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Slow Turning Lateral Vessel Bioreactor Improves Embryoid Body Formation and Cardiogenic Differentiation of Mouse Embryonic Stem Cells

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Abstract

Embryonic stem cells (ESCs) have the ability to form aggregates, which are called embryoid bodies (EBs). EBs mimic early embryonic development and are commonly produced for cardiomyogenesis. Here, we describe a method of EB formation in hydrodynamic conditions using a slow-turning lateral vessel (STLV) bioreactor and the subsequent differentiation of EBs into cardiomyocytes. EBs formed in the STLV were compared with conventional techniques, such as hanging drop (HD) or static suspension cell culture (SSC), for homogeneity of EB size, shape, proliferation, apoptosis, and *in vitro* cardiac differentiation. After 3 days of culture, a four-fold improvement in the yield of EB formation/mL, a six-fold enhancement in total yield of EB/mL, and a nearly 10-fold reduction of cells that failed to incorporate into EBs were achieved in STLV versus SSC. During cardiac differentiation, a 1.5- to 4.2-fold increase in the area of cardiac troponin T (cTnT) per single EB in STLV versus SSC and HD was achieved. These results demonstrate that the STLV method improves the quality and quantity of ES cells to form EBs and enhances the efficiency of cardiac differentiation. We have demonstrated that the mechanical method of cell differentiation creates different microenvironments for the cells and thus influences their lineage commitments, even when genetic origin and the culture medium are the same. Ascorbic acid (ASC) improved further cardiac commitment in differentiation assays. Hence, this culture system is suitable for the production of large numbers of cells for clinical cell replacement therapies and industrial drug testing applications.

Introduction

Cell transplantation is an emerging field for patients suffering from severe heart failure (White and Claycomb, 2003). Cardiac cell transplantation, including cardiomyocyte cell lines (Messina et al., 2004), fetal cardiomyocytes (Gonzales et al., 2012), skeletal myocytes (Reinecke and Murry, 2000; Invernici et al., 2008), and bone marrow–derived stem cells (Barile et al., 2011), has successfully engrafted into the adult heart. However, a limiting factor for development of cell therapy is the inadequate number of donor cells obtained that are needed for curing cardiovascular disease. Due to ethical reasons, use of cardiomyoplasty for treating heart failure is not possible (Penn and Mal, 2006).

Up to now, embryonic stem cells (ESCs) have been the most promising cell sources for cardiovascular cell therapy, because ESCs are capable of spontaneously differentiating into cardiomyocytes (Mummery et al., 2007). The spontaneously contracting cellular structures within the developing embryoid bodies (EBs) (containing myocytes) also have

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structural and functional properties similar to that of early-stage cardiac tissue (Kehat et al., 2001; Snir et al., 2003). Furthermore, transplantation of ESC-derived cardiomyocytes into rat myocardial infarctions leads to the formation of stable cardiomyocyte grafts and attenuation of the remodeling process resulting in improved cardiac function (Caspi et al., 2007; Laflamme et al., 2007). Hence, ESCs are a promising source for cell-based therapies in humans, including cardiac tissue engineering.

Even though ESCs have been shown by numerous research groups to be capable of differentiating into cardiomyocytes in mice and humans, the overall efficiency and the quantity of cells obtained by differentiation of ESCs is still rather low (Boheler et al., 2002; Mummery et al., 2003). Differentiation of ESCs into cardiomyocytes usually requires an initial aggregation step that results in the formation of spherical cell clusters referred to as EBs, which recapitulate several aspects of the developing embryo, including, similar to gastrulation *in vivo*, differentiation into cells representative of the endoderm, mesoderm, and ectoderm lineages (Desbaillets et al., 2000; Hopfl et al., 2004). The hanging drop method (HD) permits a certain level of control over EB size; however, these cultures are inherently incapable of large-scale EB production. Whereas suspension static culture (SSC) has the advantage of being easily scalable and requires less expertise, it often results in agglomeration of EBs into irregularly shaped large masses as well causing extensive cell death (Dang et al., 2002; Chen et al., 2011). Hence, the industrial application of these methods is restricted due to their complications and difficult manageability (Kurosawa, 2007).

Recent development of novel bioreactors for large-scale production of ES-derived cells applying cell expansion, differentiation, or tissue formation on biomaterial scaffolds offer a tight control of the *in vitro* environment, including the exchange of nutrients, oxygen, and metabolites (Rungarunlert et al., 2009). Stirred suspension cultures (*e.g.*, spinner flasks) have disadvantages due to the generation of shear forces, which may damage the EB cells (Chisti, 2001; He et al., 2012). Another type of bioreactor, the slow-turning lateral vessel (STLV), is characterized by EB spatial immobility with an extremely low fluid shear stress and oxygenation by diffusion. The STLV has been described to improve efficiency of EB formation and stem cell differentiation into the cells of the three germ layers. The STLV bioreactor produces EBs that are uniform in size, without necrotic centers, and with higher yields (a nearly four-fold increase in the number of EB particles in comparison to SSC with human cells) (Gerecht-Nir et al., 2004). To date, the influence of the dynamic culture parameters (*i.e.*, time of EB adherence to gelatin-coated dishes, cell seeding density, etc.) has been reported to affect cardiac differentiation from ESCs (Rungarunlert et al., 2011). However, there is inadequate information concerning the histological characteristics of EBs, which could vary due to differences in cell seeding density and day of culture formed when using the STLV. Regardless of these advances, there are still some challenges that must be addressed before this technique could be considered safe, such as improved culture conditions to obtain specific cells, including cardiomyocytes.

Moreover, many researchers have found that cytokines, growth factors, and some synthetic chemical compounds can improve cardiomyogenic differentiation rates *in vitro*. Synthetic chemicals have a particular advantage, due to longer active half-life within solutions. Hence, these chemicals are stable in *in vitro* cell culture over several days, or even weeks (Heng et al., 2004). Ascorbic acid (ASC), one such synthetic chemical compound (vitamin C), has been described to promote ESC cardiomyogenic differentiation. In ESCs, increased expression of cardiac genes, including GATA-binding protein 4 (GATA4), α-myosin heavy chain (MHC), and β-MHC in a developmentally controlled manner have been reported when using ASC (Takahashi et al., 2003).

To develop a larger-scale culture of ES-derived cells for cardiac differentiation, we used a bioprocess that directs EB formation in a scalable, fully controlled STLV following inoculation with a mouse ES single-cell suspension. EBs generated by use of the optimized STLV bioreactor were compared to SSC and HD methods for efficiency in generating EBs and subsequent cardiac differentiation. Moreover, we investigated the effect of ASC supplement on cardiomyocyte differentiation. The STLV method was found to create a more homogeneous and uniform EB population that is favorable for subsequent cardiomyocyte formation, compared with "traditional" SSC and HD methods.

Materials And Methods

Materials and general culture

Culture reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA), and all chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise specified. Cell cultured was performed at 37°C in a humidified atmosphere containing 5% CO_2. Medium was changed daily on mouse ESC cultures and every 2 days during differentiation.

Mouse ESCs and culture conditions

Parental ESCs (*Hprt*-deficient mouse ESC line HM1; 129Sv/Ola genetic background) (Magin et al., 1992) were cultured on mitomycin C–inactivated confluent mouse embryonic fibroblast (MEF) feeder layers, which were obtained from 13.5-day postcoitus mouse embryos (Nagy et al., 2006a,b). These cells were maintained in ES medium consisting of Dulbecco's modified Eagle medium (DMEM-Glutamax-I), 15% (vol/vol) fetal bovine serum (FBS; Sera Laboratories International, West Sussex, UK) supplemented with 1000 U/mL mouse leukemia inhibitory factor (LIF; ESGRO, Chemicon International), 0.1 mM β-mercaptoethanol (β-ME), 0.1 mM nonessential amino acids (NEAA), and, 50 μg/mL streptomycin, 50 U/mL penicillin. ESCs were cultured on feeder layers for at least two passages after thawing and prior to the differentiation procedure, and the cells were cultured for at least one passage in feeder-free conditions on gelatin-coated plates with LIF (2,000 U/ml) in the ES medium. Cells were passaged every 1–2 days prior to reaching 70% confluency.

EB formation

Three different methods were used for EB formation: STLV bioreactor (RCCS-4H with 110 mL of vessels, Synthecon, Cellon S.A. Bereldange, Luxembourg), the SSC method, and the HD method. For each method, the ESCs were dissociated with 0.05% trypsin–EDTA solution to form a single-cell suspension.

In the first study, we have evaluated the initial cell seeding density and time in culture effects on morphology and size of EBs in STLV and HD culture systems and on their subsequent differentiation into cardiomyocytes (Fig. 1). Two different cell amounts were used in STLV method: 3×10^5 and 5×10^5 cells/mL (STLV1 and STLV2, respectively) in 110 mL of differentiation medium. The rotation speed was 10 rotations per minute (rpm). For the HD method, 800 cells in 20 μL of differentiation medium (ES culturing medium without LIF) were pipetted on the inside surface of a bacterial Petri dish lid. To prevent drying of the cell droplets, the Petri dish bottom was filled with phosphate-buffered saline (PBS). The cells were forced to aggregate with the help of gravity by reversing the Petri dish lid. Two days later, the EBs were transferred onto poly(2-hydroxyethyl methacrylate) (poly-HEMA)–treated 100-mm bacteriological Petri dishes.

In the second study, the effects of different culture systems (STLV, SSC, and HD) on morphology and size of EBs and their subsequent differentiation into cardiomyocytes were evaluated (Fig. 2). The STLV and SSC cultures were started with an equal quantity of cells (the optimized cell seeding density derived from the first study; 3×10^5 cells/mL) and HD-derived EBs were formed using 800 cells per drop (4×10^4 cells/mL) (Fig. 2). For the SSC, 55 mL of cell suspension (cell seeding density, 3×10^5 cells/mL) was cultured on a poly-HEMA–treated 150-mm bacteriological Petri dish (351058, BD Falcon, California, USA). All EBs were kept for up to 3 days in the same culture without interruption, except for the sample collection.

In vitro *cardiac differentiation assay*

Individual EBs were placed in differentiation medium into wells of a 24-well plate containing 0.1% gelatin-coated coverslips at different time points (day 2 to day 5; D2–D5). The EBs were then cultured further, with a medium change every second day, for a period of 14 or 21 days. Beating activities were recorded daily under a phase-contrast microscope.

Morphometric analysis

To assess the efficiency of the EB formation, 10 mL of STLV and 5 mL of SSC culture medium were passed through a 100-μm cell strainer to separate individual cells from cell aggregates constituting EBs. The EBs were then transferred into Petri dishes and observed by inverted light microscopy. To investigate the total EB number for each group, the EBs present in 1 mL of STLV and SSC culture medium were counted as the yield of EB production/mL. The individual cells that passed through the cell strainer were counted as the total free cells/mL. After that, the EBs were dissociated with a 0.25% trypsin–EDTA solution to form a suspension of single cells and counted as the total cells of EBs/mL. The

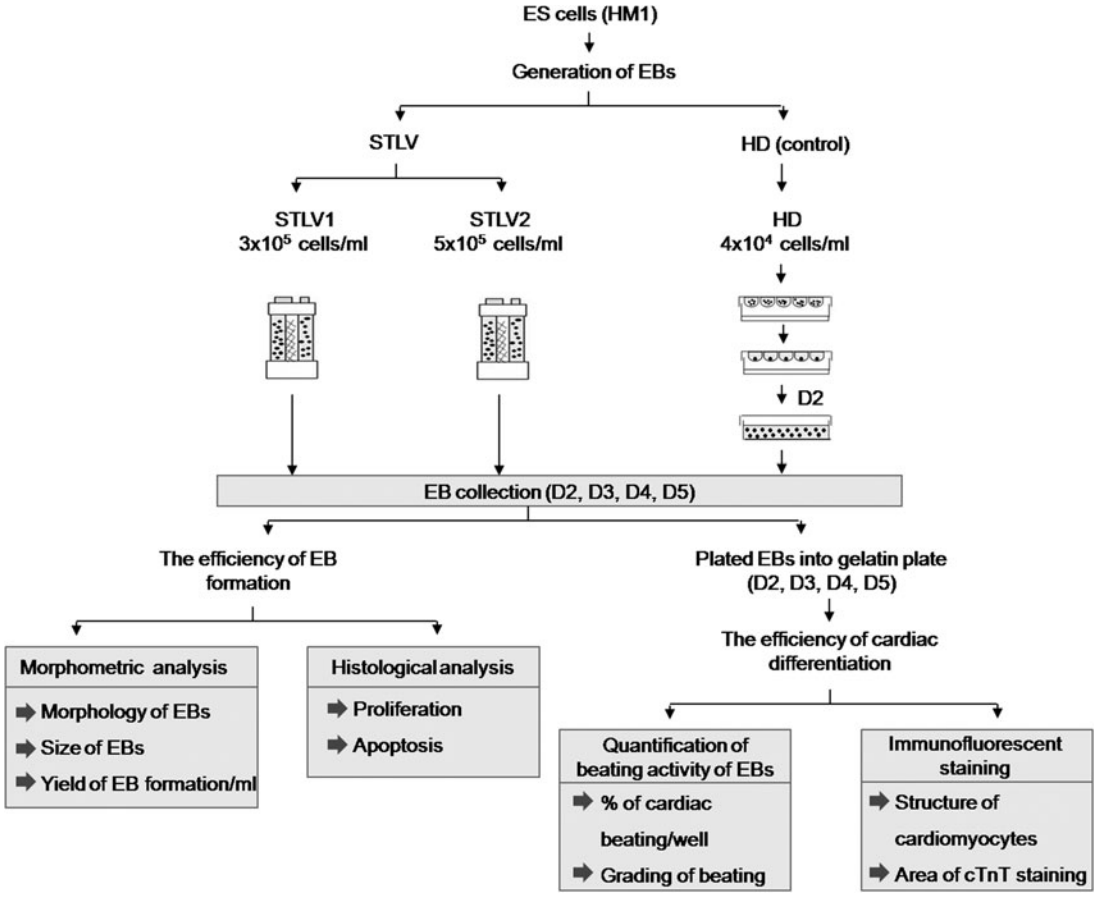

FIG. 1. Schematic presentation of *in vitro* differentiation procedure of ESCs into cardiomyocytes in STLV and HD culture systems using ESCs derived from different initial cell seeding densities: 3×10^5 and 5×10^5 cells/mL (STLV1 and STLV2, respectively) and time in culture (days 2–5).

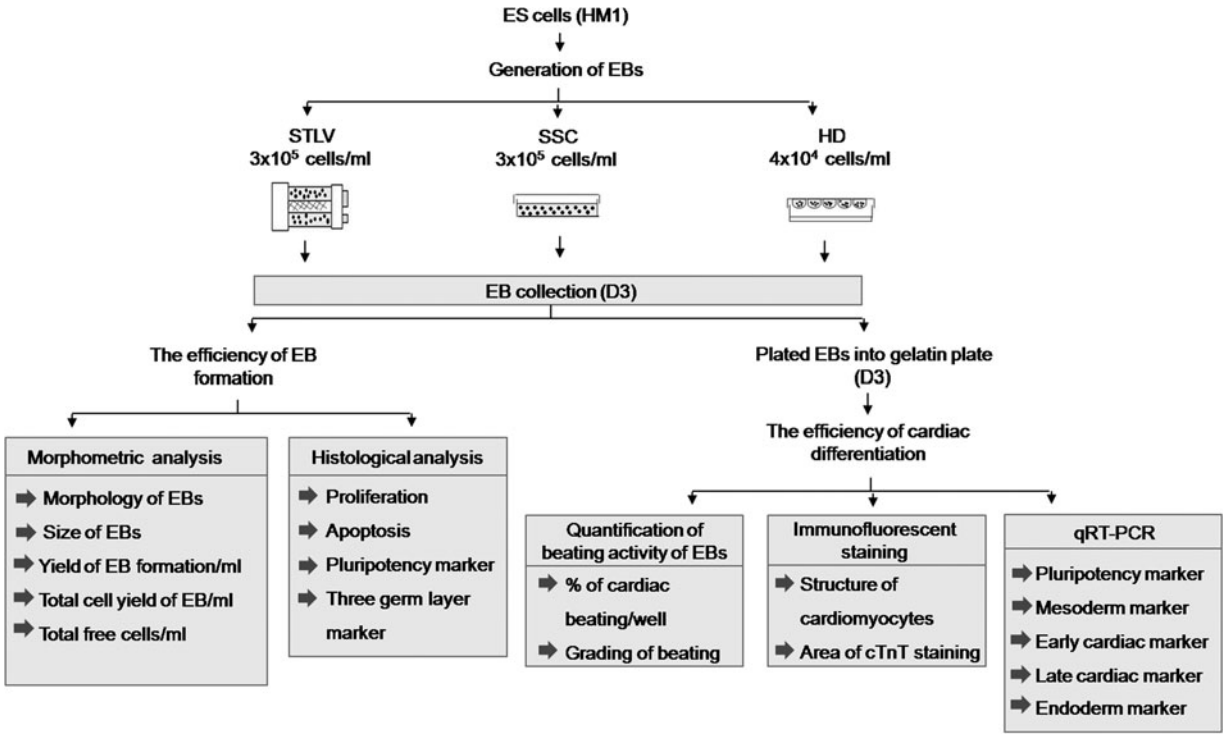

FIG. 2. Schematic presentation of *in vitro* differentiation procedure of ESCs into cardiomyocytes in different culture systems (STLV, SSC, and HD culture).

average diameter of EBs was calculated by measuring both the small and large diameters of 40 representative EBs per each replicates. The results represent the mean ± standard error of the mean (SEM) of triplicate independent experiments. An Olympus IX 71 inverted microscope with an Olympus DP70 digital camera (Olympus GmbH, Germany) was used daily for phase-contrast imaging of EBs. ImageJ image analysis software (http://rsb.info.nih.gov/ij) was applied to measure the cross-sectional area of EBs.

Histological analyses

EBs were collected, washed three times in PBS, then fixed with 4% paraformaldehyde (PFA) for 15 min, embedded in paraffin, and examined for general histological analysis. Serial sections were generated and 4-μm-thick sections were Hematoxylin & Eosin (H&E) stained for morphology assessment, with the proliferation marker Ki-67 (RM-9106, diluted 1:200; Thermo Scientific, USA) and with the apoptosis marker cleaved caspase-3 (Asp 175, 9661, diluted 1:200; Cell Signaling Technology, USA). Furthermore, the sections were stained for Oct4 (pluripotency marker, sc9081, diluted 1:100; Santa Cruz Biotechnology, USA), Brachyury (mesoderm marker, ab20680, diluted 1:200; Abcam, UK), Pax6 (ectoderm marker, diluted 1:200; Developmental Studies Hybridoma Bank, Iowa City, IA, USA), and α-fetoprotein (AFP, endoderm marker, ab3969, diluted 1:100; Abcam, UK).

Quantification of beating activity of EBs

In the first experiment, the effect of the initial cell seeding density and day of EB transfer to gelatin on the efficiency of cardiac differentiation was investigated as shown in Figure 1. Individual EBs derived from each group—3×10^5 and 5×10^5 cells/mL (STLV1 and STLV2, respectively) and 4×10^4 cells/mL (HD) as control—were placed on D2, D3, D4, and D5 into individual wells of 24-well plates (BD Biosciences, Mississauga, Canada, http://www.bdbiosciences.ca) in 1 mL of spontaneous differentiation medium. In the second study, the effect of EB culture conditions (STLV, SSC, and HD) and ASC supplement on the efficiency of cardiac differentiation was investigated as shown in Figure 2. Individual EBs derived from each group were placed on D3 into wells of a 24-well plates in 1 mL of spontaneous differentiation medium (without ASC supplement) or in 1 mL of induced differentiation medium (with ASC supplement). The EBs were then cultured further for 14 or 21 days. The beating activities were recorded daily with a Nikon TMS inverted light microscope (Nikon Instruments, Melville, NY, USA), and the quantity of EBs beating spontaneously was counted. Forty-eight EBs derived from each group and each replicate were observed, and the number of beating EBs was presented as the rate of the total plated EBs. The cardiac beatings were further evaluated by grading of cardiac beating (beating rate and the area of cardiac beating) as described earlier (Rungarunlert et al., 2011). The results are presented as the mean ± SEM of three independent experiments.

Immunofluorescent staining

After investigating the efficiency of cardiac beating the EBs were stained for cardiac troponin-T (cTnT) to measure the area of cTnT as described earlier (Rungarunlert et al., 2011). The selected EB samples were analyzed by a "double-blind" approach to avoid biases (eight EBs per one replication), and the experiments were completed in triplicates. EBs

were plated onto 0.1% gelatin-coated coverslips and fixed with 4% PFA for 15 min at room temperature, followed by permeabilization with 0.25% Triton-X100 for 10 min. After blocking with 1% bovine serum albumin (BSA) in PBS, the samples were incubated with the primary antibodies: Mouse monoclonal against cTnT (ab33589, diluted 1:200; Abcam, UK) and Oct4 (sc9081, diluted 1:100; Santa Cruz Biotechnology, USA) overnight at 4°C. Following washing, the samples were labeled with AlexaFluor 594 goat anti-mouse immunoglobulin G (IgG)-conjugated secondary antibody (A11005, diluted 1:2000; Invitrogen Life Technologies, USA) and AlexaFluor 488 goat anti-rabbit IgG (A11008, diluted 1:2000; Invitrogen Life Technologies, USA) at room temperature for 60 min. Vectashield mounting medium including 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories) was used to counterstain the nuclei. EBs were imaged with a Carl Zeiss Microscope (Carl Zeiss MicroImaging GmbH, Germany), and the area of cTnT was measured using Digital Image Processing Software (AxioVision4.8.1, Carl Zeiss MicroImaging GmbH, Germany).

RT-PCR and quantitative real-time PCR (qRT-PCR) analysis

Following the manufacturer's protocol, total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA). For each sample, RNA was reverse transcribed using the SuperScript III Reverse Transcriptase (Invitrogen Life Technologies, USA), using oligo $(dT)_{12–18}$ primer and 10 mM PCR-grade dNTP mixture (Invitrogen). For the quantitative PCR (qPCR) reaction, SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) was used, and samples were run on a Qiagen Rotor-Gene® Q controlled by Rotor-Gene Q Software. PCR reactions were performed using 5× Green GoTaq Flexi Buffer, $MgCl_2$ solution, GoTaq Pol (all from Promega, Madison, WI, USA), and dNTP Mix, PCR Grade (Invitrogen). *Gapdh* was applied as reference gene in the study. The primers and reaction conditions for the PCR reactions are presented in Table 1. The mean ± SEM results of three independent experiments are presented.

Statistical analysis

The data from at least three independently performed experiments are presented as mean ± SEM. SPSS Statistics version 18.0 was used for all statistical analyses. One-way analysis of variance (ANOVA) was used to compare the size of the EBs, the percentage of EBs beating, the grading of EB beating, and the area of cTnT between groups. The yield of EB production/mL, the total cells of EBs/mL, and the total free cells/mL between the two groups was analyzed by individual Student *t*-tests. Values with $p < 0.05$ were considered statistically significant.

Results

Effect of initial cell seeding density and time in culture, on morphology, and size of EBs in STLV and HD culture systems

In the current study, STLV cultures were initiated using two different numbers of cells (3×10^5 and 5×10^5 cells/mL), and EBs derived from HD (HD-EBs) were formed using 800 cells per drop (4×10^4 cells/mL) (Fig. 1). We have previously determined that 3×10^5 (STLV1) or 5×10^5 (STLV2) cells/mL initial seeding density and 10 rpm gives the optimal EB number and yield in STLV conditions (Rungarunlert et al., 2011). By D2, cultures formed spherical EBs with a regular shape and uniform size. EB size increased over time and showed similar gross morphology (458.1 ± 6.89, 510.3 ± 7.27, 513.8 ± 7.48, and 580.3 ± 8.49 μm diameter from STLV1 and 462.8 ± 7.07, 511.6 ± 8.69, 535.6 ± 10.51, and 547.8 ± 9.70 μm diameter from STLV2 for D2, D3, D4, and D5 respectively (Fig. 3A, B). HD-EBs grew modestly on D2 (299.2 ± 4.94 μm) and D3 (377.6 ± 5.58 μm); however, their growth accelerated by D4 (510.4 ± 10.49 μm) and D5 (624.9 ± 11.97 μm). Furthermore, HD-EBs, which appeared homogeneous in size on D2 and D3,

Table 1. Properties of the Oligonucleotide Primers Used in qRT-PCR Reactions

Primer	*Size (bp)*	*Sequence*
Oct-4	110	(Forward) 5′-AGCCGACAACAATGAGAACC-3′ (Reverse) 5′-TCTCCAGACTCCACCTCACA-3′
Brachyury	161	(Forward) 5′-TACACCTCTAATGTCCTCCCTTG-3′ (Reverse) 5′-CCATACAGTTGACTTCCCAACAC-3′
Nkx2.5	74	(Forward) 5′-ACACCCACGCCTTTCTCAGT-3′ (Reverse) 5′-AGGTCCCCAGACGCCA-3′
Tnnt2	117	(Forward) 5′-CGGATCAATGACAACCAGA-3′ (Reverse) 5′-GGGCAAGGACACAAGCAG-3′
Myh6	80	(Forward) 5′-CTACGCCTTCGTCTCTCAGG-3′ (Reverse) 5′-AGGCACTATCAGTGGCCAAG-3′
Myl2	90	(Forward) 5′- TGACCTAAGGGACACATTTGC-3′ (Reverse) 5′-ATTGGACCTGGAGCCTCTTT-3′
α-fetoprotein	129	(Forward) 5′-AACAAGGAGGAGTGCTTCCA-3′ (Reverse) 5′-GGTTGTTGCCTGGAGGTTT-3′
GAPDH	79	(Forward) 5′-AATGTGTCCGTCGTGGATCT-3′ (Reverse) 5′-CCTGCTTCACCACCTTCTTG-3′

Nkx2.5, NK2 transcription factor related, locus 5; Tnnt2, troponin T type 2 (cardiac); Myh6, myosin, heavy chain 6, cardiac muscle, alpha; Mly2, myosin regulatory light chain 2, ventricular/cardiac muscle isoform.

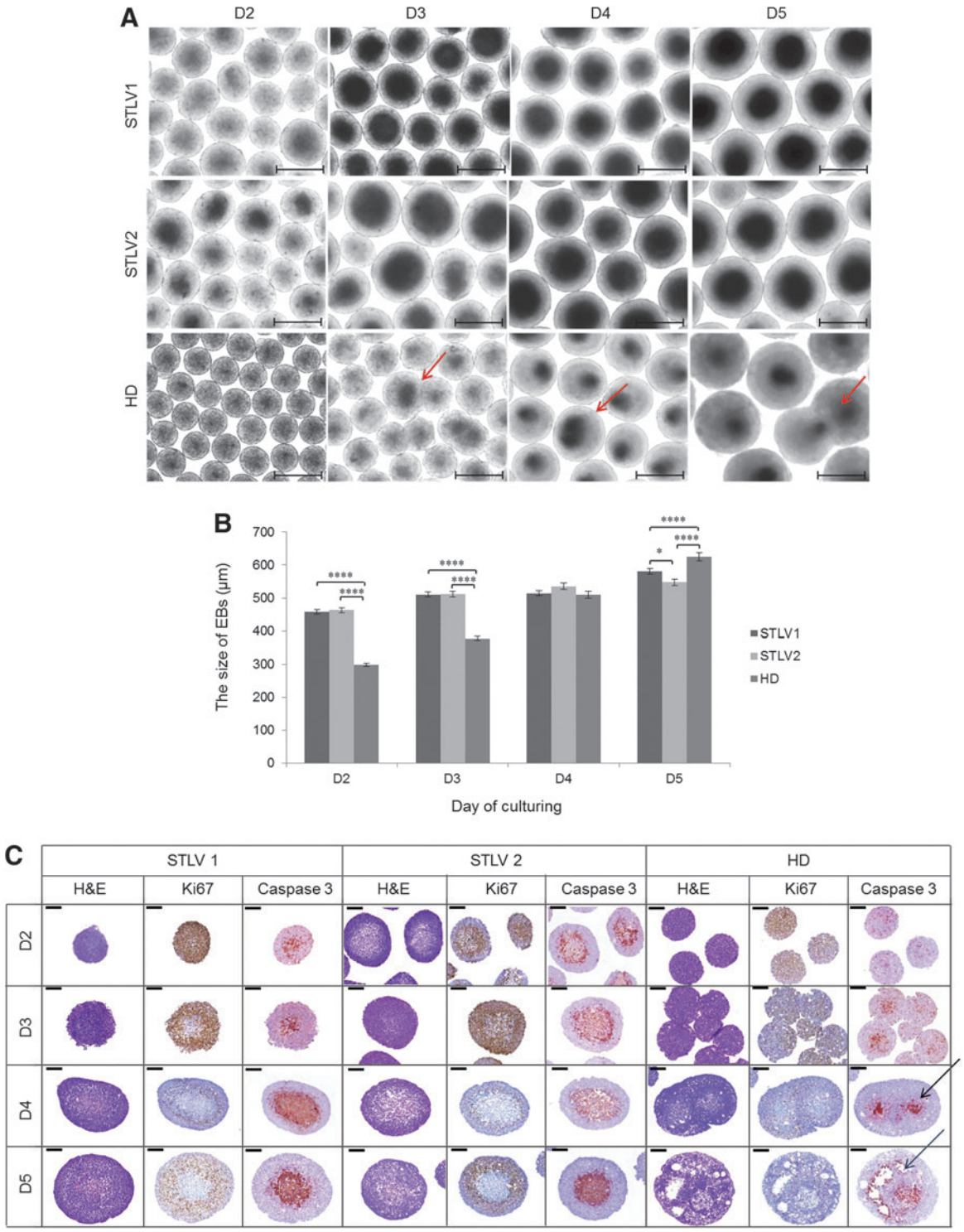

FIG. 3. Dynamic formation of EBs derived from different cell seeding densities and time in culture in both the STLV and HD culture systems. (**A**) Gross morphology of EBs. (**B**) Size of EBs. (**C**) Histological section of EB formation (H&E, Ki-67, and Caspase 3). Data presented as mean ± SEM of three independent experiments. The difference was considered significant when $p < 0.0001$ (****). Scale bars represent (**A**) 500 μm and (**C**) 100 μm. Color images available online at www.liebertpub.com/cell

became more heterogeneous at later time points (by D4 and D5). This resulted in extensive EB agglomeration, which led to the formation of large EBs (Fig. 3A; red arrows) between D3 and D5. The STLV in contrast, produced homogeneous-sized EBs, even on D4 and D5, at both cell seeding densities.

Effect of initial cell seeding density and length of culture period on proliferation and apoptosis in the STLV and HD culture systems

To elucidate the macroscopic structure of EBs derived from different initial cell seeding densities and length of

culture period in both the STLV and HD methods, cell morphology, proliferation, and apoptosis assays were performed. At D2, STLV1-EBs and STLV2-EBs were larger and more irregular than HD-EBs. By D3, D4, and D5, EBs from all groups were larger in both size and density (black spot appeared in the EB centers). However, HD-EBs began to agglomerate after D2 (observed as spotted areas within the EB center) (Fig. 3A, red arrow, and Fig. 3C, black arrow). In contrast, both STLV-EBs (STLV1-EBs and STLV2-EBs) did not appear to agglomerate. By D4, HD-EBs began to display cystic structures and by D5, at least one cyst have been observed in more than 50% of HD-EBs (Fig. 3C, blue arrow).

Ki67 was used to stain proliferating cells in EBs during differentiation. Higher levels of proliferating cells were

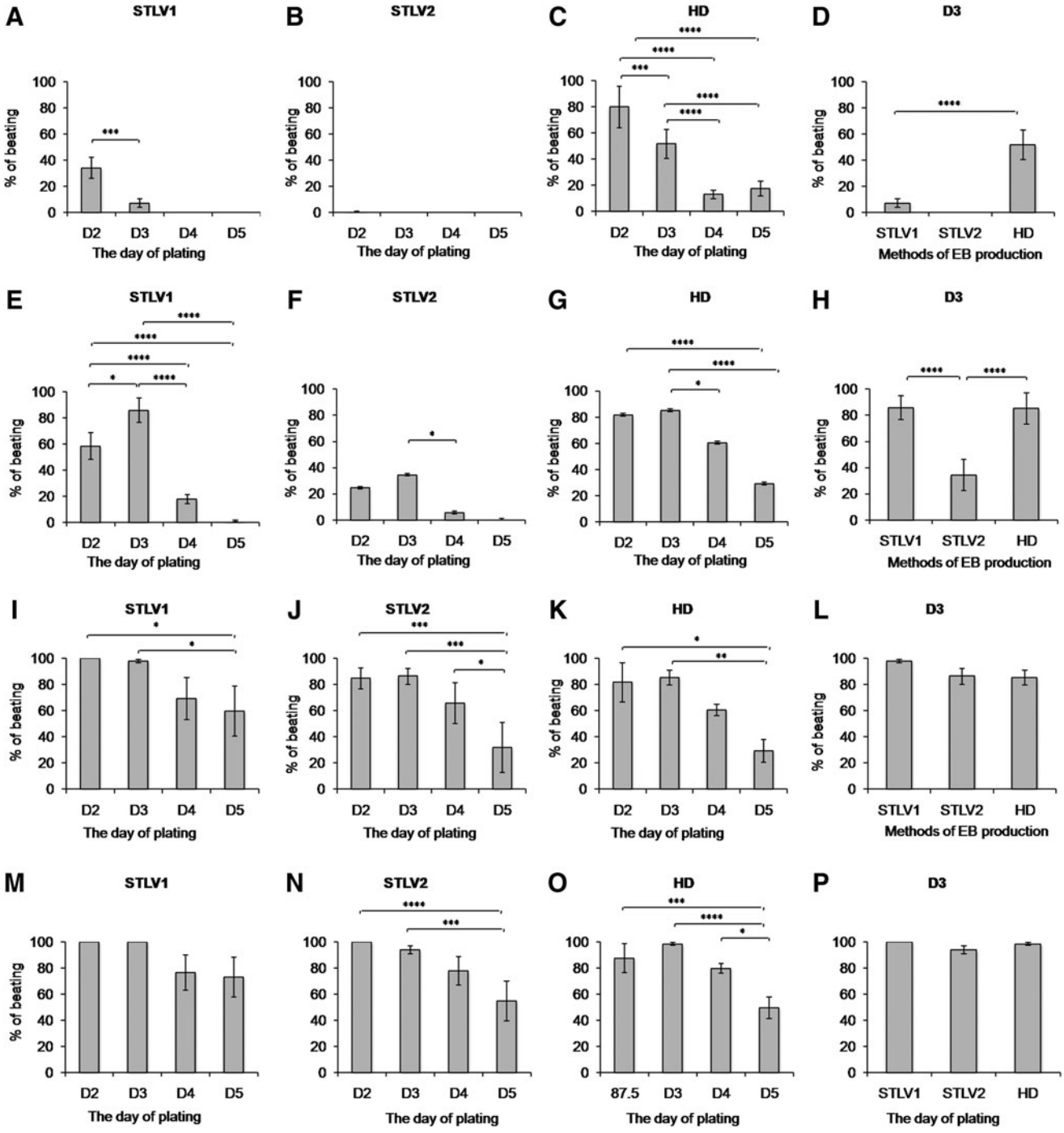

FIG. 4. Cardiac differentiation potential of EBs derived from different cell seeding densities and time of EB plating, on cardiac differentiation using the STLV and HD culture method. (**A–C**) Percentage of contracting cardiomyocytes in outgrowth of attached EBs: observed on D7 (**A–D**), observed on D8 (**E–H**), observed on D9 (**I–L**). Observed on D10 (**M–P**). Data presented as mean ± SEM of three independent experiments. The difference was considered significant when $p < 0.05$ (*), <0.01 (**), <0.005 (***), <0.0001 (****).

detected at D2 and D3 (Fig. 3C). Proliferation was less extensive at D4 and D5, and no differences were noted between the different cell seeding densities of the STLV-derived EBs (Fig. 3C). Slightly higher levels of positive Ki67 cells were found in the STLV-EBs compared to the HD-EBs, regardless of the day of culturing time. Importantly, a clear outer layer of proliferating cells became obvious in STLV-EBs from D4.

Cleaved caspase-3 staining was performed to detect apoptotic cells and revealed slight differences between STLV1 and STLV2 groups at the same time points (Fig. 3C). STLV2 started to form dense cores of apoptotic cells within EBs earlier than STLV1. In addition, a dense inner core of apoptotic cells was visible within STLV-EBs after long-term culturing (D4 and D5) (Fig. 3C). In HD-EBs sparser staining of cleaved caspase-3 was observed at D2 and D3, constituting less of the total EB area, but small, dense multiple cores of apoptotic cells were visible at D4 and D5. These data indicate that long-term culturing of EBs appears to reduce cell viability in all groups, likely due to a higher number of cells with programmed cell death.

By comparing serial sections of corresponding STLV-EBs, a proliferating outer and an apoptotic inner layer (presumably forming a cavity at a later time point) was clearly visible. On HD-EB sections, a clear outer and inner layer was absent.

Cell seeding density and day of EB transfer to gelatin influencing the efficiency of cardiac differentiation

Both in STLV and HD groups, an *in vitro* cardiac differentiation assay was used to compare EBs produced by the two different cell seeding densities and the day of transferring EBs onto gelatin plate. STLV1-EBs plated on D2 and D3 and STLV 2-EBs plated on D2 started to form rhythmically contracting areas earlier (on D7) (Fig. 4A, B) than the other groups, which started to form rhythmically contracting areas only on D8 (Fig. 4E, F). All STLV1-EBs plated on D2 and D3 were beating by days 9 and 10, respectively (100%) (Fig. 4I, M). STLV2-EBs plated on D2 were all beating by day 10 (100%) (Fig. 4N), and those plated on D3 and D4 were all beating slightly later, *i.e.*, day 14 (Fig. S1B) (Supplementary Data are available at www.liebertpub.com/cell/). When comparing the percentage of beating from different cell seeding densities, STLV1-EBs performed significantly higher than the STLV2-EBs on day 8 of cardiac differentiation (STLV1, D2–D5, 58 ± 10.1, $86 \pm 9.2\%$, $18 \pm 3.5\%$, and $1 \pm 0.7\%$ vs. STLV2, D2–D5, $25 \pm 5.5\%$, $35 \pm 12\%$, $6 \pm 2.3\%$, and 0%) as shown in Figure 4E, F, and H. In addition, EBs plated on earlier days (D2 and D3) performed significantly higher than EBs plated at later time points (D4 and D5) in both culture systems (STLVs and HD) (Fig. 4A–C, E–G, I–K). Even though, HD-EBs showed earlier spontaneous beating than the STLV-EBs (on D6) (data not shown), at later time points, not all EBs were beating (Fig. 4C, G, K, and O and Fig. S1C). Together, these results suggested that the STLV-1 EBs transferred and plated on D2 and D3 were likely the most optimal scenario for *in vitro* cardiac differentiation.

Although, all groups were capable of committing to cardiomyogenesis, the area and the frequency of beating differed. Therefore, the beating was graded and evaluated (Fig. S1E; Rungarunlert et al., 2011). EBs plated on D2 and D3 showed the fastest beating over their entire surface area (grade 4). When comparing the two different cell seeding densities, STLV1-EBs plated on D2 and D3 beat across the entire area and faster (grade 4) (even more than in the STLV-2 EBs). Furthermore, HD-EBs plated on D2–D5 exhibited a similar grading pattern as the STLV-EBs.

Finally, the areas of cTnT were measured (Fig. 5A–E) to estimate the efficacy of cardiac differentiation. STLV1-EBs produced significantly more cTnT than STLV2-EBs during *in vitro* cardiac differentiation, when STLV1-EBs and STLV2-EBs were plated on D2 and D3 (Fig. 5A, B). In addition, cTnT was expressed more centrally in the STLV1-EBs and at the edges of attached EBs and formed a filamentous network (Fig. 5F). When comparing the day of transfer of EBs onto gelatin, STLV1-EBs plated on D3 expressed significantly more cTnT than those plated on D2, D4, and D5 (Fig. 5E). EBs plated on D4 and D5 showed a more central cTnT distribution and rarely at the edges of the EBs with no filamentous connections. Additionally, STLV-EBs formed particularly more filamentous cTnT networks than the HD-EBs, which expressed cTnT mostly in the centers with no filamentous connections (Fig. 5F). A summary of these results reveals that EBs derived from STLV cultures and plated on D3 onto gelatin-coated dishes gave a higher percentage of contracting area per EB, a higher grading of beating, and higher levels of cardiac cTnT expression than the conventional HD-produced EBs. Also, the percentage of beating areas in EBs plated on D2 and D3 were higher then in those plated only on D4 or D5. Overall, the best condition is plating EBs on D3 or earlier. This observation shows the critical importance of optimizing the day of plating of the EBs onto gelatinized dishes. The following experiments were designed on the basis of the results of this initial study, indicating that STLV1 conditions are appropriate for producing more EBs, with a large and more homogeneous size and uniform shape.

Efficiency of EB formation, morphology, and size using different culture systems

To examine the effect of different culture conditions on EB development, three culture systems, including, STLV, SSC, and HD were compared. HD-derived EBs were generated using 800 cells per drop (4×10^4 cells/mL) whereas the SSC and STLV systems were both initiated with 3×10^5 cells/mL (Fig. 2). All EBs were kept for a period of maximum 3 days in the same culture without interruption, except for the sample collection. Cell aggregates started to form 1 day earlier in the SSC cultures compared to the STLV culture at 36 h. However, agglomeration of SSC-EBs also appeared on the second day (at 48 h), which was not observed in the STLV-EBs (Fig. 6A). SSC-EBs formed less defined cell aggregates, which were irregular in shape. EBs started to form at 24 h in the HD; however, three to four EBs formed in each drop. One EB per one drop appeared only at 48 h. As expected, on the basis of gross morphology, D3 STLV-EBs and HD-EBs exhibited the most homogeneous appearance: EBs were uniform, rounded, symmetric, and only a few cell agglomerations were observed (Fig. 6A). On D3, EBs had an average diameter of $518.1 \pm 10.2\,\mu$m for the STLV-EBs, and the HD-EBs were slightly smaller, with an average size of $405 \pm 5.2\,\mu$m. In contrast, SSC-EBs were heterogeneous and formed dumbbell shapes with an average small diameter of $443.2 \pm 19.4\,\mu$m and average large diameter of 607.9 ± 32.7 (Fig. 6A). On the basis of these findings, we could conclude that the STLV culture system was more effective in producing homogeneous EBs than the SSC culture system. Even though the HD culture

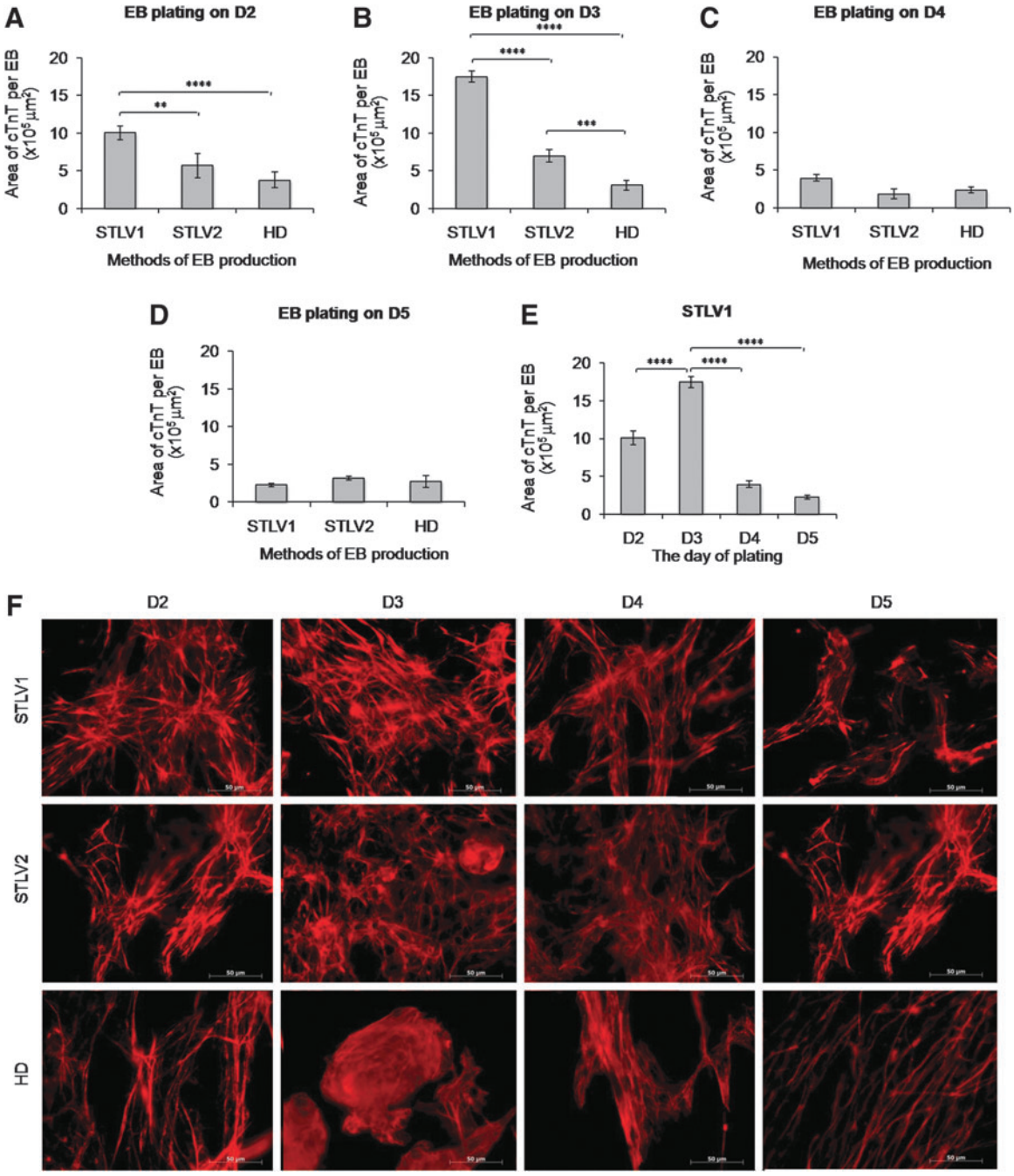

FIG. 5. Cardiac differentiation potential of EBs derived from different cell seeding densities and time of EB plating, on cardiac differentiation in both the STLV and HD culture method. (**A–E**) Area of cTnT per EBs ($\times 10^5\,\mu m^2$). (**F**) EBs immunolabeled with cTnT (red). Data are presented as mean ± SEM of three independent experiments. The difference was considered significant when $p < 0.01$ (**), < 0.005 (***), < 0.0001 (****). Scale bars, 200 μm. Color images available online at www.liebertpub.com/cell

system could produce homogeneous EBs similar to that in the STLV system, the HD method remains insufficient for mass EB production.

A four-fold improvement of EB production using the STLV culture system was significantly observed in comparison to static conditions (174 ± 5.3 and 41 ± 10.4 EBs/mL, respectively) (Fig. S2A). Also, a six-fold enhancement in total cell yield of EB/mL was significantly achieved ($39 \pm 2.1 \times 10^5$ and $7 \pm 1.4 \times 10^5$ cells/mL, respectively) (Fig. S2B). Furthermore, a nearly 10-fold diminishment in total cell yield of free cells, which were not incorporated into EBs, was significantly observed compared to the SSC method ($6 \pm 1.1 \times 10^5$ and $55 \pm 6.4 \times 10^5$ cells/mL) (Fig. S2C). The average cell density of single EBs obtained in STLV and SSC was similar on D3 ($4.2 \times 10^4 \pm 0.98$ cells/EB and $4.2 \times 10^4 \pm 1.98$ cells/EB), whereas the average cell density of a single EB derived by

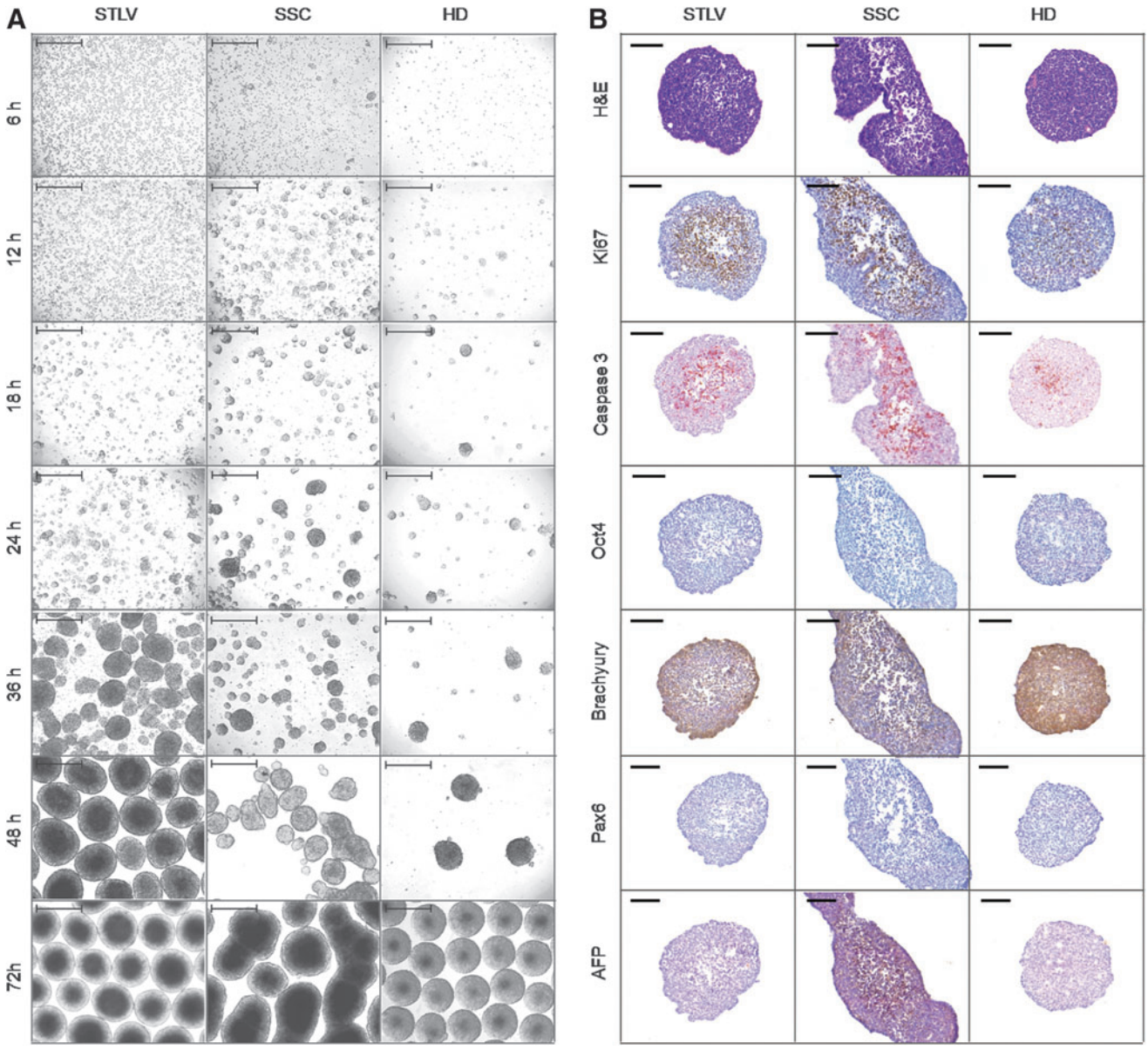

FIG. 6. Dynamic formation and the efficiency rate of EBs derived from different culture systems (STLV, SSC, and HD). (**A**) Gross morphology of EBs. (**B**) Histological section of EB formation (H&E, Ki-67, Caspase 3, Oct 4, Brachyury, Pax6, and AFP). Color images available online at www.liebertpub.com/cell

HD on D3 was smaller ($2.5\times10^4\pm0.7$ cells/EB). On the basis of these findings, our data indicated that the STLV system is efficient at producing a large scale of EBs, when compared to the SSC culture system.

Effect of EB culture conditions on proliferation, apoptosis, and pluripotency, including marker expression during differentiation

Because the histology and structure of EBs derived from different culture systems is not well known, we examined the EB formation process at daily intervals for up to 8 days on histological sections. Data indicated that the central apoptotic area was most extensive in the STLV cultures and that proliferation is localized to the outer layers of EBs, which surround the apoptotic inner core. Both Ki67 and cleaved caspase-3 staining of EB sections revealed that proliferation and apoptosis was less intense and more sparse in the core of the HD-EBs compared to the STLV- or SSC-derived EBs (Fig. 6B).

When assessing the pluripotency marker (Oct4), there was no difference in Oct4 staining between the different culture methods, which disappeared by D3. In the case of the germ layer marker, Brachyury, the STLV-EBs and HD-EBs showed high intense staining in comparison to the SSC-EBs. In the case of AFP, staining was abundant in the center of the SSC-EBs but remained sporadic in both the STLV-EBs and HD-EBs. Pax6 expression was only observed in a few cells in the core all of the EBs, which corresponds to the expected staining of D3 EBs, where only a few ectoderm cells are likely present (Fig. 6B).

Influence of EB culture conditions and ASC supplement on the efficiency of cardiac differentiation

The results from the cardiac differentiation studies are shown in Figure 7, A–F. HD-EBs formed a significantly higher percentage of rhythmically contracting areas compared to STLV-EBs and SSC-EBs on D7 (Fig. 7A). However, HD-EBs formed a similar percentage of rhythmically contracting areas compared to STLV-EBs on D8 (Fig. 7B). Moreover, HD-EBs did not reach 100% beating efficiency by D9–D17, unlike the STLV-EBs (Fig. 7C). Additionally, culturing EBs with ASC supplement improved the rate of cardiac differentiation (Fig. 7A, B). A grading system has been used to evaluate the cardiac beating based on the rate of the beating and the size of the beating area (Fig. 7F). STLV-EBs had larger beating areas, and the beating was fast (grade 4). The addition of ASC to aid in inducing cardiac differentiation also increased the beating areas and rates, regardless of the culture method used.

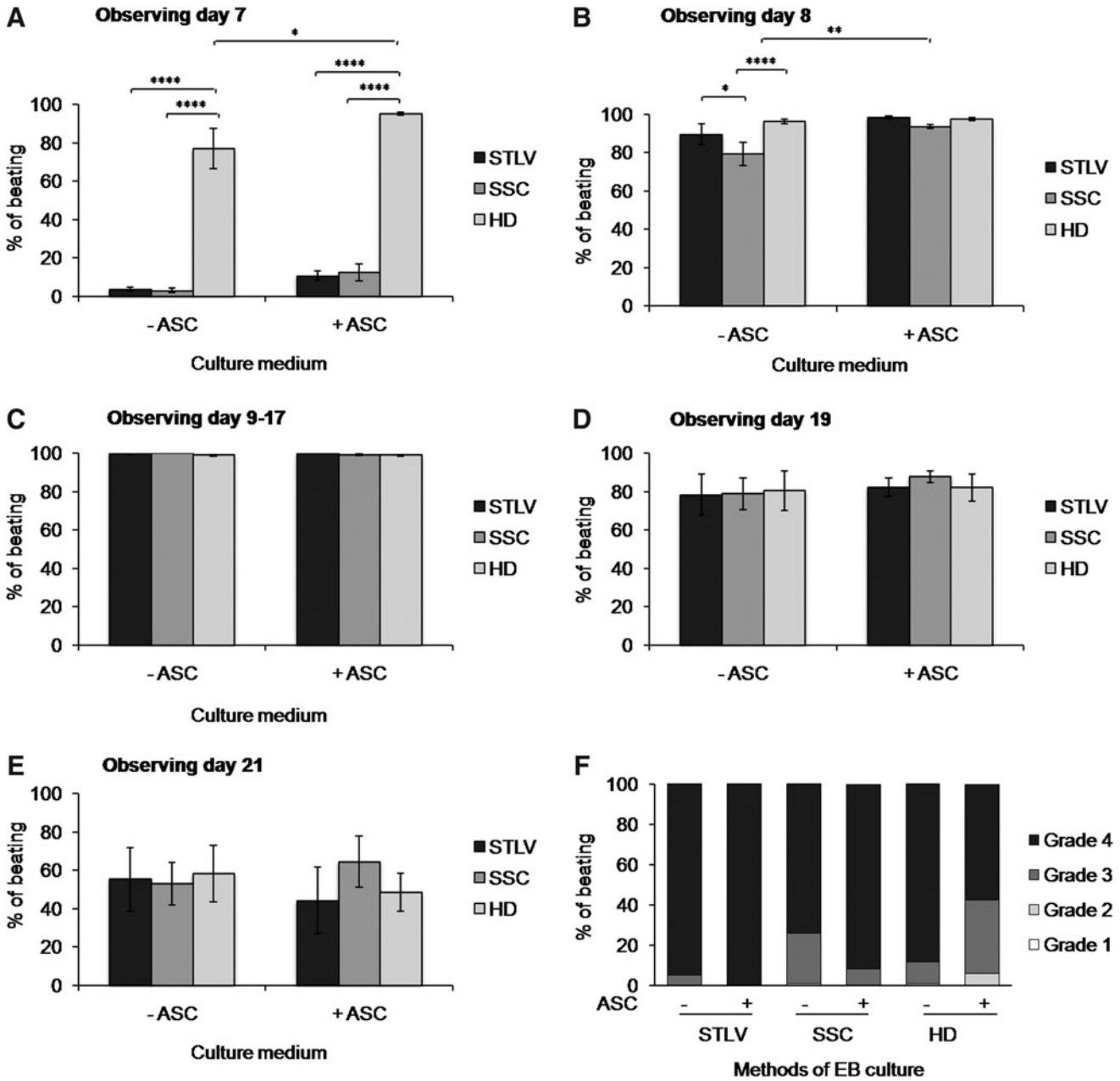

FIG. 7. Cardiac differentiation potential of EBs derived from different culture systems (STLV, SSC, and HD). (**A–E**) Percentage of contracting cardiomyocytes in outgrowths of attached EBs, when EBs were cultured in medium with or without ASC supplement. (**F**) Cardiac beating activity was evaluated by grading the area of beating foci and the beating rate. Data presented as mean ± SEM of three independent experiments. The difference was considered significant when $p < 0.05$ (*), < 0.01 (**), < 0.0001 (****).

To further evaluate the EB ability to form cardiac outgrowths amongst the different culture conditions, all EBs outgrowths were immunostained with the cardiac lineage commitment marker cTnT. Our results demonstrated that STLV-EBs were superior for producing larger areas of cTnT expression at day 14 (1.5–4.2 times higher than SSC-EBs and HD-EBs; Fig. 8A), regardless of the culture medium used. Moreover, STLV-EBs showed a higher distribution of filamentous cTnT within the central area and edges of the EBs compared to the other groups at the same time point (Fig. 8B). The expression of cTnT within SSC-EBs and HD-EBs was lower (Fig. 8B). The distribution of cTnT within the SSC-EBs and HD-EBs was predominantly in the center of the EB and rarely at the edges of the EB, with no filamentous connections observed (Fig. 8B). Furthermore, ASC supplementation increased the expression area of cTnT per EB in all groups (1.9 times in STLV, 3.7 times in SSC, and 2.3 times in HD group).

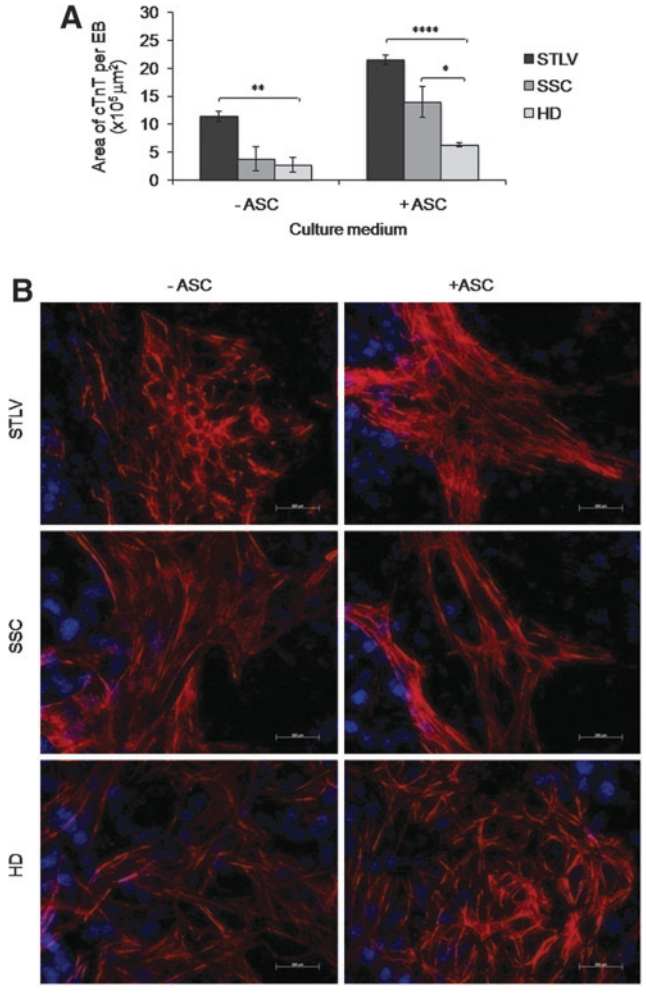

FIG. 8. Cardiac differentiation potential of EBs derived from different culture systems (STLV, SSC, and HD). (**A**) Area of cTnT per EBs ($\times 10^5$ μm^2). (**B**) EBs were immunolabeled with cTnT (red) and counterstained with DAPI (blue). Data are presented as mean ± SEM of three independent experiments. The difference was considered significant when $p < 0.05$ (*), < 0.01 (**), < 0.0001 (****). Scale bars, 200 μm. Color images available online at www.liebertpub.com/cell

Influence of EB culture conditions and ASC supplement on gene expression during cardiac differentiation

Finally, cardiac-specific gene expression of cardiac outgrowths was examined by qRT-PCR (Fig. 9) using RNA extracted from STLV-, SSC-, and HD-derived EBs at D3, D7, D14, and D21 of culture, supplemented with or without ASC. Gene expression of pluripotency (Oct-4; Fig. 9A), early mesoderm (Brachyury-T; Fig. 9B), early (Nkx2.5; Fig, 9C), and late (Tnnt2, Myh6, and Myl2; Fig. 7D–7F, respectively) cardiac markers were assayed. Moreover, AFP (which is an endoderm differentiation marker) was also investigated (Fig. 9G).

In undifferentiated cells, the level of Oct-4 was high then decreased to very low levels in all EBs by D7 (Fig. 9A). In parallel, upregulation of cardiac marker genes, namely, Nkx2.5, Tnnt2, Myh6, and Myl2 (Fig. 9C–F, respectively), has been observed. Expression of Brachyury was high at the beginning (D3–D7; Fig 9B) and showed a marked reduction later on, as expected. However, Brachyury mRNA was expressed only in HD-EBs, regardless of whether they were cultured with or without ASC. In the other groups, Brachyury levels declined gradually without any peak. This observation indicates that in the STLV-EBs and SSC-EBs all of the cells have been differentiating toward different lineages (*e.g.*, cardiac), and there was a lack of cell subpopulations to enter the primitive streak stage. The amount of Nkx2.5 transcripts (early cardiac precursor) was upregulated by D7 in all groups. STLV-EBs showed a higher expression of Nkx2.5 mRNA than the other EBs by D7 when cultured without ASC supplement and showed the highest expression of Nkx2.5 mRNA compared to the other EBs (15× induction), when cultured with ASC supplement. This indicated that STLV-EBs and ASC supplement induced cardiac differentiation by increasing Nkx2.5 mRNA (Fig. 9C).

As the cardiac differentiation progressed, a change toward the dominance of cardiomyocyte structural markers was observed, including upregulation of Tnnt2, Myh6, and Myl2 (Fig. 9D–F) peaking on D14 and with downregulation by D21 in all groups. The late cardiac marker Tnnt2 was, as expected, practically absent initially (D3) and strongly induced later. No statistical difference of Tnnt2 mRNA expression was observed between the groups (Fig. 9D). Even though no pattern of Myh6 and Myl2 mRNA expression was revealed between the different culture methods or different culture medium, STLV-EBs showed the highest expression of Myh6 mRNA compared to the other EBs, when cultured with ASC supplement. Interestingly, cultured HD-EBs preferred to differentiate into endodermal cells rather than cardiac cells, as AFP mRNA was highly expressed in HD-EBs (3–6 times vs. other groups and 3000–12,000 times vs. ESCs), when cultured with ASC supplement (Fig. 9G).

These data confirmed that STLV-derived EBs can differentiate efficiently toward terminal cardiac lineage commitment. Furthermore, our results strongly suggest that the culture condition method used affects the expression of many cardiac markers. ASC supplement also appears to improve both early and late cardiac muscle formation, especially when EBs are cultured in the STLV system.

Discussion

Differentiation of EBs mimics changes seen in embryonic development. Culture conditions and several factors that

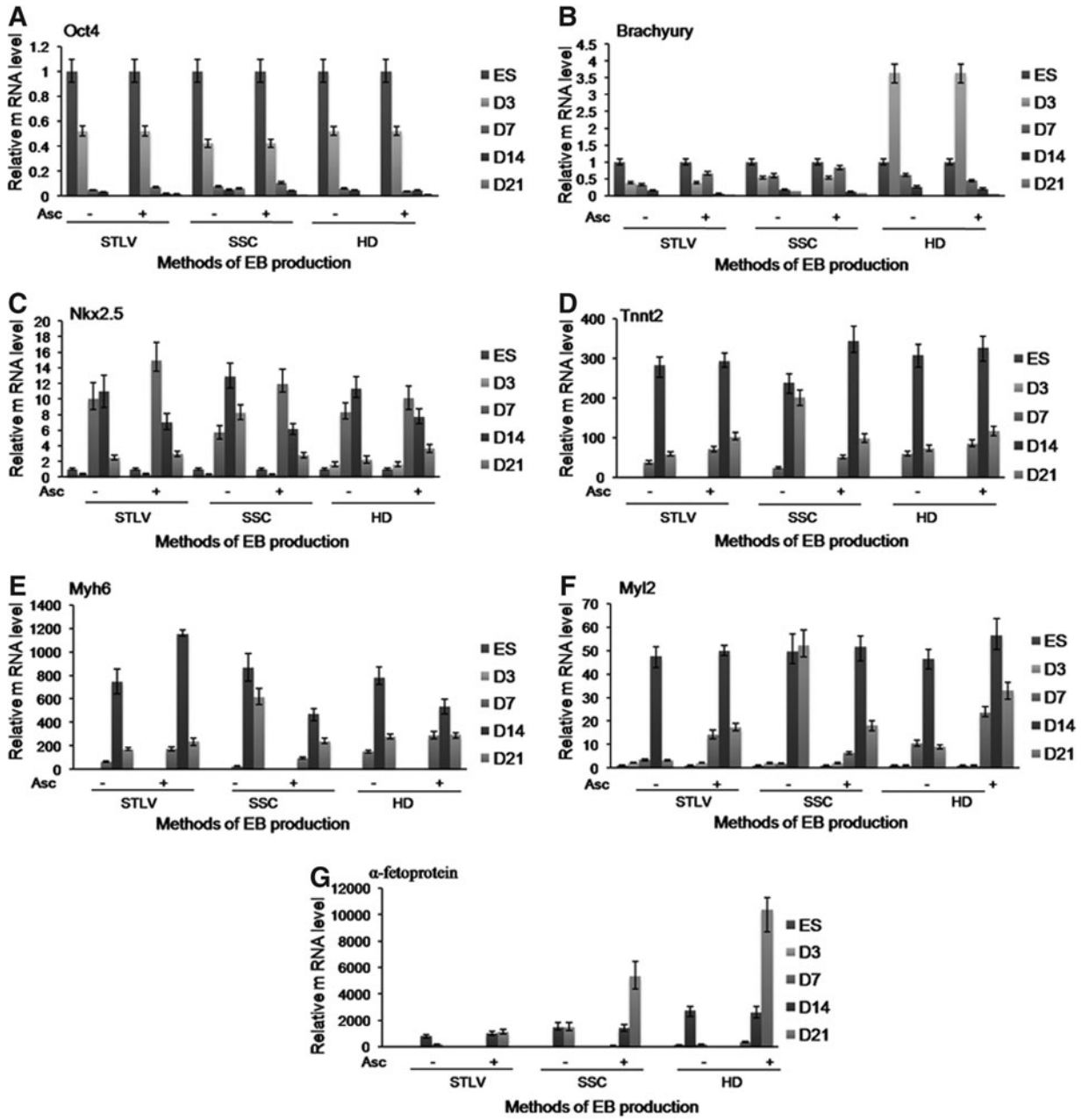

FIG. 9. qRT-PCR analysis of STLV-, SSC-, and HD-derived EBs cultured with or without ASC supplement. (**A**) Oct-4, the pluripotent marker. (**B**) Brachyury, mesodermal marker. (**C**) Nkx2.5, early cardiac marker. (**D–F**) Late cardiac markers Tnnt2 (**D**), Myh6 (**E**), and Myl2 (**F**). (**G**) AFP, endoderm marker.

partake necessary functions during early embryo development are also expected to be involved in the formation of EBs and cardiogenesis of EBs (Choi et al., 2005; Koike et al., 2007). Recently, versatile bioreactor cultures have been increasingly studied for stem cell expansion and differentiation; however, much work remains to optimize large-scale, lineage-specific differentiation for producing cardiac cells (Carpenedo et al., 2007; Rungarunlert et al., 2009). In the first experiment, we investigated the effect of varying dynamic culture parameters and their influence on cardiac differentiation of ESCs. To date, there is a limited amount of information that describes the development of specific cell lineages within EBs (Desbaillets et al., 2000). Also, the histological location of apoptosis or proliferation, which plays important roles in the early-stage embryonic development, has been rarely studied within EBs. Our studies extend knowledge about cardiac differentiation, especially in the context of histological changes within EBs, such as apoptosis and cell proliferation.

Previously, we have demonstrated that EB production, when seeding 3×10^5 and 5×10^5 cells/mL, is the most optimal for inoculating STLV cultures to grow ESC-derived EBs (Rungarunlert et al., 2011). Our initial experiment revealed that STLV culture produced more EBs, which was homogeneous in size compared to HD-EBs. In addition, STLV-EBs had a greater capacity to differentiate toward cardiac lineages than HD-EBs. For the first time, we describe the

morphology of EBs, visualized by H&E staining on sections. Co-labeling analysis for proliferation and apoptosis showed that the STLV culture resulted in more uniform EBs with similar inner and outer structures: (1) Highly proliferative outer zone (edge) and (2) apoptotic center (cavity). This mimics mouse embryo development at gastrulation stage when a cavity forms in the embryo. Thus, STLV-EB culture conditions are more superior in quality than HD-EB culture conditions.

Moreover, this is also the first report describing optimization of the time of STLV-EB adherence to gelatin-coated dishes for improving the efficiency of cardiomyocyte differentiation, similar to HD-EBs. We found that EBs plated earlier (D2 and D3) are more capable of differentiation into the cardiac lineage than EBs plated later (D4 and D5). This may pinpoint an important time point for plating EBs on adherent surfaces for even other alternate differentiation studies. Staining of cleaved caspase-3 within all EBs marked reduced cell viability and also a predisposition for cavitation, which resembles the blastocyst stage of embryo development (Fujita et al., 2008). When EBs were cultured long term, more cells underwent programmed cell death. Hence, earlier EB plating improved the efficiency of cells to spread out after EB plating and differentiate into cardiac cells compared to EBs plated later. Cavitation is the process in which cells undergo both selective cell survival and programmed cell death in a process that depends on signals from visceral endoderm in the EBs (Desbaillets et al., 2000; Karbanová and Mokry, 2002). Our results showed that HD-EBs started to form small cystic EBs on D4–D5, which preferentially differentiated into the endoderm lineage. This is in agreement with our observations of a low efficiency of HD-EBs to differentiate into cardiac cells, when plated later (D4 and D5). In standard cardiac differentiation protocols using HD culture, the initial cell seeding density is usually 400–1000 ESCs/mL, and the EBs are usually plated on gelatin-coated dishes on D5 (Chen et al., 2011; Kobolak et al., 2012; Mummery et al., 2002; Passier and Mummery, 2005), resulting a general low efficiency of cardiac differentiation. This may be due to the mistiming in plating of the EBs onto gelatin. Hence, our results strongly indicate that plating EBs earlier, on day 3, significantly improved the efficiency of cardiac differentiation, which contained high numbers of proliferating cells that preferentially formed cardiomyocytes.

In our second experiment, we found that the STLV culture system provided a stable and ideal condition for mouse ESCs to form high numbers of EB while retaining good quality and subsequently were highly efficient at differentiating into cardiomyocytes. EB formation using the STLV method was directly compared to the SSC and HD methods. Our results further showed that 3 days postseeding of mouse ESCs using the STLV culture system significantly improved the efficiency of homogeneous EB production in mouse ESCs (approximately four times higher than SSC culture), similar to Lü et al. (2008). However, variations in the size of STLV-EBs derived from human ESCs has been an unfavorable argument for use of this technique. Interruptions to the stirring and microgravity within the STLV, which can occur during changing of the medium, could be a reason for agglomeration of EBs and the formation of aggregates (Gerecht-Nir et al., 2004; Yirme et al., 2008). Our results, however, confirmed that STLV culture is reproducibly beneficial for mouse ESCs to generate high numbers of EBs, which retain quality as good as that compared to other cultures, because this culture provides: (1) Low fluid shear stress due to the horizontal whole-body rotation, (2) a chamber without gas/fluid interfaces and bubbles due to improved oxygen diffusion, (3) mild mixing of cells due to a very low rotation speed, and (4) efficient gas exchange due to the high membrane area to volume of medium ratio (Hammond and Hammond, 2001).

Furthermore, this is also the first report to describe that STLV-EBs have the highest efficiency of spontaneous cardiomyocyte differentiation over SSC-EBs and HD-EBs. We found that HD-EBs had rhythmically contracting areas within EBs 1 day earlier than STLV-EBs and SSC-EBs. However, the overall ability of HD-EB differentiation into cardiomyocytes was lower, due to lower expression and distribution of cTnT. The percentage of cardiac beating colonies/well was similar between the STLV-EBs and SSC-EBs. However, STLV-EBs showed a higher efficiency of cardiac differentiation due to a larger beating area and faster beating. Furthermore, the measurement of cTnT area per EB showed that STLV-derived EBs expressed cTnT three to five times higher than SSC- and HD-derived EBs, regardless of the culture medium. This correlates with a previous report, which described that approximately 30% of the nuclear transferred (NT)-EBs generated via STLV contained vigorously contracting cells compared to 10% of EBs generated via SSC (Lü et al., 2008).

To determine the effect of ASC on inducing cardiac differentiation, we investigated cardiac differentiation of mouse EBs via different culture methods (STLV, SSC, and HD). We initially induced the cells in the presence of ASC, followed by plating and culture in defined medium. Our results showed that ASC supplementation increased the area of cTnT expression per EB in all groups (1.9 times in STLV, 3.7 times in SSC, and 2.3 times in HD group). Our results also confirmed that ASC supplementation could induce cardiac differentiation of ESCs at a similar rate to that described by Takahashi et al. (2003). A previous report has also revealed that an approximately three-fold improvement of NT-EB beating/well could be generated via STLV when using ASC supplement (Lü et al., 2008).

The aim of our studies on EB formation and early EB differentiation was to observe the germ layer's organization timing and patterns. Differences in EB mRNA and protein expression patterns observed among different culture conditions have been correlated with cardiac differentiation potential. Our results showed a high level of Oct-4 in undifferentiated cells that had diminished by D7, in agreement with another report (Pekkanen-Mattila et al., 2010). Brachyury is upregulated in the primitive streak and induces early mesoderm formation (Pekkanen-Mattila et al., 2010); thus, is was not surprising that its mRNA level was high from D3 until D7 and diminished later. The cardiac differentiation assays detected a peak of Brachyury at D3–D4. In STLV-EBs or SSC-EBs, our data indicated a gradual decrease, without peaks, indicating the lack of cellular populations entering the primitive streak stage; rather cells gradually differentiated toward different directions, including cardiac. On the other hand, Brachyury reached its peak around D3 in HD-EBs, confirming observations described in earlier studies (Graichen et al., 2008; Pekkanen-Mattila et al., 2010).

In parallel, many of the genes upregulated in EBs were phenotypic markers of cardiac gene expression: Nkx2.5,

Tnnt2, Myh6, and Myl2. Nkx2.5 gene expression, which marks early cardiac precursors (Ranganayakulu et al., 1998; Schwartz and Olson, 1999), peaked at D7 and stayed high until D14. During mouse embryogenesis, Nkx2.5 is expressed abundantly when the heart forms and in standard *in vitro* differentiation assays peaks at D5–D8 (Akazawa and Komuro, 2005; Ma et al., 2008; Zhou et al., 2008). In our STLV groups, the continuously high expression levels of Nkx2.5 indicate the prolonged presence of cardiac precursor cells. Probably this is due to the beneficial effect of STLV conditions improving the viability of ESCs within the EBs. Interestingly, the expression of Nkx2.5 increased when ASC was added on D7. Hence, our results confirmed that ASC promotes cardiac differentiation by inducing Nkx2.5, as described by Cao et al. (2012). Gradually other cardiac markers, including those of cardiomyocyte structural constituents such as Tnnt2, Myh6, and Myl2, increased and peaked on D14. These observations are similar to previous reports (Boheler et al., 2002).

Conclusions

This study has shown that methods of EB production and the subsequent quality of EBs profoundly affected cardiogenesis. An optimized STLV system allowed a superior control of cellular agglomeration forming homogeneous sized EBs, improving further proliferation and finally cardiac differentiation of ES cells. Therefore, the STLV method could provide a positive impact on applications, including disease modeling, drug screening, and regenerative medicine.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

References

Akazawa, H., and Komuro, I. (2005). Cardiac transcription factor Csx/Nkx2-5: Its role in cardiac development and diseases. Pharmacol. Ther. 107, 252–268.

Barile, L., Cerisoli, F., Frati, G., Gaetani, R., Chimenti, I., Forte, E., Cassinelli, L., Spinardi, L., Altomare, C., Kizana, E., Giacomello, A., Messina, E., Ottolenghi, S., and Magli, M.C. (2011). Bone marrow-derived cells can acquire cardiac stem cells properties in damaged heart. J. Cell. Mol. Med. 15, 63–71.

Boheler, K.R., Czyz, J., Tweedie, D., Yang, H.T., Anisimov, S.V., and Wobus, A.M. (2002). Differentiation of pluripotent embryonic stem cells into cardiomyocytes. Circ. Res. 91, 189–201.

Cao, N., Liu, Z., Chen, Z., Wang, J., Chen, T., Zhao, X., Ma, Y., Qin, L., Kang, J., Wei, B., Wang, L., Jin, Y., and Yang, H.T. (2012). Ascorbic acid enhances the cardiac differentiation of induced pluripotent stem cells through promoting the proliferation of cardiac progenitor cells. Cell Res. 22, 219–236.

Carpenedo, R.L., Sargent, C.Y., and McDevitt, T.C. (2007). Rotary suspension culture enhances the efficiency, yield, and homogeneity of embryoid body differentiation. Stem Cells 25, 2224–2234.

Caspi, O., Huber, I., Kehat, I., Habib, M., Arbel, G., Gepstein, A., Yankelson, L., Aronson, D., Beyar, R., and Gepstein, L. (2007). Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts. J. Am. Coll. Cardiol. 50, 1884–1893.

Chen, M., Lin, Y.Q., Xie, S.L., Wu, H.F., and Wang, J.F. (2011). Enrichment of cardiac differentiation of mouse embryonic stem cells by optimizing the hanging drop method. Biotechnol. Lett. 33, 853–858.

Chisti, Y. (2001). Hydrodynamic damage to animal cells. Crit. Rev. Biotechnol. 21, 67–110.

Choi, D., Lee, H.J., Jee, S., Jin, S., Koo, S.K., Paik, S.S., Jung, S.C., Hwang, S.Y., Lee, K.S., and Oh, B. (2005). In vitro differentiation of mouse embryonic stem cells: Enrichment of endodermal cells in the embryoid body. Stem Cells. 23, 817–827.

Dang, S.M., Kyba, M., Perlingeiro, R., Daley, G.Q., and Zandstra, P.W. (2002). Efficiency of embryoid body formation and hematopoietic development from embryonic stem cells in different culture systems. Biotechnol. Bioeng. 78, 442–453.

Desbaillets, I., Ziegler, U., Groscurth, P., and Gassmann, M. (2000). Embryoid bodies: An in vitro model of mouse embryogenesis. Exp. Physiol. 85, 645–651.

Fujita, J., Crane, A.M., Souza, M.K., Dejosez, M., Kyba, M., Flavell, R.A., Thomson, J.A., and Zwaka, T.P. (2008). Caspase activity mediates the differentiation of embryonic stem cells. Cell Stem Cell 2, 595–601.

Gerecht-Nir, S., Cohen, S., and Itskovitz-Eldor, J. (2004). Bioreactor cultivation enhances the efficiency of human embryoid body (hEB) formation and differentiation. Biotechnol. Bioeng. 86, 493–502.

Gonzales, C., Ullrich, N.D., Gerber, S., Berthonneche, C., Niggli, E., and Pedrazzini, T. (2012). Isolation of cardiovascular precursor cells from the human fetal heart. Tiss. Eng. Part A. 18, 198–207.

Graichen, R., Xu, X., Braam, S.R., Balakrishnan, T., Norfiza, S., Sieh, S., Soo, S.Y., Tham, S.C., Mummery, C., Colman, A., Zweigerdt, R., and Davidson, B.P. (2008). Enhanced cardiomyogenesis of human embryonic stem cells by a small molecular inhibitor of p38 MAPK. Differentiation. 76, 357–370.

Hammond, T.G., and Hammond, J.M. (2001). Optimized suspension culture: The rotating-wall vessel. Am. J. Physiol. Renal Physiol. 281, F12–F25.

He, W., Ye, L., Li, S., Liu, H., Wang, Q., Fu, X., Han, W., and Chen, Z. (2012). Stirred suspension culture improves embryoid body formation and cardiogenic differentiation of genetically modified embryonic stem cells. Biol. Pharm. Bull. 35, 308–316.

Heng, B.C., Haider, H., Sim, E.K., Cao, T., and Ng, S.C. (2004). Strategies for directing the differentiation of stem cells into

the cardiomyogenic lineage in vitro. Cardiovasc. Res. 62, 34-–42.

Hopfl, G., Gassmann, M., and Desbaillets, I. (2004). Differentiating embryonic stem cells into embryoid bodies. Methods Mol. Biol. 254, 79–98.

Invernici, G., Cristini, S., Madeddu, P., Brock, S., Spillmann, F., Bernasconi, P., Cappelletti, C., Calatozzolo, C., Fascio, U., Bisleri, G., Muneretto, C., Alessandri, G., and Parati, E.A. (2008). Human adult skeletal muscle stem cells differentiate into cardiomyocyte phenotype in vitro. Exp. Cell Res. 314, 366–376.

Karbanová, J., and Mokry, J. (2002). Histological and histochemical analysis of embryoid bodies. Acta. Histochem. 104, 361–365.

Kehat, I., Kenyagin-Karsenti, D., Snir, M., Segev, H., Amit, M., Gepstein, A., Livne, E., Binah, O., Itskovitz-Eldor, J., and Gepstein, L. (2001). Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J. Clin. Invest. 108, 407–414.

Kobolak, J., Mamo, S., Rungsiwiwut, R., Ujhelly, O., Csonka, E., Hadlaczky, G., and Dinnyes, A. (2012). Comparative analysis of nuclear transfer embryo-derived mouse embryonic stem cells. Part I: Cellular characterization. Cell. Reprogram. 14, 56–67.

Koike, M., Sakaki, S., Amano, Y., and Kurosawa, H. (2007). Characterization of embryoid bodies of mouse embryonic stem cells formed under various culture conditions and estimation of differentiation status of such bodies. J. Biosci. Bioeng. 104, 294–299.

Kurosawa, H. (2007). Methods for inducing embryoid body formation: In vitro differentiation system of embryonic stem cells. J. Biosci. Bioeng. 103, 389–398.

Laflamme, M.A., Zbinden, S., Epstein, S.E., and Murry, C.E. (2007). Cell-based therapy for myocardial ischemia and infarction: Pathophysiological mechanisms. Annu. Rev. Pathol. 2, 307–339.

Lü, S., Liu, S., He, W., Duan, C., Li, Y., Liu, Z., Zhang, Y., Hao, T., Wang, Y., Li, D., Wang, C., and Gao, S. (2008). Bioreactor cultivation enhances NTEB formation and differentiation of NTES cells into cardiomyocytes. Cloning Stem Cells 10, 363–370.

Ma, Q., Zhou, B., and Pu, W.T. (2008). Reassessment of Isl1 and Nkx2-5 cardiac fate maps using a Gata4-based reporter of Cre activity. Dev. Biol. 323, 98–104.

Magin, T.M., McWhir, J., and Melton, D.W. (1992). A new mouse embryonic stem cell line with good germ line contribution and gene targeting frequency. Nucleic Acids Res. 20, 3795–3796.

Messina, E., De Angelis, L., Frati, G., Morrone, S., Chimenti, S., Fiordaliso, F., Salio, M., Battaglia, M., Latronico, M.V., Coletta, M., Vivarelli, E., Frati, L., Cossu, G., and Giacomello, A. (2004). Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ. Res. 95, 911–921.

Mummery, C., Ward, D., van den Brink, C.E., Bird, S.D., Doevendans, P.A., Opthof, T., Brutel de la Riviere, A., Tertoolen, L., van der Heyden, M., and Pera, M. (2002). Cardiomyocyte differentiation of mouse and human embryonic stem cells. J. Anat. 200, 233–242.

Mummery, C., Ward-van Oostwaard, D., Doevendans, P., Spijker, R., van den Brink, S., Hassink, R., van der Heyden, M., Opthof, T., Pera, M., de la Riviere, A.B., Passier, R., and Tertoolen, L. (2003). Differentiation of human embryonic stem cells to cardiomyocytes: Role of coculture with visceral endoderm-like cells. Circulation. 107, 2733–2740.

Mummery, C., van der Heyden, M.A., de Boer, T.P., Passier, R., Ward, D., van den Brink, S., van Rooijen, M., and van de Stolpe, A. (2007). Cardiomyocytes from human and mouse embryonic stem cells. Methods Mol. Med. 140, 249–272.

Nagy, A., Gertsenstein, M., Vintersten, K., and Behringer, R. (2006a). Preparing feeder cell layers from STO or mouse embryo fibroblast (MEF) cells: Treatment with mitomycin C. CSH. Protoc. 2006 (1).

Nagy, A., Gertsenstein, M., Vintersten, K., and Behringer, R. (2006b). Preparing mouse embryo fibroblasts. CSH. Protoc. 2006 (1).

Passier, R., and Mummery, C. (2005). Cardiomyocyte differentiation from embryonic and adult stem cells. Curr. Opin. Biotechnol. 16, 498–502.

Pekkanen-Mattila, M., Kerkela, E., Tanskanen, J.M., Pietila, M., Pelto-Huikko, M., Hyttinen, J., Skottman, H., Suuronen, R., and Aalto-Setala, K. (2009). Substantial variation in the cardiac differentiation of human embryonic stem cell lines derived and propagated under the same conditions—a comparison of multiple cell lines. Ann. Med. 41, 360–370.

Pekkanen-Mattila, M., Pelto-Huikko, M., Kujala, V., Suuronen, R., Skottman, H., Aalto-Setälä, K., and Kerkelä, E. (2010). Spatial and temporal expression pattern of germ layer markers during human embryonic stem cell differentiation in embryoid bodies. Histochem. Cell. Biol. 133, 595–606.

Penn, M.S., and Mal, N. (2006). Stem cells in cardiovascular disease: Methods and protocols. Methods Mol. Med. 129, 329–351.

Ranganayakulu, G., Elliott, D.A., Harvey, R.P., and Olson, E.N. (1998). Divergent roles for NK-2 class homeobox genes in cardiogenesis in flies and mice. Development 125, 3037–3048.

Reinecke, H., and Murry, C.E. (2000). Transmural replacement of myocardium after skeletal myoblast grafting into the heart. Too much of a good thing? Cardiovasc. Pathol. 9, 337–344.

Rungarunlert, S., Techakumphu, M., Pirity, M.K., Dinnyes, A. (2009). Embryoid body formation from embryonic and induced pluripotent stem cells: Benefits of bioreactors. World J. Stem Cells. 1, 11–21.

Rungarunlert, S., Klincumhom, N., Bock, I., Nemes, C., Techakumphu, M., Pirity, M.K., and Dinnyes, A. (2011). Enhanced cardiac differentiation of mouse embryonic stem cells by use of the slow-turning, lateral vessel (STLV) bioreactor. Biotechnol. Lett. 33, 1565–1573.

Schwartz, R.J., and Olson, E.N. (1999). Building the heart piece by piece: Modularity of cis-elements regulating Nkx2-5 transcription. Development 126, 4187–4192.

Snir, M., Kehat, I., Gepstein, A., Coleman, R., Itskovitz-Eldor, J., Livne, E., and Gepstein, L. (2003). Assessment of the ultrastructural and proliferative properties of human embryonic stem cell-derived cardiomyocytes. Am. J. Physiol. Heart Circ. Physiol. 285, H2355–H2363.

Takahashi, T., Lord, B., Schulze, P.C., Fryer, R.M., Sarang, S.S., Gullans, S.R., and Lee, R.T. (2003). Ascorbic acid enhances differentiation of embryonic stem cells into cardiac myocytes. Circulation 107, 1912–1916.

White, S.M., and Claycomb, W.C. (2003). Cardiac cell transplantation: Protocols and applications. Methods Mol. Biol. 219, 83–95.

Yirme, G., Amit, M., Laevsky, I., Osenberg, S., and Itskovitz-Eldor, J. (2008). Establishing a dynamic process for the formation, propagation, and differentiation of human embryoid bodies. Stem Cells Dev. 17, 1227–1241.

Zhou, B., von Gise, A., Ma, Q., Rivera-Feliciano, J., and Pu, W.T. (2008). Nkx2-5- and Isl1-expressing cardiac progenitors contribute to proepicardium. Biochem. Biophys. Res. Commun. 375, 450–453.

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