An integrated microfluidic culture device for quantitative analysis of human embryonic stem cells†

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We have successfully designed and fabricated an integrated microfluidic platform, the hESC-μChip, which is capable of reproducible and quantitative culture and analysis of individual hESC colonies in a semi-automated fashion. In this device, a serpentine microchannel allows pre-screening of dissociated hESC clusters, and six individually addressable cell culture chambers enable parallel hESC culture, as well as multiparameter analyses in sequence. In order to quantitatively monitor hESC proliferation and pluripotency status in real time, knock-in hESC lines with EGFP driven by the endogenous *OCT4* promoter were constructed. On-chip immunoassays of several pluripotency markers were carried out to confirm that the hESC colonies maintained their pluripotency. For the first time, our studies demonstrated well characterized hESC culture and analysis in a microfluidic setting, as well as a proof-of-concept demonstration of parallel/multiparameter/real-time/automated examination of self-renewal and differentiation in the same device.

Introduction

Human embryonic stem cells (hESCs),[1–3] derived from the inner cell mass of blastocyst-stage embryos, hold great potential for the treatment of many devastating diseases and injuries. This is mainly due to two distinct properties: (i) they can self-renew indefinitely and (ii) they can potentially generate all cell types in the human body. Intrinsic regulators (*e.g.*, growth factors and signaling molecules) and cellular microenvironments (*e.g.*, extracellular matrices, ECMs) play critical roles in the regulation of self-renewal and differentiation of hESCs. Conventionally, hESCs are passaged in clusters (containing approximately 20–200 cells) using well plates or culture dishes. Growth-arrested mouse embryonic fibroblast (mEF) feeder layers are co-cultured in serum replacement-containing medium to supply the essential intrinsic regulators and environmental cues. However, there have been concerns associated with xenogenic contamination that would restrict potential therapeutic applications of hESCs in clinical settings.[4,5] In order to harness the unique potential of hESCs and to improve self-renewal[6–12] and controlled differentiation of hESCs,[13–16] systematic approaches have been adopted to screen a broad range of serum- and feeder-free culture conditions to obtain a better understanding of the roles of intrinsic regulators and cellular microenvironments. The cost to perform these screening experiments is high, since they consume a considerable quantity of hESCs, ECM materials and culture media containing expensive growth factors. There is a clear need for developing a miniaturized platform on which to carry out large-scale screening in a cost-efficient fashion.

There is a growing interest to develop microfluidics-based technologies[17] for performing cell culture and analysis. Microfluidic systems offer intrinsic advantages over conventional macroscopic culture such as reduced sample/reagent consumption and precise control over the delivery of culture fluids and soluble factors. A continuous-flow microfluidic system composed of the simplest device configuration (*i.e.*, individual microchannels and the respective inlets/outlets) has been utilized to implement miniaturized cell culture and analysis.[18] In this case, bovine adrenal capillary endothelial cells were seeded in protein-coated microchannels, where culture media and assay reagents were introduced and withdrawn from the microchannels through inlets and outlets, respectively. Several challenges remain, however, to explore these continuous-flow cell culture/assay chips for systematic screenings where combinations of multiple parameters should be tested to obtain the desired

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outcomes. For example, when many cell culture conditions are screened in a microchannel network, it is inevitable that the individual conditions would be cross-contaminated through diffusion. Moreover, this multiparameter screening necessitates a delicate microfluidic delivery/mixing system for handling small amounts of culture components coordinated by an automated operation system. To overcome these challenges, different miniaturized functional modules, including isolation valves and mechanical pumps, have been developed to prevent cross contamination and to attain precise fluidic delivery and mixing. Most importantly, these miniaturized valves and pumps can be digitally controlled, thus allowing automated cell culture in a microfluidic chip.[19]

Among the exciting automated microfluidic systems, the poly(dimethylsiloxane) (PDMS)-based integrated microfluidic system represents a large-scale architecture of microchannel networks that enables the execution of sequential and parallel processes in individual devices.[20] Particularly, the biocompatible and gas-permeable properties of PDMS matrices help to retain proper physiological conditions for a wide range of mammalian cells suitable for different screening applications. The cooperation of integrated hydraulic valves confines distinct regions for testing specific screening combinations/conditions without the concern of cross contamination,[21,22] and a peristaltic pump (composed of three consecutive isolation valves) is capable of delivering, metering, and mixing of nanoliter (nL)-level fluids with great precision.[22] Over the past seven years, different PDMS-based integrated microfluidic devices have been developed for complicated chemical[23,24] and biological operations,[25–27] including recent demonstrations on culturing human mesenchymal stem cells.[28] Obviously, the characteristics of the PDMS-based integrated microfluidic system meet the needs of conducting systematic screenings of optimal hESC culture conditions. Although there are examples of the culture of human neural stem cells[29,30] and mouse embryonic stem cells[31] in different microfluidic systems, there are few reports to demonstrate the culture and manipulation of hESCs in a microfluidic platform.[32–35]

Here, we demonstrate an integrated microfluidic platform (hESC-microChip, hESC-μChip), which allows reproducible and quantitative culture and analysis of individual hESC colonies in a semi-automated fashion. Initially, several challenges were envisioned to conduct this study in a hESC-μChip. For example, hESCs are extremely sensitive to changes of intrinsic regulators, cellular microenvironments and ambient pressure/temperature. The effects of culturing hESCs into a hESC-μChip on hESCs should be addressed in these contexts. Further, hESCs have to be passaged in clusters and co-cultured in the presence of growth-arrested mEF feeder layers. Experience in handling hESC clusters in the chip and co-culturing of hESC clusters with the adherent mEF cells should be acquired. Moreover, to confirm that the chip-cultured hESC colonies maintain their pluripotency over a certain culture period, immunoassays for a number of pluripotency markers have to be carried out in sequence. Each immunoassay for chip-based operation will be optimized and some of them will be compiled in sequences. The goal of our study was not meant to unveil novel insights in hESC biology or develop a new type of microfluidic technology, but to acquire solid experience and practical knowledge of performing microfluidic hESC culture, which will constitute a useful foundation for exploring further application of microfluidic platforms in hESC research.

Experimental

hESC culture in a hESC-μChip

All hESC research described here was approved by the UCLA Embryonic Stem Cell Research Oversight Committee. A newly fabricated hESC-μChip was sterilized under UV light for 15 min prior to on-chip hESC culture. Based on a two-layer coating approach, a bovine fibronectin solution (FN, 1 mg mL^{-1} in PBS, Sigma) and a gelatin solution (0.2% in PBS) were sequentially introduced into the hESC-μChip from "Inlet 2" using Teflon tubing (Fig. S1†). γ-Irradiated mEFs (1×10^7 cells mL^{-1}) were loaded into the cell culture chambers from "Inlet 3". mEFs were cultured for 12 hr in a humidified incubator (37 °C, 5% CO_2, Thermo Fisher Scientific) before loading cells. hESCs cultured in a 6-well plate were passaged with 1 mg mL^{-1} of collagenase IV in DMEM/F12 (see the ESI†). The freshly dissociated hESC clusters were introduced into the cell culture chambers through "Inlet 1" connected to a serpentine microchannel, where every hESC colony was visually inspected (Fig. 1c). Gravity flow[36] was adopted in order to introduce hESC clusters into each cell culture chamber. To ensure the quality and uniformity of hESC colonies in our studies, only hESC clusters with desired sizes (100 ± 20 μm) and disc-shaped morphologies were selected for seeding. In general, four to six hESC colonies were accommodated in each cell culture chamber. The locations of individual hESC colonies were registered according to the ruler, allowing continuous fate mapping by an inverted microscope. The hESC-μChip-based hESC culture was carried out in a humidified incubator (37 °C, 5% CO_2). By programming the cooperation of isolation valves and peristaltic pumps, media stored in Teflon tubing was introduced into each cell culture chamber every 12 hr.

Immunocytochemistry and histology

hESC colonies were fixed by introducing paraformaldehyde (4%, Electron Microscope Science) into the cell culture chambers in the hESC-μChip. After permeabilization with Triton X-100 (0.5%, Fluka) in PBS for 30 min, a blocking solution containing normal goat serum (5%, Vector Laboratory), normal donkey serum (5%, Jackson Laboratory), bovine serum albumin (3%, Fraction V, Sigma) and *N*-dodecyl-β-D-maltoside (0.1%, Pierce)[37] was loaded into the device from "Inlet 1", and the device was incubated at room temperature for 1 hr. After rinsing with PBS containing 0.1% Tween 20 (PBS-T), the hESC colonies were incubated with human specific antibodies for OCT4 (2 μg mL^{-1}, mouse monoclonal IgG, Santa Cruz Biotechnology), NANOG (2 μg mL^{-1}, rabbit polyclonal IgG, Abcam), SSEA1 (2 μg mL^{-1}, mouse monoclonal IgM, Santa Cruz Biotechnology), SSEA4 (2 μg mL^{-1}, mouse monoclonal IgG, Santa Cruz Biotechnology), TRA-1-60 (2 μg mL^{-1}, mouse monoclonal IgM, Santa Cruz Biotechnology) or TRA-1-81 (2 μg mL^{-1}, mouse monoclonal IgM, Santa Cruz Biotechnology) for 24 hr at 4 °C. After rinsing the cell culture chambers with a blocking solution, the respective secondary antibody: Alexa Fluor 514-conjugated goat anti-mouse IgG (H + L) (10 μg mL^{-1}, Invitrogen), R-Phycoerythrin

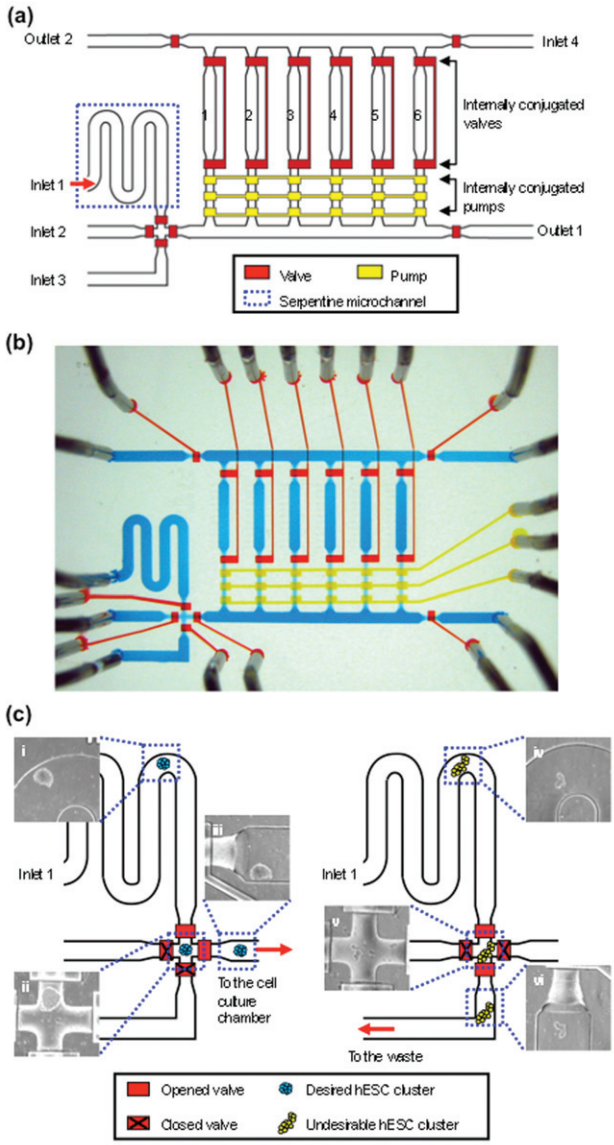

Fig. 1 Design of the hESC-μChip. (a) Schematic illustration of a hESC-μChip capable of semi-automated operation for hESC culture and analysis. The functions of different hydraulic valves are illustrated by their colors: Red for pneumatic valve operation and yellow for fluidic delivery and metering. The 6 × 1 array of cell culture chambers (with dimensions of 3000 μm (l) × 500 μm (w) × 100 μm (h) and total volume of 150 nl) are numbered 1 to 6. Each cell culture chamber is separated by hydraulic valves to achieve individual addressability. There are four inlets and two outlet channels in each device, providing accesses to hESC colonies, culture media and immunostaining reagents. (b) Optical micrograph of the actual device. Food dyes were introduced into the various microchannels to help visualize the functional components of the hESC-μChip: Red and yellow as illustrated in (a); blue indicates the fluidic channels. A ruler was fabricated alongside of each cell culture chamber to serve as a landmark that directs continuous fate mapping of individual hESC colonies by an optical microscope. For hESC culture in the hESC-μChip, freshly prepared hESC clusters were introduced into cell culture chambers through the inlet connected to a serpentine microchannel as shown in (c), where every hESC cluster was visually inspected (i,iv). To ensure the uniformity of hESC clusters used in our studies, only hESC clusters with the desired size and morphology were introduced into cell culture chambers (i–iii). Undesirable hESC clusters were removed as waste (iv–vi).

(R-PE)-conjugated goat anti-mouse IgM (10 μg mL^{-1}, BD Pharmigen), Cy5-conjugated goat anti-rabbit IgG (H + L) (7.5 μg mL^{-1}, Jackson ImmunoResearch), or Alexa Fluor 750-conjugated goat anti-mouse IgG (H + L) (20 μg mL^{-1}, Invitrogen) was loaded into the cell culture chambers to detect the bound primary antibodies. After incubating at room temperature for 1 hr, the chambers were rinsed with PBS-T. Finally, 10 μg mL^{-1} of DAPI solution was loaded for nuclear staining.

For alkaline phosphatase (AP) staining, the hESC colonies were fixed with paraformaldehyde (4%) for 30 min at room temperature. After fixation, a freshly prepared AP staining solution (1 mg mL^{-1} Fast Red TR salt in water with 0.01% AS-MX alkaline phosphate solution, Sigma) was loaded into the cell culture chambers and incubated for 30 min in the dark. Fluorescence and phase contrast images were taken with an inverted microscope (TE2000S, Nikon), and quantitatively analyzed with MetaMorph software (version 7.1.3.0; Molecular Devices) (Fig. S2†).

Results

Design and operation of hESC-μChips

A typical hESC-μChip (Fig. 1a and b) is composed of a 6 × 1 array of cell culture chambers (with dimensions of 3000 μm (l) × 500 μm (w) × 100 μm (h) and total volume of 150 nL) for accommodating individual hESC colonies. A ruler was fabricated alongside each cell culture chamber as a landmark, so that individual hESC colonies were registered for continuous fate mapping using an inverted microscope. There are four inlets and two outlet channels in each device, providing accesses to culture media and immunostaining reagents. For hESC culture in the hESC-μChip, freshly dissociated hESC clusters (obtained by digesting conventionally cultured hESC colonies with collagenase IV) were introduced into cell culture chambers through the inlet *via* a serpentine microchannel where every hESC cluster was visually inspected (Fig. 1c). To ensure the uniformity of hESC clusters in our studies, only disc-shaped clusters with diameters within 100 ± 20 μm were introduced to the cell culture chamber. In general, four to six hESC clusters were selected and seeded per chamber. To allow parallel examination of multiple variables over time, six pairs of hydraulic valves (Fig. 1a and b) conferred individual addressability to the six cell culture chambers in the device. A laptop computer was utilized to control the valves and pumps to achieve automated operation of the hESC-μChip.

To ensure general applicability of the hESC-μChips, we conducted our studies using a collection of hESC lines, including two parental hESC lines (*i.e.*, HSF1 and H1) and three genetically modified hESC lines–(i) HSF1-LG which expresses firefly luciferase and enhanced green fluorescent protein (EGFP) as a fusion protein driven by the ubiquitin promoter, (ii) HSF1-OCT4-EGFP and (iii) H1-OCT4-EGFP which express EGFP under the endogenous *OCT4* promoter. In our proof-of-concept studies, hESC-μChip-based culture experiments were carried out in the presence of γ-irradiated mEFs, using serum replacement-containing media with either 10 or 100 ng mL^{-1} of bFGF. The γ-irradiated mEFs were seeded in the protein-coated cell culture chambers for 12 hr prior to the introduction of the dissociated hESC clusters. Throughout the experiment, hESC-μChips

were stored in a humidified incubator (5% CO_2, 37 °C). The gas-permeability of PDMS allowed rapid gas exchange between the atmosphere around the hESC-μChips and the media in the cell culture chambers. The results revealed that medium with a concentration of 100 ng mL^{-1} bFGF gave better reproducibility of hESC self renewal in the device. Due to the higher surface area-to-volume ratio of the microfluidic environment, a significant amount of bFGF was absorbed on the microchannels surfaces. The use of a higher concentration of bFGF was sufficient to maintain the chip-cultured hESC colonies. Since the hESC-μChip consumes only 150 nL of medium in each culture chamber, the use of 100 ng mL^{-1} bFGF has very limited impact on experimental cost.

Optimization of hESC culture conditions

Since this digitally controlled hESC-μChip is capable of small-scale screening, we were able to utilize these devices to progressively define an optimal surface coating protocol and a cell feeding schedule which are optimized for the hESC colonies. Initially, several protein coating combinations and approaches were examined in the device in search of a recipe (Fig. S3†) which led to efficient plating of the γ-irradiated mEF layer and reproducible self-renewal of hESC colonies. We identified a layer-by-layer coating method: a layer of fibronectin (FN) was first coated onto the PDMS surfaces (by introducing 1 mg mL^{-1} FN solution into the cell culture chambers and incubated at 37 °C for 30 min), followed by sequential deposition of a gelatin layer (0.2% gelatin solution at 37 °C for 15 hr). This coating method resulted in a uniform and long-lasting FN/gelatin layer on the PDMS surface for maintaining hESC colonies. Using a hESC-μChip with six FN/gelatin-coated cell culture chambers, we then carried out a parallel examination of different cell feeding schedules. By programming the cooperation of hydraulic valves and peristaltic pumps, the medium stored in Teflon tubing was periodically introduced into each cell culture chamber at different feeding intervals (*i.e.*, 3, 6, 12, 18, 24 and 36 hr). As a result of monitoring morphology and survival rate of hESC colonies, we identified a 12-hr feeding cycle which allowed the reproducible self renewal of hESC colonies in our hESC-μChip for 6 days. By using the optimized hESC culture condition (*i.e.*, in the presence of serum replacement-containing medium, γ-irradiated mEFs and FN/gelatin coated cell culture chambers, as well as using a cell feeding cycle of 12 hr), we were able to culture HSF1, H1, HSF1-LG, HSF1-OCT4-EGFP and H1-OCT4-EGFP in the hESC-μChips for 6 days (Fig. S4†). In addition, HSF1 cells could be cultured in our μChips up to 12 days for the longest culturing periods (Fig. S5†). By chance, a single hESC (HSF1) colony was cultured in a cell culture chamber (Fig. S6†). There was no significant difference observed in contrast with the multi-colonies culture.

Chip-based immunocytochemistry to confirm hESC pluripotency

To confirm pluripotency of hESC-μChip-cultured hESCs, immunocytochemistry for a number of pluripotency markers, including alkaline phosphatase (AP), stage-specific embryonic antigen 4 (SSEA4), OCT4 (also known as POU5F1), NANOG, tumor-related antigen (TRA)-1-60 and TRA-1-81, was carried out in the same device. The digitally controlled interface allowed automated execution (Supplementary Methods) of the immunostaining processes, where multiple reagents, including paraformaldehyde (4%) for fixation, Triton X-100 (0.5%) in PBS for permeabilization of the cell membrane, and antibodies for fluorescent immunocytochemical analyses, were introduced into the cell culture chambers in sequence. It is noteworthy that mixtures of four different pluripotency markers could be introduced in individual culture chambers, allowing four fluorescence immunocytochemical analyses at the same time. Finally, the resulting hESC-μChip was mounted on either a fluorescence microscope or a confocal microscope to collect immunofluorescence micrographs. Fig. 2a and b show immunofluorescence images of hESC-μChip-cultured HSF1 and H1 colonies, respectively. These cells retained characteristic hESC morphology, and strong fluorescence signals of pluripotency markers, indicating that they maintained their stemness over the six-day culture period. Three-dimensional (3D) confocal micrographs of hESC-μChip-cultured hESCs (Fig. 2c–e, and the visualization of its 3-D structure in a movie clip in Supplemental Information) revealed 3D structures of the hESC colonies, and merged 3D confocal micrographs indicate the co-localization of different pluripotency markers.

Quantification of hESC growth in the hESC-μChip

To monitor hESCs *in vitro* and *in vivo*, HSF1-LG cells were generated by infecting HSF1 cells with lentivirus containing a mutated thermostable firefly luciferase (mtfl)[38] and EGFP as a fusion protein (LG) driven by a ubiquitin promoter (Fig. S7a–h†). The EGFP signal allows the quantification of cell growth in real time.[39] To test this idea, freshly dissociated HSF1-LG clusters were cultured in the hESC-μChip for 6 days,[40] and their EGFP signals were measured every other day (Fig. 3a). In parallel, these dissociated clusters were cultured in conventional culture dishes under similar conditions. The growth rates of hESCs were quantified by measuring the increased surface area or integrated EGFP intensities of individual hESC colonies at different time points. As shown in Fig. 3b and c, both quantification approaches gave similar results. Although inhibition of cell proliferation has been reported in other microfluidic cell culture settings,[41] possibly due to the constrained accumulation of soluble factors in the diffusion dominant microfluidic environment, the growth rates of hESC-μChip cultured colonies were not significantly different from those observed for hESCs in conventional dishes ($p = 0.21$).

Multiparameter monitoring of hESC pluripotency status

In order to monitor the pluripotent status of hESCs in real-time, we constructed OCT4-EGFP knock-in reporter lines in HSF1 and H1 cells (*i.e.*, HSF1-OCT4-EGFP and H1-OCT4-EGFP) (Fig. S8a–d†). In both cases, the linearized OCT4-EGFP knock-in construct[42] was introduced into hESCs *via* Nucleofector® (Amaxa Biosystems). These genetically modified hESCs could be passaged as their parental HSF1 or H1 cells. To ensure that EGFP expression in these hESCs faithfully represents pluripotency, both HSF1-OCT4-EGFP and H1-OCT4-EGFP were induced to differentiate in the presence of

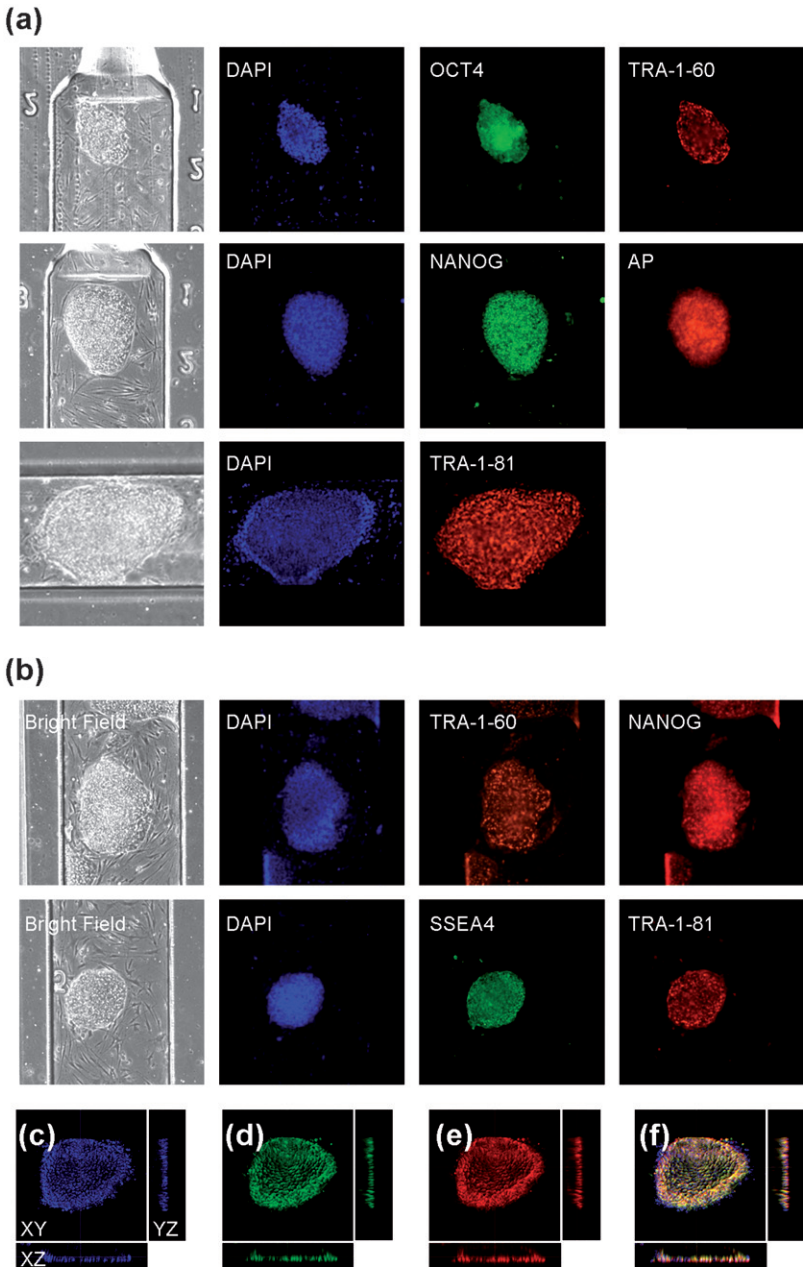

Fig. 2 On-chip immunocytochemistry to confirm hESC pluripotency. Bright-field and fluorescence micrographs of hESC-μChip-cultured hESC colonies stained with a collection of pluripotency markers: (a) Three HSF1 colonies were stained by DAPI and alkaline phosphatase (AP), as well as immunostained for OCT4, NANOG, TRA-1-60 and TRA-1-81. (b) Two H1 colonies were stained with DAPI, SSEA4, NANOG, TRA-1-60 and TRA-1-81. The characteristic morphologies and strong fluorescent signals of pluripotency markers indicate that the hESCs cultured in hESC-μChips retained their pluripotency over the six-day culture period. (c–f) Three-dimensional (3D) confocal micrographs of a genetically modified hESC colony (HSF1-LG). (c) DAPI nuclear staining, (d) EGFP expression, (e) OCT4 immunostaining and (f) the merged image. These images revealed information on the 3D structure of the hESC colonies.

fetal bovine serum (FBS, 15%) and the absence of mEFs. After about 10 days in culture, over 90% of the cells lost EGFP expression, correlating with their differentiated morphology (Fig. S8e†). Additionally, if the EGFP signal truly correlates with the endogenous OCT4 expression, this marker could be used to rescue pluripotent cells from a differentiated population. To show this, OCT-EGFP-knock-in cells were differentiated as embryoid bodies in serum containing medium. After 21 days, the EGFP positive population (approximately 3%) was sorted from the non-expressing cells (Fig. S8g†) and re-plated into conventional culture conditions. Indeed, these cells re-grew into typical ES colonies and maintained pluripotency markers (Fig. S8h–j†).

Either HSF1-OCT4-EGFP or H1-OCT4-EGFP hESCs were utilized for the demonstration of parallel examinations of controlled differentiation and proliferation in individual hESC-μChips. In a given study, differentiation of hESCs was carried out in cell culture chambers No. 1, 3 and 5, where only a layer of

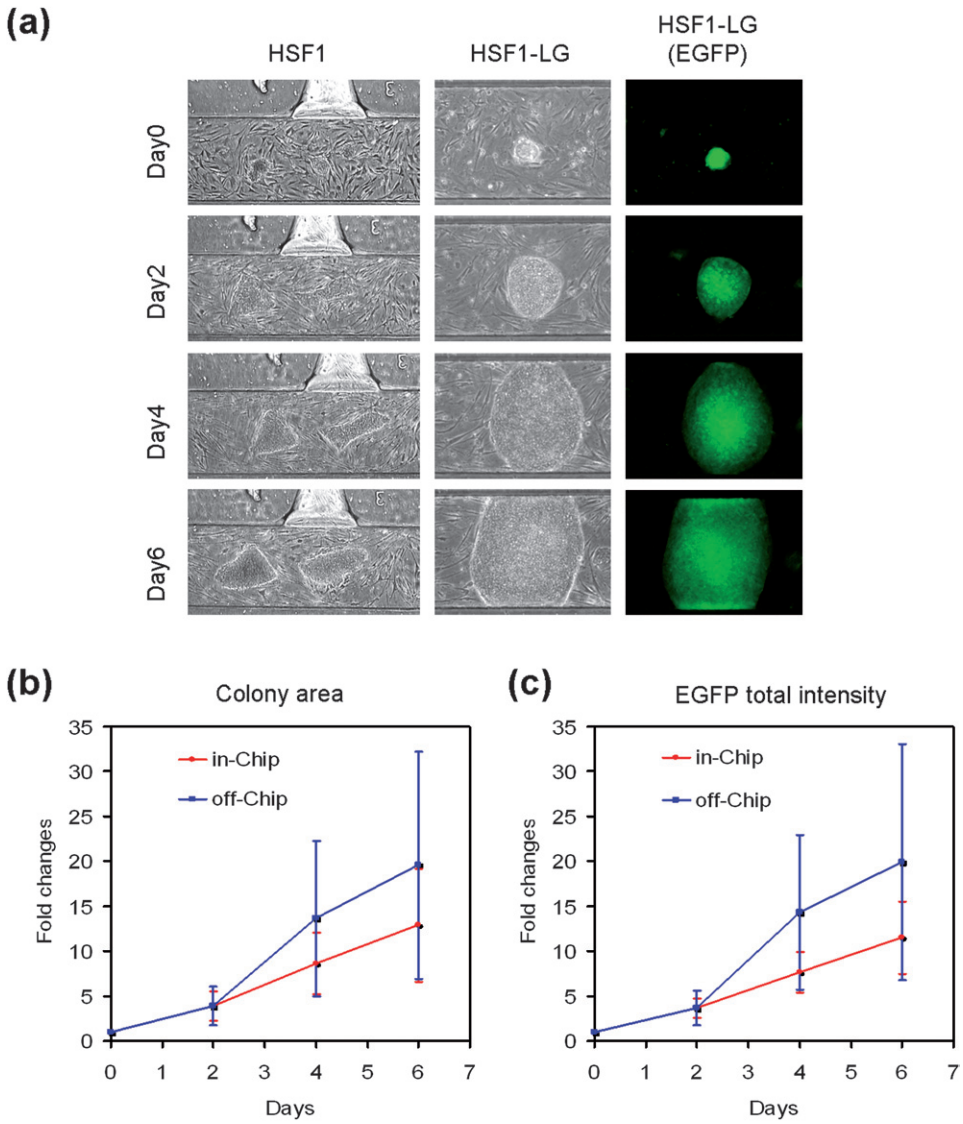

Fig. 3 Real-time quantitative monitoring of growth of hESC-μChip-cultured hESC colonies. (a) Fate mapping of hESCs cultured in a hESC-μChip with bright-field microscopy. As we show in Fig. 2, the hESC colonies still had pluripotency, even in hESC colonies attached onto the channel. And, since there are PDMS walls in the EGFP images of HSF1-LG at day 6, there is no EGFP signal from those areas. In addition, since EGFP expression in HSF1-LG is under the regulation of a ubiquitin promoter which constitutively active in any kinds of cells, EGFP intensity doesn't reflect their pluripotency. (b) Quantitative comparison of growth rate of the size of hESC colonies in conventional culture dishes and hESC-μChips. (c) Quantitative comparison of growth rate of EGFP intensity of hESC colonies in conventional culture dishes and hESC-μChips. Each bar represents the standard deviation ($n > 7$).

FN was coated and no feeder cells were applied. In parallel, proliferation of hESCs was carried out in culture chambers No. 2, 4 and 6, where FN/gelatin coating was applied and γ-irradiated mEFs were cultured. After 24 hr, the genetically modified hESCs clusters were introduced into the 6 cell culture chambers. After 3 hr, differentiation medium (containing 5 μM retinoic acid (RA) and 15% FBS) and self-renewal medium were separately introduced into the respective sets of chambers with a 12-hr feeding schedule. The EGFP signals in the differentiating or self-renewing cells were monitored every other day to record the status of their pluripotency. hESC colonies in differentiation medium gradually lost their compact morphologies and spread out. Concurrently, the EGFP signals started to diminish after 2 days, whereas the hESC colonies in the self-renewal medium grew larger accompanied by increased EGFP signal (Fig. 4a). After 4 days of culture in a hESC-μChip, immunocytochemistry for SSEA1 was performed to confirm differentiated or pluripotent status. In general, SSEA1 is the marker for pluripotency for murine ESCs, whereas only differentiated hESCs show expression.[42] As shown in Fig. 4b and 4c, hESCs in differentiation medium showed strong staining for SSEA1, correlating with the loss of EGFP signal. In contrast, hESCs in the self-renewal medium maintained strong expression of EGFP, but no expression of SSEA1 was detected. This demonstrated that a single

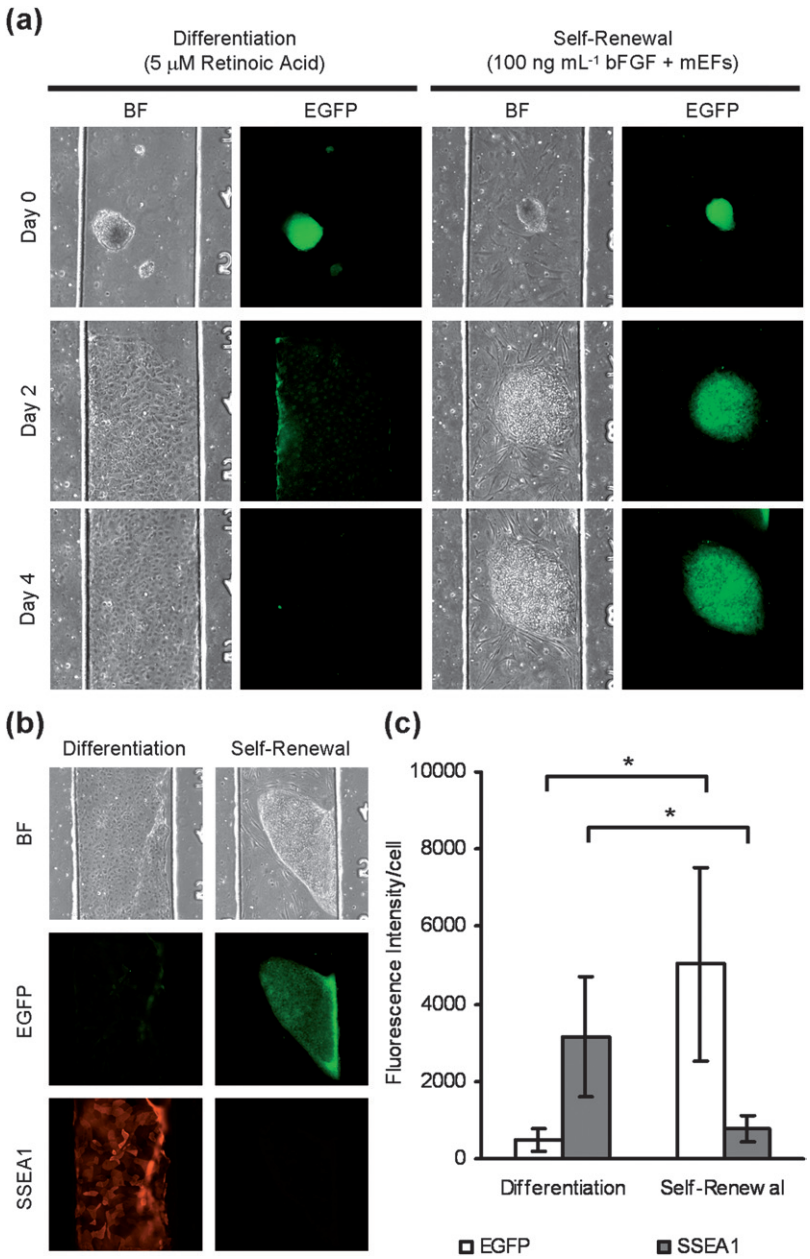

Fig. 4 A single hESC-μChip serves as a platform for parallel examination of controlled differentiation and self-renewal for hESCs. Either differentiation medium (containing 5 μM retinoic acid and 15% FBS) or self-renewal medium (100 ng mL^{-1} bFGF) was introduced into cell culture chambers No. 1/3/5 or No. 2/4/6, respectively. (a) Fate mapping of HSF1-OCT4-EGFP colonies under the differentiation and self-renewal conditions using an inverted fluorescent microscope. After 2 days, hESC colonies under the differentiation condition gradually lost their hESC morphology and EGFP signal, whereas the hESC colonies under the self-renewal condition grew larger accompanied by an increased EGFP signal. (b) HSF1-OCT4-EGFP cells were immunostained for SSEA1 (a differentiation marker) at Day 4. (c) Quantitative comparison of EGFP intensity of hESC colonies in differentiation medium and self-renewal medium. Each bar represents the standard deviation ($n > 7$).

hESC-μChip could carry out controlled self-renewal and differentiation in parallel without cross contamination.

Discussion

We have successfully demonstrated reproducible and quantitative culture and analysis of individual hESC colonies in an integrated microfluidic platform, the hESC-μChip. The six individually addressable cell culture chambers in the hESC-μChip allowed parallel examination of combinations of variables over time to obtain optimal culture conditions for self-renewal and controlled differentiation of hESCs. In addition to the intrinsic advantages of microfluidic systems, the hESC-μChip provides an opportunity to culture hESCs in different conditions in parallel as well as to run sequential phenotypical and functional analyses. Several small-scale screenings were performed to identify the optimal chip-based culture conditions that are widely applicable for a collection of hESCs, including two parental

hESC lines and three genetically modified hESC lines. Semi-automated immunoassays for a number of pluripotency markers were carried out in sequence to confirm that the chip-cultured hESC colonies maintained their pluripotency over a culture period of at least 6 days.[43] Two more hESC lines, HSF6 and H9, could also be cultured in the hESC-μChip, and maintained their pluripotency (data not shown). Finally, we were able to demonstrate parallel examination of proliferation or controlled differentiation in a single hESC-μChip. Three genetically modified hESC lines allowed quantitative monitoring of hESC proliferation and pluripotency of the hESC-μChip-cultured hESC colonies in a real-time manner.

Conventional hESC research is conducted in a collective fashion which overlooks a great deal of information on individual hESC colonies and their microenvironments over time. Lack of precise control of experimental and analytical conditions makes it difficult to interpret the results obtained from different experiments. In the hESC-μChip, there are six identical cell culture chambers providing a closely related microenvironment for multiparameter analysis. In each cell culture chamber, there is a built-in landmark to register individual hESC colonies for continuous fate mapping. The hESC-μChip is controlled by a laptop PC, allowing reproducible culture and analysis of individual hESC colonies in a semi-automated fashion. Although there were microfluidic devices developed for the culture of hESCs,[33,34,44] no quantitative and integrated culture and analysis has been reported. In conjunction with a fluorescent microscope and three genetically modified hESC lines, we demonstrated, for the first time, that the hESC-μChip is capable of integrated and quantitative culture and analysis of hESCs.

We also realized that we have a limited number of samples per chip in the hESC-μChip, and it cannot be operated in a fully automated fashion. Currently, a new generation of fully automated hESC-μChip incorporating hundreds of individual cell culture chambers is under development. We envision that the new generation hESC-μChip will be applied for high-throughput screening of feeder-free and chemically defined conditions which better regulate self-renewal and differentiation of hESCs. Furthermore, by using HSF1-LG and OCT4-EGFP knock-in cell lines, the integrated microfluidic hESC culture platform can provide a new screening system of the condition for single hESC expansion and fate mapping for individual hESCs.

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