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Technical Notes

Establishing a Dynamic Process for the Formation, Propagation, and Differentiation of Human Embryoid Bodies

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The promise of human embryonic stem cells (hESCs) to provide an unlimited supply of cells for cell therapy depends on the availability of a controllable bioprocess for their expansion and differentiation. We describe here a robust and well-defined scale up platform for human embryoid body (EB) formation, propagation, and differentiation. The efficacy of the dynamic process as compared to the static cultivation in Petri dishes was analyzed. Our optimized conditions include specific bioreactor and impeller type, seeding and propagation parameters, and scale up. Quantitative analyses of viable cell concentrations, apoptosis percentages, and EB yield revealed 6.7-fold enhancement in the generation of hESC-derived cells after 10 cultivation days. Other metabolic indices such as glucose consumption, lactic acid production and pH all pointed to efficient cell expansion in the dynamic cultures. The hydrodynamic conditions during seeding and cultivation were found to be crucial for the EB formation and propagation. The EBs' prearrangement in the static system and EB cultivation in the Glass Ball Impeller spinner flask resulted in high EB yield, a round homogenous shape, and the fastest growth rate. The appearance of representative genes of the three germ layers as well as primitive neuronal tube organization and blood vessel formation indicated that the initial developmental events in the human EBs are not interfered by the dynamic system. Furthermore, well developed endothelial networks and contracting EBs with functional cardiac muscle were also obtained after two cultivation weeks. Collectively, our study defines the technological platform for the controlled large-scale generation of hESC-derived cells for clinical and industrial applications.

Introduction

Human embryonic stem cells (hESCs) are pluripotent cells that can potentially differentiate into all existing cell types in the adult body [1,2]. However, the promise of hESCs is halted by the limited supply of cells for cellular therapy, which greatly depends on the availability of a controlled large-scale bioprocess. To realize the promise of ESC-based therapy, the culture volumes utilized in basic biology of few milliliter must be adapted to a clinically relevant scale of up to hundreds of liters [2].

In vitro hESC differentiation commonly requires an initial spontaneous formation of spherical cell clusters in suspension, called embryoid bodies (EBs). The developing EBs are composed of the cellular derivatives of all three primary germ layers—the endoderm, the mesoderm, and the ectoderm [3]. Standard methods for EB generation include hanging drop, liquid suspension, and methylcellulose culture [4]. These culture systems maintain a balance between ESC aggregation necessary for EB formation and prevention of EB agglomeration. EB agglomeration has previously been reported to have negative effects on cell proliferation and differentiation, as well as causing extensive cell death [4,5]. However, these methods are limited as far as industrial purposes are concerned due to their complexity and difficult manageability.

Stirred suspension culture systems offer attractive advantages of ready scalability and relative simplicity. Their relatively homogeneous nature make them uniquely suited for a controlled culture environment (e.g., O2 tension, pH, shear forces, medium exchange rate, etc.) [2]. The simpler process in spinner flasks equipped with paddle-impeller result in the formation of large mouse ES cell (mESC) agglomerates within a few days [4,6,7]. Attempts to increase the stirring rate so as to

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avoid agglomeration resulted in massive hydrodynamic damage to the mESCs due to extensive mixing in the vessels [8]. Methods for controlling EB formation and cell proliferation in vitro, involve mass encapsulation of ESCs in defined conditions (i.e., number of cells per EB and capsule size) such as Ca-alginate [9], agarose [10] and Matrigel [11]. Agglomeration was also avoided by performing the mouse EBs in Petri dishes for several days prior to transferring them onto spinner flasks [12], the rotation culture system of Petri dishes rotated on a horizontal rotation device [13], rotary suspension culture in disks on an orbital rotary shaker [14], or by direct seeding of the mESCs into a spinner flask equipped with a glass boll bulb-shaped impeller [7,15] and 2L Stirred Tank bioReactor (STR) equipped with a newly developed pitched-blade turbine impeller [7]. Another bioreactor that allows agglomeration-free EB formation is the slow turning lateral vessel (STLV) developed by National Aeronautics and Space Administration [5,16] which is characterized by EB immobility in space, due to an extremely low fluid shear stress (shear level of 10^{-2} dyne/cm^2) and oxygenation by diffusion [17,18].

Differentiation methods for ESC-derived cells are usually based on developing designated media composition and/or gene manipulation. Shear stress can be used as a complementary differentiation method for some differentiation pathways, such as cardiomyocytic and vascular cell differentiation.

In this paper we established the dynamic bioprocess parameters for the formation and propagation of hESC-derived cells that maintain their differentiation potential, in the scalable and well-known STR. Cell-seeding concentration, EB nucleation parameters, culture duration and mixing types are described in detail so as to serve as a scalable technological platform. Furthermore, an examination of the influence of dynamic culture on the differentiation potential of hESCs in an EB model is described.

Materials and Methods

Culture of hESCs

Undifferentiated hESCs [19] (line H9.2, passages 38–61) were cultured on inactivated mouse embryonic fibroblast (MEF) layer with culture medium consisting of 80% KnockOut® DMEM, 20% (v/v) KnockOut® SR, 1 mM glutamine, 0.1 mM mercaptoethanol, 1% nonessential amino acids stock, and 4 ng/mL basic fibroblast growth factor (all from Gibco Invitrogen Corporation, Grand Island, NY). In these conditions, most cells (~95%) were maintained in an undifferentiated state. The medium was changed daily. The cells were passaged every 4–6 days by 0.2% collagenase treatment followed by cautious mechanical dissociation, resulting in small aggregates, which were reseeded on freshly prepared feeder layers.

EB propagation in dynamic systems

In order to induce EB formation, confluent undifferentiated hESCs were dispersed into small clumps (as described above). They were then seeded at an initial concentration of $0.7 \pm 0.1 \times 10^6$ viable hESCs/mL in a static Petri dish for 2 days to allow early EB formation, with initiation nucleation foci (INF) size of a few hundred cells each.

The 2-day-old EBs were seeded into the following dynamic systems: (1) 55-mL STLV bioreactor (Synthecon, Cellon S.A. Bereldange, Luxembourg), (2) 125 mL Erlenmeyer (Corning Incorporated, Corning, NY), (3) 250 mL Glass Ball Impeller (GBI) spinner flask (CellSpin of Integra Biosciences, Fernwald, Germany), and (4) 100 mL Paddle-Impeller (PI) spinner flask (Bellco,Vineland, NJ). As control, EBs were seeded into static nonadherent 19.6-cm^2 Petri dishes (Miniplast, Ein Shemer, Israel). For operation parameters (i.e., working volume, mixing rates, etc.) see Table 1.

The EBs were grown in medium consisting of 80% KO-DMEM, supplemented with 20% fetal calf serum (Biological Industries, Beit Haemek, Israel(, 1% penicillin–streptomycin (Sigma, St. Louis, MO), 1 mM l-glutamine, and 1% nonessential amino acid stock (all from Gibco Invitrogen Corporation, Grand Island, NY). Two independent experiments were performed at each setting, in duplicates. EB formation and propagation was followed by daily visual observation and light microscopy for up to 10 days.

Table 1. Characterizations of EB-Cultured Systems

Culture system	*System type*	*Total volume (mL)*	*Working volume (mL)*	*Mixing rate (rpm)*	*Mixing EB method*	*Shear forces level*
Erlenmeyer	Stirred	125	25	105 ± 10	Orbital shaking	Mild
Spinner flask equipped with double glass boll bulb-shaped impeller (GBI)	Stirred	250	55	75[a]	Agitation	Mild
Spinner flask equipped with paddle-impeller (PI)	Stirred	100	55	105 ± 10	Agitation	Considerable
Static system—19.6-cm^2 Petri dish	Static	10	8	0	Static	Insignificant
Slow turning lateral vessel (STLV)	Dynamic with immobilized EBs	55	55	16	Static	Insignificant

[a]Maximum agitation rate available.

EB and cell concentration, size, and morphology

In order to determine the EB concentration, 1 mL of homogenously suspended EBs was removed to five wells of a 96-well plate (200 µL each). The number of EBs in each of the five wells was counted with light microscopy . To obtain a single cell suspension, 1 mL of homogenously suspended EBs was incubated for 10 min with 0.5% Trypsin-EDTA (Gibco) and resuspended to single cells. The specific growth rate was calculated by logarithmic integration, as shown by the slope of the line in the figure, on the accelerated growth phase of each growth curve (not including the lag and stationary growth phases).Viability of the culture was determined by 0.4 M trypan-blue staining (Sigma). Cell concentration was measured by Coulter Counter Z2 (Beckman, USA) and by DNA contents. All analyses were taken in duplicates.

For EB size and morphology analysis, samples were transferred into culture dishes and analyzed using inverted light microscopy. The average diameter of 20 EBs in each system was calculated.

Apoptosis detection

Apoptosis in 10-day-old EBs was detected using the In Situ Cell Death Detection Kit, AP (Roche Diagnostics GmbH, Mannheim, Germany). Percentage of the apoptotic cells was detected by incubating 1-mL EB pellet for 15 min with 0.5% Trypsin-EDTA (Gibco), followed by treatment with 4-mL Hanks solution, and fixation of the single cells on dry slides prior to using the kit. Apoptotic cells were counted using the inverted Zeiss Axiovert 200 fluorescent microscopy. All systems were sampled with duplicates of ~1,000 cells each. Location of the apoptotic cells inside whole EBs was detected by Nikon Eclipse E600 confocal microscopy, after allowing the EBs to attach to eight-well slides and staining with 150 µL/well. Slides were stored at 2–8°C before analyzing.

Immunohistochemistry

For histological analysis 10-day-old EBs were fixed in 10% neutral-buffered formalin, dehydrated in graduated alcohols (70–100%), and embedded in paraffin. For general histomorphology, 1–5 µm sections were stained with hematoxylin/eosin. Immunostaining was performed after deparaffinization, using Dako LSAB+ staining kit for ectoderm (β-3-tubulin 1:500, Chemicon International, Temecula, CA), mesoderm (CD34 1:20), and endoderm (α-fetoprotein 1:20) (both from DakoCytomation, Glostrup, Denmark). As controls, both IgG isotype and secondary antibody staining were performed. The secondary antibody was conjugated to peroxidase.

For fluorescent immunostaining of endothelial network formation and cardiac differentiation EBs were fixed with 4% paraformaldehyde, 1% tryton, and 1%tryton+2%NGS in PBS prior to exposure to CD31 (1:20; DakoCytomation, Glostrup, Denmark) and troponin I (1:800; Chemicon International, Temecula, CA) primary antibodies, overnight at 4°C. Cys 3 antibodies (Chemicon International) were used as secondary antibodies (1:50). Embryoid bodies stained for CD31 were also stained with TO-PRO-3 (1:500) for nuclease staining. Detection was analyzed using the Nikon Eclipse E600 confocal microscopy. EBs stained for troponinantibody troponin I were also stained with DAPI (1:1,000) (Boehringer, Mannheim, Germany) for nuclease staining and examined with Zeiss Axiovert 200 fluorescent microscope.

RT-PCR analysis

Total RNA was isolated from 2-, 4-, 6-, 8-, and 10-day-old EBs grown in different culture systems and from undifferentiated hESCs, using Tri-Reagent (Sigma-Aldrich), according to the manufacturer's recommended protocol. Complementary DNA was synthesized from 1 mg total RNA using M-MLV reverse transcriptase RNase H minus (Promega, Madison, WI). Polymerase chain reaction (PCR) included denaturation for 5 min at 94°C followed by repeated cycles of 94°C for 30 sec, annealing temperature for 30 sec, and extension at 72°C for 30 sec. Polymerase chain reaction primers and reaction conditions used are described in Table 2. Polymerase chain reaction products were size-fractionated using 2% agarose gel electrophoresis. DNA markers were used to confirm the size of the resultant fragments.

Real-time RT-PCR analysis

Total RNA was isolated from duplicate samples with Tri-Reagent (Sigma, St. Louis, MO) and 1 µg of total RNA was used for reverse transcription by M-MLV RT (Promega, Madison, WI). Real-Time PCR reactions were described under Supplement. performed using the 7000 Sequence Detection system in triplicate for each one of both samples. All the reactions were performed using TaqMan universal PCR master mix and primers from TaqMan gene expression assay (both from Applied Biosystems, Branchburg, NJ). The primers used are: α-myosin heavy chain (α-MHC) (Cat No HS00411908_m1), vascular endothelial (VE)-cadherin (Cat No HS00174344_m1), β-actin (Cat No HS99999903_M1). Each reaction well contained 5% of the complementary DNA sample (produced from 1 µg RNA). Relative quantification of gene expression was performed with the "7000 system SDS" software using β-actin as the internal control gene for normalization. The calculation of the relative quantification by this program was done by the $2^{-\Delta\Delta Ct}$ method.

FACS analysis

Single cells were received by EB dissociation with EDTA splitting solution (1.5 h, 37°C), followed by mechanical disruption and isolation through a 45-µm mesh. Fluorescence-activated cell sorting (FACS) analysis to phycoerythrin -conjugated VE-Cadherin/CD144 (R&D Systems Inc., Minneapolis, MN) and CD31 were performed according to the manufacturer's instructions.

Karyotype analysis

Cell division was blocked in mitotic metaphase using colcemid-spindle formation inhibitor (karyoMax colcemid solution; Gibco Invitrogen). Nuclear membranes were broken after hypotonic treatment. For chromosome

Table 2. PCR Reaction Conditions

Gene product	*Forward (F) and reverse (R) primers (5′ → 3′)*	*Reaction condition*	*Size (base pairs)*
Oct-4	F: GAGAACAATGAGAACCTTCAGGA R: TTCTGGCGCCGGTTACAGAACCA	25 cycles at 55°C in 1.5 mM $MgCl_2$	219
Rex 1	F: GCGTACGCAAATTAAAGTCCAGA R: CAGCATCCTAAACAGCTCGCAGAAT	28 cycles at 56°C in 1.5 mM $MgCl_2$	306
NF-68KD	F: GAGTGAAATGGCACGATACCTA R: TTTCCTCTCCTTCTTCACCTTC	25 cycles at 60°C in ReddyMix (ABgene, UK)	473
NEUROD1	F: CCTCGAAGCCATGAACGCAG R: GCTGTCCATGGTACCGTAAG	35 cycles at 60°C in ReddyMix (ABgene, UK)	583
CMP	F: ACGGCTGACTTCAAGACCAT R: TCAATAGGCACACCCAGACA	32 cycles at 60°C in ReddyMix (ABgene, UK)	313
CD34	F: CAACACGTGGTGGCTGATAC R: TCAAAGCTTCCTGGGAGAAA	35 cycles at 60°C in 1.5 mM MgCl2	428
Glucagon	F: AGGCAGACCCACTCAGTGA R: AACAATGGCGACCTCTTCTG	40 cycles at 55°C in ReddyMix (ABgene, UK)	308
GLUT2	F: AGGACTTCTGTGGACCTTATGTG R: GTTCATGTCAAAAAGCAGGG	32 cycles at 55°C in ReddyMix (ABgene, UK)	231

visualization, we used G-band standard staining (Giemsa; Merck, Darmstadt, Germany). The karyotypes were analyzed and reported according to the International System for Human Cytogenetic Nomenclature. At least 10 cells were examined from each sample; two samples per system.

Cardiomyocytic spontaneous differentiation

EBs from each system were seeded onto four wells in a six-well plate coated with gelatin (10–30 EBs/well) and allowed to attach, in an attempt to accelerate the cardiomyocytic spontaneous differentiation [20,21]. Two seeding days were tested: (1) 4-day-old EBs and (2) 7-day-old EBs. The EB medium was replaced every second day and contracting EBs were counted. [Ca^{2+}]i transients and length changes of spontaneously contracting EBs were measured by means of Fura-2 fluorescence and video edge detector, respectively, as previously described [22].

Statistical analysis

Results were expressed as mean ± SD. The means of two experiments were compared using Student's *t*-test for unpaired observations. A value of $p < 0.05$ was considered significantly different.

Results

Seeding conditions of hEB formation

Previous studies indicated that hESCs seeded into a spinner flask or a stirred bioreactor can quickly agglomerate into single pellets depending on the shearing forces in the dynamic system [4,5,7]. Additionally, in contrast to mEBs, hEBs cannot emerge from single cells due to low clonality and require an INF in order to start the normal EB formation process [5,10,20,21]. Hence, EB formation efficiency is determined by the size and quantity of the INF at seeding and the shear forces in the culture system throughout the seeding and propagation period. Therefore, in order to determine the seeding conditions undifferentiating hESCs were removed from their feeder layer and cultured in 5 culture systems (see Table 1).

Three seeding parameters were tested: shear force at seeding, cell-seeding concentration and INF size. To study the shear force's impact, stirred systems were seeded either directly after removal of the undifferentiated hESCs from the MEF feeder layer or after 1, 2, 4, and 6 days of EB formation in the Petri dish. We observed that direct seeding of hESCs into the dynamic cultures resulted in extensive EB agglomeration and low EB concentration, contrary to what occurred in the STLV bioreactor and Petri dish which present systems without shear forces (Fig. 1A). Seeding into the same stirred systems 1-day-old EBs formed in the Petri dish resulted in low EB yield at day 10 of cultivation (data not show). However, high and stable EB yield was established by indirect seeding, which included a 2-day cultivation period in the Petri dish prior to seeding into the stirred systems.

The effect of the initial cell concentration at seeding on the EB formation yield and final cell density was determined by seeding the hESCs in various initial concentrations: 0.15, 0.3, 0.4, 0.6, 0.8, 1.0×10^6 viable hESCs/mL into the Petri dish. All initial cell concentrations, other than the 0.15×10^6, resulted in EB formation after 24 h. Increasing the initial cell concentration up to 0.8×10^6 viable hESCs/mL yielded a higher EB concentration and higher final cell density. However, further increase of the initial cell concentration did not increase the EB concentration (data not shown). Thus, the most efficient seeding concentration was 0.8×10^6 viable hES cells/mL.

Determining the INF size was established in two stirred systems (Erlenmeyer and GBI spinner flask), seeded 2 days after EB formation in Petri dish. Undifferentiated ESC colonies were removed from the MEF feeder layer and seeded into Petri dishes at a seeding concentration of $0.7 \pm 0.1 \times 10^6$ viable hESCs/mL. Various INF aggregate

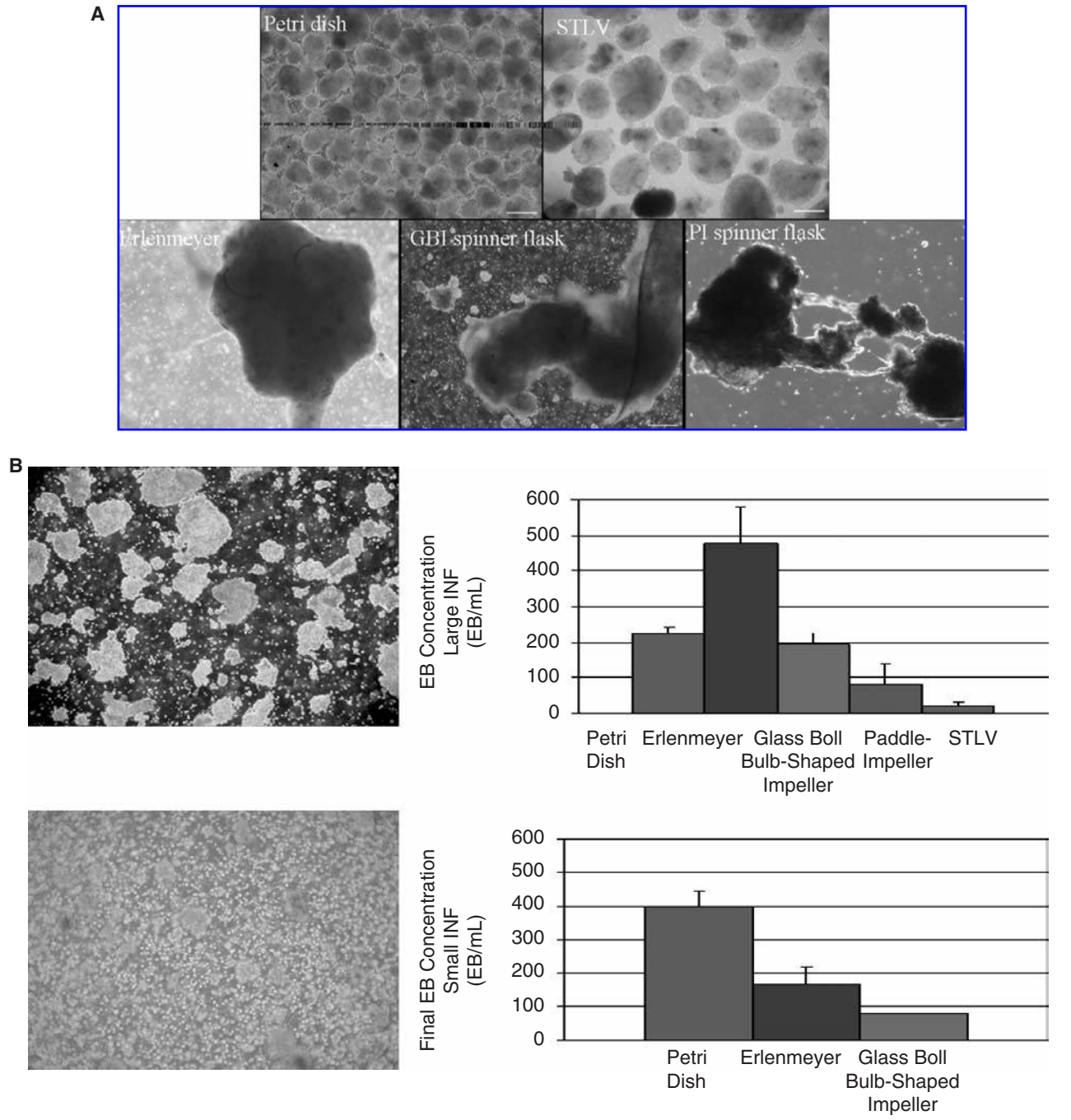

FIG. 1. hEB seeding conditions in stirred systems versus STLV and static systems. (**A**) EB formation determined by the shear forces applied at seeding. Phase contrast image of 2-day-old EBs seeded directly into the different culture systems. (**B**) Final concentration of 10-day-old EBs cultured in the GBI spinner flask (seeded at day 2) determined by initiation nucleus foci (INF) size: (**A**) large INF established by gentle mechanical breakdown with a pipette 300 μm in diameter; (**B**) small INF established by intensive mechanical breakdown with a pipette 100 μm in diameter. Culture systems seeded with $0.7 \pm 0.1 \times 10^6$ viable ESCs/mL. The results shown were obtained from two different experiments performed in duplicate. Scale bar = 300 μm.

sizes were obtained by mechanical breakdown of hESC colonies, controlled by pipette diameter. Comparison was performed between small INF (average of 5 ± 3 cells each) with high concentrations of single cells, received by intensive mechanical breakdown with a pipette 100 μm in diameter, and large INF (average of $1{,}445 \pm 115$ cells each) with a small amount of single cells, received by gentle mechanical breakdown with a pipette 300 μm in diameter.

After 10 days of cultivation at mild shear forces stirred systems (Erlenmeyer and GBI spinner flask), the large INF size experimental group yielded an 3-fold higher EB concentration as compared to the small INF size group (Fig. 1B). Furthermore, the Erlenmeyer demonstrated a stable EB concentration during the cultivation period: 489 ± 22 EBs/mL at seeding versus 479 ± 98 EBs/mL after 10 days (data not shown). However, when seeded into the Petri dish, EB concentrations in the small INF group was found 2-fold higher as compared to the experimental group with large INF size. The lowest final EB concentration was observed with the PI spinner flask (84 ± 56 EB/mL) and the STLV bioreactor (23 ± 19 EB/mL).

Consequently, the optimized seeding process for stirred systems consisted of three main conditions: (1) 2 days of EBs prearranged in static conditions prior to seeding into a stirred system; (2) a seeding concentration of $0.7 \pm 0.1 \times 10^6$ viable hESCs/mL; and (3) an INF size of a few hundred cells each. These seeding conditions yielded high EB concentrations without agglomeration.

EB size distribution

In order to evaluate the hypothesis that EB concentration, shape and size can be controlled by shear forces, we examined two system types: stirred systems with different stirring patterns and shear stress (PI and GBI spinner flasks and shaking Erlenmeyer) and the STLV bioreactor and static Petri dish which does not subject the EBs to any shear forces.

Two stirred systems, the Erlenmeyer and IGB spinner flask, revealed relatively homogenous and round EBs at day 10 of cultivation with diameters ranging between 375 ± 93 μm and 358 ± 135 μm, respectively. In contrast, EBs grown in the Petri dish and STLV bioreactor were heterogeneous, and had nonround shapes with diameter ranging between 564 ± 223μm and 491 ± 243 μm, respectively (Fig. 2). The PI spinner flask also exhibited large heterogeneous EBs (488 ± 193 μm) in correlation to the low final EB concentration presented above.

Thus, our Erlenmeyer and GBI spinner flask, which presented stirred systems with mild shear stress, provided small and more homogenous EBs, without agglomeration, due to the gentle shear forces implemented on the EBs during cultivation. These results correlate to previously published data on mEB formation in the GBI spinner flask [7].

Growth kinetics, viability, and apoptosis

Specific growth rate, final viable cell concentration, viability and apoptosis were analyzed in the stirred systems compared to the STLV and the control static system. Seeding

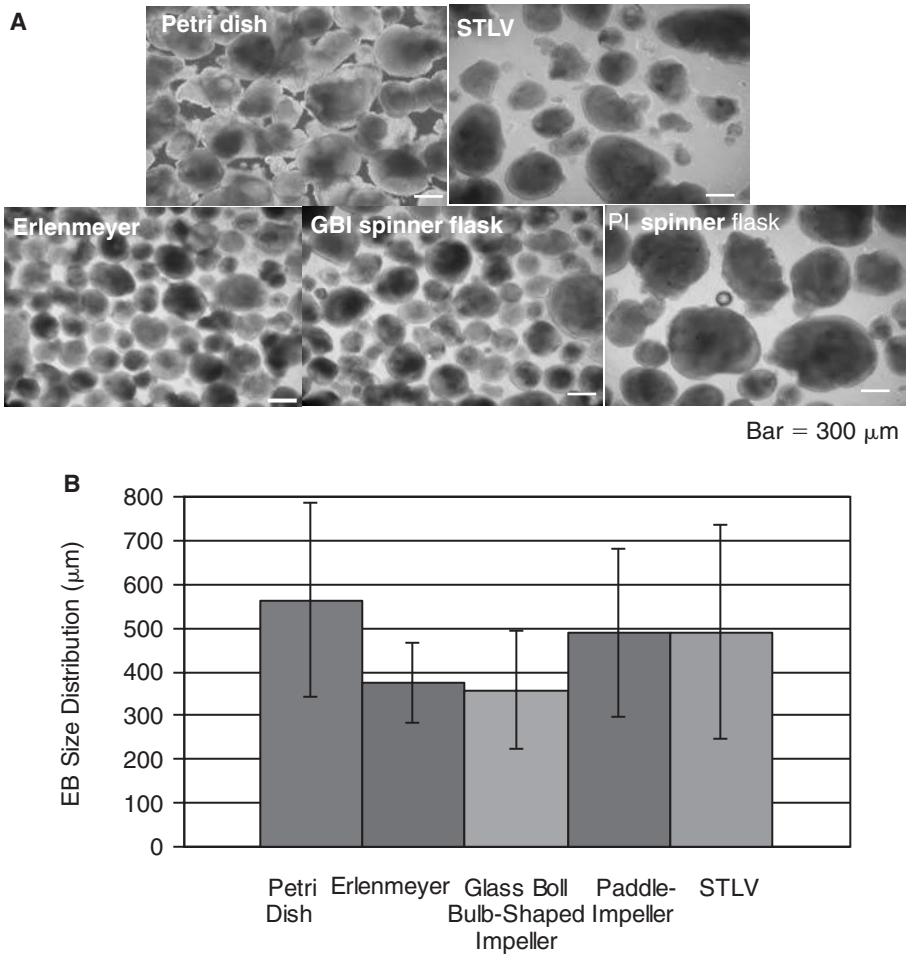

FIG. 2. hEB size distribution in stirred systems versus STLV and static systems. (**A**) Light microscopy analysis of 10-day-old EBs in the static Petri dish, STLV bioreactor, shaking Erlenmeyer, and stirred GBI and PI spinner flask. (**B**) Size distribution of 10-day-old EBs. Note the relatively homogenous small and round EBs established in the Erlenmeyer and GBI spinner flask. Culture systems seeded with large INF; $0.7 \pm 0.1 \times 10^6$ viable ESCs/mL. EBs seeded into the stirred systems were allowed 2 days of EB formation in the static Petri dish prior to seeding. The results shown are mean values (±SD) of samples obtained from two different experiments performed in duplicate. Scale bar = 300 μm.

conditions were $0.7 \pm 0.1 \times 10^6$ hESCs/m with INF size of a few hundred cells each. The STLV and the static Petri dish were seeded directly while the stirred systems were seeded after 2 days of EB formation in static conditions. EB media were replaced (80 ± 5%) every second day up to day 10 of cultivation. At every medium replacement two homogenously suspended EB samples (1 mL each) were trypsinized into single cells and cell concentration and viability were analyzed, twice for each sample. Experiments were run in duplicate and the results are presented as mean values (±SD). Figure 3A illustrates the formation phase of 2-day-old EBs in the Petri dishes, before seeding into the stirred systems. The GBI spinner flask provided the highest viable cell concentration of 5.0×10^6 viable cell/mL and the fastest specific growth rate of 2.3/day during the cultivation period. The Erlenmeyer reached 3.7×10^6 viable cell/mL with specific growth rate of 1.9/day, while the PI spinner flask yielded the lowest viable cell concentration of 1.7×10^6 cell/mL with the slowest specific growth rate of 0.53/day amongst the stirred systems. The STLV also presented low viable cell concentration (1×10^6 cell/mL) and the slowest specific growth rate of 0.3/day. The control Petri dish reached 3.2×10^6 viable cell/mL with a specific growth rate of 1.4/day. Both the GBI spinner flask and Erlenmeyer, as well as the controlled static system, allowed accelerated growth phase up to day 8 of cultivation followed by a stationary phase (Fig. 3A). Consequently, the GBI spinner flask supported the highest expansion fold (6.4) during the 10-day cultivation period, while the Erlenmeyer reached 4.8 expansion fold and the control Petri dish obtained 4.0 expansion fold. The PI spinner flask exhibited expansion folds of 2.2 while the STLV showed the lowest expansion fold of 1.2 (Fig. 3B). Metabolism analysis detected no glucose and glutamine limitations or lactate and ammonia inhibition within the entire cultivation period (data not shown).

The viability in the Erlenmeyer and GBI spinner flask was 90 ± 1% during the cultivation period similar to the Petri dish, while viability in the STLV and the PI spinner flask slightly declined during the first days to 80% and stabilized (Fig. 3C). LDH analysis shows high levels during the first two EB formation days, which were caused by death of MEFs and single ESCs. During the next 8 days the LDH concentration level remained low in correlation to the viability results (data not shown).

At the end of the propagation phase, cell apoptosis percentages of hESC-derived cells were analyzed (1,000 cells/sample). In the entire system <4% of the cells were apoptotic, except for the STLV that presented 8% apoptotic cells (Fig. 3D). Apoptosis locations in whole EBs were analyzed by immunfluorescence of entire EBs, and visualized by confocal microscopy. Ten EBs screened at each system showed random apoptotic locations, consistent with the quantitative apoptotic results, indicating no critical limitation in mass transfer (Fig. 3E). Karyotype analysis of representative EB samples from the three stirred systems, the static system, and undifferentiated hESCs, demonstrated that the cell karyotype remained stable during the whole experiment.

Our results show that EBs cultured in the GBI spinner flask underwent a 5-fold increase in the final viable cell number, compared to the STLV and 1.7-fold compared to the static control system, while viability and the survivability (measured by apoptosis) remained high. Furthermore, the cell growth curve of the GBI spinner flask had the fastest growth rate and constant logarithmic phase, without lag phases, indicating a stable culture environment compared to the control. In contrast, the PI spinner flask presented poor results compared to the control systems, correlating with published data for mEB [4,7].

EB differentiation potential into the three germ layers

Due to low growth potential in the PI spinner flask, the differentiation potential was not analyzed. Hence, differentiation was analyzed through two stirred systems, which induced shear stress on the developing EBs, in comparison to the STLV and static petri dish, where the developing EBs were not exposed to any shear stress.

Expression during the cultivation of representative genes of each of the three germ layers was further compared by reverse transcriptase (RT)-PCR. After 10 days in culture, EBs from both stirring systems expressed genes from the three germ layers. Furthermore, it appeared that faster reduction in the expression of the embryonic transcription factors Oct-4 and Rex -1 was observed in the stirred systems, compared to the static Petri dish system (Fig. 4A), while continuous increase of all differentiation genes was detected.

For detailed morphological examination of cells derived from the three germ layers, histological sections from 10-day-old EBs formed in both stirred systems were stained and compared to EBs formed in the STLV and the conventional static Petri dish system. Expression of β-tubulin III in neuronal tubes (ectoderm), CD34-positive cells surrounding blood vessels (mesoderm), and α-fetoprotein production (early endoderm) indicated a broad differentiation potential (Fig. 4B). Morphological analysis of the CD34-positive cells in the stirred systems showed formation of epithelial cells surrounding large primitive blood vessels, whereas examination of the β-tubulin III positive cells presented large neuronal rosettes.

Cardiac differentiation potential

To further validate the benefit of the stirring processes, we tried to generate ESC-derived cardiomyocytes. Since cardiomyocytic differentiation can be affected by mechanical forces [23,24].

Removing 4-day-old EBs from the culture systems and allowing spontaneous cardiomyocytic differentiation, revealed ~35% spontaneously contracting EBs from the GBI spinner flask and 18% spontaneously contracting EBs from Erlenmeyer and the Petri dish, after 15 differentiation days. The same experiment with 7-day-old EBs revealed lower contracting EB percentages during the same 15 differentiation days, although the GBI spinner flask showed the same advantage toward cardiomyocytic differentiation over all other systems (Fig. 5A).

Quantitative evaluation by real-time RT-PCR of the cardiac specific α-MHC transcripts in the suspended EB in the stirred systems during 10 cultivation days showed a clear increase after day 4 within all systems but the GBI spinner

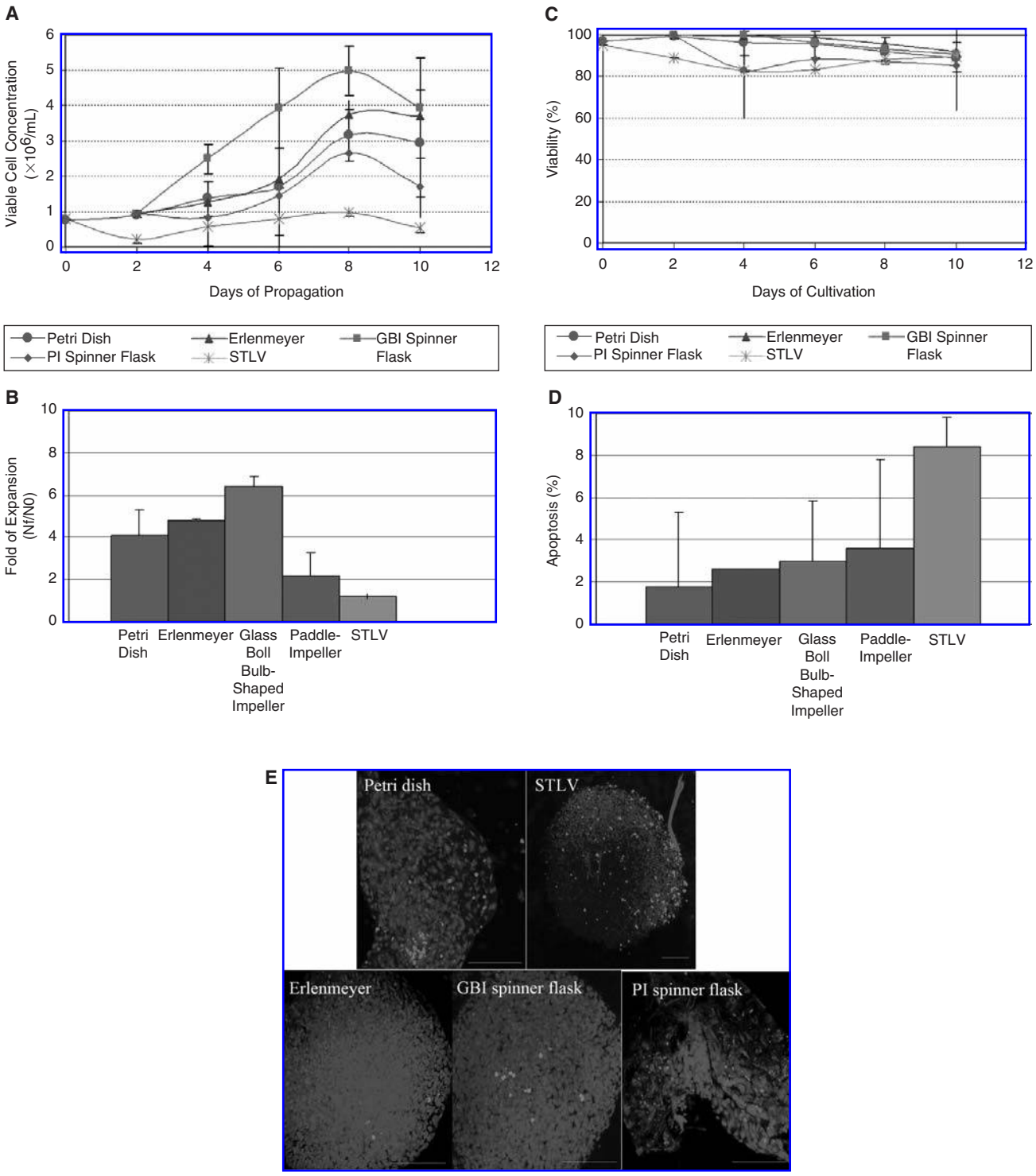

FIG. 3. Growth kinetics, viability, and apoptosis in stirred systems versus STLV and static systems. (**A**) Viable cell concentration; (**B**) folds of expansion of hEBs; (**C**) viability during 10 cultivation days; (**D**) survival potential by apoptosis percentage in 10-day-old EBs at the end of cultivation; and (**E**) Apoptosis locations in whole EBs analyzed by immunfluorescence of entire EBs and visualized by confocal microscopy. Note that the GBI spinner flask shows the longest accelerated growth phase, without lag or stationary growth phases, which lead to highest viable cell concentration and highest fold of expansion. Both viability and survivability remained high at the Erlenmeyer and GBI spinner flask during the entire cultivation period, whereas the STLV presented high apoptosis. Systems seeded with large INF; $0.7 \times 0.1 \times 10^6$ viable ESCs/mL. EBs seeded into the stirred systems were allowed 2 days of EB formation in the static Petri dish prior to seeding. The results shown are mean values (±SD) of samples obtained from two different experiments performed in duplicate. Scale bar = 50 μm.

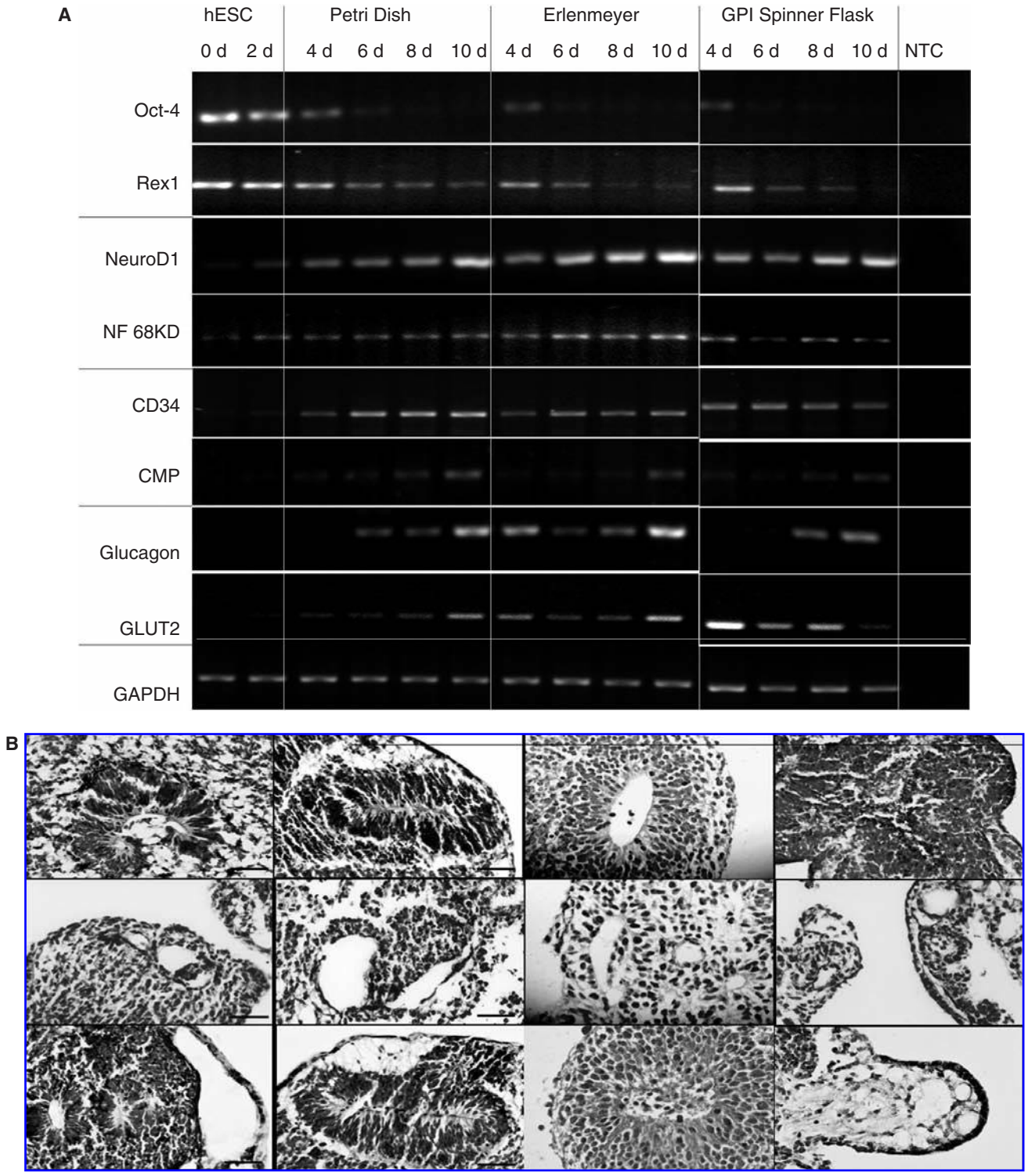

FIG. 4. Differentiation potential of hEBs in stirred systems versus STLV and static systems. (**A**) RT-PCR analysis of EBs at static and stirred systems demonstrated the expression of genes of the three germ layers in both the Erlenmeyer and the GBI spinner flask versus the static conventional system, during cultivation, along with the vanishing of the undifferentiated genes. No template (NTC) reaction for each gene is shown on the right. STLV gene expression pattern was previously reported by our laboratory [6]. No significant differences were observed in the pattern of gene expression between the stirred, the STLV, and the static culture. (**B**) Immunostaining of tissues derived from the three germ layers in the differentiating 10-day-old EBs in both stirred systems, the STLV and the static system: primitive neuronal tubes positive for β-tubulin III (ectoderm); blood vessels stained with anti-CD34 (mesoderm) and α-fetoprotein production (endoderm). Scale bar = 50 μm.

flask presented double the quantities as compared to the static system (400 vs. 200 RQ). Ten days after seeding the GBI spinner flask showed α-MHC 3-fold higher, correlating to the high percentage of contracting EBs, while both stirred systems showed an obvious advantage over the static petri dish (Fig. 5B).

hESC-derived cardiomyocytes express cardiac-specific troponin I (cTnI), a subunit of the troponin complex that provides a calcium-sensitive molecular switch for the regulation of striated muscle contraction, which can be detected only in the beating regions of the culture. In order to examine the morphology of the beating regions we determined a representative cTnI-positive area within EBs of both stirring systems compared to the control static system (Fig. 5C).

According to Dolnikov and colleagues, the functional properties of hESC-derived cardiomyocytes can be investigated by recording simultaneous traces of [$Ca2^+$]i transients and length changes of spontaneously contracting EBs, raised in suspension [22]. We found that hESC-derived cardiomyocytes from the stirring system displayed similar temporally related [$Ca2^+$]i transients and contractions compared to hESC-derived cardiomyocytes from the Petri dish. In both systems, the EBs beat spontaneously at a rate of 0.25–0.5 Hz, each [$Ca2^+$]i transient associated with a corresponding contraction, which represents the normal functionality of the excitation–contraction coupling machinery (Fig. 5D).

Endothelial differentiation potential

Mouse ESCs which differentiated into vascular endothelial cells were found to be sensitive to shear stress [25] and physical (mechanical) stress induced by weak pulsatile flow loading [26].

To study the potential of this vascularization-like process in our stirred systems, 10-day-old EBs were allowed to attach to gelatin-coated slides and 2 days later were stained with anti CD31 antibodies and analyzed using the confocal microscope. Figure 6A demonstrates a well-developed capillary network in the whole EBs in both stirred systems compared to the STLV and static system. The positive network appears the same in all systems. Quantity analysis by real-time RT-PCR of VE-cadherin during 10 cultivation days (Fig. 6B) and FACS analysis of CD31 and VE-cadherin of 12-day-old EBs (Fig. 6C) support the result that no differences were found between

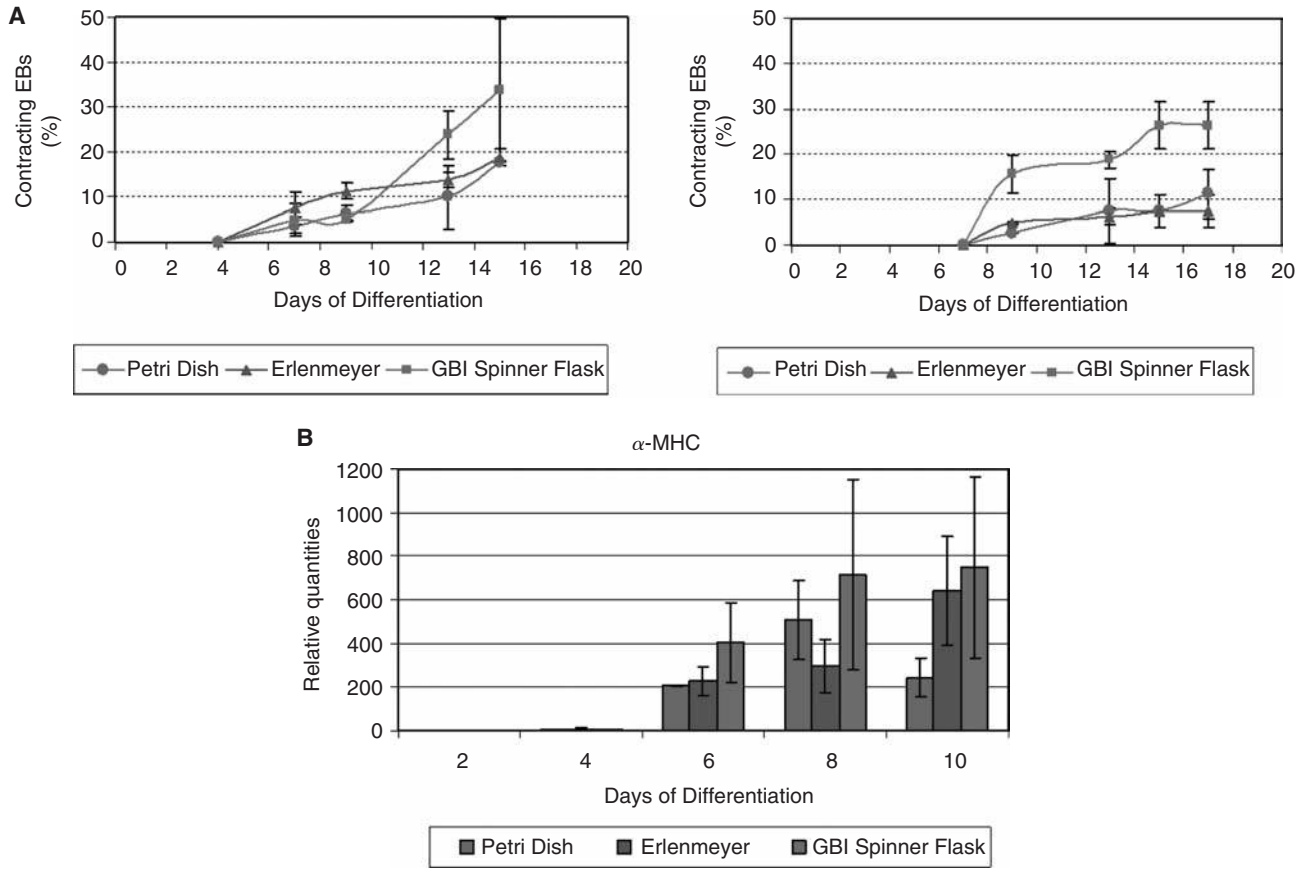

FIG. 5. Cardiac differentiation potential of hEBs under shear force conditions of stirred systems. (**A**) Percentage of contracting EBs during differentiation. EBs were transferred to gelatin-coated plates after 4 or 7 days in suspension culture to allow further differentiation into spontaneous contracting cardiomyocytes. (**B**) Real-time RT-PCR of cardiac-specific α-MHC transcripts during cultivation; note the clear correlation between the high quantity of α-MHC and the high percentage of contracting EBs in the GBI spinner flask.

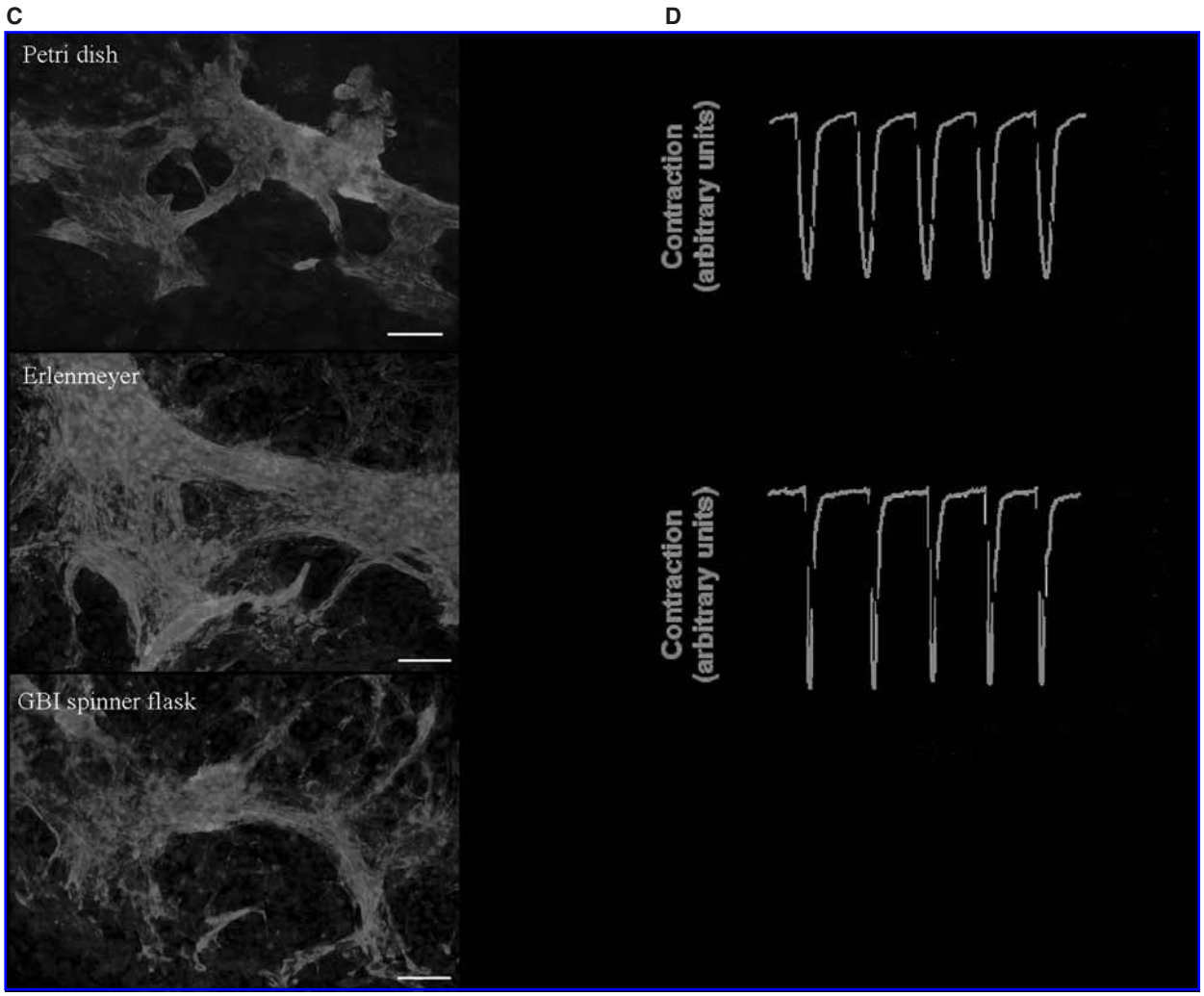

FIG. 5. *Continued.* (**C**) Fluorescence stains of contracting 17 days old EBs which were positive to cardiac-specific troponin I (cTnI). scale bar = 100 μm. (**D**) Functional properties of hESC-derived cardiomyocytes displayed by temporally related [$Ca2^{+}$]i transients and contractions.

shear stress inducing systems (i.e., the Erlenmeyer and the GBI spinner flask) and systems without shear stress (i.e., the STLV and the static petri dish), in contrast to mESC results [25].

Discussion

Large-scale production of hESC derivatives which maintain the cell's pluripotency is a technical challenge, and clinical protocols for stem cell therapy require standardization of defined culture conditions.

In this study, the stirring cultivation of hEBs in dynamic suspension conditions was investigated in several systems, in order to facilitate the development of a scaleable technological platform for the formation and propagation of hEBs that maintain their full potential of the differentiation process.

Previous studies in mESCs have shown that the conventional stirred vessels may have the disadvantage of generating shear forces and, although manageable, these forces damage the cells [4,10]. Furthermore, hEB formation in bioreactors was found to be problematic due to low clonality [5,10,27]. Stirring bioreactors with either peddle impeller or one GBI, with or without encapsulation, were inefficient [10]. The successful generation of mEBs in stirred intermediate sized vessels (i.e., spinner flask) suggested that manipulation of stirring conditions (pattern and velocity) and seeding conditions (EB formation period, cell concentration, etc.) could be exploited to establish a bioprocess [7,12,15]. Indeed, stirring speed was shown to control cell aggregate dimensions, as first demonstrated in neural mouse adult stem cells [28,29,30].

In our process, the seeding conditions which allowed efficient EB proliferation included (1) a 2-day prearrangement period in the static system prior to seeding into the stirred systems, (2) INF size of a few hundred cells each, and (3) initial hESC inoculums of $0.7 \pm 0.1 \times 10^{6}$ viable cells/mL.

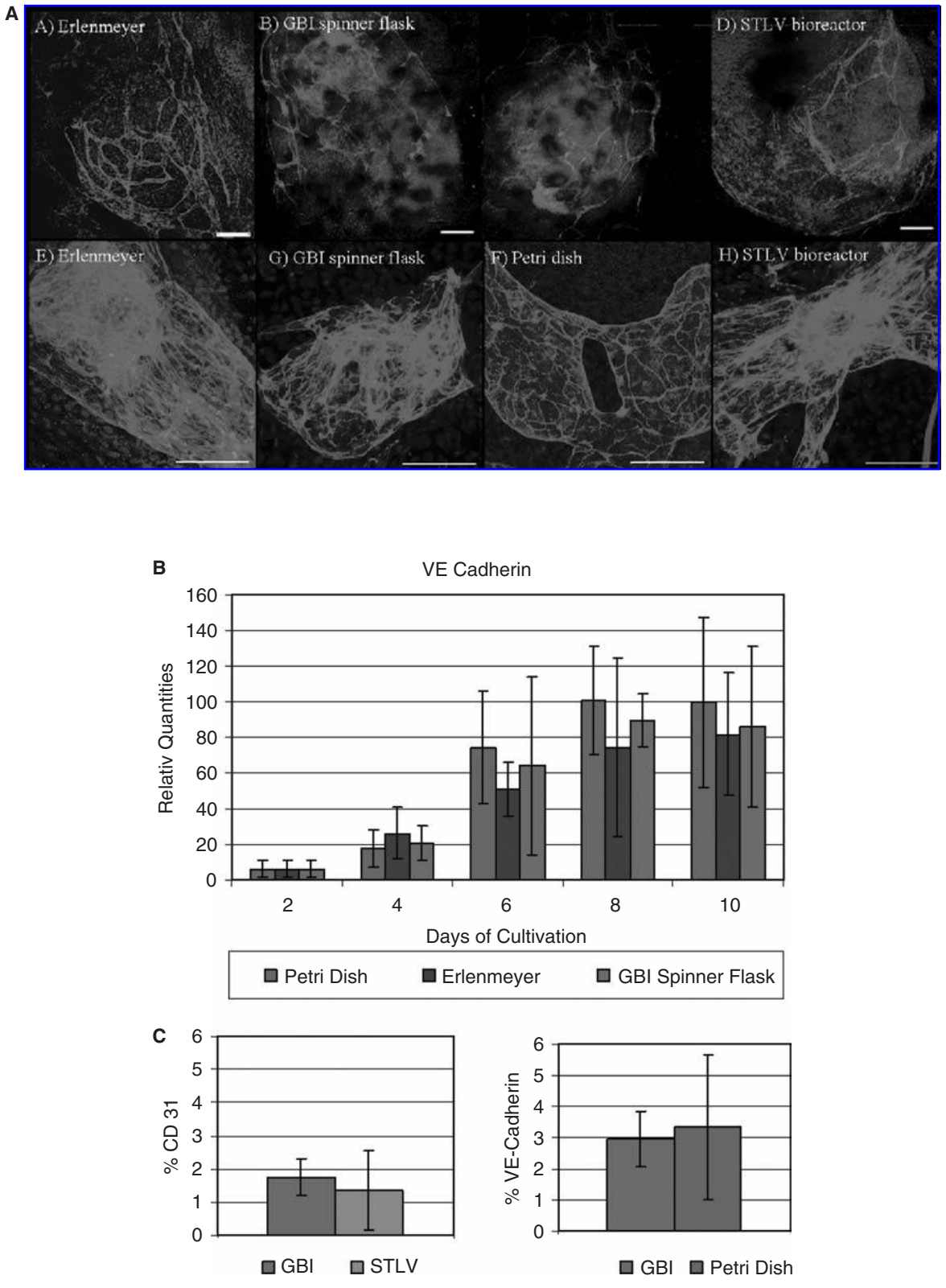

FIG. 6. Endothelial differentiation potential of hEBs under shear force conditions of stirred systems. (**A**) Confocal microscopy of 12-day-old EBs stained for CD31 showing three-dimensional network formation, vascular-like channels at the Erlenmayer and GBI spinner flask as compared to the system that did not induce shear forces (i.e., STLV and the static Petri dish). (A–D) Magnification: 17×; (E–H) Magnification: 40×; scale bar = 100 μm. (**B**) Real-time RT-PCR analysis of VE-cadherin; (**C**) FACS analysis of CD 31 and VE-cadherin. Note that no obvious difference was detected between the four systems.

The initiation period in the static system was found to be a crucial step for hEB formation in contrast to mEB in the GBI spinner flask [7]. Direct seeding resulted in accelerated agglomeration, while EBs seeded into stirred systems at day 2 yielded high cell and EB concentrations. Furthermore, direct seeding to STLV bioreactor, that not induced any shear forces on the cells [18], resulted in efficient EB formation. In agreement with Dang and colleagues, we conclude that the shear force conditions in the EB formation period accelerate the likelihood of cell and EB collisions. Thus, hESCs seeded into stirred vessels will quickly aggregate with concomitantly detrimental effects on cell yield and differentiation [4,12].

The definition of high INF size with the optimization studies on mEB seeding concentration [7,12] revealed higher inoculum's cell concentration in hEBs compared to mEBs (0.6 ± 0.2 vs. $0.1–0.2 \times 10^6$ ES cells/mL, respectively). This higher seeding concentration is due to the unique nature of hESCs that need an initiation nucleus of a small aggregate to create hEBs, while single cell cultures resulted in no EB formation in the examined conditions, unlike mEBs. Our observation corresponds to the process developed in the STLV bioreactor that was inoculated with 0.6×10^6 hESCs/mL, as previously reported by our laboratory [5]. Intensive mechanical or enzymatic cell dispension in order to create INF smaller than a few hundred of cells may also result in a high single cell concentration, leading to a low EB formation yield in the stirred systems. Finding a method to break the cells into smaller clumps without increasing the single cells concentration may have a beneficial effect on EB yield and cell-seeding concentration.

In this study, three different stirring systems characterized by induced shear forces and convection as mass transfer mechanism, were compared with STLV and static petri dish—systems which do not expose the EBs to any shear forces and have mass transfer mechanism of diffusion. Our stirring systems process resulted in a high yield (~500 EB/mL) and uniform EBs (in morphology and size), after 10 cultivation days. These results are 21-fold higher than the EB concentration obtained in the STLV bioreactor, and 2-fold higher than EB concentration in the static system. The highest EB yield was obtained in the first formation days in the Petri dish and did not change significantly in the Erlenmeyer, while in the control static system EB attachment led to a 2.5-fold decrease in EB concentration. The EB yield and small and homogenous size distribution can be explained by the moderate shear forces, which were applied in Erlenmeyer and GBI spinner flask stirring systems and prevent EB attachment. Moderate shear forces in stirred bioreactors have previously been shown to enhance cell growth by affecting cell shape and physiology, thereby resulting in improved mass transfer of nutrients into spherical structures [7,31,32].

Growth kinetics and viability were significantly higher in the GBI spinner flask compared to the PI spinner flask, STLV and static Petri dish system, while the apoptosis was lower, in correlation to the EB size, shape, and yield. These findings are in agreement with previous researches describing detrimental effects of paddle-impeller on mEB formation and high cell density obtained in spinner flask equipped with glass ball bulb-shaped impeller processed at 65 rpm [4,7,15]. In addition, 55-mL STLV bioreactor presented the slowest growth rate, and lowest final cell concentration and survival potentials. These results may be accountable to oxygen mass transfer limitation which occurs by diffusion in small surface/volume ratios existing in this bioreactor type [18]. These outcomes emphasize the importance of choosing the appropriate dynamic system and operating parameters in lab-scale processes prior to proceeding to larger-scale ones.

Most of the ESC differentiation protocols include the EB stage, which consists of two processes—proliferation and spontaneous differentiation. Additionally, most of the differentiation protocols start with the directed differentiation during the first 2 weeks of EB cultivation [7,12,20,21,33]. Here, hEBs which grew in the GBI spinner flask reached a maximum of 5.0×10^6 viable cell/mL in 8 days, with viability >90%. By day 10 the cell propagation decelerated while the cardiomyocytic and endothelial differentiation accelerated. No lag phase was observed in the GBI spinner flask system, while other systems presented different lag phase. This growth rate led to a 6.4-fold increase in cell expansion in 6 days, before the differentiation process became dominant. Optimization of the controlled bioreactor parameters (i.e., oxygen tension, pH, agitation rate and pattern, etc.) and improvement of the medium replacement methodology can lead to further cell concentration increase in that time frame.

Our study demonstrates that in both the Erlenmeyer and GBI spinner flask, representative genes from the three germ layers were expressed during the cultivation period, as well as in cells derived from all three germ layers in 10-day-old EBs. A clear decrease in the expression of undifferentiated hESC genes such as Oct-4 and Rex-1 was observed along with an increase in the expression of endoderm, mesoderm, and ectoderm genes. The stirred systems presented a high incidence of neural rosettes formation, in agreement with the increase in the NF68 and ND1 gene expressions. Rich capillary and well-developed endothelial networks were also observed.

To evaluate the differentiation potential of the stirred systems established for hEBs, we chose to study the spontaneous cardiomyocytic and endothelial differentiation. Previous spontaneous cardiac hEB differentiation studies have reported a range of 8–10% contraction [20] and 25–70% contraction in another protocol [21]. Our results clearly show that the GBI spinner flask, which applies moderate shear stress on the EBs from the beginning of their differentiation process, has an advantage in differentiation toward contracting EBs, compared to the ordinary static system, and at least 30% contracting EBs can be obtained. Respectively, the cardiac-specific α-MHC transcripts level in suspended EBs present a clear advantage of the moderate stirring systems, the GBI spinner flask and the Erlenmeyer, after 10 differentiation days. Examination of the contracting EBs demonstrates morphological development and functionality of cardiac muscle. Further development of the controlled bioreactor will allow hypoxic conditions which have previously been reported to have an advantage in mESC cardiomyocytic differentiation [34]. Our results, however, did not point to the induction of endothelial differentiation

by shear stress. Both morphology and the level of differentiation markers expression were similar in cells grown in the stirring systems and nonshearing force-inducing systems (STLV and static Petri dish).

In summary, we present a robust hEB propagation and differentiation process with a well-known stirred technological platform. The process showed an increase in cell growth and no notable decrease in differentiability, compared to the standard static system. The seeding and growth methods together with the stirred system described here can serve as corner stones for the development of a scalability process for hEBs for future industrial and clinical use.

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