Integrative Biology

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/ibiology

Insight Statement

A novel coaxial electrospray technology is developed to produce core-shell microcapsules mimicking the anatomic architecture of pre-hatching embryos, the native home of embryonic stem cells. This is achieved in one step using two biocompatible aqueous fluids without the use of any evaporative and cytotoxic organic solvent that is commonly used to form core-shell microcapsules for drug encapsulation by conventional electrospray. Significantly higher pluripotency was observed in the stem cells when cultured in the miniaturized 3D liquid space of the biomimetic microcapsule core compared to cells under the conventional 3D (in hydrogel) and 2D (in petri dishes) culture, which renders the stem cells higher capability of directed differentiation into the cardiac lineage.

Coaxial electrospray of liquid core-hydrogel shell microcapsules for encapsulation and miniaturized 3D culture of pluripotent stem cells

Shuting Zhao[1,2], Pranay Agarwal[1,2], Wei Rao[1,2], Haishui Huang[1,2,3], Renliang Zhang[8], Zhenguo Liu[4], Jianhua Yu[5,6], Noah Weisleder[2,7], Wujie Zhang[9], and Xiaoming He[1,2,5,*]

[1]Department of Biomedical Engineering, [2]Davis Heart and Lung Research Institute, [3]Department of Mechanical Engineering, [4]Division of cardiovascular medicine, [5]Comprehensive Cancer Center, [6]Division of Hematology, [7]Department of Physiology and Cell Biology, The Ohio State University, Columbus, OH, 43210, USA

[8]Department of Neurology, Jinling Hospital, Nanjing University School of Medicine, Nanjing 210002, China

[9]Biomolecular Engineering Program, Department of Physics and Chemistry, Milwaukee School of Engineering, Milwaukee, WI 53202, USA

*Correspondence should be addressed to:
Xiaoming He, Ph.D.
Department of Biomedical Engineering
The Ohio State University
1080 Carmack Road
Columbus, OH 43210
Phone: (614) 292-6992
Fax: (614) 292-7301
E-mail : He.429@osu.edu

Electronic Supplementary Information Available (Table S1, Figures S1-S5, and Movie S1)

Abstract

A novel coaxial electrospray technology is developed to generate microcapsules with a hydrogel shell of alginate and an aqueous liquid core of living cells using two aqueous fluids in one step. Approximately 50 murine embryonic stem (ES) cells encapsulated in the core with high viability (92.3 ± 2.9%) can proliferate to form a single ES cell aggregate of 128.9 ± 17.4 μm in each microcapsule within 7 days. Quantitative analyses of gene and protein expression indicate that ES cells cultured in the miniaturized 3D liquid core of the core-shell microcapsules have significantly higher pluripotency on average than the cells cultured on 2D substrate or in the conventional 3D alginate hydrogel microbeads without a core-shell architecture. The higher pluripotency is further suggested by their significantly higher capability of differentiation into beating cardiomyocytes and higher expression of cardiomyocyte specific gene markers on average after directed differentiation under the same conditions. Considering its wide availability, easiness to set up and operate, reusability, and high production rate, the novel coaxial electrospray technology together with the microcapsule system is of importance for mass production of ES cells with high pluripotency to facilitate translation of the emerging pluripotent stem cell-based regenerative medicine into the clinic.

Keywords: Coaxial electrospray; miniaturization; 3D culture; pluripotency; differentiation

1. Introduction

Pluripotent stem cells such as embryonic stem (ES) cells have been intensively explored in recent years as a promising cell source for tissue regeneration, which is particularly valuable for treating diseases due to the loss of static or permanent cells (e.g., cardiomyocytes) that do not replicate much at all in response to injury.[1-4] This is because pluripotent stem cells are capable of self-renewal to maintain pluripotency that allows them being coaxed (or directed) to differentiate into any desired type of somatic cells under an appropriate cue. The pluripotency of ES cells, however, is regulated by their niche or culturing microenvironment that is still elusive today.[5-7]

As ES cells naturally form aggregates in their native home of a pre-hatching stage embryo consisting of a miniaturized (~70-150 μm dependent on species) aqueous 3D core of embryonic cells and a hydrogel shell known as the zona pellucida (Fig. S1),[8-12] an aqueous liquid microenvironment allowing ES cells to form aggregates without much resistance might be desired for maintaining their pluripotency (or stemness). As a result, pluripotent stem cells have been commonly cultured either on 2D substrates overlapped with or in 3D suspension of bulk liquid culture medium. The former is non-physiological, which has been shown to result in altered gene and protein expression in many non-pluripotent or non-stem cells compared to 3D culture.[13-14] Hanging drop, low-attachment plates, and micro-patterned wells have been commonly used to provide a bulk and open 3D liquid environment for culturing pluripotent stem cells.[15-23] Although useful for research purpose, these culture methods suffer difficulty to scale up, cell damage due to shear stress, and/or limited control of aggregate size and shape, which hinders the clinical application of pluripotent stem cells. In recent years, both macro and microencapsulation of cells in solid-like hydrogels of various biomaterials for 3D culture have been studied intensively.[24-27] For pluripotent stem cells, however, encapsulating them in a miniaturized 3D aqueous liquid-like space might be desired for maintaining their stemness.

Alginate has been one of the most widely used natural biomaterials for cell encapsulation because of its excellent biocompatibility and mild condition of gelling through ionic crosslinking that is not harmful to living cells.[28-29] Electrospray has been extensively used for cell microencapsulation using alginate probably due to its easiness to set up and operate, reusability, and high production rate with a single device. However, current electrospray technology is limited to either producing homogeneous, solid-like microbeads that lack an aqueous liquid core or relying on the use of highly evaporative organic solvents that are immiscible with aqueous solution to generate a core-shell structure.[30-32] The latter is not applicable for encapsulating living cells due to the high cytotoxicity of organic solvents although it works well for drug encapsulation.[33-35] For the former, a non-liquid alginate hydrogel core is not native to pluripotent stem cells and has been shown to interfere ES cell proliferation to form aggregates of controllable size and shape.[36-39] Although several studies have been conducted to create an aqueous liquid core by liquefying the non-liquid core material, the multiple steps of encapsulating and liquefying are time-consuming and difficult to control and may cause damage to the encapsulated cells.[38-41] More recently, two studies reported the use of nonplanar microfluidics to produce microcapsules with a liquid core and hydrogel shell in one step for cell microencapsulation and culture.[42-43] However, no study has been reported to produce liquid core-shell microcapsules for cell microencapsulation using electrospray. Moreover, there is no report regarding how culturing pluripotent stem cells in the miniaturized 3D liquid core could affect the expression of genes and proteins in the cells and the directed differentiation of pluripotent stem cells into a specific lineage as compared to the conventional 3D hydrogel culture approach.

In this study, we developed a novel coaxial electrospray technology for one-step generation of hundreds of thousands of microcapsules with a hydrogel shell of alginate (that mimics zona pellucida) and an aqueous liquid core of ES cells with high viability (> 90%) in less than one

hour. This is achieved by using two aqueous fluids without any cytotoxic organic solvent that is usually needed by contemporary electrospray technology to produce core-shell structured microcapsules for drug encapsulation. Starting from ~50 single ES cells in each microcapsule (compared to more than one thousand cells needed for the conventional hanging drop method[20,44]), cell aggregates of uniform size and shape (spheroidal) with high pluripotency could be obtained within 7 days. The pluripotency and capability of directed cardiac differentiation of the aggregated ES cells were found to be significantly higher (on average) than that of the ES cells under the conventional 2D culture or cultivated in conventional 3D microbeads of homogeneous alginate hydrogel, which has not been reported before. Considering its easiness to set up (simply by replacing the single lumen needle with a coaxial needle in the widely used conventional electrospray or spinning systems) and operate and several other aforementioned advantages, the novel coaxial electrospray technology together with the biomimetic microcapsule system for miniaturized 3D culture of pluripotent stem cells developed in this study is valuable for mass production of ES cells with high pluripotency to facilitate translation of the emerging pluripotent stem cell-based regenerative medicine into the clinic.

2. Material and methods

2.1. Materials

Knockout® DMEM and serum for ES cell culture were purchased from Life Technologies. Leukemia inhibitory factor (LIF) was purchased from Millipore. Trypsin/EDTA, regular DMEM (high glucose), and the live/dead viability/cytotoxicity kit for mammalian cells were purchased from Invitrogen. Fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Hyclone. Primary antibodies were purchased from Abcam. Secondary antibodies for immunohistochemistry were purchased from Invitrogen. Alginate was purchased from Sigma and

purified by washing in chloroform with charcoal and dialyzing against deionized water, followed by freeze-drying.[45] The purpose of purification is to remove potential impurities that could compromise cell viability and induce immune response *in vivo*.[46-48] All other chemicals were purchased from Sigma unless specifically indicated otherwise.

2.2. Cell culture

R1 murine ES cells from ATCC were cultured in ES cell medium made of Knockout® DMEM supplemented with 15% Knockout® serum, 1000 U/ml LIF, 4 mM *l*-glutamine, 0.1 M 2-mercaptoethanol, 10 μg/ml gentamicin, 100 U/ml penicillin, and 100 μg/ml streptomycin in gelatin coated tissue culture flasks with medium being changed daily. For cardiac differentiation, ES cells were cultured in regular DMEM supplemented with 25 ng/ml BMP-4, 5 ng/ml bFGF, 100 U/ml penicillin, and 100 mg/l streptomycin for 3 days, followed by maintaining in regular DMEM supplemented with 20% FBS. All cells were cultured at 37 ºC in a humidified 5% CO_2 incubator.

2.3. Coaxial electrospray of core-shell microcapsules

The coaxial electrospray system and a cross-sectional view of the coaxial needle are illustrated in Fig. 1A and B, respectively. The system consists of two syringe pumps for pushing the core fluid with living cells and shell fluid of alginate through the concentric inner (28 G) and outer (21 G) lumens in the coaxial needle, respectively. Under an open electric field from a voltage generator, concentric drops of the two coaxial fluids at the needle tip were broken up into microdrops and sprayed into the gelling bath containing 100 mM calcium chloride solution to instantly gel alginate in the shell fluid before the two fluids got mixed. The core fluid contained ES cells at 5×10^6 per ml, 0.25 M aqueous mannitol (instead of medium or physiologic saline according to our previous study[38]), and either 2% sodium alginate (w/v) for making microcapsules with an alginate hydrogel core or 1% (w/v) sodium carboxymethyl cellulose for

making microcapsules with a liquid core. The shell fluid consisted of 2% purified alginate (w/v) in 0.25 M aqueous mannitol solution. After encapsulation, the microcapsules with ES cells in the gelling solution were washed with 0.5 M mannitol solution for 7 min and suspended in ES cell medium after removing mannitol for further culture.[38]

2.4. Cell viability, proliferation, and pluripotency

Viability of the encapsulated ES cells was determined using the live/dead assay kit. Proliferation of the encapsulated ES cells was monitored as the formation of cell aggregates in the microcapsule core. The pluripotency of aggregated ES cells on day 7 was examined by qRT-PCR for expression of both pluripotency and differentiation genes and by immunohistochemical staining for pluripotency protein expression. In brief, ES cell aggregates formed in the hydrogel or liquid core were released from microcapsules by incubating in 55 mM sodium citrate solution (adjusted to be isotonic using sodium chloride) for 30 s and washing in 1x PBS for 3 min. RNAs in the released or 2D culture cell aggregates were isolated using an RNeasy Plus Mini Kit (Qiagen) following the manufacture's instruction. Synthesis of complementary DNAs (cDNAs) was performed using the iScript^{TM} cDNA synthesis kit (Bio-Rad) according to the manufacture's instruction. A Bio-Rad CFX96 real time PCR machine was used to quantify gene expression. Relative gene expression was calculated using the $\Delta\Delta C_t$ method built in the Bio-Rad software.[38] The expression of five pluripotency genes including Oct-4, Sox-2, Nanog, Klf2, and SSEA-1 as well as five differentiation genes including Nestin, Sox-7, Brachyrury (T), Nkx2.5, and cTnT (see Table S1 for their primers) was studied. Moreover, the expression of three pluripotency protein markers including Oct-4, Nanog, and Sox-2 was exanimated using flow cytometry. Briefly, cell aggregates from the three different culture conditions were collected and dissociated into single cells, followed by fixation in 4% paraformaldehyde for 15 min at 4 ºC. The fixed samples were then blocked to prevent non-specific binding using 3% BSA with 0.1% Triton for

30 min and further incubated first with primary antibodies for 20 min at room temperature and then with secondary antibodies for 30 min at room temperature in the dark. Afterward, all the samples were re-suspended in 0.2 ml ice-cold PBS and analyzed using a BD LSR-II flow cytometer. The controls of these flow cytometry studies were the corresponding isolated cells from the three different culture conditions incubated with rabbit IgG only (i.e., secondary antibody without a fluorescence probe). The data were further analyzed using FlowJo. For immunohistochemical staining of Oct-4 and SSEA-1 proteins, the aggregates from the aqueous liquid core were fixed with 4% paraformaldehyde for 15 min at room temperature and then incubated with 3% bovine serum albumin at room temperature for 30 min to block non-specific binding. The primary antibodies for Oct-4 and SSEA-1 were then applied by overnight incubation at 4 ºC and the corresponding secondary antibodies were added for 1 h for fluorescence labeling at room temperature in the dark. The samples were further stained for nuclei by incubating with 5 μM Hoechst 33342 for 15 min before examination using an Olympus FV1000 confocal microscope.

2.5. Cardiac differentiation

After 7 days culture, the aggregates formed in hydrogel core as well as liquid core were released using isotonic solution of 55 mM sodium citrate, plated in gelatin-coated dish, and cultured with differentiation medium containing BMP-4 and bFGF for 3 days, followed by maintaining in regular DMEM supplemented with 20% FBS for up to 2 weeks. For the 2D aggregate/colony differentiation, ES cells were plated on the gelatin-coated dish and cultured in complete ES cell growth medium for 3 days to form colonies/aggregates that were induced to differentiate using the same differentiation medium for 3 days, followed by maintaining in regular DMEM supplemented with 20%. The cumulative percentage of beating aggregates or colonies on each day out of the total number of aggregates plated was counted and calculated

starting from the day of initiating differentiation. To stain cTnT for flow cytometry analysis, cells before and after differentiation (on day 14) were collected by trypsin and processed using the same methods described in the previous section for examining pluripotency proteins. Then they were further incubated with cTnT antibody for 45 min at 4 ºC in the dark. All the samples were resuspended in 0.2 ml ice-cold PBS and studied using BD LSR-II flow cytometer and the results were further analyzed using FlowJo. For gene expression study, RNAs of the cell aggregates obtained from all the three different culture conditions before and after differentiation were obtained using the RNeasy Plus mini kit (Qiagen). The cDNAs were then synthesized for cTnT, Nkx2.5, and Brachyury (or T) genes with their primers also giving in Table S1. Immunohistochemical analyses were done by fixing the attached cell aggregates from the 3D liquid core on day 13 when the beating reaches the plateau, staining the fixed samples with antibodies for cTnI, α-actinin, and connexin 43 according to the manufacture's protocol, and examining with an Olympus FV1000 confocal microscope.

2.6. Statistical analysis

All data were reported as mean ± standard deviation. Student's two-tailed t-test assuming equal variance was calculated using Microsoft® Excel to assess statistical significance ($p < 0.05$).

3. Results and discussion

3.1. Coaxial electrospray of cell-laden core-shell microcapsules in one step

The coaxial electrospray setup is illustrated in Fig. 1A and B. The core and shell aqueous fluids were injected into the inner and outer lumen of a coaxial needle, respectively. Under an open electric field, drops of the two fluids at the tip of the coaxial needle were broken up and sprayed into the gelling bath of 100 mM calcium chloride ($CaCl_2$) solution to instantly gel alginate in the shell fluid. In order to form a core-shell structure, mixing between the core and

shell fluids must be minimized before alginate is gelled, which was achieved in this study by adding 1% sodium carboxymethyl cellulose in the core fluid to raise its viscosity. Cellulose, a major polysaccharide in plant cell wall, was chosen to be the viscosity modifier because of its non-toxic nature to mammalian cells.[49-50] The high viscosity of both the cellulose-based core fluid and alginate-based shell fluid together with the fast gelling kinetics of alginate in calcium chloride solution is crucial to the formation of microcapsules with a liquid core and hydrogel shell. Typical differential interference contrast (DIC) and confocal fluorescence micrographs demonstrating the core-shell morphology of the resultant microcapsules (no cells) of ~300 μm (in diameter) are shown in Fig. S2, where the alginate hydrogel shell was made fluorescent by adding 1% FITC-labeled dextran (500 kD) in the shell fluid to make the microcapsules. For cell microencapsulation, ES cells were suspended in the core fluid at a density of 5 x 10^6 cells/ml and electrospray was done under the following conditions: core flow rate, 47 μl/min; shell flow rate, 90 μl/min; and voltage, 1.8 kV. The core fluid was 2% sodium alginate and 1% cellulose solution for making microcapsules with a hydrogel and liquid core, respectively. The resultant cell-laden core-shell microcapsules are 315 ± 31 μm in outer diameter (slightly larger than microcapsules without cells) and their typical size distribution is shown Fig. 1C. Most of the cell-laden microcapsules are from 285 to 345 μm.

Typical morphology of the resultant microcapsules with an ES cell-laden hydrogel and liquid core on day 0, 3, and 7 is shown in Fig. 2A-C and G-I, respectively. The corresponding fluorescence images of ES cells in the hydrogel and liquid core are given in Fig. 2D-F and J-L, respectively. Approximately 50 ES cells were encapsulated in the core of each microcapsule with high viability (92.3 ± 2.9% and 90.4 ± 1.2% for liquid and hydrogel core, respectively) on day 0, which indicates the mild nature of the coaxial electrospray process. The encapsulated cells in the liquid core proliferated and started to form multiple small aggregates on day 3 that eventually

merged together to form one single aggregate of 128.9 ±17.4 μm in the liquid core of each microcapsule on day 7 as shown in Fig. 2G-L. However, ES cells in the hydrogel core formed relatively smaller aggregates with many dead single cells on day 3 and eventually formed multiple irregular aggregates in each microcapsule on day 7 as shown in Fig. 2A-F. The non-uniform size and irregular shape of ES cell aggregates in the microcapsules with a hydrogel core are probably due to the cross-linked alginate fibers that prevent ES cells from continuously growing to merge into single aggregates, which would not occur in the liquid core or in a pre-hatching embryo, the native home of ES cells. Typical images of a larger field showing more single ES cell aggregates with high cell viability in microcapsules with a liquid core on day 7 are given in Fig. 2M-N. The high viability of cells during the extended culture in the microcapsules with a liquid core is probably due to the relatively more efficient transport of nutrients and oxygen to all cells in the liquid core as a result of the miniaturized dimension, compared to macrocapsules or bulk gel that have been used for 3D cell culture today.[51-52]

The fact that only ~ 50 ES cells are sufficient to obtain a cell aggregate of ~130 μm on day 7 (more than one thousand ES cells are usually needed initially with the commonly used hanging drop method for ES cell culture)[20,44] makes it particularly attractive to stem cell-based personalized medicine, for which the number of cells available are usually limited. Another major advantage of using the coaxial electrospray technology is its high efficiency for mass production of ES cell aggregates to meet their high demand for tissue regeneration in clinical applications, considering the fact that ~100,000 microcapsules can be produced in ~20 minute in one step and that many ES cell aggregates can be obtained within 7 days. Lastly and importantly, the proliferation of ES cells in the liquid core enclosed by an alginate hydrogel shell resembles the proliferation of embryonic (including ES) cells in the miniaturized 3D aqueous core encompassed by zona pellucida (the hydrogel shell of embryo) of a pre-hatching stage embryo during embryo

development from one cell (also called zygote, the totipotent cell) to two cells, four cells, and eventually a single merged aggregate of many cells inside the zona pellucida called morula (Fig. S1).[8-12] The morula further develops into pre-hatching blastocyst consisting of a single aggregate of embryonic (including ES) cells known as the inner cell mass, a liquid-filled cavity, and a layer of trophoblasts underlining the inside wall of zona pellucida (Fig. S1).[8-12] The formation of the trophoblast layer facilitates the embryo to hatch (i.e., release out of the zone pellucida) and implant into the uterus wall and the differentiation of pluripotent ES cells in the inner cell mass to into multipotent lineage/organ specific stem cells and somatic cells (Fig. S1).[8-12] Therefore, the core-shell microcapsule system generated in one step in this study using coaxial electrospray could provide a biomimetic, miniaturized 3D liquid culture microenvironment for pluripotent stem cells to better maintain their pluripotency than the 3D solid-like hydrogel and conventional 2D culture methods, which is discussed below.

3.2. ES cells cultured in the biomimetic core-shell microcapsules have significantly higher pluripotency on average than the cells under 3D hydrogel and conventional 2D culture

To investigate the pluripotency of aggregated ES cells on day 7, we conducted quantitative RT-PCR (qRT-PCR) analyses of 5 pluripotency marker genes including Klf2, Nanog, Oct-4, Sox-2, and SSEA-1 as well as five differentiation markers including Nestin for ectoderm, Sox-7 for endoderm, Brachyrury (or T) for mesoderm, Nkx2.5 for early cardiac commitment, and cTnT for late cardiac differentiation, as shown in Fig. 3. The aggregated ES cells obtained by culturing in the miniaturized 3D liquid core have significantly higher expression of Nanog (1.73 times) and Klf2 (1.6 times) genes on average than that obtained by the conventional 2D culture (see Fig. S3 for the morphology of the ES cell colonies/aggregates formed under 2D culture), although the expression is not significantly different in terms of Oct-4, Sox-2, and SSEA-1 genes. The latter is possibly because the specific factors that control the expression of the three genes might not be

significantly different in the microenvironment of the two different culture conditions. Moreover, the ES cell aggregates formed in the 3D liquid core also have significantly higher expression of Klf2 (1.74 times), Nanog (2.55 times), and Sox-2 (1.83 times) on average than the cells cultured in 3D alginate hydrogel core. Furthermore, the aggregated ES cells obtained from the miniaturized 3D liquid core have significantly lower expression of Nestin (10.4 times), Sox-7 (7.1 times), Brachyrury (T) (1.70 time), Nkx2.5 (3.1 times), and cTnT (7.1 times) than that from 2D culture. Similar trend was observed for all the five differentiation marker genes when compared to the aggregated ES cells obtained from the 3D hydrogel core culture. These data indicate that the miniaturized 3D liquid microenvironment should help ES cells to maintain an undifferentiated state, rather than commit to any specific germ layer or lineage. In other words, the miniaturized 3D aqueous liquid microenvironment should promote stemness and suppress spontaneous differentiation at gene level.

To study pluripotency of ES cells under the three different culture conditions at the level of protein expression, we conducted flow cytometry analyses on three representative pluripotency protein markers selected according to the gene expression data: Oct-4, Sox-2, and Nanog that are not significantly different among all the three culture conditions, significantly different between the two 3D culture conditions only, and significantly different between the 3D liquid core and all the two conventional culture conditions, respectively. The data are given in Fig. 4, which shows that ES cell aggregates formed in the miniaturized 3D liquid core have significantly higher expression of both Nanog (1.7 times) and Sox-2 (1.62 times) proteins on average compared to the 2D cultured cells. The expression of Nanog (3.95 times) and Sox-2 (3.52 times) on average is also significantly higher in the ES cell aggregates obtained in 3D liquid than hydrogel core. It is not significantly different for the three culture conditions in terms of the expression of Oct-4 protein, which is consistent with the gene expression data for Oct-4 in Fig. 3A. These results are

not only consistent with what has been reported in recent years that gene and protein expression in cells cultured under 2D and bulk 3D conditions could be very different[27,53-55] but also indicate that a miniaturized 3D liquid microenvironment is important to culturing pluripotent stem cells. The high pluripotency of the aggregated ES cells obtained in the miniaturized 3D liquid core is further visually confirmed at the level of protein expression by immunohistochemical staining of Oct-4 and SSEA-1 (Fig. S4). Therefore, the biomimetic, miniaturized 3D culture in the pre-hatching embryo-like core-shell microcapsules indeed is beneficial for culturing ES cells to maintain their pluripotency.

3.3. ES cells cultured in the miniaturized 3D liquid core have significantly higher capability of directed cardiac differentiation on average than the cells under conventional 2D and 3D culture

To further study the effect of culture condition on their capability of directed differentiation into the cardiac lineage (instead of spontaneous differentiation into all three germ-layer lineages), the aggregated ES cells obtained after 7 days culture under the three different conditions were induced using BMP-4 and bFGF for 3 days, followed by maintaining the cell aggregates in regular medium for up to 14 days. Both BMP-4 and bFGF have been used to induce mesoderm specification and cardiac commitment from stem cells.[56-59] The successful differentiation into cardiac lineage was first confirmed by the cumulative percentage of beating foci as shown in Fig. 5A. For the aggregates obtained from the miniaturized 3D liquid core, beating was first observed on day 4 (i.e., 4 days after initiating differentiation) and the cumulative percentage of beating aggregates reached a maximum of ~67.2% on day 12. Starting from day 6, the beating percentage for the differentiated aggregates from the miniaturized 3D liquid core is significantly higher than that from 2D culture (9.75% maximum on day 12) and 3D hydrogel core (48.3% maximum on day 12). The morphology of a typical beating aggregate is shown in Fig. 5B as well as Movie S1. The morphology of cells in the beating aggregates suggests the presence of cardiomyocytes

together with cardiac stromal cells such as fibroblasts, similar to the mixture of multiple types of cells naturally residing in the heart.

The differentiation of ES cell aggregates obtained from the three culture conditions was further studied at the gene expression level using qRT-PCR for cardiac troponin T (cTnT), Nkx2.5, brachyury (or T) genes: the cTnT gene is specifically expressed in cardiomyocytes, Nkx2.5 is only present in the heart from the time of cardiac specification, and the brachyury (or T) gene is a mesoderm marker. As shown in Fig. 5C, compared to the corresponding cells before differentiation, the expression of cTnT and Nkx2.5 in the differentiated cells is significantly (up to 414 and 16.3 times, respectively, for the 3D liquid core) higher on average, indicating successful directed differentiation of the ES cells into the cardiac lineage. Moreover, the expressions of these two cardiac markers are significantly much higher on average in cells differentiated from ES cell aggregates obtained from the 3D liquid core than that from 2D culture (18.1 time higher for cTnT) and 3D hydrogel core (5.5 times higher for cTnT). The expression of the mesoderm marker T gene in the cells differentiated from ES cell aggregates obtained from the miniaturized 3D liquid core is lower than that in the 3D alginate hydrogel core, which is probably because more of the cells from miniaturized 3D liquid core passed the mesoderm stage and committed specifically into the cardiac lineage on day 14 (the day for the qRT-PCR study) than the cells from 3D hydrogel core. Interestingly, we found that although aggregated ES cells from the miniaturized 3D liquid core have relatively lower expression of Brachyrury, Nkx2.5, and cTnT before differentiation (as shown in Fig. 3B), they have much higher expression of these markers on average after differentiation. These data suggest that high pluripotency maintained in miniaturized 3D liquid core before differentiation is indeed beneficial for further directed lineage specification.

The percentage of potential cardiomyocytes among the cells differentiated from ES cell

aggregates cultured in miniaturized 3D liquid core was estimated to be 42.2 ± 3.0% based on flow cytometry analysis using cTnT as the marker protein for cardiomyocytes (Fig. 5D), which is significantly higher on average than that differentiated from ES cell aggregates obtained from 2D culture (17.0 ± 0.0%). Although it is also higher than that for the 3D hydrogel core culture condition (33.4 ± 14.0%), the difference is not significant because of the big variation of the latter. Moreover, it is also close to the percentage of cardiomyocytes in native cardiac tissue.[60-61] In addition, immunohistochemical staining was performed to visualize the expression of cTnI, α-actinin, and connexin 43 (Cx43), proteins that are critical for cardiomyocytes to function (beat) properly. Both cTnI and α-actinin are key proteins in the sarcomere (the force generation unit) of cardiomyocytes and Cx43 is in the gap junction between cardiomyocytes that is crucial for synchronized beating in mature heart tissue.[62-64] As shown in Fig. S5, all these important proteins were highly expressed in the cells differentiated from ES cell aggregates obtained from the miniaturized 3D liquid core on day 13 of differentiation, which ultimately should promote the aggregates to beat. Therefore, the differentiated cell aggregates may closely mimic native cardiac tissue and may be transplanted directly to treat cardiac diseases such as myocardial infarction (MI), which warrants further investigation in a separate future study. These results demonstrate that our core-shell microcapsules can be used as a biomimetic, miniaturized 3D system for culturing pluripotent stem cells to maintain their pluripotency for further directed cardiac differentiation.

4. Conclusions

In summary, we successfully developed a novel and efficient coaxial electrospray technology to produce microcapsules with an alginate hydrogel shell and an aqueous liquid (rather than hydrogel) core of ES cells. The encapsulated ES cells could survive well and proliferate to form a

single spheroidal aggregate of uniform size in the core of each microcapsule within 7 days. Moreover, the aggregated ES cells obtained by culturing in the miniaturized 3D liquid core were found to have higher pluripotency on average than the cells under 2D culture or cultivated in the conventional 3D microbeads consisting of homogeneous alginate hydrogel, and they can be much more efficiently directed/coaxed to differentiate into cardiac cells. Considering its wide availability, easiness to set up and operate, reusability, and high production rate (several hundred thousand microcapsules can be generated in less than one hour), the novel coaxial electrospray technology for cell microencapsulation and biomimetic microscale biomaterial system for miniaturized 3D culture of pluripotent cells are valuable to facilitate translation of the emerging pluripotent stem cell-based regenerative medicine into the clinic.

Disclosure

The authors declare no conflict of interests.

Acknowledgements

This work was partially supported by grants from NSF (CBET-1154965) and NIH (R01EB012108).

References

1. T. J. Nelson, A. Martinez-Fernandez, A. Terzic, Induced pluripotent stem cells: developmental biology to regenerative medicine. *Nat Rev Cardiol* 2010, *7*. 700-710, DOI: Doi 10.1038/Nrcardio.2010.159.
2. M. Mimeault, R. Hauke, S. K. Batra, Stem cells: A revolution in therapeutics - Recent advances in stem cell biology and their therapeutic applications in regenerative medicine and cancer therapies. *Clin Pharmacol Ther* 2007, *82*. 252-264, DOI: Doi 10.1038/Sj.Clpt.6100301.
3. G. Keller, Embryonic stem cell differentiation: emergence of a new era in biology and medicine. *Gene Dev* 2005, *19*. 1129-1155, DOI: Doi 10.1101/Gad.1303605.
4. K. R. Boheler, J. Czyz, D. Tweedie, H. T. Yang, S. V. Anisimov, A. M. Wobus, Differentiation of pluripotent embryonic stem cells into cardiomyocytes. *Circ Res* 2002, *91*. 189-201, DOI: Doi 10.1161/01.Res.0000027865.61704.32.
5. J. Kramer, C. Hegert, K. Guan, A. M. Wobus, P. K. Muller, J. Rohwedel, Embryonic stem cell-derived chondrogenic differentiation in vitro: activation by BMP-2 and BMP-4. *Mechanisms of development* 2000, *92*. 193-205.
6. Y. S. Pek, A. C. A. Wan, J. Y. Ying, The effect of matrix stiffness on mesenchymal stem cell differentiation in a 3D thixotropic gel. *Biomaterials* 2010, *31*. 385-391, DOI: Doi 10.1016/J.Biomaterials.2009.09.057.
7. U. Ripamonti, S. Ma, A. H. Reddi, The Critical Role of Geometry of Porous Hydroxyapatite Delivery

System in Induction of Bone by Osteogenin, a Bone Morphogenetic Protein. *Matrix* 1992, *12*. 202-12.
8. J. K. Choi, X. He, In vitro maturation of cumulus-oocyte complexes for efficient isolation of oocytes from outbred deer mice. *PLoS One* 2013, *8*. e56158, DOI: 10.1371/journal.pone.0056158.
9. B. Mitchell, R. Sharma, *Embryology: An illustrated colour bood text, 2nd Ed.* Churchill Livingston-Elseview: 2005.
10. T. W. Sadler, *Langman's medical embryology 12th Ed.* Lippincott Williams & Wilkins: Philadelphia, PA, 2012.
11. R. Lewis, D. Gaffin, M. Hoefnagels, B. Parker, in *Life (5th Ed.)*. McGraw-Hill Higher Education: Columbus, OH, 5th edn., 2004.
12. D. T. Scadden, The stem-cell niche as an entity of action. *Nature* 2006, *441*. 1075-9, DOI: 10.1038/nature04957.
13. E. Cukierman, R. Pankov, D. R. Stevens, K. M. Yamada, Taking cell-matrix adhesions to the third dimension. *Science* 2001, *294*. 1708-12, DOI: 10.1126/science.1064829.
14. L. G. Griffith, M. A. Swartz, Capturing complex 3D tissue physiology in vitro. *Nat Rev Mol Cell Biol* 2006, *7*. 211-24, DOI: 10.1038/nrm1858.
15. B. S. Yoon, S. J. Yoo, J. E. Lee, S. You, H. T. Lee, H. S. Yoon, Enhanced differentiation of human embryonic stem cells into cardiomyocytes by combining hanging drop culture and 5-azacytidine treatment. *Differentiation* 2006, *74*. 149-159, DOI: Doi 10.1111/J.1432-0436.2006.00063.X.
16. D. E. Kehoe, D. H. Jing, L. T. Lock, E. S. Tzanakakis, Scalable Stirred-Suspension Bioreactor Culture of Human Pluripotent Stem Cells. *Tissue Eng Pt A* 2010, *16*. 405-421, DOI: Doi 10.1089/Ten.Tea.2009.0454.
17. A. Faulkner-Jones, S. Greenhough, J. A. King, J. Gardner, A. Courtney, W. M. Shu, Development of a valve-based cell printer for the formation of human embryonic stem cell spheroid aggregates. *Biofabrication* 2013, *5*. DOI: Doi 10.1088/1758-5082/5/1/015013.
18. J. Park, C. H. Cho, N. Parashurama, Y. W. Li, F. Berthiaume, M. Toner, A. W. Tilles, M. L. Yarmush, Microfabrication-based modulation of embryonic stem cell differentiation. *Lab Chip* 2007, *7*. 1018-1028, DOI: Doi 10.1039/B704739h.
19. A. M. Bratt-Leal, R. L. Carpenedo, T. C. McDevitt, Engineering the embryoid body microenvironment to direct embryonic stem cell differentiation. *Biotechnol Prog* 2009, *25*. 43-51, DOI: 10.1002/btpr.139.
20. H. Kurosawa, Methods for inducing embryoid body formation: in vitro differentiation system of embryonic stem cells. *J Biosci Bioeng* 2007, *103*. 389-98, DOI: 10.1263/jbb.103.389.
21. M. Shafa, B. Day, A. Yamashita, G. Meng, S. Liu, R. Krawetz, D. E. Rancourt, Derivation of iPSCs in stirred suspension bioreactors. *Nat Methods* 2012, *9*. 465-6, DOI: 10.1038/nmeth.1973.
22. D. A. Fluri, P. D. Tonge, H. Song, R. P. Baptista, N. Shakiba, S. Shukla, G. Clarke, A. Nagy, P. W. Zandstra, Derivation, expansion and differentiation of induced pluripotent stem cells in continuous suspension cultures. *Nat Methods* 2012, *9*. 509-16, DOI: 10.1038/nmeth.1939.
23. Y. S. Hwang, B. G. Chung, D. Ortmann, N. Hattori, H. C. Moeller, A. Khademhosseini, Microwell-mediated control of embryoid body size regulates embryonic stem cell fate via differential expression of WNT5a and WNT11. *Proc Natl Acad Sci U S A* 2009, *106*. 16978-83, DOI: 10.1073/pnas.0905550106.
24. N. S. Hwang, S. Varghese, Z. Zhang, J. Elisseeff, Chondrogenic differentiation of human embryonic stem cell-derived cells in arginine-glycine-aspartate modified hydrogels. *Tissue Eng* 2006, *12*. 2695-2706, DOI: Doi 10.1089/Ten.2006.12.2695.
25. H. Park, J. S. Temenoff, Y. Tabata, A. I. Caplan, A. G. Mikos, Injectable biodegradable hydrogel composites for rabbit marrow mesenchymal stem cell and growth factor delivery for cartilage tissue engineering. *Biomaterials* 2007, *28*. 3217-3227, DOI: Doi 10.1016/J.Biomaterials.2007.03.030.
26. G. Orive, R. M. Hernandez, A. R. Gascon, R. Calafiore, T. M. S. Chang, P. De Vos, G. Hortelano, D. Hunkeler, I. Lacik, A. M. J. Shapiro, J. L. Pedraz, Cell encapsulation: Promise and progress. *Nat Med* 2003, *9*. 104-107, DOI: Doi 10.1038/Nm0103-104.
27. H. Liu, J. Lin, K. Roy, Effect of 3D scaffold and dynamic culture condition on the global gene expression profile of mouse embryonic stem cells. *Biomaterials* 2006, *27*. 5978-89, DOI:

10.1016/j.biomaterials.2006.05.053.
28. K. Y. Lee, D. J. Mooney, Alginate: properties and biomedical applications. *Prog Polym Sci* 2012, *37*. 106-126, DOI: 10.1016/j.progpolymsci.2011.06.003.
29. A. D. Augst, H. J. Kong, D. J. Mooney, Alginate hydrogels as biomaterials. *Macromol Biosci* 2006, *6*. 623-33, DOI: 10.1002/mabi.200600069.
30. W. Zhang, X. He, Encapsulation of living cells in small (approximately 100 microm) alginate microcapsules by electrostatic spraying: a parametric study. *J Biomech Eng* 2009, *131*. 074515, DOI: 10.1115/1.3153326.
31. Y. H. Lee, F. Mei, M. Y. Bai, S. Zhao, D. R. Chen, Release profile characteristics of biodegradable-polymer-coated drug particles fabricated by dual-capillary electrospray. *J Control Release* 2010, *145*. 58-65, DOI: 10.1016/j.jconrel.2010.03.014.
32. J. Kim, P. Sachdev, K. Sidhu, Alginate microcapsule as a 3D platform for the efficient differentiation of human embryonic stem cells to dopamine neurons. *Stem cell research* 2013, *11*. 978-989, DOI: 10.1016/j.scr.2013.06.005.
33. F. Chen, Y. Zhan, T. Geng, H. Lian, P. Xu, C. Lu, Chemical transfection of cells in picoliter aqueous droplets in fluorocarbon oil. *Anal Chem* 2011, *83*. 8816-20, DOI: 10.1021/ac2022794.
34. Y. Wu, I. C. Liao, S. J. Kennedy, J. Du, J. Wang, K. W. Leong, R. L. Clark, Electrosprayed core-shell microspheres for protein delivery. *Chem Commun (Camb)* 2010, *46*. 4743-5, DOI: 10.1039/c0cc00535e.
35. L. Zhang, J. Huang, T. Si, R. X. Xu, Coaxial electrospray of microparticles and nanoparticles for biomedical applications. *Expert Rev Med Devices* 2012, *9*. 595-612, DOI: 10.1586/erd.12.58.
36. D. B. Shenoy, A. A. Antipov, G. B. Sukhorukov, H. Mohwald, Layer-by-layer engineering of biocompatible, decomposable core-shell structures. *Biomacromolecules* 2003, *4*. 265-72, DOI: 10.1021/bm025661y.
37. T. Maguire, E. Novik, R. Schloss, M. Yarmush, Alginate-PLL microencapsulation: effect on the differentiation of embryonic stem cells into hepatocytes. *Biotechnol Bioeng* 2006, *93*. 581-91.
38. W. Zhang, S. Zhao, W. Rao, J. Snyder, J. K. Choi, J. Wang, I. A. Khan, N. B. Saleh, P. J. Mohler, J. Yu, T. J. Hund, C. Tang, X. He, A Novel Core-Shell Microcapsule for Encapsulation and 3D Culture of Embryonic Stem Cells. *J Mater Chem B Mater Biol Med* 2013, *2013*. 1002-1009, DOI: 10.1039/C2TB00058J.
39. X. Wang, W. Wang, J. Ma, X. Guo, X. Yu, X. Ma, Proliferation and differentiation of mouse embryonic stem cells in APA microcapsule: A model for studying the interaction between stem cells and their niche. *Biotechnol Prog* 2006, *22*. 791-800.
40. S. Sakai, I. Hashimoto, K. Kawakami, Production of cell-enclosing hollow-core agarose microcapsules via jetting in water-immiscible liquid paraffin and formation of embryoid body-like spherical tissues from mouse ES cells enclosed within these microcapsules. *Biotechnol Bioeng* 2008, *99*. 235-43.
41. S. Sakai, S. Ito, K. Kawakami, Calcium alginate microcapsules with spherical liquid cores templated by gelatin microparticles for mass production of multicellular spheroids. *Acta Biomater* 2010, *6*. 3132-7, DOI: 10.1016/j.actbio.2010.02.003.
42. C. Kim, S. Chung, Y. E. Kim, K. S. Lee, S. H. Lee, K. W. Oh, J. Y. Kang, Generation of core-shell microcapsules with three-dimensional focusing device for efficient formation of cell spheroid. *Lab Chip* 2011, *11*. 246-52, DOI: 10.1039/c0lc00036a.
43. P. Agarwal, S. Zhao, P. Bielecki, W. Rao, J. K. Choi, Y. Zhao, J. Yu, W. Zhang, X. He, One-step microfluidic generation of pre-hatching embryo-like core-shell microcapsules for miniaturized 3D culture of pluripotent stem cells. *Lab Chip* 2013, *13*. 4525-33, DOI: 10.1039/c3lc50678a.
44. Y. C. Tung, A. Y. Hsiao, S. G. Allen, Y. S. Torisawa, M. Ho, S. Takayama, High-throughput 3D spheroid culture and drug testing using a 384 hanging drop array. *Analyst* 2011, *136*. 473-8, DOI: 10.1039/c0an00609b.
45. J. Choi, P. Agarwal, H. Huang, S. Zhao, X. He, The crucial role of mechanical heterogeneity in regulating follicle development and ovulation with engineered ovarian microtissue. *Biomaterials* 2014, *35*. 5122-5128, DOI: DOI: 10.1016/j.biomaterials.2014.03.028.

46. D. R. Cole, M. Waterfall, M. McIntyre, J. D. Baird, Microencapsulated islet grafts in the BB/E rat: a possible role for cytokines in graft failure. *Diabetologia* 1992, *35*. 231-7.
47. P. de Vos, C. G. van Hoogmoed, B. J. de Haan, H. J. Busscher, Tissue responses against immunoisolating alginate-PLL capsules in the immediate posttransplant period. *J Biomed Mater Res* 2002, *62*. 430-7, DOI: 10.1002/jbm.10345.
48. W. Dou, D. Wei, H. Li, H. Li, M. M. Rahman, J. Shi, Z. Xu, Y. Ma, Purification and characterisation of a bifunctional alginate lyase from novel Isoptericola halotolerans CGMCC 5336. *Carbohydrate polymers* 2013, *98*. 1476-82, DOI: 10.1016/j.carbpol.2013.07.050.
49. J. C. Duarte, Lignin Biodegradation and Transformation. *Fems Microbiol Rev* 1994, *13*. 121-121, DOI: Doi 10.1016/0168-6445(94)90075-2.
50. D. M. Updegraf, Semimicro Determination of Cellulose in Biological Materials. *Anal Biochem* 1969, *32*. 420-&, DOI: Doi 10.1016/S0003-2697(69)80009-6.
51. C. K. Griffith, C. Miller, R. C. A. Sainson, J. W. Calvert, N. L. Jeon, C. C. W. Hughes, S. C. George, Diffusion limits of an in vitro thick prevascularized tissue. *Tissue Eng* 2005, *11*. 257-266, DOI: Doi 10.1089/Ten.2005.11.257.
52. M. Zhang, D. Methot, V. Poppa, Y. Fujio, K. Walsh, C. E. Murry, Cardiomyocyte grafting for cardiac repair: Graft cell death and anti-death strategies. *J Mol Cell Cardiol* 2001, *33*. 907-921, DOI: Doi 10.1006/Jmcc.2001.1367.
53. M. Amit, I. Laevsky, Y. Miropolsky, K. Shariki, M. Peri, J. Itskovitz-Eldor, Dynamic suspension culture for scalable expansion of undifferentiated human pluripotent stem cells. *Nature protocols* 2011, *6*. 572-9, DOI: 10.1038/nprot.2011.325.
54. R. Zweigerdt, R. Olmer, H. Singh, A. Haverich, U. Martin, Scalable expansion of human pluripotent stem cells in suspension culture. *Nature protocols* 2011, *6*. 689-700, DOI: 10.1038/nprot.2011.318.
55. M. P. Storm, C. B. Orchard, H. K. Bone, J. B. Chaudhuri, M. J. Welham, Three-dimensional culture systems for the expansion of pluripotent embryonic stem cells. *Biotechnol Bioeng* 2010, *107*. 683-95, DOI: 10.1002/bit.22850.
56. P. W. Burridge, S. Thompson, M. A. Millrod, S. Weinberg, X. A. Yuan, A. Peters, V. Mahairaki, V. E. Koliatsos, L. Tung, E. T. Zambidis, A Universal System for Highly Efficient Cardiac Differentiation of Human Induced Pluripotent Stem Cells That Eliminates Interline Variability. *PLoS One* 2011, *6*. DOI: DOI 10.1371/journal.pone.0018293.
57. S. J. Kattman, A. D. Witty, M. Gagliardi, N. C. Dubois, M. Niapour, A. Hotta, J. Ellis, G. Keller, Stage-Specific Optimization of Activin/Nodal and BMP Signaling Promotes Cardiac Differentiation of Mouse and Human Pluripotent Stem Cell Lines. *Cell Stem Cell* 2011, *8*. 228-240, DOI: Doi 10.1016/J.Stem.2010.12.008.
58. S. S. Y. Wong, H. S. Bernstein, Cardiac regeneration using human embryonic stem cells: producing cells for future therapy. *Regen Med* 2010, *5*. 763-775, DOI: Doi 10.2217/Rme.10.52.
59. P. B. Zhang, J. A. Li, Z. J. Tan, C. Y. Wang, T. Liu, L. Chen, J. Yong, W. Jiang, X. M. Sun, L. Y. Du, M. X. Ding, H. K. Deng, Short-term BMP-4 treatment initiates mesoderm induction in human embryonic stem cells. *Blood* 2008, *111*. 1933-1941, DOI: Doi 10.1182/Blood-2007-02-074120.
60. B. I. Jugdutt, Ventricular remodeling after infarction and the extracellular collagen matrix: when is enough enough? *Circulation* 2003, *108*. 1395-403, DOI: 10.1161/01.CIR.0000085658.98621.49.
61. K. Song, Y. J. Nam, X. Luo, X. Qi, W. Tan, G. N. Huang, A. Acharya, C. L. Smith, M. D. Tallquist, E. G. Neilson, J. A. Hill, R. Bassel-Duby, E. N. Olson, Heart repair by reprogramming non-myocytes with cardiac transcription factors. *Nature* 2012, *485*. 599-604, DOI: 10.1038/nature11139.
62. R. G. Gourdie, N. J. Severs, C. R. Green, S. Rothery, P. Germroth, R. P. Thompson, The spatial distribution and relative abundance of gap-junctional connexin40 and connexin43 correlate to functional properties of components of the cardiac atrioventricular conduction system. *Journal of cell science* 1993, *105 (Pt 4)*. 985-91.
63. L. F. Lemanski, Role of tropomyosin in actin filament formation in embryonic salamander heart cells. *The Journal of cell biology* 1979, *82*. 227-38.
64. Z. Ai, A. Fischer, D. C. Spray, A. M. Brown, G. I. Fishman, Wnt-1 regulation of connexin43 in cardiac myocytes. *The Journal of clinical investigation* 2000, *105*. 161-71, DOI: 10.1172/JCI7798.

Figure captions

Figure 1. A schematic illustration of the coaxial electrospray system for generating microcapsules with a liquid core and alginate hydrogel shell in one step using two aqueous fluids: (A), an overview of the complete setup; (B), a cross-sectional view of the coaxial needle design; and (C), typical size (outer diameter) distribution of cell-laden microcapsules generated using the coaxial electrospray system for this study.

Figure 2. Survival and proliferation of ES cells encapsulated in microcapsules with a hydrogel (A-F) and aqueous liquid (G-N) core: typical phase contrast and fluorescence micrographs showing high viability of all encapsulated ES cells on day 0 (A, D, G, and J), higher viability of ES cells in the liquid than hydrogel core on days 3 (B, E, H and K) and 7 (C, F, I, L, M and N), and the formation of either multiple ES cell aggregates of non-uniform size and irregular shape in hydrogel core (C and F) or single spheroidal aggregates of uniform size with high viability in liquid core (I-L and M-N) on day 7.

Figure 3. Pluripotency of ES cells obtained under three different culture conditions at the gene level: (A), qRT-PCR data of pluripotency genes showing that on average, the aggregated ES cells in miniaturized 3D liquid core have significantly higher expression of Klf2 and Nanog compared to the cells under 2D culture and significantly higher expression of klf2, Nanog, and Sox-2 compared to the cells cultured in 3D hydrogel core and (B), qRT-PCR data of typical germ layer markers and cardiac markers showing that ES cells from miniaturized 3D liquid core have significantly lower expression of Nestin (ectoderm), Sox-7 (endoderm), Brachyrury (or T, mesoderm), Nkx2.5 (early stage cardiac marker), and cTnT (late stage cardiac marker). *: $p < 0.05$

Figure 4. Expression of pluripotency protein markers of ES cells obtained under three different culture conditions: (A), typical flow cytometry peaks for the expression of Nanog, Oct-4, Sox-2 in ES cells from 2D culture (dark blue), 3D hydrogel core (red), and miniaturized 3D liquid core (green), compared to their corresponding controls (grey) and (B), quantitative analysis of mean intensity of the peaks given in panel A, showing cells obtained from miniaturized 3D liquid core have significantly higher expression of Nanog and Sox-2 on average than 3D hydrogel and 2D culture. *: $p < 0.05$

Figure 5. Directed cardiac differentiation of ES cells obtained under three different culture conditions induced by BMP-4 and bFGF: (A), cumulative percentage of beating foci on each day showing that the aggregates from the miniaturized 3D liquid core has significantly more beating foci than that from 3D hydrogel and 2D culture; (B), typical phase contrast image of a spontaneously beating aggregate (Movie S1) with the beating boundary being indicated by dotted line; (C), qRT-PCR data showing relative expression of cardiac specific genes (Nkx2.5 and cTnT) and mesoderm gene marker (brachyury or T) after directed differentiation into the cardiac lineage: The data were after normalizing to the corresponding expression before differentiation for the three different culture conditions shown in Fig. 3B and the values for relative expression before differentiation are therefore all one; and (D), flow cytometry data showing ~17%, 33.4%, and 42.2% of cells differentiated from ES cell aggregates obtained under 2D, 3D hydrogel, and miniaturized 3D liquid culture, respectively, are positive for cTnT, a protein marker specific of cardiomyocytes. *: $p < 0.05$ and **: $p < 0.01$