three cell types until the end of the experiment. We hypothesized that the inclusion of higher percentage of cardiomyocytes might improve the functional properties of the engineered heart tissues. In our previous study (Iyer, et al., 2008) we determined that when 40% enriched cardiomyocytes were seeded into cardiac organoids along with FB and EC, there were only 10–20% of cardiomyocytes in the organoids at the end of cultivation. Thus, in this study we also explored seeding 60% and 80% of enriched cardiomyocytes into the organoids in order to obtain a physiological percentage of cardiomyocytes at the end of cultivation (~33%). Similarly, in order to further ensure a physiological cell composition, we decided to use primary cardiac fibroblasts rather than the NIH3T3 fibroblasts we used in our previous tri-culture work (Iyer, et al., 2008)

The objectives of this study were as follows: 1) to systematically vary the seeding fraction of CM, FB and EC in cardiac organoids in Pre-culture and Simultaneous Tri-culture, 2) perform spatial and temporal cell tracking studies in order to better understand how and why the three cell types assemble into healthy, elongated organoids when pre-cultured, but not when simultaneously tri-cultured.

2. Materials and Methods

2.1 Microfabrication and sterilization of PEG templates

The technique for PEG template microfabrication has been previously described (Iyer, et al., 2008). Briefly, liquid poly(ethylene glycol) (PEG) diacrylate monomer was mixed with 0.5% v/v hydroxy methyl propiophenone photoinitiator (HMPP, Sigma) to create a pre-polymer solution. The pre-polymer was pipetted onto a polypropylene mesh (McMaster-Carr) comprised of perpendicularly arranged fibers, which served as negatives for microchannels. The pre-polymer was crosslinked around the mesh by exposure to a handheld 4W 365nm UVB light source (UVL-21, Ultraviolet Products) for 30 seconds at a distance of 1 cm through a circular mask, resulting in PEG discs patterned with three-dimensional microchannels 100–200µm diameter and 3–4 mm in length. The discs were sterilized in 100% ethanol, washed in PBS, and then soaked in CM/FB medium (composition described below) for 24–48 hours. Discs were coated with Matrigel® Matrix (Beckton-Dickinson) and placed in 96-well titer dishes at 4°C for 24 hours. The discs were then placed at 37°C for one hour prior to cell seeding to allow the Matrigel® to undergo gelation.

2.2 Cells

2.2.1 Neonatal Rat Heart Isolation—Neonatal (1 to 2 day-old) Sprague-Dawley rats were euthanized according to the procedure approved by the University of Toronto Committee on Animal Care. The hearts were removed, quartered and the cells were isolated by an overnight treatment with trypsin (4°C, 6120 units/mL in Hank's Balanced Salt Solution, HBSS) followed by serial collagenase digestion (220units/ml in HBSS) as described in previous work (Radisic, et al., 2004). The supernatant from 5 collagenase digests of the tissues was collected and centrifuged at 750 RPM (94 × g) for 4 minutes, resuspended in culture medium and pre-plated into T75 flasks (Falcon) for two 1 hour intervals to separate the non-adherent cells (enriched cardiomyocytes) from the adherent cells (non-myocytes). Primary cardiac fibroblasts (FB) were used in all Pre-culture and Simultaneous Tri-culture experiments and were obtained by cultivating the cells adhered to the T75 flask during the pre-plating step for up to 7 days. The CM and FB were cultured in the same medium, termed "CM/FB medium", consisting of Dulbecco's Modified Eagle Medium (DMEM) with 4.5g/L glucose, 4mM L-glutamine, 10% certified fetal bovine serum (FBS), 100U/mL penicillin, 100µg/mL streptomycin, and 10mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid buffer (HEPES, Gibco/Invitrogen).

2.2.2 Cell lines—D4T endothelial cells (kind gift of P. Zandstra), an embryoid body-derived mouse endothelial cell line(Choi, et al., 1998), were maintained in EC medium consisting of Iscove's Modified Dulbecco's Medium, 5% certified FBS, 100U/mL penicillin, and 100µg/mL streptomycin (Gibco/Invitrogen). Cells were passaged at 70% confluence every 5–7 days.

2.3 Cell Ratios

To determine the effect of varying the CM percentage in each sample, enriched cardiomyocytes were seeded at 40%, 60%, 80% and 100% of the total cell number. The total cell number was fixed at 2×10^5 cells/disc. In the case of Pre-culture and Simultaneous Tri-culture, the remaining cells (1.2×10^5 cells/disc, 0.8×10^5 cells/disc and 0.4×10^5 cells/disc) were seeded according to the ratio 0.2/0.7 FB/EC, as in the 40% Pre-culture condition which we previously reported (Iyer, et al., 2008). The final seeding percentages of cells used in each group are given in Table 1 below. Enriched cardiomyocytes alone were used as a control.

It is important to note that the cardiomyocytes used in these experiments are actually a heterogeneous mixture of cells from the neonatal heart which has been enriched for cardiomyocytes by two 1-hour pre-plating steps, with final cell fractions of $81 \pm 14\%$ CM, $3 \pm 3\%$ EC, and $16 \pm 3\%$ FB, as we previously reported (Iyer, et al., 2008).

2.4 Cell Tracking

As shown in Figure 1, the three cell types used in all Pre-culture and Simultaneous Tri-culture experiments were fluorescently labeled to facilitate cell tracking. CellTracker™ Red CMPTX (Molecular Probes Cat. No. C34552) was used for EC, CellTracker™ Green (Molecular Probes, Cat. No. C2925) was used for FB and a nuclear stain, 4'-6-diamidino-2-phenylindole (DAPI) was used for labeling of CM. Thus tri-colour images would show ECs in red, FB in green, and CM nuclei in blue. The CellTracker dyes were solubilized in DMSO to a concentration of 10mM and then further diluted in serum free culture medium (DMEM) to create a working concentration of 10 μ M. The three cell types (EC, FB, CM) were aliquoted in suspension into three separate tubes in ratios corresponding to the various myocyte percentages (see “Cell ratios” and Table 1 below) and centrifuged at 1500 RPM for 7 minutes ($RCF = 377 \times g$) to pellet the cells. The resulting pellet was reconstituted in 100 μ L of the 10 μ M working dye solution. The dye/cell suspension was transferred to a well of a 96-well titer plate for incubation. An additional 100 μ L of DMEM was added to the tube to rinse out any residual cells/dye and then added to the microwell, diluting the dyes to a final concentration of 5 μ M and DAPI to a final concentration of 20 μ g/mL. The well plate was then incubated at 37°C in 5% CO₂ for 30 mins for the CellTracker dyes and for 1 hr for DAPI labeling. After incubation, the dye/cell suspension was transferred into a tube, centrifuged at 1500 RPM ($RCF = 377 \times g$) for 7 min, and the pellet was thoroughly washed in 3 mL of DMEM.

For Pre-cultures, the non-myocytes alone were mixed and recentrifuged before seeding, while for Simultaneous Tri-cultures, all three cell types (myocytes and non-myocytes) were mixed and recentrifuged before seeding (see Figure 1). As in our previous studies, the non-myocytes (ECs and FBs) in Pre-cultures were seeded in PEG microchannels and cultivated for 2 days prior to myocyte addition. In the case of Enriched CM alone, no mixing was required and the cell pellet was seeded directly. The discs were incubated for 30 minutes at 37°C to allow cells to attach to the Matrigel, and then fed with 200 μ L of CM/FB medium, as previously reported (Iyer, et al., 2008).

Fluorescence imaging to track cells was performed by flipping the discs over and imaging under fluorescence microscopy at days 1 and 4 after seeding the CM on an inverted fluorescence microscope (Olympus America, Model # IX81) equipped with a humidified, temperature controlled environmental chamber. Images were obtained using QED Imaging In Vitro Software version 3.1.0 (Media Cybernetics Inc.). Since cells tended to become dislodged during the flipping/imaging process, independent samples were imaged at each time point.

2.5 Assessments

2.5.1 Live/Dead Staining—Live/dead staining was performed as previously described (Iyer, et al., 2008). Briefly, the PEG discs were incubated in a solution of propidium iodide and carboxyfluorescein diacetate-succinimidyl ester for 30–45 minutes at 37°C. As a result, non-viable cell nuclei were labeled red while viable cell cytoplasm were labeled green. The cells in the microchannels were imaged on a fluorescence microscope (Leica DMIRE2), and $n=3-6$ samples were imaged per group. The viability refers only to cells in the areas represented by the long intersecting microchannels and not in the wider, square areas

separating them, where there is no Matrigel and cells necessarily die in larger numbers without a substrate to attach to.

2.5.2 Cryosectioning and Immunofluorescence—For immunostaining we used organoids that were not labeled with cell tracker dyes. Organoids were cryosectioned and immunostained as described previously. Briefly, discs were fixed in 4% PFA for one hour and immersed in a 30% w/v solution of sucrose for 1–2 hours. The discs were placed face down in cryomolds, embedded in O.C.T. medium, and snap frozen in liquid nitrogen for 30 seconds. The discs were stored at -80°C for at least 1 hour before cryosectioning in a cryostat operating at an ambient and specimen temperature of -24°C . $10\mu\text{m}$ -thick face sections were mounted on positively charged specimen slides (VWR) and stored at -20°C for at least 24 hours. The specimens were thawed at room temperature, fixed in acetone, and again air dried before blocking with 10% normal goat serum (NGS) or normal horse serum (NHS) in PBS and stained with primary antibody (at 4°C for 24–48hrs) and secondary antibodies (at room temperature for 1 hour). Primary antibodies included rabbit or mouse anti-cardiac troponin I (Chemicon, 1:150), rabbit anti-PECAM-1/CD31 (Abcam, 1:50), and mouse anti-vimentin-Cy3 (Sigma, 1:50). FITC-conjugated goat anti-rabbit (Vector Laboratories, 1:100) or goat anti-mouse antibody were used as secondary antibodies for troponin-I and CD31. DAPI was used as a nuclear counterstain (final concentration of $1\mu\text{g}/\text{mL}$) and was added to the secondary antibody solution. A drop of mounting medium was added to the specimen and a glass coverslip was placed on top. Fluorescence microscopy was carried out on an inverted fluorescence microscope (Olympus America, Model # IX81) controlled by QED Imaging In Vitro Software version 3.1.0 (Media Cybernetics Inc.). Cell fractions were determined by manual counting, which was performed by two blinded, independent observers. Numbers of cardiomyocytes, non-myocytes, or endothelial cells were determined by counting the number of Troponin-I-positive, Vimentin-positive, or CD31-positive cells in each fluorescence image. These were normalized to the total cell number per image, as determined by DAPI counterstaining, and plotted as average percentages over several images per sample, with $n=3$ –6 samples per group.

2.5.3 Functional Properties—Contractile function of cardiac organoids was measured using either an S88X or an S48 Grass Stimulator (Grass Technologies/Astro-Med Inc) as described previously (Iyer, et al., 2008, Radisic, et al., 2004). Briefly, the Excitation Threshold (ET) and Maximum Capture Rate (MCR) of the tissues were determined by subjecting the tissues to monophasic, square pulses of 2 ms pulse width and measuring the minimum amplitude at which synchronous contractions could be observed. MCR was measured by doubling the ET and measuring the maximum frequency of synchronized contraction with the stimulator output. Thus, a lower ET and higher MCR implied a more electrically responsive tissue. If the organoids could not be excited with the voltage of up to 10 V/cm, they were deemed as non-beating (denoted n/a). The frequency of beating samples was defined as the number of beating samples divided by the total number of samples tested. Functional data was collected from $n=6$ to $n=12$ independent samples.

2.5.4 Statistics and Data Representation—Results in bar graphs are plotted as means with error bars representing one standard deviation. Statistically significant differences, unless otherwise indicated, are denoted with horizontal bars or asterisks. All statistical calculations were performed using SigmaStat 3.0 (SPSS Inc). One-way ANOVA in conjunction with the Holm-Sidak test was used to compare 3 or more groups or 2 groups versus control while t-test was used to compare two groups. Normality of data and equality of variance were checked for all comparisons. Where normality failed, one-way ANOVA on ranks was performed using a Mann-Whitney test. Where equality of variance failed,

ANOVA on ranks in conjunction with Dunn's test and multiple pairwise comparisons was performed. A p-value less than 0.05 was considered statistically significant for all tests.

3. Results

We performed cell tracking studies at day 1 and day 4 in culture since we expected that the highest degree of cellular reorganization will occur within days after seeding. Figure 2 shows the results of cell tracking studies performed on Pre-cultures grown at three different seeding percentages of enriched cardiomyocytes (40%, 60%, and 80% of the total cell number). Cardiac FB are labeled green, D4T EC are labeled red, and neonatal CM appear as blue nuclei. In general cells appear more elongated in the 60% group and the 80% group compared to the 40% group on day 1, but the differences are less evident by day 4. A higher proportion of FB (green) is seen in the 40% group compared to more EC (red) and CM (blue nuclei) in both the 60% and 80% groups. The morphology of the CM is not clearly discernible because the DAPI labels only nuclei and not the cytoplasm. In the 60% group even on day 1, elongated cells could be seen (white arrows), suggesting that this condition might be more conducive to formation of healthy tissues. In general, FB (green) and endothelial cells (red) appear to co-localize into thin, rope-like structures (white arrows) while CM (blue nuclei) organize into thicker organoid-like structures.

Figure 3 depicts the results of cell tracking studies on Simultaneous Tri-cultures. A comparison with Pre-cultures reveals marked differences in morphology. In Simultaneous Tri-culture, cells appear rounded and organized into spherical clusters (white arrows). Clusters were not apparent on day 1 but become more apparent on day 4, suggesting the cells organized into clusters over time. FB and CM clearly organized at the periphery of these clusters for the 60% and 80% Simultaneous Tri-culture groups. In all three groups, EC (red) were primarily observed to organize into the centers of clusters, which was especially striking in the 60% and 80% groups. This localization of EC into clusters is also consistent with immunofluorescence data shown in previous studies(Iyer, et al., 2008).

Live/Dead staining (Figure 4) performed on day 7 of cultivation indicated overall high viability in all groups except at the 80% seeding percentage of enriched cardiomyocytes, where more dead cells were evident in the microchannels. Consistent with our cell tracking studies (Figure 2 and Figure 3) and previous data for the 40% group (Iyer, et al., 2008), Pre-cultures had more elongated cells compared to Simultaneous Tri-culture, where cells appeared rounded and sparsely distributed. As the seeding percentage of enriched cardiomyocytes was increased to 60%, however, the Simultaneous Tri-culture condition improved in morphology showing evidence of cell elongation. In the 80% group, the Simultaneous Tri-culture showed a somewhat sparse appearance (as in the 40% fraction) but the cells appeared healthier and comparable in morphology to the Pre-culture 80% group.

By visual observation, spontaneous contractions were noted in all groups except for the Simultaneous Tri-culture group at the 40% seeding percentage of enriched cardiomyocytes, consistent with our previous work (Iyer, et al., 2008). Visual observations in the Pre-culture groups indicated that the spontaneous contractions in the 60% and 80% groups were of larger amplitude than in the 40% group, suggesting that a higher seeding percentage of enriched cardiomyocytes might aid in improving the functional properties of the engineered organoids. In the 60% and 80% groups of Simultaneous Tri-culture, we observed only sparse areas of spontaneous, low amplitude contractions.

Electrical excitability parameters of organoids were evaluated in response to field stimulation (Figure 5). As the seeding percentage of enriched cardiomyocytes increased (from 40 to 80%) in Simultaneous Tri-culture, so did the success rate for formation of

beating organoids. At 40%, the organoids were completely non-contractile as in our previous studies (Iyer, et al., 2008). The ET remained significantly higher and the MCR remained significantly lower in the Simultaneous Tri-culture 60% and 80% groups compared to the Enriched cardiomyocyte control, indicating inferior functional properties. In contrast, the frequency of beating samples was always high and did not change in the Pre-culture groups. In the Pre-culture group a trend of improved functional properties (i.e. decreasing ET) was observed with increasing seeding cardiomyocyte percentage. The Pre-culture 80% group had an ET that was not significantly different than that of Enriched cardiomyocytes and the MCR of all Pre-culture groups was comparable to that of Enriched cardiomyocytes seeded alone.

At the end of cultivation, double immunofluorescence staining for cardiac Troponin-I (TnI, Green) as a cardiomyocyte marker and Vimentin (Vim, Red) as a non-myocyte marker was performed, with DAPI as a counterstain (Blue), while ECs were identified by staining for CD31 (Figure 6 and 7). The Enriched cardiomyocyte control group is also included for comparison – note that there are Vim-positive and CD31+ cells in this group due to contamination with non-myocytes from the native heart isolate. As we stated in the Methods section, the Enriched cardiomyocytes obtained by two pre-plating steps consisted of $81 \pm 14\%$ CM, $3 \pm 3\%$ EC, and $16 \pm 3\%$ FB (Iyer, et al., 2008).

In general, cells in the Enriched cardiomyocyte controls appeared elongated and formed compact organoids. Pre-cultures contained Vimentin positive (non-myocyte) areas surrounded by elongated myocytes. As the seeding percentage of cardiomyocytes increased, so did the areas positive for TnI. Overall, Simultaneous Tri-cultures (Figure 7) exhibited the same or a higher degree of Vimentin staining compared to Pre-culture. In the Simultaneous Tri-culture 40% group, the majority of cells were positive for Vimentin, consistent with our previous observations (Iyer, et al., 2008). Staining for CD31 was overall sparse in the Pre-culture groups (Figure 6) in contrast to Simultaneous Tri-culture that clearly had a large population of CD31-positive cells (Figure 7).

In order to better quantify the fractions of the three cell types from immunofluorescence data, the percentages of the three cell populations were determined by counting (Figure 8). Note that numbers of fibroblasts were not quantified but can be derived by subtraction of the CD31-positive population from the Vim-positive population, since the Vim-positive subpopulation is the sum of all the non-myocytes. The percentage of Vim-positive cells on day 7 decreased as a function of the seeding percentage of enriched cardiomyocytes, which was expected since the number of fibroblasts seeded was lower as the seeding cardiomyocyte percentage increased. The percentage of CD31-positive cells remained fairly constant even as the seeding percentage of enriched cardiomyocytes increased. However, the percentage of Vim-positive cells on day 7 increased compared with the percentage of fibroblasts seeded at the beginning of cultivation, which clearly indicated that fibroblasts proliferated with time in this system during culture. The percentage of TnI-positive cells on day 7 was conversely much lower than the seeded percentage at the beginning of cultivation, which may have been due to inability of these cells to proliferate and possible cell death early in the culture.

In the Pre-culture groups, there was an increasing trend in the percentage of TnI positive cells with an increase in seeding percentage of enriched cardiomyocytes, although it was not statistically significant at $p < 0.05$; in the Simultaneous Tri-culture group the increase was significant (Figure 8). A corresponding decreasing trend was observed in the non-myocyte percentage (Vim+) and the endothelial cell percentage (CD31+), since the total seeding cell number was held constant. Importantly, during culture, the percentage of cardiomyocytes in the Enriched cardiomyocyte group also decreased in comparison to the originally seeded

percentage (from 81% to 42%) while the percentage of endothelial cells increased (from 3% to 5%) as well as the percentage of all non-myocytes (Vim+, from 19% to 58%). Interestingly, at the end of cultivation, the 60% and 80% Pre-culture groups had a cellular composition comparable to that found in the native myocardium (i.e. ~1/3 of cardiomyocytes and ~2/3 non-myocytes) (Nag, 1980, Banerjee, et al., 2006)

4. Discussion

In normal heart tissue, cardiomyocytes are elongated and have a length-to-width ratio (aspect ratio) of around 7:1 (Bray, et al., 2008). In diseased heart tissue, this aspect ratio can be significantly reduced due to hypercontracture of the myofilaments, resulting in rounded cells with poor contractile properties (Bray, et al., 2008, Harding, et al., 1989). Fibroblasts have also been shown to exhibit elongation and spreading *in vivo* and take on a much more rounded morphology in the absence of an underlying substratum (Couchman, et al., 1983). Similarly, endothelial cells exhibit elongation and orientation in the direction of blood flow in arterial vessels (Reidy and Langille, 1980). We have shown previously that engineered heart tissues stimulated with an electric field exhibited a differentiated phenotype characterized by thick, elongated and aligned myofibres expressing cardiac markers while non-stimulated constructs contained mostly rounded cells (Radisic, et al., 2004). The stimulated constructs also exhibited superior contractile properties to the non-stimulated constructs. An elongated morphology and spreading of these three cell types is, therefore, essential for cell survival, proliferation, and differentiation *in vivo*, and is an equally important hallmark in the heart tissues engineered *in vitro*.

Our ultimate goal is to engineer a vascularized heart tissue of cell composition and functionality resembling the native heart. By inclusion of two more important cardiac cell types, we hypothesized that the structure of the engineered tissues would ultimately recapitulate the architecture of the native myocardium and, in doing so, also give rise to improved function. The enriched cardiomyocyte condition, which is frequently used in the cardiac tissue engineering literature, represents a cellular mixture depleted of endothelial cells and fibroblasts. Our experimental groups are thus compared to the enriched cardiomyocyte group, as a typical cell composition used in the literature.

In our previous study (Iyer, et al., 2008) we used a fixed seeding percentage of cardiomyocytes (40%), and could only determine the positions of the cells at the end of the experiment through immunofluorescence staining. Our main intention in the current study was to improve upon our previous findings by determining the relative positions of the different cell types at various time points during cultivation as well as by varying the seeding percentage of the cardiomyocytes to introduce more beating cells into the organoids.

As in our previous tri-culture studies (Iyer, et al., 2008), we chose to use a microfabricated system for our tri-culture studies because it allowed us to carry out these experiments in a cost efficient and time-saving manner. The microchannels are three-dimensional with a height of nearly 200µm or 10–20 cell diameters. This is in contrast to a Matrigel-coated tissue culture poly(styrene) (TCPS) surface, which is inherently two-dimensional. The microscale approach also allowed us to carry out the studies without worrying about oxygen diffusion limitations, which are only significant for much thicker tissues. The PEG microchannels are transparent, thus allowing us to perform cell tracking studies on 3D cardiac organoids.

Fluorescent dyes have been used for labeling and tracking a number of primary cell types and cell lines, both *in vitro* and *in vivo* (Wang, et al., 2005, Horan, et al., 1990, Parish, 1999). These dyes can remain photostable for several days without compromising the

viability of the cells (Wang, et al., 2005, Horan, et al., 1990, Parish, 1999). The fluorescent compound 4'-6-diamidino-2-phenylindole (DAPI) has been used extensively as a counterstain for cell nuclei due to its high affinity for the minor groove of DNA (Kapuscinski, 1995), and more recently been used to label and track cardiomyocytes in engineered heart tissues (Zimmermann, et al., 2006). Cell tracking data provided important spatial and temporal information on the localization of various cell types in tissue engineered cardiac organoids. The benefit of using live cell imaging (rather than immunofluorescence staining of tissue sections) to obtain such information is that the cells are not disrupted or heavily processed before they are imaged. Since the cell tracking dyes diffuse evenly throughout the cytoplasm, it is possible to obtain a clear picture of changes in overall cell shape and elongation with time. The use of the cell tracker dyes and DAPI in combination with the micro-scale approach to cardiac organoid culture allowed for rapid selection of favorable tissue culture conditions that could be determined over the course of time, instead of only at the endpoint of the experiment. This gave us important spatiotemporal information about the localization of the three cell types and also helped us to perform a check, in real time, on our findings from immunofluorescence data.

From our cell tracking studies, it was evident that Pre-cultured cells appeared significantly more elongated overall (Figure 2). However the greatest degree of elongation could be seen in the 60% seeding percentage of enriched cardiomyocytes. There was also no evidence of cell clustering in any of the Pre-culture conditions and these structures eventually formed cardiac organoids. In contrast, Simultaneous Tri-cultures displayed cells which were mostly rounded in morphology (Figure 3). The cells organized into spherical clusters containing endothelial cells at their centers and cardiomyocytes and fibroblasts at the outer peripheries. These findings are consistent with those we previously published in which immunofluorescence for the 40% Simultaneous Tri-culture condition showed a high level of CD31 expression at the center of clustered areas (Iyer, et al., 2008). This result demonstrates that the timing of the non-myocyte seeding (sequentially, rather than simultaneously) greatly affected the organization of multiple cell types in engineered cardiac tissues.

Qualitatively, differences could be seen in the viability of the organoids. Here, viability refers to the relative numbers of green (live) and red (dead) cells present in the areas represented by the intersecting microchannels. The square-shaped areas outside of the microchannels were excluded from the analysis since they do not support formation of organoids and do not contain any Matrigel. Higher viability was exhibited in the Pre-culture groups than in the Simultaneous Tri-culture groups, except at the 80% seeding percentage of cardiomyocytes, where both groups exhibited comparable viabilities (Figure 4). An important observation was the clustering and lack of spontaneous contractility in the Simultaneous Tri-culture group seeded with 40% enriched cardiomyocytes. This was not surprising, since we had previously shown similar results for Simultaneous Tri-cultures seeded with NIH 3T3 fibroblasts. Thus replacing NIH 3T3 fibroblasts by primary cardiac fibroblasts had no significant effect on the 40% Simultaneous Tri-culture group. Functional properties of Simultaneous Tri-culture group improved as the seeding percentage of cardiomyocytes increased (Figure 5), however they remained inferior to the Enriched cardiomyocyte group. In contrast, functional properties of Pre-culture at 60% and 80% were comparable to the Enriched cardiomyocytes.

Immunofluorescence microscopy (Figure 6 and Figure 7) of Pre-culture and Tri-culture for the three groups showed an increasing number of cardiomyocytes as the seeding percentage of enriched cardiomyocytes increased, though the percentages had decreased significantly in comparison to the seeding percentages (40%, 60%, 80%). In addition, the percentages of non-myocytes decreased as the seeding percentage of enriched cardiomyocytes was increased. However, overall proliferation of non-myocytes was observed in all groups. Cells

were generally elongated in all of the Pre-culture groups, but appeared most elongated in the 60% condition. Interestingly, for the Tri-culture groups, cells appeared more elongated as the seeding percentage of cardiomyocytes increased. While staining for CD31 was sparse in the Pre-culture groups, many CD31+ cells were seen in the Simultaneous Tri-culture groups. Since the same percentage of D4T endothelial cell was seeded in both Tri-culture and Pre-culture, this suggests that the endothelial cells proliferated more when simultaneously tri-cultured than when pre-cultured. This observation may provide an early clue to a potential mechanism involving a soluble factor, which, if dysregulated, would lead to a higher proliferation rate of endothelial cells in the mixture. We will continue to explore this possibility in ongoing and future mechanistic studies.

Taking into account that the Enriched cardiomyocytes used for cell seeding consisted of 81% CM, 3% of EC and 16% FB and were seeded at the percentages given in Table 1, it is possible to calculate the real cell seeding percentages for our three main groups. For the 40% groups, we actually introduced 32% CM, 48% EC and 19% FB. In the 60% groups, the actual percentages were 49% CM, 33% EC and 19% FB; while in the 80% groups the real cell seeding percentages were 65% CM, 18% EC and 17% FB. At the end of cultivation, the final cell percentages in Pre-culture varied only slightly between the groups and the majority of these groups did not exhibit significant differences from the Enriched cardiomyocyte group, resulting in a final cell composition closely resembling that of the native heart (~1/3 cardiomyocytes and ~2/3 non-myocytes) (Nag, 1980, Banerjee, et al., 2006). It is possible that this physiological cell ratio is, in fact, optimized for cell viability and function in 3D. It can further be concluded that the fibroblasts must proliferate until these physiologically similar cell ratios are achieved, since cardiomyocytes are terminally differentiated and cannot divide to alter to the seeded cell ratios. The percentage of endothelial cells in the Pre-culture group was in the range from 10% – 16%, lower in comparison to the initial seeding percentages, but higher than in the native cell isolate (e.g. 3% as reported by Naito et al (Naito, et al., 2006)) and significantly higher than the EC percentage in the Enriched cardiomyocyte controls. This indicates that Pre-culture may be a good approach for introducing endothelial cells into cardiac tissue constructs with the view of enhancing vascularization *in vitro* and *in vivo*, while maintaining contractile function.

Surprisingly, Simultaneous Tri-culture yielded a significantly lower percentage of cardiomyocytes (5% – 25%) and significantly higher percentage of non-myocytes (47% – 95%) at the end of cultivation compared to the Pre-culture (Figure 8) even though the non-myocytes had two more days to proliferate under Pre-culture conditions compared to the Simultaneous Tri-culture conditions. In contrast, during Pre-culture, FBs and ECs deposit extracellular matrix components and soluble factors that may promote attachment and survival of subsequently seeded cardiomyocytes ultimately leading to improved functional properties. Since the final percentage of ECs in Simultaneous Tri-culture (Figure 8, 32% – 18%) resembled the actual seeding percentage of ECs as indicated above (48%-18%) it is possible to conclude that the majority of proliferation among the non-myocytes was due to fibroblast proliferation, and that this occurred at the expense of cardiomyocytes in these groups.

In the current study, we used mouse D4T endothelial cells instead of primary rat microvascular endothelial cells, since we knew that this cell line could be grown in the cardiac culture medium used in this study without any detrimental effects to the cells (Iyer, et al., 2008). Inclusion of a new endothelial cell type would require de novo optimization of the culture medium composition. Thus, there is a possibility that the differences in the species of the three cell types may play a role in the ability of the cells to communicate by way of soluble factors. Since we have used primary rat cardiac fibroblasts and primary rat cardiomyocytes in combination with a mouse endothelial cell line, it is possible that the

endothelial cells secreted species-specific cues which the cardiomyocytes could not recognize and vice versa. This potential limitation will be further explored in future studies in which we will use only primary cells of rat origin.

5. Conclusions

We investigated cell ratio modulation of cardiac tri-cultures and conducted cell tracking studies in order to better visualize the localization of the cells *in vitro*. Through the use of commercially available cytoplasmic cell tracker dyes and DAPI, a nuclear counterstain, we were able to follow the positions of cells with time for up to 4 days. This gave us important visual clues as to how cells organize in cardiac organoids engineered from simultaneously versus sequentially seeded tri-cultures. Specifically, endothelial cells were seen to aggregate into clusters surrounded by cardiomyocytes and fibroblasts when simultaneously tri-cultured, while in contrast, Pre-cultures contained thin rope-like structures that were composed of elongated endothelial cells and fibroblasts that were anchored to organoids composed mainly of cardiomyocytes. Live/dead staining showed clustering in the 40% myocyte percentage for Tri-culture, consistent with our previous work, but also showed improved overall cell viability and morphology at higher myocyte percentages in the case of both Tri-culture and Pre-culture. We also varied the seeding percentage of enriched cardiomyocytes while keeping the total cell number constant. It was demonstrated that the functional properties of organoids could be improved by increasing the seeding percentage of enriched cardiomyocytes from 40% to 80%. Surprisingly, even the Tri-culture condition which we previously reported to be non-functional when seeded with 40% enriched cardiomyocyte fraction could be paced by an electrical stimulus when the seeding percentage of enriched cardiomyocytes was increased, though the functional properties were still inferior to those of the Enriched cardiomyocyte group. In contrast, all Pre-cultures could respond to an electrical stimulus regardless of the seeding percentage of enriched cardiomyocytes, and had comparable functional properties to Enriched cardiomyocytes even though they contained fewer myocytes. Immunofluorescence staining revealed that the percentage of non-myocytes in Simultaneously Tri-cultured organoids increased at the end of 7 days of cultivation in comparison to the seeding percentages, while the percentage of cardiomyocytes decreased. Since the endothelial cell percentage on day 7 did not vary greatly from the initially seeded percentages in the Tri-culture group, this was likely due to fibroblast proliferation. While fibroblast proliferation also occurred in the Pre-culture groups, all of these groups attained final cell ratios on day 7 that were very similar to the physiological composition of the native neonatal rat heart (~1/3 myocytes and ~2/3 non-myocytes), suggesting that this ratio may be optimized for cardiac tissue engineering.

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References

1. Nag AC. Study of non-muscle cells of the adult mammalian heart: a fine structural analysis and distribution. *Cytobios/Cytobios*. 1980; 28:41–61.
2. Banerjee I, Yekkala K, Borg TK, Baudino TA. Dynamic Interactions between Myocytes, Fibroblasts, and Extracellular Matrix. *Ann N Y Acad Sci/Ann N Y Acad Sci*. 2006; 1080:76–84.
3. Seghezzi G, Patel S, Ren CJ, Gualandris A, Pintucci G, Robbins ES, Shapiro RL, Galloway AC, Rifkin DB, Mignatti P. Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis. *J Cell Biol/J Cell Biol*. 1998; 141:1659–1673.

4. Kuzuya M, Kinsella JL. Induction of endothelial cell differentiation in vitro by fibroblast-derived soluble factors. *Exp Cell Res/Exp Cell Res*. 1994; 215:310–318.
5. Bursac N, Papadaki M, Cohen RJ, Schoen FJ, Eisenberg SR, Carrier R, Vunjak-Novakovic G, Freed LE. Cardiac muscle tissue engineering: toward an in vitro model for electrophysiological studies. *Am J Physiol/Am J Physiol*. 1999; 277:H433–H444.
6. Naito H, Melnychenko I, Didie M, Schneiderbanger K, Schubert P, Rosenkranz S, Eschenhagen T, Zimmermann WH. Optimizing engineered heart tissue for therapeutic applications as surrogate heart muscle. *Circulation/Circulation*. 2006; 114:172–178.
7. Caspi O, Lesman A, Basevitch Y, Gepstein A, Arbel G, Habib IH, Gepstein L, Levenberg S. Tissue engineering of vascularized cardiac muscle from human embryonic stem cells. *Circ Res/Circ Res*. 2007; 100:263–272.
8. Levenberg S, Rouwkema J, Macdonald M, Garfein ES, Kohane DS, Darland DC, Marini R, van Blitterswijk CA, Mulligan RC, D'Amore PA, Langer R. Engineering vascularized skeletal muscle tissue. *Nat Biotechnol/Nat Biotechnol*. 2005; 23:879–884.
9. Iyer RK, Chiu LL, Radisic M. Microfabricated poly(ethylene glycol) templates enable rapid screening of triculture conditions for cardiac tissue engineering. *J Biomed Mater Res A/J Biomed Mater Res A*. 2008
10. Radisic M, Park H, Shing H, Consi T, Schoen FJ, Langer R, Freed LE, Vunjak-Novakovic G. Functional assembly of engineered myocardium by electrical stimulation of cardiac myocytes cultured on scaffolds. *Proc Natl Acad Sci U S A/Proc Natl Acad Sci U S A*. 2004; 101:18129–18134.
11. Choi K, Kennedy M, Kazarov A, Papadimitriou JC, Keller G. A common precursor for hematopoietic and endothelial cells. *Development/Development*. 1998; 125:725–732.
12. Bray MA, Sheehy SP, Parker KK. Sarcomere alignment is regulated by myocyte shape. *Cell Motil Cytoskeleton/Cell Motil Cytoskeleton*. 2008; 65:641–651.
13. Harding SE, Vescovo G, Jones SM, Bennett G, Yacoub M, Poole-Wilson PA. Morphological and functional characteristics of myocytes isolated from human left ventricular aneurysms. *J Pathol/J Pathol*. 1989; 159:191–196.
14. Couchman JR, Hook M, Rees DA, Timpl R. Adhesion, growth, and matrix production by fibroblasts on laminin substrates. *J Cell Biol/J Cell Biol*. 1983; 96:177–183.
15. Reidy MA, Langille BL. The effect of local blood flow patterns on endothelial cell morphology. *Exp Mol Pathol/Exp Mol Pathol*. 1980; 32:276–289.
16. Wang XQ, Duan XM, Liu LH, Fang YQ, Tan Y. Carboxyfluorescein diacetate succinimidyl ester fluorescent dye for cell labeling. *Acta Biochim Biophys Sin (Shanghai)/Acta Biochim Biophys Sin (Shanghai)*. 2005; 37:379–385.
17. Horan PK, Melnicoff MJ, Jensen BD, Slezak SE. Fluorescent cell labeling for in vivo and in vitro cell tracking. *Methods Cell Biol/Methods Cell Biol*. 1990; 33:469–490.
18. Parish CR. Fluorescent dyes for lymphocyte migration and proliferation studies. *Immunol Cell Biol/Immunol Cell Biol*. 1999; 77:499–508.
19. Kapuscinski J. DAPI: a DNA-specific fluorescent probe. *Biotech Histochem/Biotech Histochem*. 1995; 70:220–233.
20. Zimmermann WH, Melnychenko I, Wasmeier G, Didie M, Naito H, Nixdorff U, Hess A, Budinsky L, Brune K, Michaelis B, Dhein S, Schwoerer A, Ehmkec H, Eschenhagen T. Engineered heart tissue grafts improve systolic and diastolic function in infarcted rat hearts. *Nat Med/Nat Med*. 2006; 12:452–458.

1. Incubate in respective dyes for 30-60 minutes

2. Mix all 3 cell types (Tri-Culture) or Seed non-myocytes followed by myocytes 2 days later (Pre-culture)

3. Seed onto matrigel-coated microchannels of PEG disc

PEG disc

Figure 1. Cell Tracking Procedure

Cardiac FB, D4T EC, and neonatal rat CM were incubated in green cytoplasmic dye, red cytoplasmic dye, and blue nuclear dye, respectively. For FB and EC, cells were incubated for 30 minutes at a dye concentration of 5 μM , while for CM, cells were incubated for 1 hour in a 1:5 dilution of DAPI. For Simultaneous Tri-cultures, the three cell types were mixed and seeded onto microchannels, while for Pre-culture, the non-myocytes were seeded two days prior to the myocytes. Organoids were imaged by fluorescence microscopy on days 1 and 4 after seeding the CM.

Figure 2. Pre-culture displays an even distribution of elongated cells

The total cell seeding number was kept constant at 2×10^5 cells/disc and the seeding EC:FB ratio was fixed at 0.7:0.2. The seeding percentage of CM was as indicated in the figure labels. Cardiac FB are green, EC are red and CM nuclei are blue nuclei. The image was taken from an area close to the center of the intersecting point of the microchannels, as shown schematically in the top-left of the figure (red circle indicates approximate area where image was taken from, white areas represent microchannels).

Figure 3. Simultaneous Tri-cultures contain, rounded cells which organize into clusters over time
 The total cell seeding number was kept constant at 2×10^5 cells/disc and the seeding EC:FB ratio was fixed at 0.7:0.2. The seeding percentage of CM was as indicated in the figure labels. Cardiac FB are green, EC are red and CM nuclei are blue nuclei.

Figure 4. Cardiac organoids display high cell viability

Live/dead staining was performed on organoids cultivated for 7 days with a constant cell density of 2×10^5 cells/disc under various seeding percentages of enriched cardiomyocytes (40%, 60%, 80%, 100%). Live cells are stained green and dead cells are stained red.

Figure 5. Pre-culture groups exhibit superior functional properties to Simultaneous Tri-culture groups

Excitation Threshold (ET) and Maximum Capture Rate (MCR) of the tissues was measured in response to electrical field stimulation using monophasic, square pulses.

Figure 6. Immunostaining for cell specific markers in Pre-culture groups

Double staining for cardiac Troponin-I (Green, CM) and Vimentin (Red, FB) and staining for CD31/PECAM-1 (Green, EC) with DAPI as a counterstain (Blue) was performed to enable the quantification of cell percentages at the end of cultivation, and to allow for visualization of structural differences in organoid morphology.

Figure 7. Immunostaining for cell specific markers in Simultaneous Tri-culture groups
Double staining for cardiac Troponin-I (Green, CM) and Vimentin (Red, FB) and staining for CD31/PECAM-1 (Green, EC) with DAPI as a counterstain (Blue) was performed to enable the quantification of cell percentages on at the end of cultivation, and to allow for visualization of structural differences in organoid morphology.

Figure 8. The percentages of different cell types in cardiac organoids based on immunostaining
 Cell percentages were determined by manual counting of immunofluorescence images. Manual counting was performed by two independent observers and the numbers were averaged and plotted as percentage of the total cell number per image as determined by DAPI counterstaining.

Table 1

Percentages of cells seeded in CM ratio experiments

Group	%Enriched CM	%EC	%FB
Tri-/Pre-culture 40%	40%	47%	13%
Tri-/Pre-culture 60%	60%	31%	9%
Tri-/Pre-culture 80%	80%	16%	4%
Enriched cardiomyocytes	100%	0%	0%