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Cardiogenesis of Embryonic Stem Cells with Liquid Marble Micro-Bioreactor

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A liquid marble micro-bioreactor is prepared by placing a drop of murine embryonic stem cell (ESC) (Oct4B2-ESC) suspension onto a polytetrafluoroethylene (PTFE) particle bed. The Oct4B2-ESC aggregates to form embryoid bodies (EBs) with relatively uniform size and shape in a liquid marble within 3 d. For the first time, the feasibility of differentiating ESC into cardiac lineages within liquid marbles is being investigated. Without the addition of growth factors, suspended EBs from liquid marbles express various precardiac mesoderm markers including Flk-1, Gata4, and Nkx2.5. Some of the suspended EBs exhibit spontaneous contraction. These results indicate that the liquid marble provides a suitable microenvironment to induce EB formation and spontaneous cardiac mesoderm differentiation. Some of the EBs are subsequently plated onto gelatin-coated tissue culture dishes. Plated EBs express mature cardiac markers atrial myosin light chain 2a (MLC2a) and ventricular myosin light chain (MLC2v), and the cardiac structural marker α-actinin. More than 60% of the plated EBs exhibit spontaneous contraction and express mature cardiomyocyte marker cardiac troponin T (cTnT), indicating that these EBs have differentiated into functional cardiomyocytes. Together, these results demonstrate that the liquid-marble technique is an easily employed, cost effective, and efficient approach to generate EBs and facilitating their cardiogenesis.

1. Introduction

Adult hearts have a very limited capacity for self-regeneration after myocardial infarction (MI; heart attack). Transplanted stem cells or progenitor cells have the capacity to repair infarcted myocardium.[1,2] Pluripotent embryonic stem cell (ESC), isolated from the inner cell mass of a developing blastocyst,[3] possess the ability to self-renew, and have the potential to differentiate into various cell lineages, including all three germ layers[4] and cardiomyocytes.[5] The ability to engineer these ESC genetically, together with their ability to differentiate into cardiomyocytes in vitro, makes them valuable and promising cell sources for cell therapy, tissue engineering, and regenerative medicine.[6] Forming 3D embryo-like cell aggregates, known as embryoid bodies (EBs), is a key step for the in vitro differentiation of ESC.[7] Indeed, an EB consists of three germ layers (ectodermal, mesodermal, and endodermal tissues) that emulate the features of a developing embryo,[4,8] thereby providing a valuable tool for various embryogenesis studies.[9,10]

Several methods have been employed to form EBs from ESC and to subsequently differentiate them into cardiomyocytes. These include hanging-drop culture,[9] spinner flask,[11] centrifuge-forced aggregation,[12] and suspension culture in a low-adherence vessel.[4,7] The hanging drop method is the most commonly used technique for EB formation, in which an ESC suspension was placed on the inner surface of a Petri dish lid. EBs can be formed after inverting the lid due to the balance of gravitational and surface tension forces. Changing the droplet volume and seeding density can tune the size of the EBs. However, the hanging drop method is labor intensive and time consuming. It is also practically impossible to perform medium exchange using this method. In addition, the drop volume is limited to less than 50 µL, thereby making it incapable of supporting large-scale production.[8] Rotation-based methods, such as the spinner flask and centrifuge-forced aggregation methods, can facilitate the large-scale production. However, these methods require costly equipment; moreover, the shear stress induced by the rotation may reduce cell viability, and disrupt cell–cell signaling and the subsequent cell differentiation. The method based on suspension culturing using conventional low-adherence vessels has limited control over size, shape, and uniformity of EBs.[13]

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The liquid marble was first described by[14] and consists of a drop of liquid encapsulated by hydrophobic powder particles. These particles adhere to the surface of the liquid drop, isolating the liquid core from the supporting surface, while allowing gas exchange between the interior liquid and the surrounding environment.[15] A liquid marble can be rolled around similar to a droplet of mercury.[16] As the shells of liquid marbles are made from discrete particles, the shells can be opened, allowing materials such as reagents and products to be introduced into or extracted from the liquid marble; this unique property therefore facilitates chemical and biochemical reactions to be controlled within liquid marbles.

In addition, reagent consumption can be reduced due to the small size of a liquid marble. The chance of contamination is low as a result of the indirect contact between the liquid core and the supporting surface, thus providing an advantage for a variety of applications.[15,17–19] The use of liquid marbles as miniaturized bioreactors is particularly attractive because of the capability to contain chemical and biological reactions.[18,20–23] Our previous studies reported the production of 3D cancer-cell spheroids using liquid marble micro-bioreactors.[20] This liquid-marble method is advantageous for spheroid production, as it allows the production of spheroids with homogeneous size and shape at a larger scale compared with the hanging drop method, as well as facilitating medium exchange. Unlike rotation-based methods, the liquid-marble method does not induce shear stress on the spheroids, thereby producing viable spheroids.[13]

Herein, we report the use of the liquid marble as a micro-bioreactor to produce EBs from ESC and, for the first time, we study the feasibility to further differentiate the EBs into lineage-specific cells. The in vitro cardiac differentiation ability of the resulting EBs was assessed by examining gene expression, protein expression, and contraction characteristics. We demonstrate that liquid marbles provide a promising platform to facilitate EB differentiation into cardiac lineages.

2. Result and Discussion

2.1. Pluripotency and Propagation of EB

Formation of 3D aggregates called EBs is an important step that precedes the initiation of in vitro differentiation of ESC into various cell types.[24] Under the in vitro conditions, an EB is known to simulate the events of a developing embryo. In a previous study, we reported the possibility of using liquid marbles (LMs) as a facile and efficient micro-bioreactor for in vitro EB formation, the viability of cells in EB obtained using LM was much higher than those obtained using conventional suspension
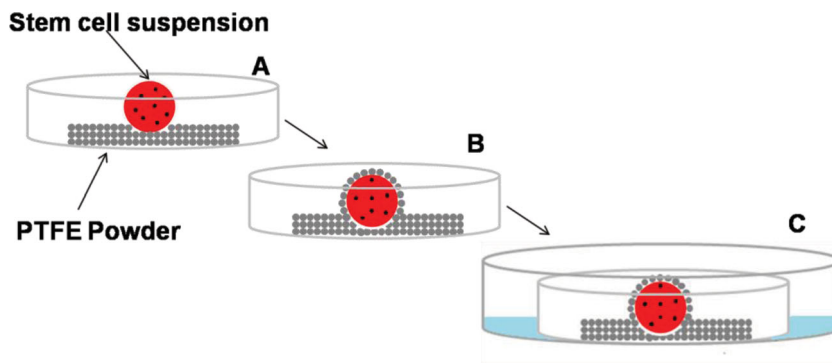

Figure 1. Schematic illustration of the steps involved in preparing a liquid marble bioreactor. A) 300 mL of cell suspension is placed onto a hydrophobic PTFE powder bed, B) The Petri dish was then rolled gently to allow the PTFE particles to cover the cell suspension to form the liquid marble. C) Placing the marble dish inside a bigger Petri dish with sterilized water to prevent evaporation

culture method.[13] In that study, liquid marble micro-bioreactor was formed by self-assembled polytetrafluoroethylene (PTFE) powder around a droplet of ESC suspension as illustrated in **Figure 1**, ESC were cultivated inside liquid marbles and cell aggregates were obtained from day 3. All three germ layers developed spontaneously within the cell aggregates, indicating that the ESC formed EBs successfully inside the liquid marbles.[13] In our present study, we use liquid marbles as micro-bioreactors to generate EBs from Oct4B2-ESC and investigate, for the first time, the capability of liquid marble to facilitate cardiac differentiation. **Figure 2** illustrates the steps involved in cardiac differentiation. The morphology of cells harvested from the liquid marbles was assessed using optical microscopy. The Oct4B2-ESC contain a pluripotency marker that drives the expression of GFP, hence GFP expression is a direct indicator of cell pluripotency.[25]

The pluripotency of cells forming the EBs was monitored by examining their GFP expression. Representative images in **Figure 3** show that after cultivating Oct4B2-ESC in liquid marbles for 3 d, these cells aggregated to form EBs, and that these EBs exhibited a compact and round shape with relatively uniform size.

As evident from Figure 3A,B, the ES cells, while aggregating to form clusters destined to form EBs, retained their GFP expression, indicating that the cells do not lose their pluripotency. The day 10 analysis of the EBs revealed an increase in size suggesting the proliferation of cells within the LM. There was a decrease in the GFP flourescence observed, stipulating the loss of pluripotency and that there was an initiation of differentiation of ES cells within the LM (Figure 3D).
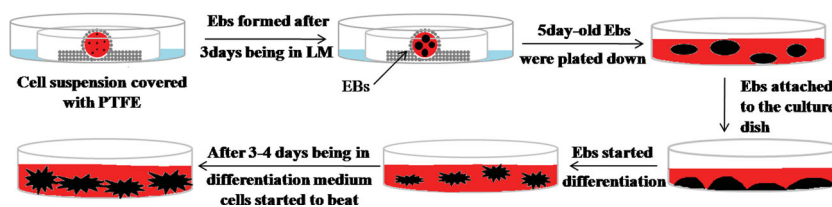

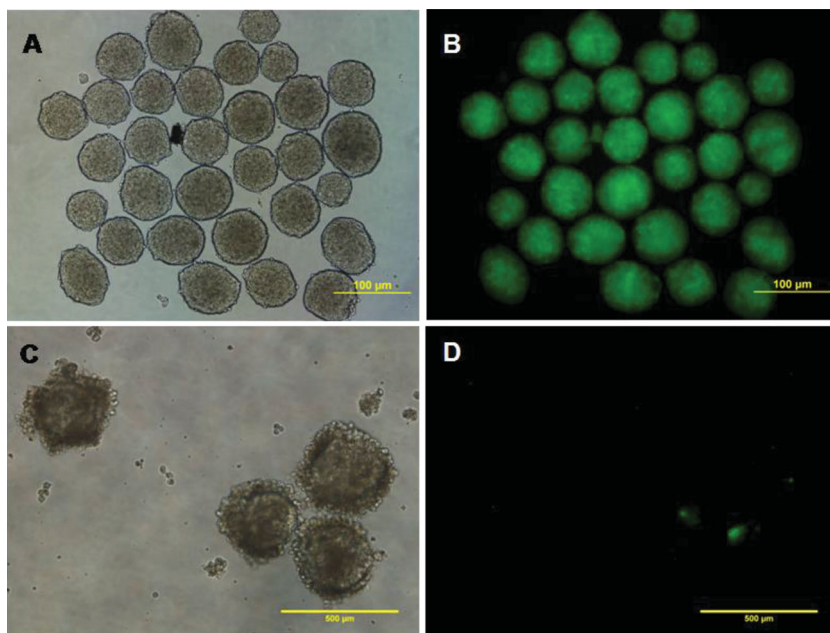**Figure 2.** Schematic steps leading to cardiac differentiation.

Figure 3. Representative phase-contrast and fluorescence microscopy images showing the morphology of EBs. These EBs were formed by allowing Oct4B2-ESC to aggregate inside liquid marbles for A,B) 3 d (scale bars represent 100 µm) and C,D) 10 d (scale bars represent 500 µm). B,D) Fluorescence microscopy images show the expression of GFP under the control of Oct4.

cardiomyocytes (small and rounded with rarely developed sarcomeres) to terminally differentiated atrial-/ventricular-like (elongated with high content of organized myofibrils) cells.

It is worth mentioning that a double Petri dishes set-up was used to minimize evaporation of liquid marble in this study. With this set-up, very little change in liquid marble size was observed over the period of cell culture study. For future work, the influence of evaporation on the liquid marble size, thus its volume, can be monitored using a video camera and image-processing technique. A calibration curve of volume change versus liquid marble size can be established, such that any volume loss due to evaporation can be quantified using images of the liquid marble and the calibration curve, and equivalent amount of fresh medium can be added to replace any volume loss. However, this is beyond the scope of the current study, as this work aims to demonstrate the feasibility of generating cardiomyocytes in liquid marble micro-bioreactor.

Oct4B2 cells were allowed to form EBs in liquid marbles for 10 d; samples were collected from the liquid marbles at days 0 (control), 3, 7, and 10. Fluorescence-activated cell sorting (FACS) analysis was performed on the dissociated cells to quantify the number of GFP+ cells in an EB in order to examine their pluripotency. Representative FACS analysis profiles in **Figure 4** show that 98.8% and 92.4% of cells expressed GFP at days 0 and 3, respectively. The GFP expression gradually reduced to 20% and 9.4% at days 7 and 10, respectively. Thus, the loss of GFP expression as suggested from the FACS analysis and the flourescence data implies the ability of LM to allow proliferation and differentiation of Oct4B2 cells. This novel system is thus capable of providing a suitable microenvironment for the growth and differentiation of ESCs.

It has been well documented that during the development of contractile cardiomyocytes, progenitor cells need to first anchor to a substrate followed by cell spreading, withdrawal from the cell cycle, and fusion with nascent myotubes before their ultimate differentiation into cardiomyocytes.[26] In order to promote cardiogenesis of our LM-induced EBs, 5-day-old EBs were plated onto gelatin-coated six wells and allowed to differentiate further. Upon EB adhesion, the cells began to migrate and grew outwards from the periphery of the EB to form a monolayer, as shown in the representative images in **Figure 5**A,B. In the outgrown areas shown in Figure 5C, it was observed that cells exhibited heterogeneous cell morphology. At the start of cardiac differentiation, cells were small and rounded, which upon further differentiation changed to elongated, spindle-shaped cells. This observation was in-line with the results of the electrophysiological measurements conducted by Hescheler et al.[27] wherein the heterogeneous population of cardiomyocytes undergoes a shift from early stage

2.2. Reverse Transcription PCR Analysis of Cardiac Marker Expression During Cardiogenesis

To characterize the differentiation pathway of EBs generated from a liquid marble, the gene expression of EB-derived cells was qualitatively determined using reverse-transcription-polymerase chain reaction (RT-PCR). Cells from a suspended EB obtained from a liquid marble and plated EBs were collected at different time points and were characterized using a series of cardiac markers. Flk-1 expression is found in mesodermal progenitor cells that have the ability to further differentiate into cardiac muscles.[28,29] Gata4 is a regulator of early cardiogenesis,[30] it expresses in precardiac mesoderm and subsequently expresses in the endocardial and myocardial tissues of developing heart and heart tube; overexpression of Gata4 is known to up-regulate the expression of transcription factors Nkx2.5.[31] The cardiac specific transcription factor Nkx-2.5 is the key regulator of cardiac-specific transcription involved in cardiogenesis, which is generally observed in the pluripotent stem cell-derived cardiomyocytes.[32] Therefore, Flk-1, Gata4, and Nkx2.5 were all employed as precardiac mesoderm markers in this study. β-actin was used as housekeeping marker gene. Atrial myosin light chain 2a (MLC2a) and ventricular myosin light chain (MLC2v) are both cardiac structural proteins, and are often employed as mature cardiac cell markers.[33,34] Sarcomeric protein α-actinin is a cardiac structural protein that crosslinks actin filaments within the Z-disc of cardiac muscle.[34,35] MLC2a, MLC2v, and α–actinin were thereby used as mature cardiomyocyte markers in this study. **Figure 6**A shows that cells from suspended EBs expressed Gata4 from days 3 to 10. Flk1 and Nkx2.5 expressions could also be detected in suspended EBs from days 3 to 10. The positive expression of Flk1, Gata4, and
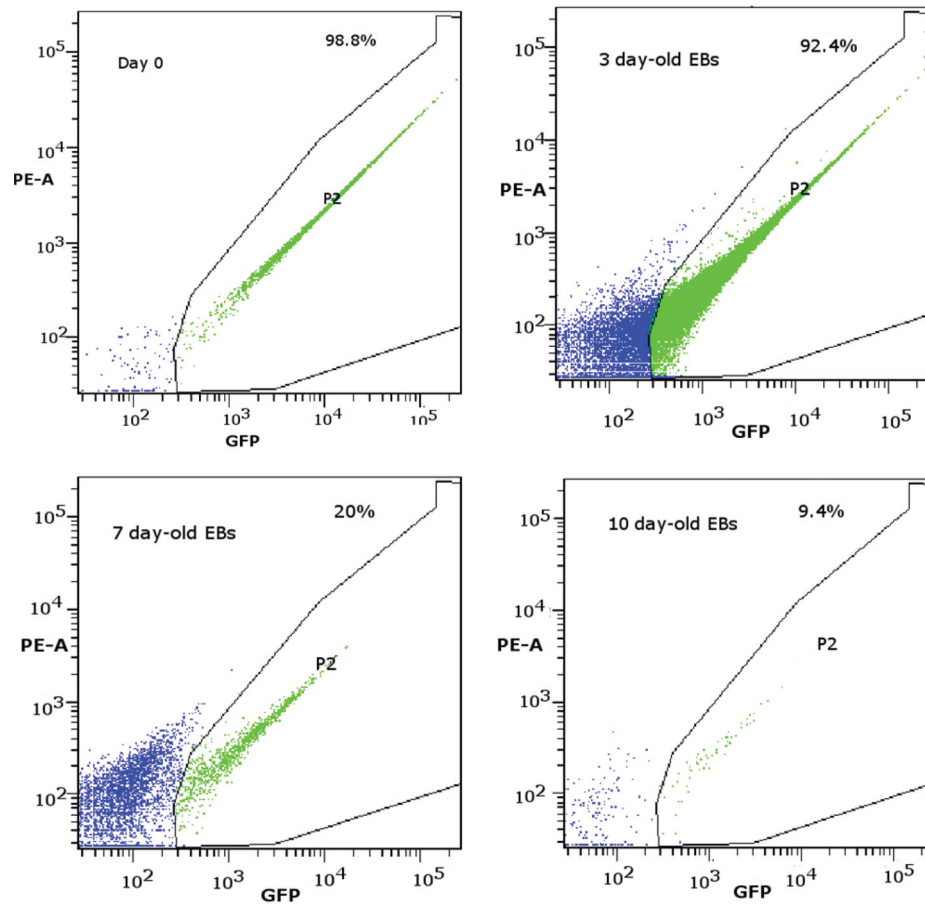
Figure 4. FACS analysis: GFP expression of LM induced EBs of Oct4B2 at days 0 (control), 3, 7, and 10. Numbers indicate the percentage of GFP+ cells. GFP is indicated on x-axis, and autofluorescence on y-axis. GFP− population is represented by blue dots, while GFP+ events are represented by green dots.

Nkx2.5 suggests that liquid marbles provide a suitable environment to induce cardiac mesoderm differentiation in ESC. Figure 6B shows the RT-PCR analysis of the plated-down EBs. Nkx2.5, Gata4, MLC2a, MLC2v, and α-actinin expressions were detected from days 6 to 15, revealing that these cells had differentiated into precardiac mesoderm and mature cardiomyocytes after plating-down. Similar to the ESC differentiation seen in a hanging drop,[36] removing leukemia inhibitory factor (LIF) from the medium in our culture system, signs of ESC differentiation were detected in suspended EBs inside liquid marbles as well as in outgrown cells in plated-down culture. The use of the liquid marble allows the formation of EBs, which, in turn, enables cell-to-cell interactions. This cell-to-cell interaction is known to stimulate the expression of early cardiac lineage markers.[34] Nevertheless, the liquid-marble method permits the use of larger drop volumes compared to the hanging drop method and is therefore more advantageous for larger scale studies.

2.3. Quantitative Real-Time PCR Analysis of Cardiac Markers Expression During Cardiogenesis

EBs were allowed to form inside liquid marbles for 5 d, and were plated in gelatin-coated plates with differentiation medium that contained no growth factors. The gene expression of these cells was quantitatively determined using real-time PCR by harvesting cells at days 6, 8, 10, 12. The time-dependent
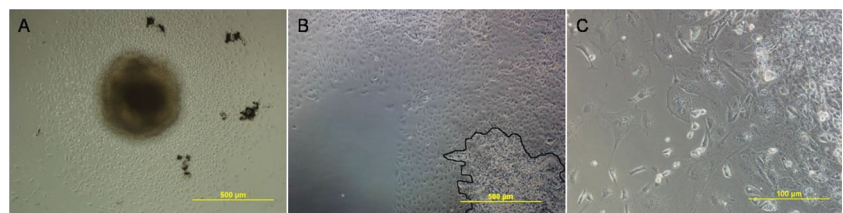

Figure 5. Phase-contrast images of a cardiac explants outgrowth. A) Representative image showing a plated EB attached to a gelatin-coated plate after 1 d, B) representative image of plated EB outgrowth at day 4, scale bars represent 500 μm. The black line shows the border of flattened EBs after plating down. C) Representative image of the plated EB depicting the heterogeneous cell morphology (small and round, spindle shaped cells), at day 8, scale bar represent 100 μm.
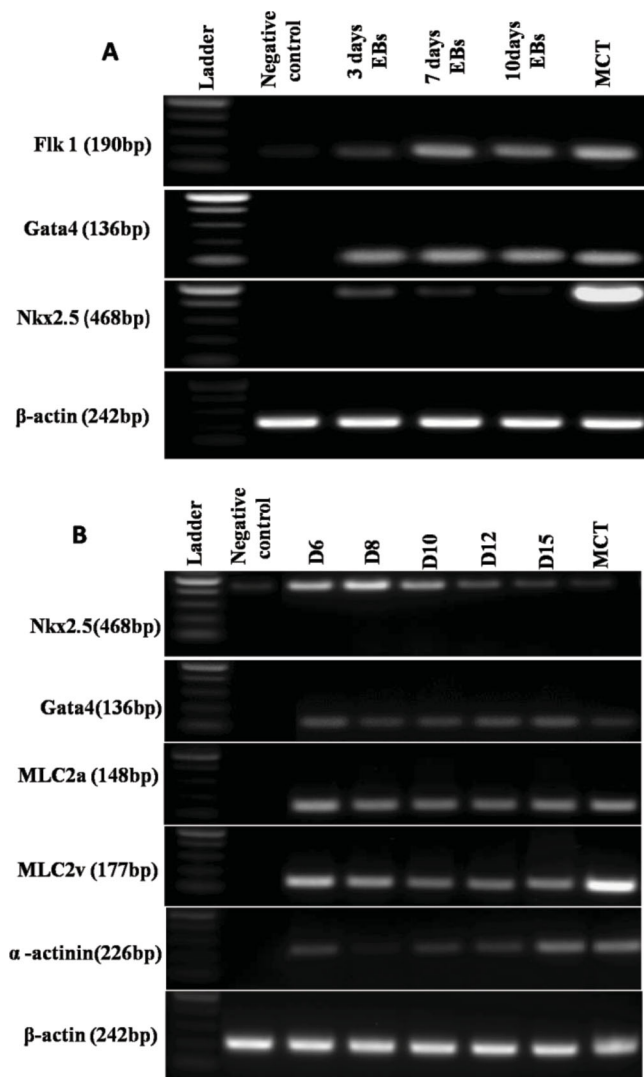
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Figure 6. A) Representative RT-PCR analysis for precardiac mesoderm markers expression in cells from EB suspensions obtained from liquid marbles after 3, 7, and 10 d of culture. B) Representative RT-PCR analysis for precardiac mesoderm and mature cardiomyocytes markers expression from EBs after plating down for 6, 8, 10, 12, and 15 d. β-actin was used as a housekeeping gene. Cells obtained from murine cardiac tissue (MCT) were used as a positive control. Undifferentiated ES cells were used as a negative control.

expressions of various lineage and cardiac markers during the course of differentiation are shown in **Figure 7**. β-actin was used as a constitutive housekeeping gene for real-time PCR and used to normalize changes in specific gene expressions. Octamer-binding transcription factor 4 (Oct4) was used as both a positive control for pluripotent cells and a negative control for cardiac cells. The pluripotent or undifferentiated ESCs express high level of Oct4 gene, whereas cells that are undertaking cardiac differentiation express negligible levels of Oct4 gene. Oct4 expression was down regulated from day 6, which suggested that the ESC had lost their pluripotency. The expression of precardiac mesoderm markers Flk-1, Gata4, and Nkx2.5 were up regulated after plating down the EBs for 6 d compared with undifferentiated ES cells. The expression of Flk-1 decreased

from day 8 onward, while the expression of Gata4 and Nkx2.5 decreased from day 10 onward. These results together suggested that the ESC had differentiated into cardiac mesoderm and subsequently differentiate into other cell types. The formation of cardiac mesoderm is a prerequisite intermediate step for cardiomyocytes differentiation from pluripotent stem cells. Expressions of MLC2a, MLC2v, and α–actinin were first detected at day 6. The MLC2a expression increased at day 10, and decreased gradually from day 12. The MLC2v expression increased at day 8, and decreased gradually from day 10. The α–actinin expression remained constant from days 6 to 12, and increased sharply at day 15, thus suggesting that stable sarcomeric structural protein was formed within the cell culture. The expression of MLC2a, MLC2v, and α–actinin, together indicated that plated EBs had differentiated into cardiomyocytes spontaneously. It is noted that spontaneous beating was observed in EBs after plating down for 4 d. The results are consistent with those reported in other studies, where Nkx2.5, MLC2v, and MLC2a are expressed in beating cardiomyocytes that had differentiated from ES cells.[31] Altogether, the results indicate that EBs derived from liquid marbles had differentiated into mesoderm and subsequently into mature cardiomyocytes.

2.4. Immunocytochemistry

Five-day-old EBs were collected from liquid marbles and plated down. Immunostaining was performed on EBs that exhibited spontaneous contraction to detect the presence of the cardiac-specific proteins. As shown in representative images in **Figure 8** (top panel), the expression of the cardiac transcription factor Nkx2.5 (AlexaFlour594, stained red) was detected after plating down the EB for 7 d. Nkx2.5 is a transcription factor that is expressed in myocardiogenic progenitor cells during myocardial development;[32,37] Nkx2.5 is the earliest known marker for cardiogenesis in the veterbrate embryo.[38–40] Representative images in Figure 8 (bottom panel) show that cells expressed cardiac troponin T (cTnT) markers (AlexaFlour594, stained red) after plating down for 12 d. cTnT is a marker for mature cardiomyocytes,[34] EB-derived cells expressed cardiac-specific protein markers, thus further confirming that this liquid marble method can generate functional EBs that can further differentiate into mature cardiomyocytes.

2.5. Beating Cardiac Cells

In order to observe contractile, EBs were kept inside liquid marbles for 7 d. At day 7, EBs were retrieved and suspended in a low-adhesion dish. After 8 h, it was observed that only ≈4.5% of EBs exhibited spontaneous contraction (Supporting Information 1), the contraction only lasted for a few hours, probably because once the ESC had differentiated into lineage-specific progenitor cells, they needed to anchor onto a substrate for optimal differentiation, as reported by Engler et al.[26] Nevertheless, this observation confirmed the differentiation potential of EBs into generate cardiomyocyte-like cells inside liquid marbles. To examine if EBs generated from liquid marbles can further differentiate into beating cardiac cells, 5-day-old EBs
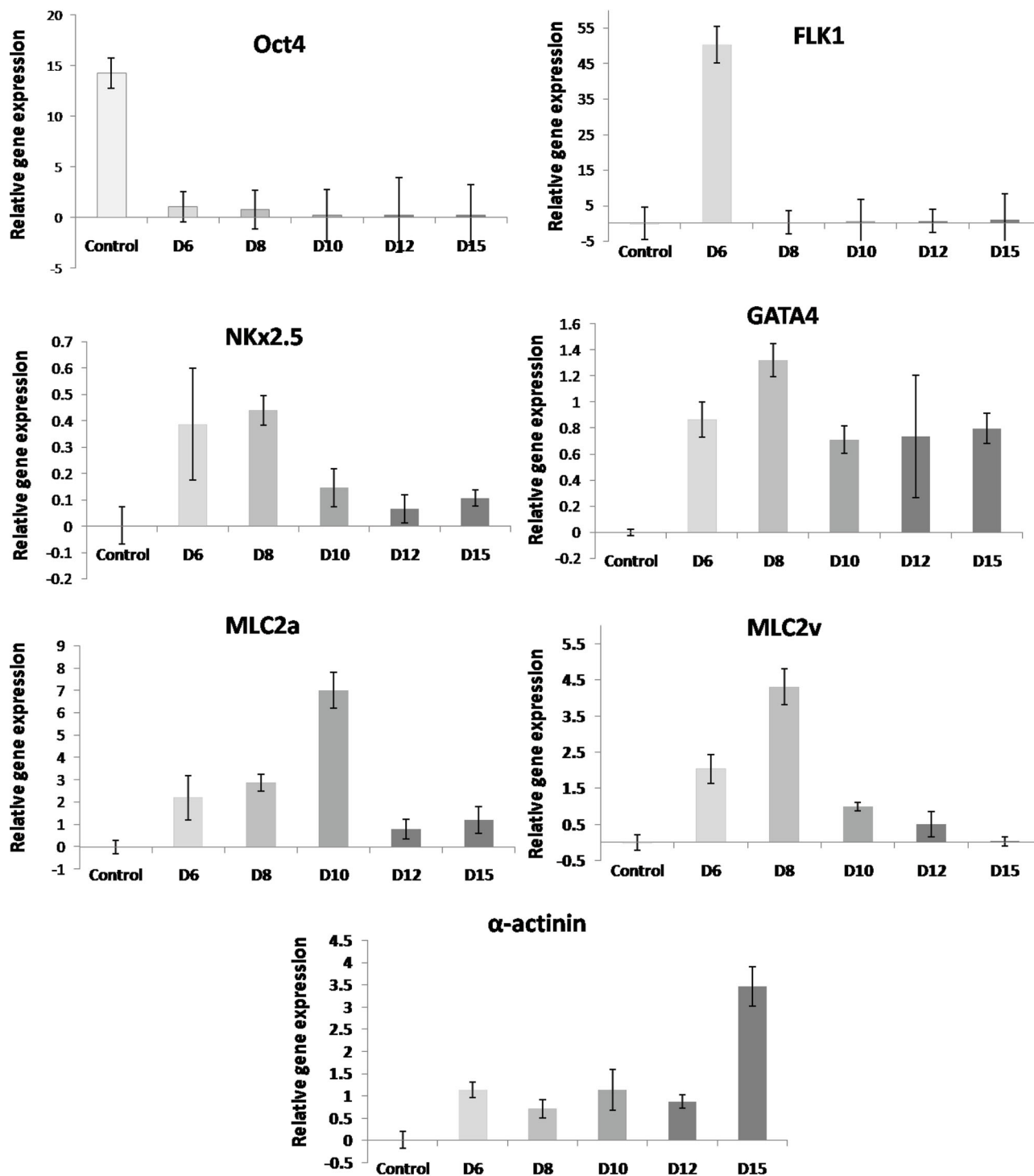
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Figure 7. Real-time PCR analysis of gene expression over 15 d of cardiomyogenesis after plating down 5-d old EBs derived from liquid marbles. Cardiomyogenesis was characterized by a continuous decrease in pluripotency marker (Oct4) expression over the course of differentiation, followed by an initial increase in the expression of the precardiac mesoderm markers (Flk1, Nkx2.5, and Gata4) expression, which eventually decreased. The increase in the expression of mature cardiomyocytes markers (MLC2v, MLC2a, and α-actinin) expression as seen resulted in the formation of mature cardiomyocytes.

were removed from the liquid marble and plated down in a gelatin-coated wells and the activities of the EBs were monitored. These EBs adhered and proliferated well on the substrate.

Spontaneous rhythmic beating was detected in the outgrown EBs after plating down for 4 d. Supporting Information 2,3 show the videos of beating cells derived from EBs after plating
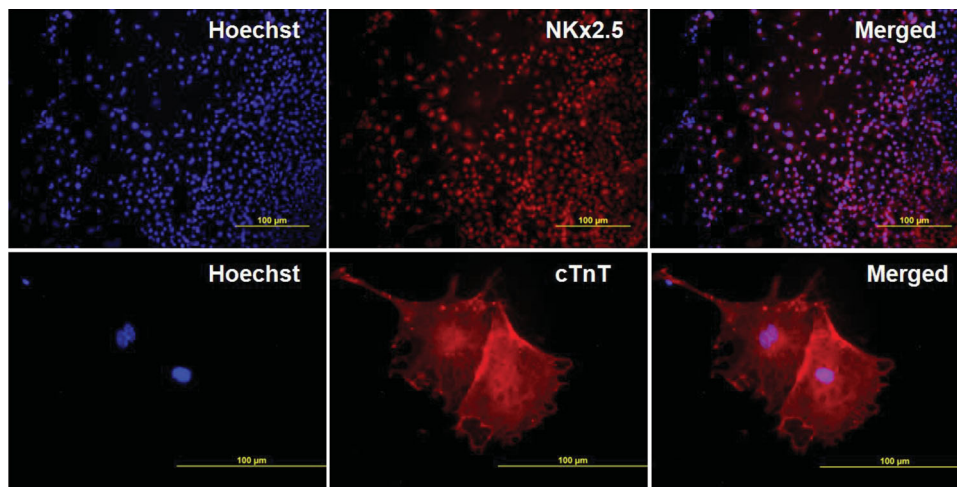
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Figure 8. Immunostaining for Nkx2.5 (after 7 d) and cTNT (after 12 d) of plated down cells. Nuclei were counterstained with Hoechst (blue). Scale bars represent 100 μm.

down for 4 and 7 d, respectively. The rhythmic beating was considered as a functional cardiac marker.[41] For brevity, EBs that differentiated into beating cells are denoted as beating EBs. **Figure 9** A,B show the mean intensity of beating obtained from representative areas of beating foci after plating down the EBs for 4 and 7 d, respectively. At day 4, the beating was relatively regular with minor arrhythmia. At day 7, the beating became more regular. The beating frequency was calculated by measuring the time interval between two consecutive peaks in the

mean intensity patterns. The frequency increased from 0.76 s^{-1} at day 4, to 1.29 s^{-1} at day 7, as shown in Figure 9C. This was probably because the cells were initially beating asynchronously after migrating out from the EB; these cells became more synchronized given the longer culture time.

After plating the EBs for 4 d, approximately 16% of the EBs underwent differentiation into rhythmic beating cells (Figure 9D). The percentage of beating EBs increased to 48% after 7 d. The percentage of beating EBs continued to increase
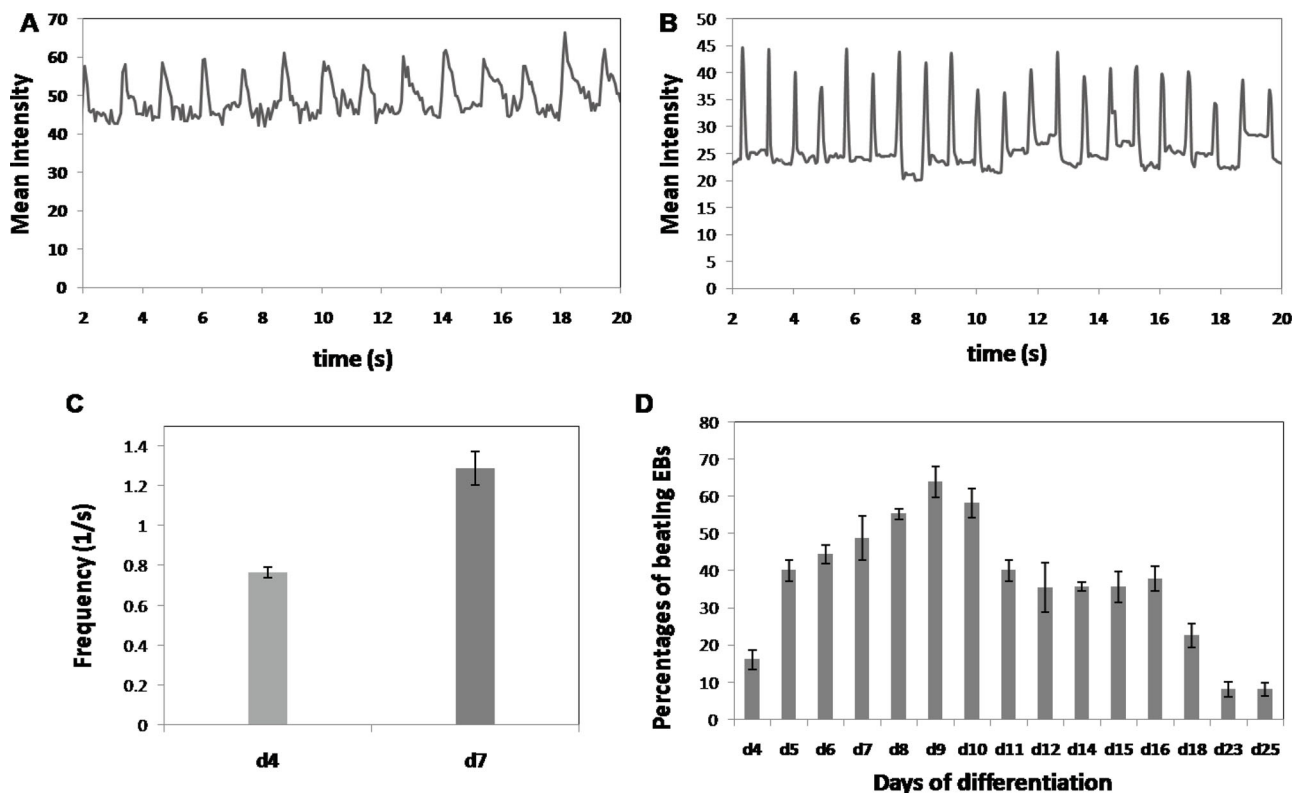

Figure 9. Mean intensity patterns obtained from representative areas in contractile EBs after plating for A) 4 and B) 7 d. C) The beating frequency of EBs at days 4 and 7. D) The percentages of contractile EBs at different time points.

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over time reaching a maximum of 64% after 9 d. After 10 d, the rhythmic beating activity declined, possibly due to the overgrowth of cells causing peeling of cells from the plate, although approximately 35% of the EBs still exhibited rhythmic beating activity from days 12 to 16. Approximately 8% of EBs still exhibited rhythmic beating at day 25. As expected, a larger number of EBs exhibited spontaneous contraction in plated culture compared to suspension culture. This observation is consistent with other studies that found that EBs differentiate extensively after attaching to a substrate.[42,43] The observation of spontaneous rhythmic beating again confirmed that liquid-marble-derived EBs differentiated into functional cardiac cells. Taken together, these results indicate that the liquid marble is a promising platform for EB generation, and for facilitating further differentiation into cells with a cardiac lineage.

3. Conclusions

In this study, we investigated the capability of liquid marbles to induce EB formation and subsequent differentiation into cardiomyocytes. Liquid marbles were prepared by inoculating ESC onto a bed of hydrophobic PTFE particles. ESC aggregated to form uniform EBs after inoculation, and subsequently differentiated into cardiac mesoderm cells without the use of growth factors. Further on plating, these EBs further differentiated into contractile cardiomyocytes. The contraction of cardiomyocytes was synchronized with longer time in culture. The liquid-marble method was found to be advantageous for EB formation as it is cost effective and simple; it also allows larger scale EB production compared with the hanging drop method. Overall, this study shows that liquid marbles can serve not only as a novel platform to induce the formation of EBs but also to facilitate cardiogenesis. The cardiomyocytes generated via this liquid marble strategy could provide a continuous source of donor cardiomyocytes for cell replacement therapy in damaged hearts. Furthermore, this technology would be highly beneficial to provide cardiomyocytes for use in cardiac drug discovery programs and safety testing. Since the quantity of cells required for the above-mentioned applications is very high, it becomes imperative to develop defined and efficient in vitro protocols, which would then provide the stringent levels of safety and quality control making stem cell transplantation therapy realizable. Our study provides a step up this ladder and gives a new promise and hope in cardiovascular research

4. Experimental Section

Tissue Culture: Feeder free murine Oct4B2-ESC (129/Sv) containing the Oct4-GFP-IRES-puromycin and hygromycin resistance cassettes were used for this work. For expansion, ESC were cultured in Dulbecco's modified Eagle medium (cat#11995; Gibco) supplemented with 10% FBS (JRH Biosciences, Australia), 1% non-essential amino acids (NEAA) (cat. #11140-050; Invitrogen), 1% GlutaMAX (cat#35050061; Invitrogen), 0.5% penicillin–streptomycin (cat#15070–063; Invitrogen), 0.1×10^{-3} M β-mercaptoethanol (cat. #21985-023; Invitrogen), and 1000 U mL^{-1} ESGRO leukemia inhibitory factor (mLIF, Chemicon, Australia). The medium was filtered through a 0.22-μm filter for sterilization and was stored at 4 °C for up to a fortnight. Cells were cultured on 0.1%

gelatin-coated six-well plates (BD Falcon) at 37 °C in a humidified 5% CO_2 incubator and were passaged every 2–3 d. The GFP expression of the cells was monitored using an IX71 Olympus epifluorescence microscope.

Preparation of Cell Containing Liquid Marble Micro-Bioreactor: ESC were cultured to 70%–80% confluency; cells were then washed with Dulbecco's phosphate buffered saline (DPBS; Sigma) and dispersed into single cells using Tryple express (Gibco, Life Technologies, Australia). To form liquid marbles, 2×10^4 ESCs were suspended in 300 μL of differentiation medium. The differentiation medium consisted of high-glucose DMEM (Gibco, Life Technologies, Australia) supplemented with 10% FBS (JRH Biosciences, Australia), 1% non-essential amino acids, 1% GlutaMAX (Gibco, Life Technologies, Australia), 0.5% penicillin–streptomycin (Gibco, Life Technologies, Australia), 0.1×10^{-3} M β-mercaptoethanol (Gibco, Life Technologies, Australia) and without mLIF. This drop of cell suspension was placed onto a PTFE (35 μm particle size; Sigma, Australia) powder bed inside a Petri dish (60 mm diameter) using a micropipette. When the drop of cell suspension was rolled on the hydrophobic powder bed, the powder particles wrapped the surface of the drop, thus leading to the formation of a liquid marble. The strong hydrophobic and chemically inert properties of PTFE powder are the desirable properties for making liquid marble biological reactors for this work. Two critical requirements to the reactor shell, apart from air permeability, are that the shell should resist cell adhesion and the marble shell should be mechanically strong and maintain its integrity for the duration of the experiment. Our recent work[13,20] showed that PTFE powder marble shell satisfies both requirements. The selection of 35 μm PTFE powder was based on the balanced liquid marble shell mechanical strength and its ability to resist cell adhesion so as to encourage the formation of stem cell EBs.[13] Arbatan et al. characterized the mechanical strength of the liquid marble by measuring the force by which a glass cover slip cut through the powder-covered water surface.[44] They found that liquid marble shell formed by 35 μm powder has a sufficient mechanical strength to sustain the reactor integrity, while also produces the most uniform stem cell EBs.[13] The Petri dish containing the liquid marble was then placed inside a larger Petri dish (100 mm diameter) containing sterile water to minimize evaporation from the liquid marbles, following which the set of dishes were covered and kept inside a humidified incubator. Periodically, the size of the liquid marble was being checked visually. The ESCs were allowed to aggregate to form EBs within the liquid marble over a period of 5 d. Figure 1 illustrates the process of forming the liquid marbles. The color of the medium in liquid marble was monitored on a daily basis, no color change was observed over the first 5 d. From day 5 onward, whenever a color change was observed in the liquid marble, 50–100 μL of spent medium was removed and replaced by the same amount of fresh medium using a micropipette.

EB Morphology and Pluripotency Characterization: ESC-containing liquid marbles were prepared and allowed to incubate for a period of 10 d. EB samples were taken from the liquid marbles at days 3, 7, and 10. The morphology and GFP expression of EBs were monitored using optical and epifluorescence microscopy (Olympus 1 × 70 microscope). Both phase-contrast and epifluorescent images were captured. The collected EBs were further dissociated into single cells using Tryple express; the GFP expression of these cells was quantified using FACS at different time points during EB formation.

In Vitro Cardiac Differentiation: For cardiac differentiation, 5-day-old EBs were removed from the marbles and transferred to 0.1% gelatin-coated 24-well plates and cultured in differentiation medium for further analysis. The plated EBs were examined daily for contractile activity based on videos captured at 15 fps using a camera through an optical microscope. The detailed description of the protocol is illustrated in Figure 2.

Reverse-Transcription and Real-Time Polymerase Chain Reaction: The gene expression analysis of the EBs and the cells undergoing differentiation were carried out quantitatively using the RT-PCR. Liquid-marble-suspended EBs and plated-down EBs were both subjected to RT-PCR analysis using various differentiation markers. EBs were

Table 1. PCR primer sequences and the NCBI accession numbers for the corresponding genes.

Gene name	Forward/reverse (5′ 3′)	NCBI accession no.
Flk-1	GGCGGTGGTGACAGTATCTT/ CTCGGTGATGTACACGATGC	NM_010612.2
Nkx2.5	ACACCCACGCCTTTCTCAGTCAAA/ CGACAGGTACCGCTGTTGCTTGAA	NM_008700.2
Gata4	TCTCACTATGGGCACAGCAG/ GCGATGTCTGAGTGACAGGA	NM_008092
MLC2a	TCAGCTGCATTGACCAGAAC/ AAGACGGTGAAGTTGATGGG	NM_022879.2
MLC2v	AAAGAGGCTCCAGGTCCAAT/ CCTCTCTGCTTGTGTGGTCA	NM_010861.3
α-actinin	ATGAGGATTGGCTGCTTT/ TGTTCCACCCGGTCTTG	NM_013456
β-actin	CACCACACCTTCTACAATGAGC/ TCGTAGATGGGCACAGTGTGGG	NM_007393.3
Oct4	GTTCAGCCAGACCACCATCT/ CCAGGGTCTCCGATTTGCAT	NM_013633

allowed to form inside the liquid marbles for 3, 7, and 10 d, and were then retrieved from the LM for RT-PCR. To allow further differentiation, some EBs were retrieved from liquid marble after 5 d and plated down on gelatin-coated wells for another 15 d. The plated EBs were analysed for differentiation markers at time points D6, 8, 10, 12, and 15 d. Cells were harvested with Tryple express, the resulting cell pellets were snap chilled at −80 °C prior to analysis. For RT-PCR, ribonucleic acid (RNA) was isolated from cells using the RNeasy kit (Qiagen, Australia) according to the manufacturer's instructions. RNA quality and concentration were measured using a NanoDrop ND-1000 (NanoDrop Technologies, Australia). The isolated RNA was subjected to RQ1 DNase (Ambion, Australia) treatment to remove any contaminating genomic deoxyribonucleic acid (DNA). Complementary DNA (cDNA) was generated using the Superscript III enzyme (Life Technologies, Australia) according to the manufacturer's protocols. The cDNA samples were subjected to PCR amplification with mouse cardiac-specific primers. The primer sequences were obtained from the online NCBI Primer-Blast databank and are listed in **Table 1**. The PCR products were size fractionated using 1% agarose gel electrophoresis at 110 V for 1 h. For quantification, real-time PCR was performed. Real-time PCR analysis was performed using a 7900HT fast real-time PCR system (Applied Biosystems, Australia) at standard reaction conditions using the Power SYBR Green PCR Master Mix (Applied Biosystems, Australia). Briefly, after 2 min denaturation at 95 °C, 35 PCR cycles were carried out at 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s, followed by a dissociation stage. Relative mRNA levels were calculated using the ΔΔCT method[45] and were analysed using SDS Version 2.4.1 software, β-actin was used as internal reference gene. The results were reported as fold change values compared to murine cardiac tissue (MCT). The experiment was replicated three times.

Immunocytochemistry: The protein markers on the cells were characterized using immunostaining. Incubated cells were first fixed in 4% paraformaldehyde for 30 min and washed three times with 1% bovine serum albumin (BSA) in +/+DPBS. Fixed cells were incubated with blocking solution (5% goat serum, 1% BSA in +/+DPBS) for 1 h at room temperature. Cells were then incubated overnight with NKx2.5 primary antibody (Santa Cruz, ThermoFisher Scientific, Australia) and cTnT primary antibody (Abcam, Sapphire Bioscience, Australia) diluted (1:500) in blocking solution at 4 °C. Next, the cells were washed three times with 1% BSA in +/+DPBS and then incubated at room temperature for 1 h with Alexa 594-labeled secondary antibodies (Molecular Probes, Life technologies, Australia) at a concentration of 1:400 in blocking solution. Cells were washed three times with 1% BSA in DPBS for

10 min. Nuclei were counterstained with Hoechst (1 μg mL^{-1}; Sigma) dye. Cells were analyzed by epifluorescence microscopy (IX71 Olympus microscope, Australia).

Image Analysis: After plating 5-day-old EBs for another 4 d in gelatin-coated wells, beating foci appeared in the outgrowing EBs. Videos of the beating foci were captured using the microscope camera system; these videos were converted into image sequences using NIS viewer elements software (Nikon, USA). The images were converted to gray scale. The contraction rhythm of the EBs was evaluated using a modified image processing method described in Arshi et al.[46] As the EB underwent rhythmic beating, the cell cluster color changed from dark to light. Ten different areas were selected from each video, the contracting motion through changes in gray scale intensity in each area was analyzed using ImageJ software (NIH, USA), and the resulting mean intensity was plotted against time.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author

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