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Microfluidic culture of single human embryonic stem cell colonies

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Abstract

We have developed a miniaturized microfluidic culture system that allows experimentation on individual human embryonic stem cell (hESC) colonies in dynamic (flow applied) or static (without flow) conditions. The system consists of three inlet channels that converge into a cell-culture channel and provides the capability to spatially and temporally deliver specific treatments by using patterned laminar fluid flow to different parts of a single hESC colony. We show that microfluidic culture for 96 h with or without flow results in similar maintenance of hESC self-renewal, the capability to differentiate into three germ cell lineages, and to maintain a normal karyotype, as in standard culture dishes. Localized delivery of a fluorescent nucleic acid dye was achieved with laminar flow, producing staining only in nuclei of exposed cells. Likewise, cells in desired regions of colonies could be removed with enzymatic treatment and collected for analysis. Re-coating the enzyme treated area of the channel with extracellular matrix led to re-growth of hESC colonies into this region. Our study demonstrates the culture of hESCs in a microfluidic device that can deliver specific treatments to desired regions of a single colony. This miniaturized culture system allows *in situ* treatment and analysis with the ability to obtain cell samples from part of a colony without micromanipulation and to perform sensitive molecular analysis while permitting further growth of the hESC colony.

Introduction

Human embryonic stem cells (hESCs) are derived from the blastocyst inner cell mass and provide a potentially unlimited supply of cells that may be directed to differentiate toward specific lineages.¹ The study of hESCs will enhance the understanding of early embryo development as well as advance the efficiency of drug discovery and screening, while providing a source of material for cell-based therapeutics. The ability of hESCs to sense and respond to their culture microenvironments and differentiate into many different types of cells presents challenges as well as opportunities to develop innovative culture technologies such as the use of microfluidics.

Microfluidic technologies have the potential to regulate both the chemical and mechanical environment of cell culture in ways not possible with conventional dishes and macroscopic cell culture-ware. The experimental strengths of microfluidic technologies with application to stem cell biology have been demonstrated in studies with human neural stem cells,² mesenchymal stem cells,^{3–5} and mouse ESCs^{6,7} to study cell growth, cell differentiation and to control the formation of embryoid bodies from mouse ESCs.⁸ However, the use of microfluidics in hESC studies is still limited to a few reports,^{9–11} at least in part, due to demands associated with their *in vitro* culture needs. Human ESC growth is optimal when the cells are handled as colonies rather than individual cells.¹² This prompts unique challenges not encountered with other cell types, which are often dissociated and introduced or removed from channels as suspensions of single cells. Human ESCs are also sensitive to enzymatic dissociation, which can lead to karyotype instability if used over time.^{13,14} The removal or sampling of cells from hESC colonies grown inside microchannels also represents a challenge. Human ESCs have unique extracellular matrix requirements,^{1,12,15} as well as growth in optimal osmolality and nutrient levels¹⁶ and require gentle handling so they are not exposed to excess shear stress. Thus the design of a microfluidic device should be able to fulfill all the above requirements of the *in vitro* culture of hESCs. In addition, it will be necessary for the microfluidic device to have good optical access along with the ability to perform spatiotemporal patterning of the chemical environment where hESC colonies are cultured.

Here, we describe a micro-culture system that permits experimentation on individual hESC colonies in both dynamic and static microfluidic culture conditions that promote cell self-renewal and differentiation. By using patterned laminar flow we also demonstrate patterned delivery of reagents and dissociation enzymes to select regions of individual hESC colonies. Cell samples obtained in this manner from portions of individual hESC colonies were analyzed at the molecular level without affecting the viability and re-growth of the remaining cells of the colony.

Materials and methods

Microfluidic device fabrication

The microfluidic device design albeit with some modifications follows the one previously used by Takayama and collaborators.^{17–19} The device has three inlet-channels that converge into a cell-culture channel. The channel's size was customized to accommodate and facilitate the introduction and movement of hESC clusters, and colony formation. The height, width and length of each channel were 200 μm , 1 mm and 1 cm, respectively and the entire device fits in a 100 mm cell culture dish (BD Falcon Cell Culture Dishes, #353803; BD Biosciences; San Jose, CA) (Fig 1). Microchannels were fabricated from poly-(dimethylsiloxane) (PDMS) formed from prepolymer (Sylgard 184, Dow Corning, Midland, MI) at a ratio of 10:1 base to curing agent using a soft lithographic method.²⁰ Relief features of the mold were composed of SU-8 (Microchem, Newton, MA). To allow introduction of solutions and cells into channels, access holes were punched through PDMS to form three inlets and one outlet. The PDMS

prepolymer was cast against the mold and put into an oven at 60 °C for 2 hours. The PDMS channels were bonded to a 100 mm tissue culture dish using a thin layer of liquid PDMS prepolymer as mortar.²¹ In this procedure, a mixture of toluene and PDMS prepolymer with a volume ratio of 3:2 was spincoated (1500 rpm for 60s) onto a glass slide to cover the surface with a thin layer of PDMS (~5 µm), which served as mortar to hold channels and dish together. This mortar was transferred to the PDMS channel by contacting all flat areas of the embossed PDMS surfaces with the thin layer of PDMS mortar while keeping channel areas free of PDMS mortar. The PDMS channel was then placed onto the dish and left at room temperature for 10 min to remove air bubbles. Plastic reservoirs were then bonded to 3 inlets and one outlet using the PDMS prepolymer. The combined device was cured at 60 °C for 2 hours.

Cell culture

Human embryonic stem cell lines hESBGN-01 (NIH code: BG01; BresaGen, Inc., Athens, GA), and H9 (NIH code: WA09; WiCell Research Institute, Madison, WI) were grown on irradiated mouse embryonic fibroblasts (MEFs) in gelatin-coated tissue culture dishes at 37 °C in 5% CO₂ in air. Culture medium consisted of Dulbecco's modified Eagle medium (DMEM)/F12 (Invitrogen, Carlsbad, CA) supplemented with 20% knockout serum replacer (Invitrogen), 0.1 mM β-mercaptoethanol, 1.0 mM L-glutamine, 1% nonessential amino acids (Invitrogen), and 4 ng/ml human recombinant FGF-2 (Invitrogen).¹⁵ Undifferentiated hESC colonies were passaged by mechanical dissection into small clumps (100–150 µm).

Cell culture in microchannels was performed with either media previously conditioned by irradiated MEFs or differentiation media. To obtain conditioned media (CM), irradiated MEFs (8×10^6 cells) were seeded on gelatin coated culture dishes (150 mm; Corning Incorporated, Corning, NY). Twenty-four hours after plating, MEF culture media was replaced with hESC culture media (60 ml), and media collected the following day. Then MEFs were fed again with hESC culture media daily and collected for 3 days. Conditioned media (CM) was frozen at -20 °C until use. Before use in hESC culture, it was supplemented with an additional 0.1 mM β-mercaptoethanol, 2.0 mM L-glutamine, and 4 ng/ml FGF-2. Differentiation media consisted of DMEM/F12, 20% neurobasal (Invitrogen), 0.8% N2 (Invitrogen), 0.2% B27 (Invitrogen), and 1% sodium pyruvate.

hESC culture in a microfluidic device

Fluids were introduced into microchannels by gentle aspiration at the outlet, or by adding different levels of fluids to the inlet and outlet reservoirs to create pressure differences using gravitational forces. The microfluidic device was rendered hydrophilic by exposure to oxygen plasma (SPI supplies, West Chester, PA) for 5 min. Shortly afterwards, channels were filled with PBS to maintain their hydrophilic state and devices were sterilized with UV light for 30 min. Microchannels were then coated with Matrigel (BD BioSciences, San Jose, CA) by first removing PBS by aspiration, followed by introduction of Matrigel solution (1:20 in cold DMEM/F12; 300 µl per inlet) into microchannels and incubation at room temperature for at least 2 h. To remove excess Matrigel, the channel system was washed three times with PBS, followed by MEF-CM. Finally, 200 µl of MEF-CM was added to each inlet and the outlet, and equilibrated for 1 h at 37 °C in 5% CO₂.

To introduce clumps of hESCs into the cell-culture channel, an additional 200 µl of MEF-CM was added to the outlet well to create gravity-driven flow. Clumps were then placed at the outlet well bottom close to the cell-culture channel entrance, and were carried into the channel by flow. Once clumps were in the desired localization, flow was neutralized by adding MEF-CM into each inlet well. To avoid media evaporation mineral oil was added to each inlet and outlet, and the microfluidic devices were placed at 37 °C in 5% CO₂ and air during 24 h to

allow hESC colony formation. During long-term static culture of hESCs in chip and standard dishes, media was partially replaced daily.

To create a dynamic flow, media was supplied from syringes to reservoirs through silicone tubes by using a multi-syringe infusion pump (KDS220, KD Scientific; Holliston, MA). To prevent bubble formation inside microchannels and keep the flow stable, one end of the silicone tube was connected to a syringe needle with the other end reaching into the inlet reservoir but not connected to the microfluidic device. The outlet plastic reservoir was removed to keep the outlet media level lower than in inlets. The media volume difference between each of the three inlet reservoirs and the outlet reservoir was maintained by the infusion of media into the inlet reservoirs resulting in a stable gravity-driven flow rate of 0.5 ml/h. The average flow speed and Reynolds (Re) number in the inlet channels were 0.7 mm/s, and 0.233, respectively.

Measurement of hESC colony area

Colony area from micrographs was measured using ImageJ 1.38 software (<http://rsb.nih.gov/ij>). Microfluidic devices were randomly selected for either dynamic or static culture 24 h after introducing colonies. Colony area was measured at that time and after 48 h in culture. Mean ($\pm$ SEM) colony diameter between culture systems were compared statistically using Student's t-test and differences considered significant with $p \leq 0.05$.

Experiments using laminar flow

SYTO 9 green fluorescent nucleic acid dye (Invitrogen) or 0.05% Trypsin-EDTA (Invitrogen) solutions were used in laminar flow experiments. These solutions were administered through one inlet while the other two contained culture media. SYTO 9 was diluted in culture medium to a concentration of 100 nM. For partial colony collection by enzymatic treatment, one inlet was first rinsed with PBS, to wash out all culture media content in the corresponding side of the cell-culture channel, and then trypsin-EDTA solution was added, until cells were completely removed. Culture media was then administered to stop the enzymatic activity of trypsin-EDTA. Both experiments were repeated at least three times.

Immunostaining

Cells were fixed in 2% paraformaldehyde for 30 min at room temperature and then permeabilized with 0.1% Triton X-100 for 10 min. The antibodies used were OCT3/4 (1:100; Santa Cruz Biotechnology, Inc, Santa Cruz, CA), SOX2 (1:200; Chemicon, Anderson, CA), SSEA4 (1:100; Developmental Studies Hybridoma Bank, IO) and TRA-1-60 (1:100; Chemicon) and were diluted in 1% normal donkey serum and incubated with cells overnight at 4 °C. Primary antibody was detected using the corresponding secondary antibody from Jackson Immuno-Research (West Grove, PA). Primary antibody was omitted as a negative control. Cell nuclei were stained with Hoechst No. 33258 (Invitrogen). Samples were imaged using phase-contrast and epifluorescence microscopy.

Extraction and purification of total RNA

Cells were pelleted by centrifugation at $800 \times g$ in RNase-free in 1.5 ml siliconized microcentrifuge tubes (Ambion, Austin, TX). Total RNA extraction was performed with RNeasy® Micro Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. RNA quality was checked using RNA 6000 Nano Assays performed on the Bioanalyzer 2100 Lab-on-a-Chip system (Agilent Technologies, Palo Alto, CA).

Reverse-transcription PCR analysis

RT-PCR amplification of 10ng of purified RNA was performed for each RNA sample. The reaction consisted of 50µl volume containing a SuperScript™ One-Step RT-PCR with

platinum® Taq (Invitrogen) following the standard protocol and 0.2 μ M of each primer (Table 1). Tubes were placed in a Perkin Elmer Model 480 DNA Thermal Cycler (Perkin Elmer, USA). The reaction included cDNA synthesis at 55 °C for 30 min followed by a 2-min denaturing step at 94 °C and PCR for 40 cycles of denaturation (94 °C, 15 sec), annealing (56 °C, 30 sec), and extension (72 °C, 30 sec) followed by a final extension step of one cycle at 72 °C for 10 min. The size of each PCR product was evaluated with ethidium bromide-stained 2% agarose gels visualized under ultraviolet light and documented with a Polaroid GelCam DS-34 Film Camera (Polaroid, USA).

Real-time RT-PCR analysis

Total RNA was reverse-transcribed using MultiScribe™ Reverse Transcriptase System (Applied Biosystems; Foster city, CA). The ABI 7300 PCR and Detection System (Applied Biosystems) with SYBR® Green PCR Master Mix (Applied Biosystems) was used in real time-PCR. PCR was conducted in triplicate. Primers are listed in Table 1. Human β -Actin was amplified as an internal standard. The relative quantification of SOX3 gene expression was performed using the Comparative Ct Method ($\Delta\Delta$ Ct).

Cytogenetic analysis

Karyotype analysis of hESCs was performed by cytogeneticists at Cell Line Genetics (Madison, WI). Chromosomes were prepared using standard protocols and analysis was done using the GTL-banding method on at least 20 cells.

Results and discussion

Figure 1 shows the schematic design of the microfluidic system used to generate multiple laminar flow streams of different liquids in the cell-culture channel. Some experiments were performed with two or three laminar fluid streams by adding media to either two or three of the inlets, respectively. An average flow rate of 0.5 ml/h was supplied to each individual inlet channel from a syringe pump. Thus, the flow rate in the cell-culture channel was approximately either 1 or 1.5 ml/h, depending on the number of parallel streams.

Achieving successful culture of hESC colonies presented unique challenges that were employed in the design of the microfluidic device. Like most cells, hESCs require adhesion to an extracellular matrix (ECM) for survival and growth.^{1,12} For this reason, channels were coated with Matrigel, a basement membrane preparation rich in ECM proteins, that supports proliferation of undifferentiated hESCs¹⁵ (Fig. 1B). In addition, hESCs are vulnerable to apoptosis upon cellular detachment and dissociation. They undergo extensive cell death particularly after complete dissociation, and the cloning efficiency of dissociated single hESCs is generally $\leq 1\%$.^{1,12,22} Therefore, hESC clusters of ~100–150 μ m were used to establish colonies for both standard culture dish and microfluidic culture (Fig. 2A). The microfluidic device channels were 1 mm in width and 200 μ m in height, allowing the introduction and displacement of hESC clusters along the microchannels. These adaptations permitted 100% (30/30 attempts) establishment of undifferentiated hESC colonies, and multiple colonies were obtained in one chip when several clusters were introduced. Established colonies had well defined borders and small cells with a high nucleus:cytoplasm ratio, characteristics of undifferentiated hESC colonies (Fig. 2A). Immunohistochemistry was used to confirm the expression of pluripotent stem cell markers and transcription factors such as OCT3/4, SOX-2, SSEA-4, and TRA-1-60, which are associated with the undifferentiated state of hESCs (Fig. 2B).

The use of flow in the microfluidic device generated shear stress on the hESC colonies. It has been reported that the minimum shear needed to remove cells cultured on a surface is 6.5 dyn/

cm,^{2,23} whereas shear stress levels in the range of 15–30 dyn/cm² cause damage to attached cells.²⁴ Here, the shear stress to which hESC colonies were exposed was 0.6 dyn/cm², and did not affect cell adhesion. The effect of mechanical forces on cell growth,²⁵ differentiation,²⁶ and signal transduction²⁷ have also been documented in several cell types. It has been suggested that mechanical forces do not affect hESC pluripotency.²⁸ Here it was observed that a flow rate of 1–1.5 ml/h (0.36–0.6 dyn/cm²) did not induce hESC differentiation. All hESC colonies cultured in both dynamic (15/15) and static (15/15) conditions remained undifferentiated and expressed OCT3/4, a marker of ES pluripotency (Fig. 3). Cell differentiation could be induced in both dynamic (5/5) and static (5/5) culture in the device as observed by an up-regulation of *SOX3*, a neural progenitor marker by 0.8 fold and a significant reduction in OCT3/4 expression on differentiated cells (Fig. 3). Further analysis demonstrated that undifferentiated hESCs cultured in the microfluidic device retained a normal karyotype, were able to form embryoid bodies, and differentiated into ectoderm, mesoderm and endoderm derivatives, similar to hESCs growth in standard culture dish (data not shown).

Cell proliferation was not different between dynamic and static culture. After 48 h of continuous culture in dynamic conditions, colonies had an area of $3.04 \pm 0.33 \text{ mm}^2$ (mean $\pm$ SEM) and a fold increase in size of 1.48 ± 0.12 , which was not statistically different than $2.89 \pm 0.8 \text{ mm}^2$ in area, and 1.70 ± 0.24 fold increase in colony size in static conditions. These results contrast with previous reports by Fong and collaborators, who suggested that perfusion culture enhances hESC number compared with static conditions.²⁹ However, comparisons between both studies are difficult due to differences in culture conditions and methodologies used to evaluate cell proliferation. In our experiments flow rate was maintained continuously for 48 h, which results in constant nutrient delivery and removal of cell secreted factors and waste, while in the previous study,²⁹ media was perfused for 120 min at 12 h intervals, which may create a beneficial microenvironment due to exposure to autocrine growth factors that influence cell propagation.³⁰ We concluded that dynamic flow culture in our microfluidic device, while it may remove some autocrine factors, it did not induce hESC differentiation. Moreover, cells retained normal karyotype, and cell proliferation was compared to static culture. Therefore, this miniaturized culture system provides a useful platform for experimentation under dynamic conditions without interfering with the characteristics of hESCs and where results are caused by specific treatments and not by flow.

The importance of this microfluidic device is its capability to deliver and remove specific factors and treatments by using patterned laminar fluid flows to different parts of a single hESC colony, which can be used to test spatially regulated signaling, drug screening, or studies that involve intracellular compartments and subcellular heterogeneity. Such experiments are difficult to perform in standard static culture. As an example, bilaminar flow was created to partially expose an individual colony to a fluorescent nucleic acid dye SYTO 9, and as result only the nuclei of exposed cells were stained (Fig. 4A). Furthermore, desired regions of undifferentiated hESC colonies were removed by trypsin/EDTA treatment using laminar flow (Fig. 4B). The detached cells were then collected and analyzed to demonstrate the expression of *OCT3/4* and *NANOG*, genes present in undifferentiated hESCs (Fig. 4C). Interestingly, the remaining part of the colonies continued growing selectively in the Matrigel coated area, but not in the trypsin-treated area likely due to proteolytic cleavage of ECM proteins from the Matrigel coating on the channel floor. Cell re-colonization of this area was not observed after further culture until Matrigel re-coating was performed (Fig. 5). This suggests that cell migration and proliferation of hESCs is highly dependent on the presence of exogenously administered ECM. It also highlights additional requirements of hESCs in contrast with other cell types such fibroblasts that migrate and proliferate in-channel regardless of ECM.³¹ In fact, using Matrigel re-coating, we were able to repeatedly re-expand and collect cells from one single colony, prolonging the total culture time in chip to 10 days.

Conclusion

We present a microfluidic device that supports the growth of undifferentiated hESCs. We demonstrate that dynamic culture at flow rates up to 1.5 ml/h did not affect adhesion, proliferation, self-renewal, pluripotency, or karyotype of hESCs. Using laminar flow it is possible to target portions of hESC colonies for treatment with subcellular spatial selectivity and perform experiments where a single colony serves as its own experimental control, thereby decreasing sample variability. This may allow the study of propagation signals from one end to other end of a colony, which is similar to normal development where gradients exist. This microfluidic system is compatible with live-cell imaging, and *in situ* and/or *out situ* analysis via collection of specific areas of a colony. We believe that this experimental platform is useful in drug screening and studies of dynamics, migration, spatially regulated signaling, and differentiation of hESCs. This type of platform may be particularly interesting given the recent discovery of novel small molecules that mediate cell-fate acquisition and the differentiation of a number of stem cell types.^{32–35}

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Fig. 1.

Design of a microfluidic device adapted for hESC culture. A) Schematic representation of the microfluidic device, with three inlets channels that converge into a single main channel. The channels size was customized to accommodate and facilitate the introduction and movement of hESC clusters and posterior colony formation. Each channel feature is 200 μm in height, 1 mm in width and 1 cm in length, and the entire device fits in a 100 mm tissue culture dish. This microfluidic device is able to generate laminar flow in the cell-culture channel. B) Channels were coated with Matrigel to provide extracellular matrix (ECM) components that allow hESC attachment and colony formation. The photograph shows a microfluidic device stained with Commassie blue to demonstrate the ECM coating. The insert shows a microfluidic device without ECM coating.

Fig. 2. Characterization of hESCs cultured in a microfluidic device in dynamic and static conditions. A) Phase contrast micrographs illustrating a representative colony of undifferentiated hESCs cultured on mouse embryonic fibroblasts (a). Colonies were cut in to pieces of approximately 100 μm diameter (b), and the clusters of cells were introduced into the cell-culture channel. Micrographs (c and d) show a representative hESC colony cultured in chip for 24 and 48 h, respectively. B) Immunohistochemistry (IHC) was performed to confirm the undifferentiated state of colonies cultured for 96 h by the expression of OCT3/4, SOX2, SSEA-4 and TRA-1-60, markers of undifferentiated hESCs. Nuclear staining by DAPI and merged images are shown next to each marker.

Fig. 3.

Culture of hESCs in a microfluidic device in dynamic and static conditions. Human hESC colonies were cultured in dynamic (with flow; a–d) or static (without flow; e–h) conditions with either MEF-CM (a, b, e and f) or cell differentiation media (c, d, g and h) for 96 h. The undifferentiated state was evaluated by OCT3/4 expression, which is strongly expressed when cultured in MEF CM (b and f), but lost when cultured with differentiation media (d and h). DAPI was used for nuclear staining.

Fig. 4.

Application of laminar flow to hESC colonies in a microfluidic device. A) Nuclear staining of cells on a hESC colony was performed with laminar flow. Two out of three inlets carried culture media, and the third contained SYTO 9, a green fluorescent nucleic acid dye. Light microscopy image of the entire hESC colony is shown in (a), while cell nuclei selectively stained only in cells present on the channel side carrying SYTO 9 is shown in (a'). White dotted line indicates the channel walls, and red dotted line indicates the interface between media and SYTO 9 created by laminar flow. B) Selective cellular removal of hESC colonies by 0.05% trypsin-EDTA treatment using laminar flow. In this experiment two inlets were used, and the left inlet carried the 0.05% trypsin-EDTA solution, while the right inlet carried MEF-CM. Progressive cellular

removal is observed from top to bottom of micrographs, as indicated by seconds ("). Red dotted line indicates the interface between trypsin-EDTA solution and MEF-CM created by laminar flow. C) Dissociated cells were collected and analyzed by PCR to detect *OCT3/4* and *NANOG*, both markers of pluripotency expressed in undifferentiated hESCs. *GAPDH* was used as a control gene. Scale bar in micrographs represents 500 μm .

Fig. 5. Selective growth of hESCs on surfaces coated with ECM proteins. (A and D) Light microscopy images of hESC colonies growing in cell-culture channels coated with ECM (Matrigel). (B and E) The left side of the cell-culture channel was treated with 0.05% trypsin-EDTA solution to selectively remove half of the colonies. The trypsin treatment proteolytically cleaved both the cell surface proteins from hESCs and ECM coating of the cell-culture channel floor. (C) Forty-eight h after trypsin treatment, the remaining half of the colony maintained cell proliferation exclusively on the side of the cell-culture channel that was not treated with trypsin. Cell growth on the side of the channel exposed to trypsin was only observed after re-coating with ECM and after 48 h of further cell culture as observed in micrograph (F). Scale bar represents 500 μm .

Table 1

List of primers used for RT-PCR and Real Time RT-PCR

Gene	Forward primer	Reverse primer	Amplicon (bp)
GAPDH	accacagagagactgggcatgg	cacattgggggttaggaacac	170
OCT3/4	ctgcagctgggggttcgggca	ctggctgcagaaagtgggtggaggga	168
NANOG	cggcttcctcctctctctctatc	atcgatttcactcatcttcacacgtc	953
SOX3	ccatcgcatcgcactctca	agctaaacaaggcgtcccaa	277
β-Actin	atctggcaccacacctctctacaaatgagctgcg	cgtcatactctctctgtgtgctgacacatctgc	837