Video Article

In Vitro Differentiation of Human Mesenchymal Stem Cells into Functional Cardiomyocyte-like Cells

Peter Szaraz*[1,2], Yarden S. Gratch*[1], Farwah Iqbal[1,2], Clifford L. Librach[1,2,3,4,5]

[1]Create Fertility Centre

[2]Department of Physiology, University of Toronto

[3]Department of Obstetrics and Gynecology, University of Toronto

[4]Department of Physiology, University of Toronto

[5]Department of Obstetrics and Gynecology, Women's College Hospital

*These authors contributed equally

Correspondence to: Peter Szaraz at peter@createivf.com

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Abstract

Myocardial infarction and the subsequent ischemic cascade result in the extensive loss of cardiomyocytes, leading to congestive heart failure, the leading cause of mortality worldwide. Mesenchymal stem cells (MSCs) are a promising option for cell-based therapies to replace current, invasive techniques. MSCs can differentiate into mesenchymal lineages, including cardiac cell types, but complete differentiation into functional cells has not yet been achieved. Previous methods of differentiation were based on pharmacological agents or growth factors. However, more physiologically relevant strategies can also enable MSCs to undergo cardiomyogenic transformation. Here, we present a differentiation method using MSC aggregates on cardiomyocyte feeder layers to produce cardiomyocyte-like contracting cells.

Human umbilical cord perivascular cells (HUCPVCs) have been shown to have a greater differentiation potential than commonly investigated MSC types, such as bone marrow MSCs (BMSCs). As an ontogenetically younger source, we investigated the cardiomyogenic potential of first-trimester (FTM) HUCPVCs compared to older sources. FTM HUCPVCs are a novel, rich source of MSCs that retain their *in utero* immunoprivileged properties when cultured *in vitro*. Using this differentiation protocol, FTM and term HUCPVCs achieved significantly increased cardiomyogenic differentiation compared to BMSCs, as indicated by the increased expression of cardiomyocyte markers (*i.e.,* myocyte enhancer factor 2C, cardiac troponin T, heavy chain cardiac myosin, signal regulatory protein α, and connexin 43). They also maintained significantly lower immunogenicity, as demonstrated by their lower HLA-A expression and higher HLA-G expression. Applying aggregate-based differentiation, FTM HUCPVCs showed increased aggregate formation potential and generated contracting cells clusters within 1 week of co-culture on cardiac feeder layers, becoming the first MSC type to do so.

Our results demonstrate that this differentiation strategy can effectively harness the cardiomyogenic potential of young MSCs, such as FTM HUCPVCs, and suggests that *in vitro* pre-differentiation could be a potential strategy to increase their regenerative efficacy *in vivo*.

Video Link

The video component of this article can be found at https://www.jove.com/video/55757/

Introduction

Congestive heart failure (CHF) persists as a leading cause of morbidity and mortality worldwide. CHF often occurs following the massive loss of cardiomyocytes and the development of cell-free scar tissue as the pathological result of a myocardial infarction (MI)[1]. While the heart is a partially self-renewing organ, the resident stem and progenitor cell pool responsible for executing tissue regeneration significantly diminishes in abundance and function in aged patients, often becoming insufficient for optimal recovery after injury. Thus, there is great interest in developing experimental treatments that involve the transplantation of healthy donor cells into the damaged myocardium. It is imperative that the donor cells not only restore the structure of the tissue, but also achieve the functional recovery of the affected myocardium.

The native heart employs heart tissue-resident and endogenous bone marrow-originated stem cells for post-injury repair[2,3,4]. Regenerative cells- host- and donor-derived alike- must have the capacity to obtain the appropriate phenotype and function in the microenvironment of the remodeling myocardium, along with the ability to efficiently and safely replace the lost cells. *In vitro* differentiation methods have been used extensively to achieve high-efficiency, stem cell-based cardiomyocyte production[5,6]. The expression profile of cardiac lineage markers is used to define the process of stem cell differentiation towards the cardiac lineage[7]. Early differentiation markers, such as NKX2.5, myocyte enhancer factor 2C (Mef2c), and GATA4[8,9], can be an indication of the initiation of the cardiomyogenic process. Mature cardiomyocyte markers

commonly used to assess differentiation efficacy are signal regulatory protein α (SIRPA)[10], cardiac troponin T (cTnT)[11], heavy chain cardiac myosin (MYH6)[8,12,13], and connexin 43 (Cx43)[14,15,16]. The methods using embryonic stem cells (ESCs) and pluripotent stem cells (PSCs) have been thoroughly optimized and discussed regarding the details of inductive factors, oxygen and nutrient gradients, and the exact timing of action[5,6,7,17,18]. Nonetheless, ESC- and PSC-based technologies still present multiple ethical and safety concerns, along with suboptimal electrophysiological and immunological features[19,20]. Hosts transplanted with these cells often experience immunorejection and require permanent immunosuppression. This is mainly due to mismatching major histocompatibility complex (MHC) molecules in the host and donor and to the resulting T-cell response[21]. While individual MHC class I matching is a possible solution, a more accessible clinical practice would require a cell source that is universally immunoprivileged to overcome the concern of rejection.

As an alternative cell source for use in clinical applications, MSCs and in particular, BMSCs, have been investigated for use in tissue regeneration since their initial description in 1995[22]. MSCs are believed to be resident regenerative cells that can be found in nearly any vascularized tissue[23]. Upon isolation from the desired source, MSCs can easily be expanded in culture, have extensive paracrine capacity, and often possess immunoprivileged or immunomodulatory properties[24,25]. Their safety and efficacy have already been shown in several pre-clinical studies, in particular for cardiac regeneration[3,26].

Many MSC differentiation strategies utilize pharmacological agents, such as 5-azacytidine[22] and DMSO[27], and growth or morphogenic factors, like BMPs[5,7,28,29] or angiotensin-II[30], with variable efficiency. These strategies, however, are not based on the obstacles that a naïve regenerative cell is likely to encounter after homing or being delivered to the site of injury *in vivo.* More physiologically relevant strategies, while more difficult to define and manipulate, are based on the premise that MSC differentiation can be induced through signals from the tissue microenvironment itself. Previous studies have shown that exposure to the cardiac cell lysates[31] or ventricular myocardium[32,33], or direct contact with primary cardiomyocytes *in vitro*[15,34], can increase the expression of cardiac markers in MSCs. Others have demonstrated spontaneous cardiomyogenesis after treating cardiac injuries with MSCs[35,36,37,38], although in part, the fusion of BMSCs and cardiomyocytes[39,40] generated the nascent myocardium. To our knowledge, functional, spontaneously contracting cardiomyocytes from human MSCs (hMSCs) of any tissue source have not yet been reported.

The current consensus is that all MSCs arise from perivascular cells[23]. Young MSCs with pericyte properties can be isolated from the perivascular region of human umbilical cord tissue[41,42,43]. In comparison to BMSCs, HUCPVCs possess increased differentiation potential and several other regenerative advantages, both *in vitro*[41,44] and *in vivo*[45,46,47]. Notably, the source being the maternal-fetal interface, HUCPVCs have significantly lower immunogenicity compared to adult sources of MSCs. Our research focuses on the characterization and pre-clinical applications of FTM HUCPVCs, the youngest source of MSCs investigated, which we have previously shown to have increased proliferative and higher multilineage differentiation capacities, including in the cardiomyogenic lineage[41].

Here, we present a protocol that combines aggregate formation and primary cardiac cell feeder layers as inductive forces to attain the complete cardiomyogenic differentiation of MSCs. Aggregates provide a 3D environment, which better models conditions *in vivo* compared to 2D adherent cultures. Utilizing cardiac feeder layers provides an environment that is representative of the ultimate transplantation site for the MSCs. We demonstrate that younger sources of MSCs isolated from pre- or post-natal umbilical cords have a higher capacity to form aggregates and to reach the cardiac phenotype compared to adult BMSCs, while still maintaining their immune-privilege. Besides the steep elevation of cardiac lineage marker genes and the induced expression of intracellular (*i.e.,* cTnT and MYH6) and cell-surface proteins (*i.e.,* SIRPA and Cx43) specific for cardiomyocytes, we show that the differentiation potential of FTM HUCPVCs can be harnessed with this method and that they can give rise to spontaneously contracting cardiomyocyte-like cells.

Protocol

All studies involving animals were conducted and reported according to ARRIVE guidelines[48]. All studies were performed with institutional research ethics board approval (REB number 454-2011, Sunnybrook Research Institute; REB 29889, University of Toronto, Toronto, Canada). All animal procedures were approved by the Animal Care Committee of the University Health Network (Toronto, Canada), and all animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals, 8th edition (National Institutes of Health 2011).

1. Tissue Culture

1. Culture FTM HUCPVCs, term HUCPVCs (previously established, $n \geq 3$ independent lines for each)[42] and commercially available BMSCs in alpha-minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and a 1% penicillin/streptomycin (P/S) cocktail. Culture rat primary cardiomyocytes and MSC-cardiomyocyte co-cultures in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12) containing 10% FBS and 1% P/S.
   NOTE: Sterilize the medium using a 0.2-µm filter. Store prepared medium solutions at 4 °C for up to 3 weeks.
2. Maintain cell cultures in humidified incubators (95% relative humidity, 37 °C, and 5% CO_2) and passage at 70-80% confluency, determined by phase-contrast microscopy. Use appropriate volumes of medium for the size of tissue culture dish used (*e.g.,* 10 mL in a 10-cm dish and 2 mL per well in 6-well tissue culture plate). Use these culture conditions for the duration of the protocol.
3. Dissociate MSC monolayers for passaging or MSC-cardiomyocyte co-culture establishment using a dissociation enzyme solution (2 mL/well in a 6-well plate) and incubate at 37 °C for 4 min.
4. Transfer the dissociated cells to a 15-mL tube and centrifuge at 400 x g for 5 min.
5. Aspirate the supernatant without disrupting the cell pellet and resuspend the cells in 1 mL of a culture medium appropriate for counting using an automated cell counter. Seed the cells as described in the following protocol sections.

2. Preparation of Primary Rat Cardiomyocyte-MSC Co-cultures

1. Obtain heart tissue for primary cardiomyocyte isolation.

1. Euthanize rat pups (5-6 days postnatal) using CO_2 asphyxiation. Set CO_2 chambers to 20% gas replacement (flow rate = 0.2 x chamber volume per min). Confirm exitus by the absence of the pinch reflex.
2. Remove the atria with the connecting major blood vessels using sterilized instruments (*i.e.,* forceps and curved scissors)[41]. Transfer the hearts to 50-mL tubes containing sterile PBS with1% P/S (PBS-P/S) on ice.
3. Cut the ventriculi in half and let the blood wash out in a 10-cm dish with 10 mL of PBS-P/S on ice. Cut the ventricular walls into small pieces (diameter = 2-3 mm) using curved scissors.
4. Transfer the heart pieces from 10-12 animals to a 50-mL tube using a serological pipette and let them settle.
5. Remove as much PBS-P/S as possible without removing any heart pieces. Add 10 mL of new PBS-P/S.

2. Digest the heart tissue to isolate the cardiomyocytes.
   1. Allow the heart pieces to settle. Replace the PBS-P/S with 10 mL of 0.15% trypsin in PBS and shake at 37 °C for 10 min.
   2. Discard the supernatant. Repeat the digestion described in step 2.2.1 three more times, but decant the supernatants into 50-mL collection tubes containing 10 mL of 100% FBS.

3. Centrifuge the cells (400 x g, 5 min) and aspirate the supernatant. Resuspend the cells in DMEM-F12 containing 10% FBS and 1% P/S and seed onto a 6-well plate (1 x 10^5 cells/cm^2, 2 mL of medium per well).
4. After 1 h, transfer the medium containing non-attached cells to a 50-mL tube and discard the attached cells. Count the cells in suspension and re-plate them into new 6-well plates (1 x 10^5 cells/cm^2, 2 mL of DMEM-F12 containing 10% FBS and 1% P/S per well).
5. Inhibit cell proliferation with bromodeoxyuridine (BrdU).
   Caution: BrdU is a strong teratogen and suspected mutagen. Please ensure proper training is provided and refer to the safety data sheet before use.
   1. Once cells have attached, replace the medium in the 6-well plate with DMEM-F12 containing 10% FBS, 1% P/S (2 mL of medium per well), and 5 µM BrdU. Incubate for 16 h (37 °C, 5% CO_2).
   2. Remove the BrdU-containing medium and replace with DMEM-F12 containing 10% FBS and 1% P/S (2 mL of medium per well).

6. Prepare pre-stained MSCs.
   1. Once MSC cultures are at 70-80% confluency in 10-cm dishes, remove the culture medium and add 3 mL of cell dissociation solution. Incubate the dish at 37 °C and 5% CO_2 for 5 min.
   2. Transfer the dissociated cells to a 15-mL tube and centrifuge at 400 x g for 5 min.
   3. Aspirate the supernatant without disrupting the cell pellet and resuspend the cells in 1 mL of DMEM-F12 containing 10% FBS and 1% P/S for counting using an automated cell counter.
   4. Dilute the cells to a concentration of 1 x 10^6 MSC/mL of DMEM-F12 containing 10% FBS and 1% P/S.
   5. Incubate the MSCs with viable, non-transferable fluorescent dye (5 µM, 30 min, 37 °C, 5% CO_2) in 1.5-mL centrifuge tubes for 1 h.
   6. Centrifuge the tubes at 400 x g for 5 min. Aspirate the supernatant and resuspend the pellet in DMEM-F12 containing 10% FBS and 1% P/S for a cell concentration of 1 x 10^6 MSC/mL. Repeat this a total of 3 times.

7. Transfer the MSCs onto cardiomyocytes (step 2.5.2) at a concentration of 10 x 10^4 cells per well of the 6-well plate.

3. Preparation of Aggregate Co-cultures

1. Prepare a single-cell suspension of MSCs (2 x 10^4 cells/mL of medium, passage # ≤ 6) in alpha-MEM supplemented with 10% FBS and 1% P/S (see step 2.6).
   NOTE: Refer to section 1 of the protocol for the passaging of cells. Alternatively, pre-stain MSCs as per step 2.6.
2. Initiate aggregate formation by placing 25-µL drops of cell suspension (500 cells) on the inner surface of the lids of 10-cm tissue culture dishes (up to 50 drops per lid). Place the lids on their bottom counterparts containing PBS-P/S. Incubate at 37 °C and 5% CO_2.
   NOTE: Place 5-7 mL of PBS-P/S into the culture dish below the hanging drops to avoid drop evaporation.
3. Observe aggregate formation in the drops after 3 days using a stereomicroscope. If over 40 out of 50 drops contain formed aggregates, collect the drops from the lids using a 1-mL micropipette and transfer the aggregates directly onto primary rat cardiomyocyte monolayers (prepared in steps 2.1-2.7; 10 drops/well). Avoid vigorous pipetting to preserve aggregate integrity.
4. Keep aggregate co-cultures in the incubators for up to 2 weeks, changing the full volume of medium (2 mL of DMEM-F12 containing 10% FBS and 1% P/S per well) every 72 h.
   1. Daily observe aggregates attaching on feeder cell layers using bright-field microscopy. Record contracting aggregates when observed.

5. Prepare aggregates for analysis.
   1. Remove the medium and add 2 mL of PBS per well of a 6-well tissue culture dish. Remove the PBS and add 2 mL of dissociation solution per well. Incubate for 3 min at 37 °C and 5% CO_2.
   2. Centrifuge at 400 x g for 5 min to obtain a cell pellet. Resuspend in medium, as specified for the applications described in the subsequent steps (see steps 4.1, 5.1, and 6.1) and pass through a 70-µm cell strainer.

4. Flow Cytometry (FC) and Fluorescence-activated Cell Sorting (FACS)

1. Incubate cell suspensions (1 x 10^5 cells in 200 µL of PBS containing 3% FBS) with fluorophore-conjugated (FITC or APC) primary antibodies (*i.e.,* CD49f, Cx43, TRA-1-85, HLA-A, HLA-G, and SIRPA for FC or TRA-1-85 for FACS; 1:40) at 4 °C for 30 min, protected from light.
2. Centrifuge (400 x g, 5 min) and resuspend the cells in 1 mL of PBS with 3% FBS for FC or PBS with 0.5% FBS for FACS.
   NOTE: The FC of MSCs was optimized by Hong *et al.*[41].
3. Maintain the cells at 4 °C in the dark until they are ready to be analyzed by FC (at least 1 x 10^4 events) or FACS. Sort the cells as described[41]. Re-plate TRA-1-85 high-positive sorted cells in 6-well plates (1 x 10^4 cells/well, 2 mL of DMEM-F12 containing 10% FBS and 1% P/S) within 1 h.

NOTE: For the gating strategy of the TRA-1-85 human cell surface antigen, see the **Supplementary Figure**.

5. Immunocytochemistry (ICC) and Microscopy

1. Re-plate the cell suspensions obtained from the co-cultures (step 3.5.2) or FACS (section 4) onto chamber slides (1 x 10^4 cells/well, 2 mL of DMEM-F12 containing 10% FBS and 1% P/S per well). Let the cells attach overnight in a tissue culture incubator (see section 1 for the conditions).
2. Fix the cells using 3 mL of 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature. Wash 3 times with 3 mL of PBS containing 1% bovine serum albumin (BSA; PBS-BSA) for 5 min per wash.
   Caution: Wear appropriate personal protective equipment when handling PFA.
3. Permeabilize the cells in 3 mL of PBS-BSA with 0.1% Triton X-100. Incubate at room temperature for 10 min for intracellular antigens (*i.e.,* alpha sarcomeric actinin (aSarc) and Cx43), or 25 min for intra-nuclear antigens (*i.e.,* Mef2c and human nuclear antigen (HuNu)). Wash 3 times with 3 mL of PBS-BSA for 5 min per wash.
4. Block the samples against non-specific antibody reactions with 3 mL of PBS containing 5% normal goat serum (NGS) and 1% BSA for 15 min at room temperature. Wash 3 times with 3 mL of PBS-BSA for 5 min per wash.
5. Incubate the cells in the primary antibodies (*i.e.,* Mef2c, aSarc, Cx43, and HuNu) diluted 1:200 in 3 mL of PBS-BSA at 4 °C overnight.
6. Wash 3 times with 3 mL of PBS-BSA for 5 min per wash and incubate with secondary antibodies for 30 min at room temperature. Wash 3 times with 3 mL of PBS-BSA for 5 min per wash.
7. Store the stained specimens in 3 mL of of mounting medium.
8. Acquire images using a fluorescence microscope. Use a 10X objective (NA = 0.3), and a 20X objective (NA = 0.45) for lower-magnification imaging. Use fluorescence filter cubes and wavelengths for GFP (ex = 470/22 nm, em = 525/50 nm) and RFP (ex = 531/40 nm, em = 593/40 nm) for the secondary antibodies used (see **the Materials and Equipment Table**).
9. Quantify images using imaging software (see the **Materials and Equipment Table** for the recommended software). Normalize the fluorescence intensity readings to the secondary control acquisitions.

6. RNA Isolation and Quantitative RT-PCR

1. Prepare RNA samples from undifferentiated MSC cultures or MSCs sorted from co-cultures using column-based RNA isolation, according to the manufacturer's instructions. Prepare 1 x 10^4 to 1 x 10^6 cells in 0.7 mL of cell lysis buffer (provided with the RNA isolation kit) per sample.
2. Prepare cDNA from up to 2 µg of RNA per 100-µL RT reaction.
3. Perform qPCR using 10 ng of cDNA per reaction (40 cycles, 60 °C annealing/extending temperature).
   1. Use primers for human MY6H and cTnT in a 500-nM concentration and 1-100 ng of cDNA per reaction (see the **Materials and Equipment Table**). Use GAPDH, ACTB, and HPRT as internal housekeeping normalizers. Use commercially available human-induced pluripotent stem cell-derived cardiomyocytes as a positive control.
      NOTE: Express the fold-change of expression compared to undifferentiated MSC-derived cDNA samples.

Representative Results

HUCPVCs Display Higher Aggregate-formation Potential and CD49f Expression Levels Compared to BMSCs:

To induce the differentiation of hMSCs (*i.e.,* FTM HUCPVCs, term HUCPVCs, and BMSCs), single-cell suspensions of undifferentiated MSCs or MSC-containing hanging drops (**Table 1**) were transferred onto rat primary cardiomyocyte monolayers to establish direct co-cultures or aggregate co-cultures, respectively. The three different MSC types displayed similar abundance and appearance in direct co-cultures (**Figure 1A**). 2-3 days after transferring hanging drops onto cardiac cells in aggregate co-cultures (**Table 1**), FTM and term HUCPVCs appeared in larger aggregates (310 µm ± 30 µm and 240 µm ± 40 µm, respectively) compared to those seen for BMSCs (180 µm ± 30 µm; **Figure 1B**). It was previously reported that integrin expression, in particular CD49f (integrin subunit α6), can critically impact the aggregate formation potential of human cells[49,50]. FC analysis of CD49f expression in undifferentiated MSCs showed that 96 ± 3% of FTM HUCPVCs, 89 ± 6% of term HUCPVCs, and 53 ± 7% BMSCs were positive for cell-surface CD49f (**Figure 1C**). This correlates intuitively with the observed differences in aggregate size.

Both direct and aggregate co-cultures were dissociated 7 days after co-culture and were processed for FC and ICC (**Table 1**). We performed FC analysis using an antibody specific to human cell-surface marker TRA-1-85 (CD147). This Ok blood group marker is exclusively expressed by cells of human origin. The analysis identified high-positive (TRA-1-85^{high}, human), low-positive (TRA-1-85^{low}, undetermined), and negative (TRA-1-85^{neg}, rat) cell populations (**Supplementary Figure 1A**). To further clarify the identity of the cell populations, ICC for HuNu was performed (**Supplementary Figure 1B**). As shown by fluorescence microscopy, TRA-1-85^{high} cells presented with single or multiple HuNu-positive nuclei, while over 70% of TRA-1-85^{low} cells had several nuclei with various ratios of HuNu-positive and -negative labeling (**Supplementary Figure 1B**). This observation is consistent between all 3 MSC types and implies that cell fusion takes place in cardiomyocyte co-cultures generating TRA-1-85^{low} chimeric cells.

SIRPA and Cx43 Expression Is Induced in HUCPVCs but Not in BMSCs in Cardiomyocyte Co-cultures:

FC on TRA-1-85^{high} cells showed the upregulation of cardiomyocyte-associated marker SIRPA in FTM HUCPVCs (41.2 ± 7%) and term HUCPVCs (43 ± 29%), but not in BMSCs (5.3 ± 2.6%; **Figure 2A**) from direct cardiomyocyte co-cultures. Cx43 upregulation was significantly ($p < 0.01$) higher in FTM HUCPVCs (35 ± 13%) compared to term HUCPVCs (19.2 ± 7.8%) or BMSCs (13.2 ± 5.8%) in direct co-cultures (**Figure 2B**).

Aggregate co-culture conditions further elevated SIRPA levels in term HUCPVCs (57.9 ± 14.7%) and BMSCs (17.6 ± 3.4%), but not in FTM HUCPVCs (35.6 ± 6.9%; **Figure 2A**). Cx43 positivity of term HUCPVCs in aggregate co-cultures reached a significantly ($p < 0.01$) higher level (72.3 ± 2.6%) than that of FTM HUCPVCs (37.2 ± 13%) or BMSCs (3.8 ± 1.9%; **Figure 2B**).

Differentiating FTM and term HUCPVCs both upregulated Cx43, with term HUCPVCs achieving a higher proportion of cells testing positive with FC analysis. However, fluorescence microscopy demonstrated that, while Cx43-positive puncta localized in the cell membrane of FTM HUCPVCs, a predominantly cytoplasmic distribution was observed in term cells (**Figure 2C**). To see the localization of Cx43-positive puncta in FTM HUCPVCs more clearly, high-magnification fluorescence microscopy was performed on sorted human cells after co-culturing (**Figure 2D**). This confirmed the high abundance of Cx43 in the plasma membrane (white arrows).

Mef2c nuclear cardiomyogenic differentiation marker was analyzed by ICC in undifferentiated human MSC cultures and human MSC, rat cardiomyocyte co-cultures (**Figure 2**). HuNu staining was performed concurrently to identify human MSC-originated cells in the cultures. Undifferentiated human MSC cultures did not show Mef2c-positive cells (**Figure 2E, undifferentiated**).Mef2c/HuNu double-positive cells were identified in co-cultures, with HUCPVCs presenting more double-positive nuclei than BMSCs (**Figure 2E, differentiated, and Figure 2F**). Statistical analysis has confirmed a significantly higher number ($p < 0.01$) of Mef2c-positive human nuclei in FTM and term HUCPVC-containing co-cultures compared to BMSCs (**Figure 2F**).

Cardiomyocyte-specific Regulatory and Structural Proteins Are Upregulated by Co-culture Differentiation in HUCPVCs:

TRA-1-85^{high} cells were sorted from hMSC, rat cardiomyocyte co-cultures, and the expression levels of human cTnT and MY6H genes were analyzed by qPCR (**Figure 3A**). Housekeeping genes ACTB, HPRT, and GAPDH were used as internal normalizers. The up-regulation of the cTnT mRNA level was detected in both FTM (148 ± 71-fold) and term (81.7 ± 32-fold) HUCPVCs compared to the expression levels in their undifferentiated counterparts. MYH6 mRNA expression was also found to be strongly induced during differentiation in both FTM (6,200 ± 4,800-fold) and term (8,100 ± 3,500-fold) HUCPVCs. Expression levels of cTnT and MYH6 in BMSCs were below detection level. Primer specificities were confirmed by testing cTnT and MYH6 primers on human heart-derived cDNA and human cell-free rat primary cardiomyocyte culture-derived cDNA (**Figure 3B**).

To confirm the terminal cardiomyogenic differentiation of FTM HUCPVCs, ICC for HuNu and aSarc was performed (**Figure 4**). Undifferentiated FTM HUCPVCs tested negative for aSarc (**Figure 4A**).Secondary controls andsingle primary-antibody staining confirmed antibody reaction specificities in co-cultures (**Figure 4B, C, and D**). Both FTM HUCPVC, rat cardiomyocyte direct co-cultures (**Figure 4E**) and re-plated FTM HUCPVC aggregate co-cultures (**Figure 4F**) revealed a high abundance of aSarc-positive cells containing exclusively HuNu-positive (human) nuclei (white arrows).

The abundance of aSarc/HuNu double-positive cells in FTM HUCPVC-containing direct and aggregate co-cultures were 18.5 ± 4% and 36 ± 7%, respectively (**Figure 4E and F**).

FTM HUCPVC Aggregates Display Spontaneous Contractions Within 1 Week of Co-culture On Rat Cardiomyocyte Feeder Layers:

Three of the 5 FTM HUCPVC lines investigated during our studies were consistently found to produce spontaneously pulsating aggregates within 5-7 days in co-culture with rat primary cardiomyocytes (**Videos 1, 2, and 3**). In the same culture conditions and for up to 3 weeks, contracting term HUCPVC or BMSC aggregates were not found. Live-imaging and recording fluorescent pre-stained FTM HUCPVC aggregates revealed that contracting human cells first became active in the inner parts of the aggregates, as opposed to the marginal regions (**Video 1**). Moreover, aggregates became wholly and synchronously motile after 7 days and remained active despite the cardiomyocyte feeder layer no longer displaying physical activity (**Videos 4 and 5**).

FTM HUCPVCs Remain Immunoprivileged After Cardiomyogenic Differentiation:

To assess the immunophenotypical characteristics of hMSCs in culture, we analyzed the abundance of immunogenicity-related HLA-A- and immunoprivilege-related HLA-G-positive cells by flow cytometry. In undifferentiated cultures of hMSCs, both HUCPVC types displayed a significantly ($p < 0.01$) lower frequency of HLA-A-positive cells (23.5 ± 15.2% and 32.3 ± 23% versus 70.3 ± 8.1%, respectively) and a significantly ($p < 0.01$) higher frequency of HLA-G-positive cells (18.2 ± 6.4% and 14.7 ± 6% versus 4.2 ± 2.8, respectively; **Figure 5A**) compared to BMSCs.

During direct co-culture-based cardiomyogenic differentiation, HLA-A expression remained significantly ($p < 0.01$) lower in FTM (40.8 ± 18.2%) and term (57.8% ± 26.1%) HUCPVCs in comparison to BMSCs (90.7% ± 7.8%; **Figure 5B**). Although FTM HUCPVCs achieved a terminally differentiated state in aggregate co-cultures that even granted them the ability of spontaneous contraction, the expression of HLA-A was still found to be significantly ($p < 0.01$) lower (53.6 ± 22.8%) than those of term (85.4 ± 3%) HUCPVCs and BMSCs (87.3 ± 3%; **Figure 5C**). Concurrently, HLA-G expression was significantly ($p < 0.01$) higher in both direct co-cultures of HUCPVCs (FTM: 23.8 ± 6.2%, term: 21 ± 4.7%) and aggregate co-cultures (FTM: 42.8 ± 30% and term: 29.6 ± 6%) when compared to BMSCs (3.2 ± 1.7% and 3.7 ± 0.7%, respectively).

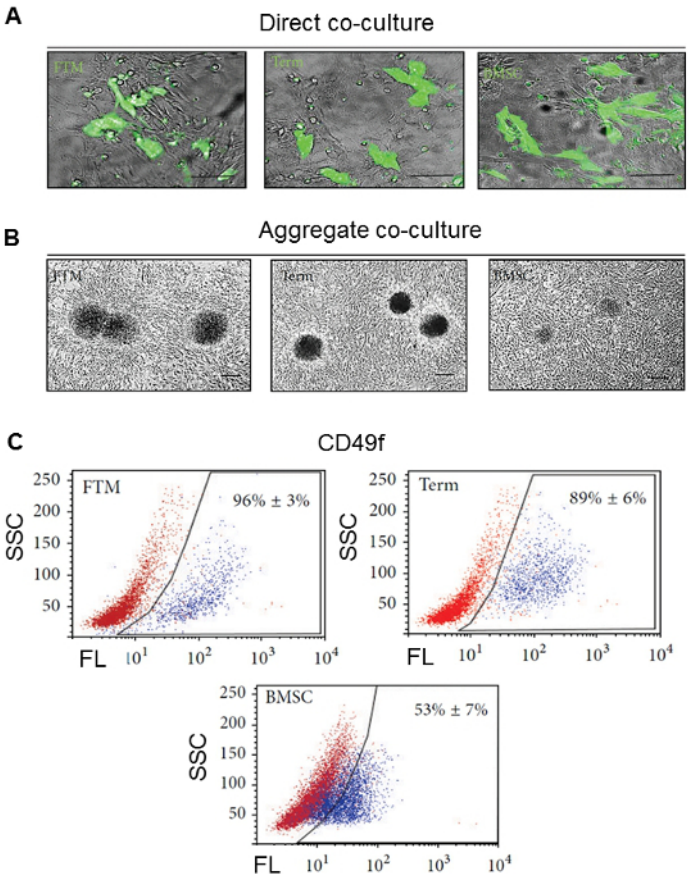


Figure 1: Cardiomyogenic induction of MSCs using primary rat cardiomyocyte co-culture strategies.
(A) Bright-field, fluorescent microscopy overlay of direct co-cultures from pre-stained hMSCs (green) and rat primary cardiomyocytes on day 3. FTM: FTM HUCPVCs, term: term HUCPVCs, BMSC: bone marrow MSCs. Scale bar = 100 µm. **(B)** Bright-field microscopy images of MSC aggregate co-cultures on day 4. Scale bar = 200 µm. **(C)** Flow cytometry analysis of CD49f-positive cell populations in undifferentiated MSC cultures. FL: fluorescence of CD49f labelling. SSC: side scatter axis. ± values refer to the standard deviation (SD). The figure was adapted from Szaraz *et al.*[51], with permission. Please click here to view a larger version of this figure.

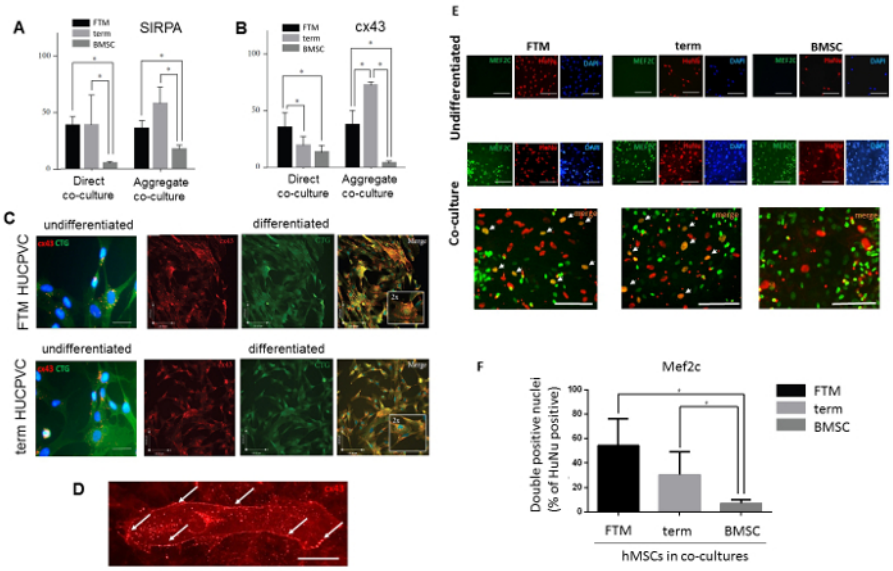
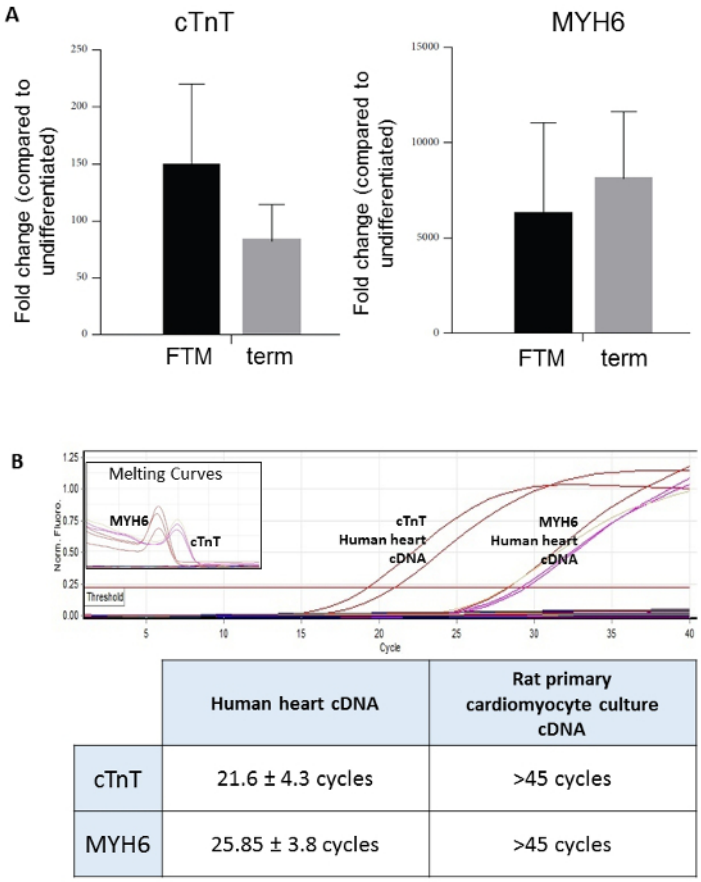


Figure 2: Analysis of cardiomyogenic differentiation markers in differentiated hMSCs.
Flow cytometry analysis of MSCs: cardiomyocyte marker signal regulatory protein α (SIRPA) **(A)** and gap junction protein connexin 43 (Cx43) **(B)** levels expressed as the percent of overall human cell counts in differentiated (direct co-culture, aggregate co-culture) FTM HUCPVCs (FTM), term HUCPVCs (term), and BMSCs. The asterisk (*) indicates statistically significant difference ($p < 0.01$). **(C)** Cx43 staining on differentiated FTM and term HUCPVCs. Cells were counterstained with cytoplasmic fixable fluorescent dye (CTG, green). Inner box: 2x magnification of representative fields showing the intracellular distribution of Cx43-positive puncta. Scale bars: x = 110 µm; y = 100 µm. Blue: Hoechst (DAPI filter). **(D)** High-magnification image of Cx43 staining on FTM HUCPVC sorted after co-culture differentiation. The arrows show plasma membrane-localized Cx43-positive particles. Scale bar = 50 µm. **(E)** Fluorescence microscopy images of FTM HUCPVCs, term HUCPVCs, and BMSCs in the undifferentiated state (upper row) and in direct co-cultures (middle row) for 7 days. Immunostaining performed for monocyte enhancer factor 2C (Mef2c; green) and human nuclear antigen (HuNu; red). Blue: DAPI. Merged images show double-positive nuclei (yellow, white arrows) in co-cultures. Scale bar = 200 µm. **(F)** Quantification of Mef2c/HuNu double-positive nuclei in hMSC, rat cardiac co-cultures. The numbers are expressed as the percent of HuNu-positive nuclei. The asterisk (*) indicates a statistically significant difference ($p < 0.01$). The error bars refer to the standard deviation (SD). The figure was adapted from Szaraz *et al.*[51], with permission. Please click here to view a larger version of this figure.



	Human heart cDNA	Rat primary cardiomyocyte culture cDNA
cTnT	21.6 ± 4.3 cycles	>45 cycles
MYH6	25.85 ± 3.8 cycles	>45 cycles

Figure 3: Quantitative PCR analysis of cTnT and MYH6.
(A) Fold-increase of cardiac troponin (cTnT) and heavy chain cardiac myosin (MYH6) mRNA levels in TRA-1-85^{high} FTM and term HUCPVCs sorted from direct co-cultures, compared to the undifferentiated state. **(B)** Representative graphs of dCt quantification of qPCR primer specificity testing on human and rat cDNA samples. The error bars refer to standard deviation (SD). The figure was adapted from Szaraz *et al.*[51], with permission. Please click here to view a larger version of this figure.

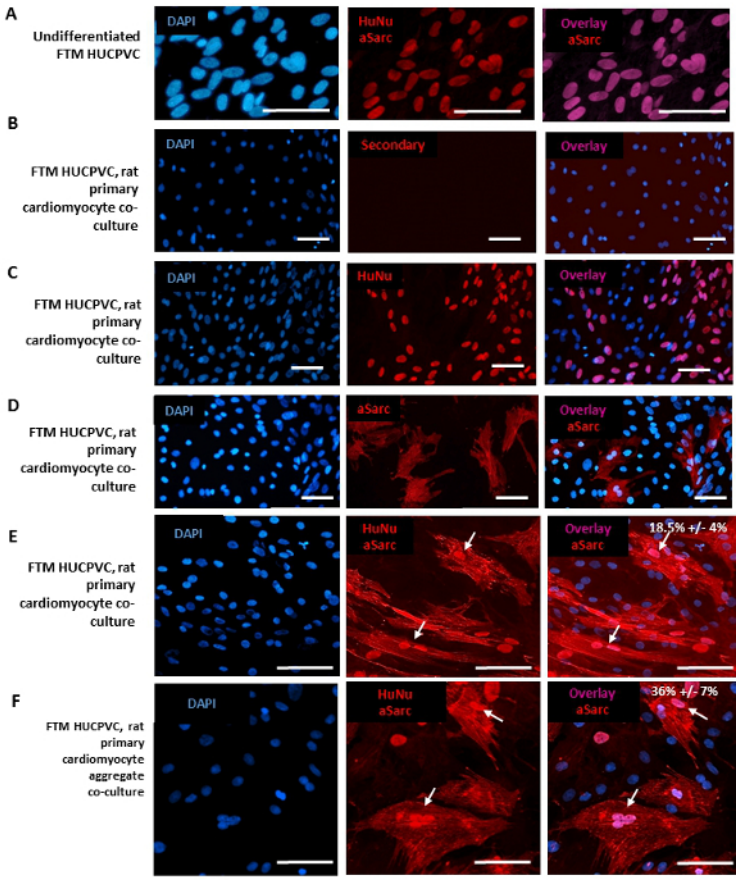


Figure 4: Alpha sarcomeric actinin staining of differentiated FTM HUCPVCs.
(A) HuNu staining (red) of undifferentiated FTM HUCPVC cultures. **(B)** Secondary antibody control (red) on FTM HUCPVC, rat primary cardiomyocyte co-cultures. **(C)** HuNu staining (red) on FTM HUCPVC, rat primary cardiomyocyte co-cultures. **(D)** Alpha sarcomeric actinin (aSarc, red) staining of primary rat cardiac cultures. **(E)** HuNu/aSarc co-staining of rat primary cardiac cell, FTM HUCPVC direct co-cultures and **(F)** re-plated aggregate co-cultures. White arrows indicate human nuclei. DAPI = blue. Scale bar = 100 µm. The figure was adapted from Szaraz *et al.*[51], with permission. Please click here to view a larger version of this figure.

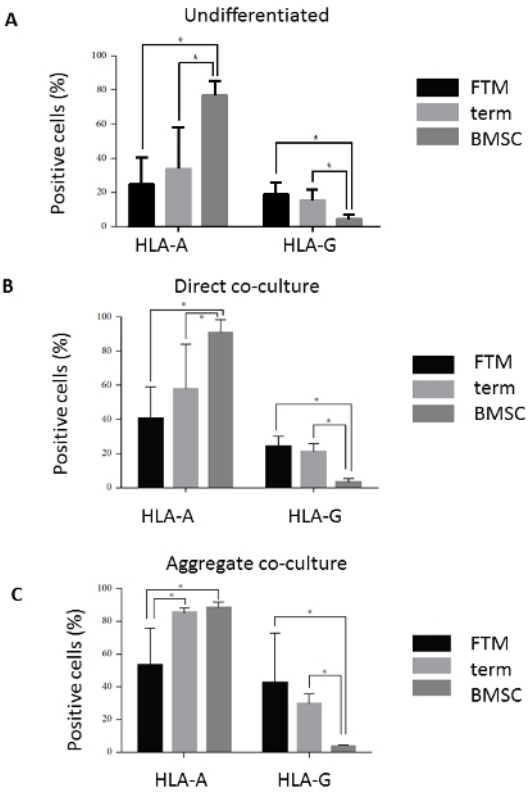
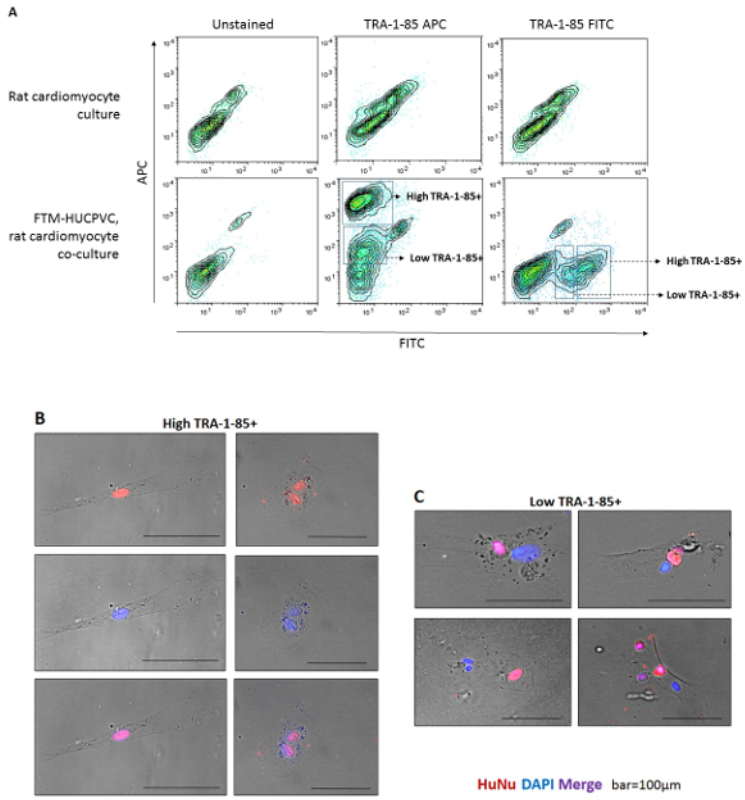


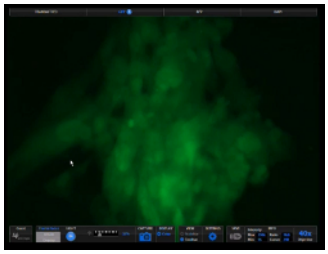
Figure 5: Immunological characteristics of MSCs after cardiac differentiation.
(A) FC analysis of HLA-A and HLA-G expression in undifferentiated human MSC cultures. FC analysis of HLA-A and HLA-G expression in **(B)** direct co-cultures and **(C)** aggregate co-cultures of differentiated FTM HUCPVCs, term HUCPVCs, and BMSCs. Values expressed as the percent positive from the overall human cell number in corresponding cultures. The asterisk (*) indicates a statistically significant difference ($p < 0.01$). The error bars refer to standard deviance (SD). The figure was adapted from Szaraz *et al.*[51], with permission. Please click here to view a larger version of this figure.

Timeline				
	Day 0	**Day 3**	**Day 5**	**Day 7**
Rat cardiac cells	Isolate and plate primary cardiac cells	Remove unattached cells/ change media	Aging cardiac feeder layer becomes inactive	Collect aggregates or lift co-cultures. Sort human cells, process for FC, ICC, qPCR
Human MSCs	Prepare hanging drops	Transfer aggregates to feeder layer	Observe and record contracting aggregates	

Table 1: Schematic timeline and sequence of events during aggregate-based differentiation.



Supplementary Figure 1: Human cell detection in FTM HUCPVC, rat cardiomyocyte co-culture samples.
(A) Flow cytometry analysis and gating strategy distinguishes human cells from mixed species populations using primary, fluorophore-conjugated TRA-1-85 human cell-surface marker-specific antibodies (FITC: x-axis, APC: y-axis). The inner boxes mark high and low TRA-1-85-positive cell populations, identified from co-cultures. **(B)** Immunocytochemistry analysis of high and low TRA-1-85-positive cell populations sorted from co-cultures using HuNu-specific antibody (red). Scale bar = 100 µm. The figure was adapted from Szaraz *et al.*[51], with permission. Please click here to view a larger version of this figure.



Supplemental Videos: FTM HUCPVC aggregates display spontaneous contractions. Please click here to view this video. (Right-click to download.)

Discussion

The cardiac differentiation of stem cells has been under development for over 2 decades, with several different strategies being used to generate cardiomyocyte-like cells from MSC sources. Many of these strategies, however, are inefficient, and the conditions used are often not representative of the environment transplanted cells encounter *in vivo*.

In contrast to existing methods, the protocol presented here utilizes a combination of primary cardiac feeder layers and MSC aggregate formation. The primary cardiac feeder layers provide a differentiation environment similar to that which the hMSCs are exposed to upon transplantation. The aggregates generate an environment with oxygen and nutrient gradients, which can have an inductive effect on initiating lineage commitment. PSC- and ESC-based cardiac differentiation strategies using hypoxic preconditioning and aggregate formation have established this inductive effect on stem cells[5,52,53]. Additionally, a 3D structure better resembles the cell-to-cell connections provided *in vivo*. Using this method, we successfully produced synchronously contracting hMSC aggregates-the first time an hMSC type was able to achieve this, to our knowledge. Importantly, only the youngest hMSC cell source applied, FTM HUCPVCs, exhibited this ability.

A significant advantage of using either naïve or pre-differentiated MSCs over ESCs or PSCs for cardiac regeneration lies in their immunomodulatory or immunoprivileged properties. However, even in the case of MSCs, immunomodulatory properties vary with their age and source[54]. BMSCs have been shown to change their immunogenic properties post-differentiation, which often leads to their rejection upon

implantation[55]. As previously described, MSCs from the umbilical cord origin possess special immune privilege[56] due to their *in utero* origin. Using the protocol described above, even after differentiation into cardiomyocyte-like cells, term and FTM HUCPVCs exhibited high levels of HLA-G (that actively attenuates the innate immune response) and maintained low levels of HLA-A (that determines T-cell mediated cytotoxicity).

This differentiation protocol has the advantage of conveniently allowing for multiple levels of analysis. First, the live imaging of functional, contracting aggregates is more feasible than distinguishing single cells integrated into a feeder layer. As the protocol utilizes mixed-species co-cultures, human-specific primers are applied to qPCR analysis, while FACS and ICC provide the rapid identification of phenotype- specifically, cardiac marker expression (**Figure 2**). Using the protocol as described, elevated levels of SIRPA, cTnT, MYH6, aSarc, Mef2c, and Cx43 were detected. In conjunction with HuNu, it is possible to identify the expression of Mef2c and aSarc in differentiated hMSCs and to distinguish from the rat cardiomyocyte-derived feeder layers or fused cells. While the identification of contracting aggregates on the feeder layer is relatively easy, collecting or harvesting them can pose a limitation. Microscopes equipped with a micromanipulator can be employed to handle this challenge. Alternatively, human cells can be efficiently sorted from co-cultures using human-specific anti-TRA-1-85 antibodies. While the co-cultures also present the challenge of potential cell fusion, this too can be overcome using a combination of nuclear and cell-surface human marker tracers (**Supplementary Figure 1**).

A possible modification of our differentiation method is to adjust the formation of aggregates. Our results suggest that aggregate size can be a critical parameter for optimal differentiation. Assuming that aggregation is selective for more adhesive cells, it can act as a pre-selection step for CD49f-positive hMSCs. Enhanced MSC sphere formation has been linked to increased CD49f expression[49], and CD49f is also associated with higher stemness because it is directly connected to SOX2 and OCT4[49]. We observed lower numbers of CD49f-positive cells in older sources of hMSCs compared to FTM HUCPVCs, which could explain the diminished aggregate size observed. We theorize that this selection can be compensated for by increasing the number of hMSCs per aggregate to achieve a higher aggregate size, thus possibly increasing the efficiency of aggregation-induced differentiation.

Future applications might require modifications to the protocol as well. It is imperative that clinical applications of pre-differentiated hMSCs take place in xeno-free conditions. The contracting aggregates produced using this protocol could also be used for the creation of engineered heart muscle tissues. To produce a clinically applicable product, a cGMP-compliant workflow is necessary. While xeno-free culture medium is available for hMSCs, providing an animal-free feeder layer might be challenging. However, applying pre-differentiated feeder layers of other stem cell sources, such as iPSCs, is a possible solution.

In conclusion, the differentiation method described can be applied to produce functional cardiomyocyte-like cells from young sources of hMSCs in a convenient and reproducible manner.

Disclosures

Dr. Clifford L. Librach is joint holder of the patent: *Methods of isolation and use of cells derived from first trimester umbilical cord tissue*, granted in Canada and Australia.

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References

1. Badano, L. P. *et al.* Prevalence, clinical characteristics, quality of life, and prognosis of patients with congestive heart failure and isolated left ventricular diastolic dysfunction. *J Am Soc Echocardiogr.* **17** (3), 253-261 (2004).
2. Leri, A., Kajstura, J., & Anversa, P. Cardiac stem cells and mechanisms of myocardial regeneration. *Physiol Rev.* **85** (4), 1373-1416 (2005).
3. Orlic, D. *et al.* Mobilized bone marrow cells repair the infarcted heart, improving function and survival. *Proc Natl Acad Sci U S A.* **98** (18), 10344-10349 (2001).
4. Schuster, M. D. *et al.* Myocardial neovascularization by bone marrow angioblasts results in cardiomyocyte regeneration. *Am J Physiol Heart Circ Physiol.* **287** (2), H525-532 (2004).
5. Zandstra, P. W. *et al.* Scalable production of embryonic stem cell-derived cardiomyocytes. *Tissue Eng.* **9** (4), 767-778 (2003).
6. Boheler, K. R. *et al.* Differentiation of pluripotent embryonic stem cells into cardiomyocytes. *Circ Res.* **91** (3), 189-201 (2002).
7. Kattman, S. J. *et al.* Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. *Cell Stem Cell.* **8** (2), 228-240 (2011).
8. Dixon, J. E., Dick, E., Rajamohan, D., Shakesheff, K. M., & Denning, C. Directed differentiation of human embryonic stem cells to interrogate the cardiac gene regulatory network. *Mol Ther.* **19** (9), 1695-1703 (2011).
9. Stennard, F. A. *et al.* Cardiac T-box factor Tbx20 directly interacts with Nkx2-5, GATA4, and GATA5 in regulation of gene expression in the developing heart. *Dev Biol.* **262** (2), 206-224 (2003).
10. Dubois, N. C. *et al.* SIRPA is a specific cell-surface marker for isolating cardiomyocytes derived from human pluripotent stem cells. *Nat Biotechnol.* **29** (11), 1011-1018 (2011).
11. Panteghini, M. Present issues in the determination of troponins and other markers of cardiac damage. *Clin Biochem.* **33** (3), 161-166 (2000).
12. Burridge, P. W. *et al.* Improved human embryonic stem cell embryoid body homogeneity and cardiomyocyte differentiation from a novel V-96 plate aggregation system highlights interline variability. *Stem Cells.* **25** (4), 929-938 (2007).
13. Ovchinnikov, D. A. *et al.* Isolation of contractile cardiomyocytes from human pluripotent stem-cell-derived cardiomyogenic cultures using a human NCX1-EGFP reporter. *Stem Cells Dev.* **24** (1), 11-20 (2015).

14. Moscoso, I. *et al.* Differentiation "in vitro" of primary and immortalized porcine mesenchymal stem cells into cardiomyocytes for cell transplantation. *Transplant Proc.* **37** (1), 481-482 (2005).
15. Ramkisoensing, A. A. *et al.* Gap junctional coupling with cardiomyocytes is necessary but not sufficient for cardiomyogenic differentiation of cocultured human mesenchymal stem cells. *Stem Cells.* **30** (6), 1236-1245 (2012).
16. Kempen, M. *et al.* Expression of the electrophysiological system during murine embryonic stem cell cardiac differentiation. *Cell Physiol Biochem.* **13** (5), 263-270 (2003).
17. Mummery, C. L. *et al.* Differentiation of human embryonic stem cells and induced pluripotent stem cells to cardiomyocytes: a methods overview. *Circ Res.* **111** (3), 344-358 (2012).
18. Puceat, M. Protocols for cardiac differentiation of embryonic stem cells. *Methods.* **45** (2), 168-171 (2008).
19. Naito, H. *et al.* Optimizing engineered heart tissue for therapeutic applications as surrogate heart muscle. *Circulation.* **114** (1 Suppl), I72-78 (2006).
20. Zimmermann, W. H. *et al.* Heart muscle engineering: an update on cardiac muscle replacement therapy. *Cardiovasc Res.* **71** (3), 419-429 (2006).
21. Hulot, J. S. *et al.* Considerations for pre-clinical models and clinical trials of pluripotent stem cell-derived cardiomyocytes. *Stem Cell Res Ther.* **5** (1), 1 (2014).
22. Wakitani, S., Saito, T., & Caplan, A. I. Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. *Muscle Nerve.* **18** (12), 1417-1426 (1995).
23. Caplan, A. I. Adult Mesenchymal Stem Cells: When, Where, and How. *Stem Cells Int.* **2015** 628767 (2015).
24. Burchfield, J. S., & Dimmeler, S. Role of paracrine factors in stem and progenitor cell mediated cardiac repair and tissue fibrosis. *Fibrogenesis Tissue Repair.* **1** (1), 4 (2008).
25. Hsiao, S. T. *et al.* Comparative analysis of paracrine factor expression in human adult mesenchymal stem cells derived from bone marrow, adipose, and dermal tissue. *Stem Cells Dev.* **21** (12), 2189-2203 (2012).
26. Tomita, S. *et al.* Autologous transplantation of bone marrow cells improves damaged heart function. *Circulation.* **100** (19 Suppl), II247-256 (1999).
27. Skerjanc, I. S. Cardiac and skeletal muscle development in P19 embryonal carcinoma cells. *Trends Cardiovasc Med.* **9** (5), 139-143 (1999).
28. Hou, J. *et al.* Combination of BMP-2 and 5-AZA is advantageous in rat bone marrow-derived mesenchymal stem cells differentiation into cardiomyocytes. *Cell Biol Int.* **37** (12), 1291-1299 (2013).
29. Yoon, J. *et al.* Differentiation, engraftment and functional effects of pre-treated mesenchymal stem cells in a rat myocardial infarct model. *Acta Cardiol.* **60** (3), 277-284 (2005).
30. Xing, Y., Lv, A., Wang, L., & Yan, X. The combination of angiotensin II and 5-azacytidine promotes cardiomyocyte differentiation of rat bone marrow mesenchymal stem cells. *Mol Cell Biochem.* **360** (1-2), 279-287 (2012).
31. Yuan, Y. *et al.* [Differentiation of mesenchymal stem cells into cardio myogenic cells under the induction of myocardial cell lysate]. *Zhonghua Xin Xue Guan Bing Za Zhi.* **33** (2), 170-173 (2005).
32. Toma, C., Pittenger, M. F., Cahill, K. S., Byrne, B. J., & Kessler, P. D. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation.* **105** (1), 93-98 (2002).
33. Yannarelli, G. *et al.* Donor mesenchymal stromal cells (MSCs) undergo variable cardiac reprogramming in vivo and predominantly co-express cardiac and stromal determinants after experimental acute myocardial infarction. *Stem Cell Rev.* **10** (2), 304-315 (2014).
34. Rangappa, S., Entwistle, J. W., Wechsler, A. S., & Kresh, J. Y. Cardiomyocyte-mediated contact programs human mesenchymal stem cells to express cardiogenic phenotype. *J Thorac Cardiovasc Surg.* **126** (1), 124-132 (2003).
35. Bakogiannis, C. *et al.* Circulating endothelial progenitor cells as biomarkers for prediction of cardiovascular outcomes. *Curr Med Chem.* **19** (16), 2597-2604 (2012).
36. Deb, A. *et al.* Bone marrow-derived cardiomyocytes are present in adult human heart: A study of gender-mismatched bone marrow transplantation patients. *Circulation.* **107** (9), 1247-1249 (2003).
37. Laflamme, M. A., Myerson, D., Saffitz, J. E., & Murry, C. E. Evidence for cardiomyocyte repopulation by extracardiac progenitors in transplanted human hearts. *Circ Res.* **90** (6), 634-640 (2002).
38. Quaini, F. *et al.* Chimerism of the transplanted heart. *N Engl J Med.* **346** (1), 5-15 (2002).
39. Alvarez-Dolado, M. *et al.* Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. *Nature.* **425** (6961), 968-973 (2003).
40. Nygren, J. M. *et al.* Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. *Nat Med.* **10** (5), 494-501 (2004).
41. Hong, S. H. *et al.* Ontogeny of human umbilical cord perivascular cells: molecular and fate potential changes during gestation. *Stem Cells Dev.* **22** (17), 2425-2439 (2013).
42. Sarugaser, R., Ennis, J., Stanford, W. L., & Davies, J. E. Isolation, propagation, and characterization of human umbilical cord perivascular cells (HUCPVCs). *Methods Mol Biol.* **482** 269-279 (2009).
43. Sarugaser, R., Lickorish, D., Baksh, D., Hosseini, M. M., & Davies, J. E. Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors. *Stem Cells.* **23** (2), 220-229 (2005).
44. Kadivar, M. *et al.* In vitro cardiomyogenic potential of human umbilical vein-derived mesenchymal stem cells. *Biochem Biophys Res Commun.* **340** (2), 639-647 (2006).
45. Baksh, D., Yao, R., & Tuan, R. S. Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. *Stem Cells.* **25** (6), 1384-1392 (2007).
46. Wu, K. H. *et al.* Cardiac potential of stem cells from whole human umbilical cord tissue. *J Cell Biochem.* **107** (5), 926-932 (2009).
47. Yannarelli, G. *et al.* Human umbilical cord perivascular cells exhibit enhanced cardiomyocyte reprogramming and cardiac function after experimental acute myocardial infarction. *Cell Transplant.* **22** (9), 1651-1666 (2013).
48. Kilkenny, C., Browne, W. J., Cuthill, I. C., Emerson, M., & Altman, D. G. Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. *J Pharmacol Pharmacother.* **1** (2), 94-99 (2010).
49. Yu, K. R. *et al.* CD49f enhances multipotency and maintains stemness through the direct regulation of OCT4 and SOX2. *Stem Cells.* **30** (5), 876-887 (2012).
50. Lee, R. H. *et al.* The CD34-like protein PODXL and alpha6-integrin (CD49f) identify early progenitor MSCs with increased clonogenicity and migration to infarcted heart in mice. *Blood.* **113** (4), 816-826 (2009).

51. Szaraz, P. *et al.* In Vitro Differentiation of First Trimester Human Umbilical Cord Perivascular Cells into Contracting Cardiomyocyte-Like Cells. *Stem Cells Int.* **2016** 7513252 (2016).
52. Bauwens, C., Yin, T., Dang, S., Peerani, R., & Zandstra, P. W. Development of a perfusion fed bioreactor for embryonic stem cell-derived cardiomyocyte generation: oxygen-mediated enhancement of cardiomyocyte output. *Biotechnol Bioeng.* **90** (4), 452-461 (2005).
53. Jing, D., Parikh, A., & Tzanakakis, E. S. Cardiac cell generation from encapsulated embryonic stem cells in static and scalable culture systems. *Cell Transplant.* **19** (11), 1397-1412 (2010).
54. Hare, J. M. *et al.* Comparison of allogeneic vs autologous bone marrow-derived mesenchymal stem cells delivered by transendocardial injection in patients with ischemic cardiomyopathy: the POSEIDON randomized trial. *JAMA.* **308** (22), 2369-2379 (2012).
55. Huang, X. P. *et al.* Differentiation of allogeneic mesenchymal stem cells induces immunogenicity and limits their long-term benefits for myocardial repair. *Circulation.* **122** (23), 2419-2429 (2010).
56. Hare, J. M. *et al.* A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction. *J Am Coll Cardiol.* **54** (24), 2277-2286 (2009).

