Microfluidic platform for controlling the differentiation of embryoid bodies†

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Embryonic stem (ES) cells are pluripotent cells, which can differentiate into any cell type. This cell type has often been implicated as an eminent source of renewable cells for tissue regeneration and cellular replacement therapies. Studies on manipulation of the various differentiation pathways have been at the forefront of research. There are many ways in which ES cells can be differentiated. One of the most common techniques is to initiate the development of embryoid bodies (EBs) by in vitro aggregation of ES cells. Thereafter, EBs can be induced to undergo differentiation into various cell lineages. In this article, we present a microfluidic platform using biocompatible materials, which is suitable for culturing EBs. The platform is based on a Y-channel device with two inlets for two different culturing media. An EB is located across both streams. Using the laminar characteristics at low Reynolds number and high Peclet numbers, we have induced cell differentiation on half of the EB while maintaining the other half in un-induced stages. The results prove the potential of using microfluidic technology for manipulation of EBs and ES cells in tissue engineering.

Introduction

Embryonic stem (ES) cells are derived from the inner cell mass of the blastocyst of a developing embryo.[1] They are pluripotent cells which could virtually give rise to any cell type including neural cells, cardiomyocytes and hepatocytes through cellular differentiation.[2] ES cells are self-renewable and for that reason, they have often been implicated as an eminent source of renewable cells for tissue regeneration and cellular replacement therapies. Hence, it is not surprising that the control and manipulation of the various differentiation pathways in ES cells have been at the forefront of research.

There are many ways to differentiate ES cells. One of the most common techniques is to initiate the development of embryoid bodies (EBs) by in vitro aggregation of ES cells.[3,4] Thereafter, EBs can be induced to undergo differentiation into the various cell lineages.[4–7] To date, no attempt was ever made to sub-differentiate a single EB into more than one lineage. This is mainly compounded by the limitations of conventional culturing techniques, which do not permit an EB to be induced by more than one differentiation media at the same time.

Microfabricated culturing systems can circumvent this limitation and permit the control of the differentiation process by manipulating the environment of the culture in both 'time and space', which is almost impossible to achieve using conventional culturing protocols. The behaviour of fluids at the microscale is different from that in the macroscale. For microfluidic systems some gripping and counterintuitive properties appear. When two fluids streams come together in a microchannel, a parallel laminar flow can be generated without turbulence.[8] The Reynolds number, which represents the ratio between inertial force and viscous force, is in microscale often less than unity. At these low Reynolds numbers, the Peclet number, which represents the ratio between advection and diffusion, is still in the order of 1000. Thus a microchannel would allow two liquids to follow side by side without major intermixing. This unique property of liquid in a microchannel enables possibilities and new tools for biological research.

With recent advances in their design and fabrication, microfluidic platforms have been applied in research areas including stem cell and developmental biology. Manipulation of microenvironments, such as temperature, pH, mechanical stimuli and growth factor gradient can be achieved by a microfluidic platform, which will be useful in identifying factors involved in stem cell proliferation and differentiation.[9] Recently, a gradient-generating microfluidic platform has been applied to study the effects of a continuous gradient of epidermal growth factor, fibroblast growth factor 2, and platelet-derived growth factor on the growth and differentiation of neural stem cells.[10] Interestingly, colony formation of murine embryonic stem cells can be influenced by different flow rates generated by a microfluidic platform although the mechanism behind the flow rate dependent effects remains uncertain.[11] Furthermore, the temperature gradient created by a microfluidic platform revealed the importance of temperature on development of the *Drosophila* embryo.[12] Cell fusion between mouse embryonic stem cells and mouse embryonic fibroblasts was demonstrated with a newly designed microfluidic device.[13] A microfluidic platform has been also shown to facilitate individual gene expression profiling on neural stem cells.[14,15] To further improve the capability of the microfluidic platform, 3D microfluidic cell culture systems have been developed, which offers a similar *in vitro* 3D microenvironment as *in vivo*.[16,17] Clearly, the microfluidic platform has become a new tool for stem cell and developmental biology research from 2D to

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3D culture because it offers a wide range of micro-environmental control and it is a powerful tool to probe the separate effects of environmental and spatial conditions on cell fate.[9]

In most of the previous experimental investigations on EB differentiation, EBs were only subjected to a single differentiation condition.[18–21] Here we demonstrate that by independently cultivating the two halves of an EB in two separate media resulting from laminar co-flow in a microchannel, cell differentiation can be induced on half of the EB while the other half of the EB maintained in un-induced stages can be achieved.

Materials and methods

Mouse ES cell culture

E14 Mouse ES (mES) cells were cultured in dishes coated with 0.1% w/v gelatin (Sigma) and maintained in a complete medium consisting of Dulbecco's Modified Eagle's Medium (DMEM; Gibco) containing 20% fetal bovine serum (FBS; Hyclone), 0.1 mM 2-mercaptoethanol (Sigma), 1% MEM non-essential amino acids (Gibco), 100 U/ml penicillin (Invitrogen), 100 U/ml streptomycin (Invitrogen) and 1000 U/ml ESGRO-LIF (Chemicon), in a humidified incubator (37 °C in an atmosphere of 5% CO_2), with the medium changed every 2 days. To induce EB formation, mES cells were dissociated into a single-cell suspension by 0.05% trypsin and 0.04% EDTA in PBS and plated onto non-adherent bacterial culture dishes and maintained in the complete DMEM medium without LIF for 3 days on a 3D shaking platform in a humidified incubator (37 °C in an atmosphere of 5% CO_2).

Induction of cell differentiation

For the differentiation assay of EB, a three-days EB grown in bacterial culture dish were plated onto 60 mm tissue culture dishes coated with 0.5% w/v gelatin and maintained in the complete DMEM medium without LIF in a humidified incubator (37 °C in an atmosphere of 5% CO_2). After two days of incubation, the EB attached and spread onto the tissue culture dishes, and then the medium was changed into either differentiation medium [complete L15 medium (Gibco) added with 10 μM retinoic acid (RA; Sigma)], or fresh complete L15 medium in a humidified incubator (37 °C) with medium changed every 2 days. After 5 days of incubation, the EB was then collected and lysed with lysis buffer (PIERCE). 20 μg of EB lysate (5–10 EBs) was used in each lane of western blot analysis.

Western blot analysis

Mouse anti-PCNA (Santa Cruz), mouse anti-neurofilament 68 kDa (NF68) (Abcam), and mouse anti-GAPDH (Santa Cruz) antibodies were used in these experiments. Total proteins were extracted with M-PER mammalian protein extraction reagent (Thermo Scientific). 20 μg of EB lysate (5–10 EBs) was loaded on each lane and then separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane (Whatman). The membranes were incubated with antibodies to specific protein followed by incubation with secondary antibodies: polyclonal goat anti-mouse immunoglobulins/HRP (Dako), polyclonal mouse anti-goat immunoglobulins/HRP (Dako) or polyclonal rabbit anti-goat immunoglobulins/HRP (Dako), and developed with chemiluminescence reagent (Thermo Scientific).

Fabrication of the PMMA/PDMS microfluidic platform

A Y-shape channel was cut on a rectangular (76.2 × 25.4 mm) piece of polymethylmethacrylate (PMMA). A CO_2 laser machining system (Universal M-300 Laser Machining Platform, Universal Laser Systems Inc., Arizona, USA) was used for cutting the 1 mm-thick PMMA sheet (Goodfellow, England). Two layers of polydimethylsiloxane (PDMS) at the top and at the bottom of the Y shape channel were used for sealing of the device (Fig. 1A). The PDMS sheet with the thickness of 0.5 mm was prepared by spin coating the PDMS mixture (Dow Corning Corporation, USA) at 500 rpm for 30 seconds. The prepolymer and the curing agent of PDMS were mixed with a weight ratio of 10:1. The thin PDMS layer was then cured in an oven for 4 hours at 90 °C. Holes were cut in the top layer of PMMA and PDMS to provide an inlet and outlet for the media. The microfluidic chip consisted of two PMMA layers (1mm thick), two layers of PDMS (0.5 mm thick) and one glass slide. Thus, the internal dimension (width and height) of the microchannel was 1 mm × 1 mm. The inlet and outlet of the channel were cut as a "Y" and a "J" shape for connecting to the inlets and outlet of the holder (Fig. 1B) respectively. Two holders were used to fasten the microfluidic chip by screws at both ends. The PMMA holder with sealing rings for the inlets of the microfluidic device was connected to a peristaltic pump (Cole Parmer, USA) by tubing with 0.5 mm inner diameter (Cole Parmer, USA), whereas another holder served as the outlet, which was connected to a waste bottle (Fig. 1C).

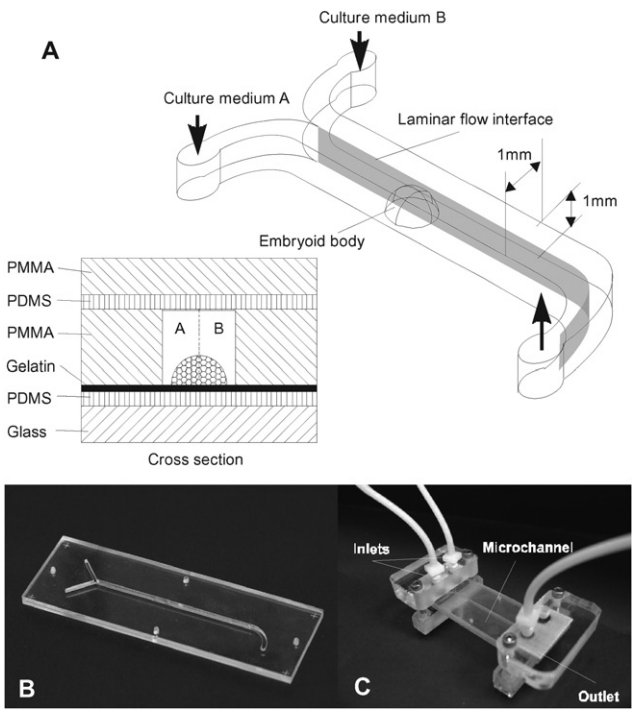

Fig. 1 Schematic diagram illustrating the components of the microfluidic device (A). Photographs of the microfluidic chip (B) and the assembled microfluidic device (including holders, microfluidic chip and tubings) (C).

Measuring the laminar flow generated by different flow rates in the microfluidic channel

Two different media were prepared. Medium A contains complete L15 with a green fluorescence dye (Alexa 488). Medium B contains complete L15 without the green fluorescence dye (Alexa 488). Subsequently, medium A and B were pumped into the microfluidic device at the same flow rate. The fluorescence intensity of the medium passing through the microfluidic device was imaged by a coupled charge device (CCD) camera attached to the fluorescence microscope at a 1 second interval. Flow rates of 50 μl/min, 100 μl/min, 150 μl/min, and 200 μl/min were examined. The fluorescence intensity across the microchannel (Fig. 2A) was measured using the line-scan function of the Zeiss Axiovision software and plotted as a X-Y scatter graph shown in Fig. 2B. At 50 μl/min, the estimated Reynolds number and Peclet number are on the order of 1 and 1000. Thus flow is laminar, but advection dominates over diffusion. The culture media do not mix across their interface.

Immunofluorescence staining of EBs

After 5 days incubation, the microfluidic device was dissembled and the EB was fixed with 4% (w/v) paraformaldehyde for 30 min and then permabilized with 0.5% Triton X-100 (Amersham) in phosphate buffered saline (PBS) for 30 min. The EB was blocked with 5% bovine serum albumin (BSA) in PBS for 30 min and sequentially exposed to primary and secondary antibodies: mouse anti neurofilament 160 kDa (NF160) protein antibodies (Chemicon; 1/1000 dilution; room temperature 1 hr), rabbit anti mouse Ki67 antibodies (Novacastra; 1/500 dilution; room temperature 1 hr), Hexa Fluro 488 goat anti-mouse IgG (Invitrogen; 1/1000 dilution; room temperature 1 hr), Alexa Fluro 594 goat anti-rabbit IgG (Invitrogen; 1/1000 dilution; room temperature 1 hr). Cell nuclei were then counter-stained with DAPI and the coverslip was mounted with ProLong® Gold antifade reagent (Invitrogen). Fluorescent images were obtained by an inverted microscope equipped with a motorized stage (Carl Zeiss).

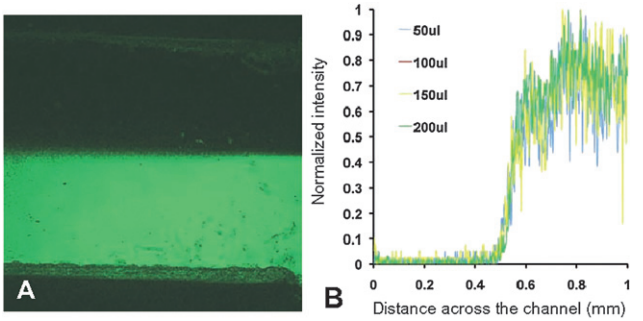

Fig. 2 Measuring the mixing effect of two media in the microchannel at different flow rates. The laminar flow generated by different flow rates in the microchannel was visualized by addition of a green fluorescence dye (Alexa 488) into one of the media (A). Flow rates of 50 μl/min, 100 μl/min, 150 μl/min, and 200 μl/min were examined. The normalized fluorescence intensities across the microchannel were measured and plotted against the width of the microchannel (B). The results showed that all the flow rates tested could maintain a parallel laminar flow.

Results and discussion

Determination of the minimum flow rate of the medium

To optimize the parallel laminar flow with minimum consumption of the culturing medium, different flow rates were examined. It was found that all the tested flow rates could maintain a parallel laminar flow without intermixing between the fluorescence labelled medium and the non-labelled medium (Fig. 2A and B). Hence, a flow rate of 50 μl/min was chosen in the subsequent experiments to minimize the consumption of the culturing medium.

Biocompability of PDMS for the growth and differentiation of embryoid bodies

The microfluidic chip was assembled as shown in Fig. 1 to test the ability of PDMS to support the growth and differentiation of the embryoid body. A three-days EB (Fig. 3Aa) was transferred by a pipette tip into the microchannel, and the whole microfluidic chip was immersed in the complete DMEM in a 100 mm tissue culture dish. The EB was incubated at 37 °C for another 2 days (Fig. 3Ab). When the EB was attached onto the gelatin coated PDMS layer of the microfluidic chip, another two layers of PDMS and PMMA were placed on the PMMA layer as illustrated in Fig. 1. A peristaltic pump was connected to the two inlets of the microchannel, which provided a continuous flow of either complete L-15 medium (Gibco), to maintain the normal growth of EB, or complete L15 medium with 10 μM RA, to induce cell differentiation of EB, at a flow rate of 50 μl/min, respectively. The microfluidic device was then incubated in the live imaging system (Carl Zeiss). The growth of EB was monitored by time-lapse microscopy (Carl Zeiss) (Fig. 3B). After 5 days of incubation at 37 °C, the microfluidic device was disassembled and the EB in the microchannel was analyzed by immunofluorescence staining using antibodies against Ki67 as proliferation marker and NF160 as cell differentiation marker. It was found that there was strong Ki67 staining on the EB incubated with complete L15 medium, suggesting that growth of EB was not affected by PDMS. Most importantly, addition of RA induced cell differentiation of EB in the microfluidic channel as suggested by the positive immunostaining of NF160 (Fig. 3C). Taken together, PDMS has no adverse effect on the growth and differentiation of EB.

To investigate the effects of RA on changes in rate of cell proliferation and induction of cell differentiation during EB development, western blot analysis was performed. Our results showed that the level of PCNA, the proliferation marker, in EBs cultured in complete L15 medium was higher than those in complete L15 medium with 10 μM RA after 5 days of incubation (Fig. 3D and 3E) (the two-tailed P value equals 0.0040). Besides, NF68, which are intermediate filaments found specifically in neurons, was only detected in the EBs maintained in complete L15 medium with 10 μM RA for 5 days (Fig. 3D and 3E). These results suggested that addition of RA is proficient and effective to induce cell differentiation and slow down the rate of cell proliferation on EB in the microfluidic device.

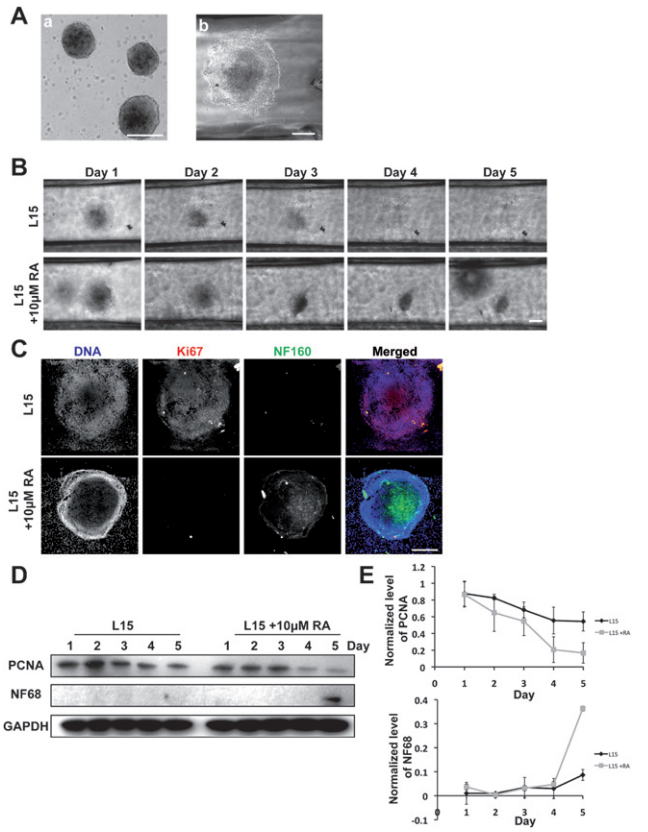

Fig. 3 Culturing of the embryoid bodies in the microfluidic device. The optical micrograph of EBs was taken from the non-adherent bacterial culture dish after three days incubation (Aa). A 3-day EB was transferred into the microchannel and cultured with complete L15 medium without LIF for 2 days in a humidified incubator. Bright field micrograph of the EB was attached onto the surface of the microchannel after incubation (Ab). The microfluidic device was assembled and connected with a peristaltic pump. The EB was then maintained in the microfluidic device under a constant flow rate (50 μl/min) with complete L15 medium or complete L15 medium with addition of 10 μM RA for 5 days. Time-lapse images of EBs cultured in the microfluidic device from day 1 to day 5 with either complete L15 medium or complete L15 medium with 10 μM RA respectively (B). After 5 days incubation, EB in the microchannel cultured with complete L15 medium or complete L15 medium with 10 μM RA were fixed and stained with anti-NF160 and anti-Ki67 antibodies, cell nuclei were counter-stained with DAPI (scale bar: 200 μm) (C). The expression of NF160 was only detected on the EB maintained with complete L15 medium with 10 μM RA. Scale bar, 200 μm. EBs maintained with complete L15 and complete L15 with 10 μM RA from day 1 to 5 were collected. 20 μg of EB lysate (from 5–10 EBs) was then analysed by western blot analysis using antibodies specific for PCNA and NF68 using GAPDH as control (D). Expression level of PCNA and NF68 were quantified and then normalized by the level of GAPDH at the same point. The average normalized expression levels of PCNA and NF68 were then plotted against number of incubation days. Data are mean ± standard deviation of four independent western blot analyses (E).

Induction of neural differentiation on half of the EB while maintaining the other half in un-induced stage

We then tested the idea whether the microfluidic device could be used to induce cell differentiation on half of the EB while maintaining the rest in un-induced stages, a three-day EB was seeded into the microfluidic device as mentioned above. The EBs were supplied with culturing media *via* the two inlets of the microfluidic device at the flow rate of 50 μl/min each. One stream consists of complete L-15 medium to maintain the normal growth of EB. The other stream consists of complete L-15 medium with 10 μM RA, to induce cell differentiation of EB. The microfluidic device was incubated in the live imaging system (Carl Zeiss) at 37 °C. The growth of EB was monitored by time-lapse microscopy (Carl Zeiss) (Fig. 4A). At the end of the experiment, the EB was analyzed by immunofluorescence staining using antibodies against Ki67 as proliferation marker or NF160 as cell differentiation marker.

A red fluorescence dye was added into one of the media to further confirm that the laminar flow was not disturbed during the incubation. The fluorescence intensity across the channel was measured (data not shown). Neurofilament (NF160) was only found in one half of the EB, while the other half has strong staining of Ki67 (Fig. 4B and ESI Fig. 1).† Consistently, we found that NF68 was expressed in the EB supplied with complete

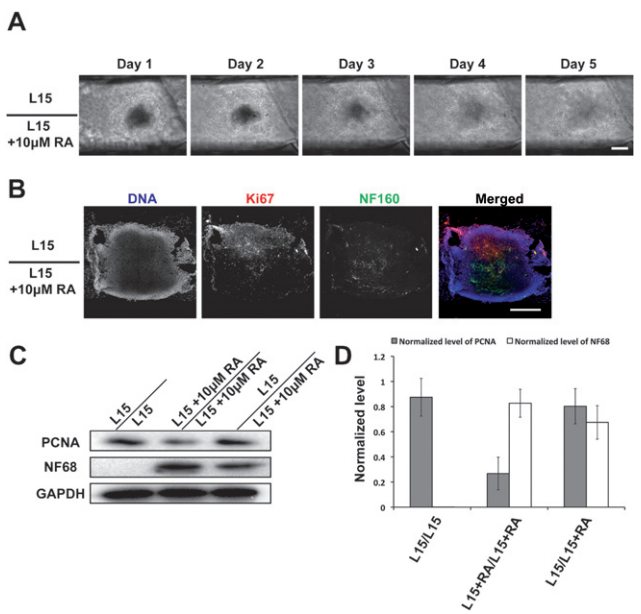

Fig. 4 Induction of neural differentiation on half of EB in the microfluidic device. Time-lapse images of EB cultured under the laminar flow of complete L15 medium and complete L15 with 10 μM RA at a constant flow rate of 50 μl/min (A). After 5 days incubation, the EB in the microchannel was fixed and stained with anti-NF160 and anti-Ki67 antibodies, cell nuclei were counter-stained with DAPI (scale bar: 200 μm) (B). The expression of NF160 was detected mainly at the half of EB that was cultured with complete L15 medium with 10 μM RA, while strong staining of Ki67 was observed on the half of EB maintained with complete L15 medium. For Western blot analysis, three-day EBs were transferred onto the microchannel, the microfluidic device was assembled and then the EBs were maintained with supplying complete L15 medium, complete L15 medium with 10 μM RA or under the laminar flow of complete L15 medium and complete L15 with 10 μM RA at a constant flow rate respectively. After 5 days of incubation, EBs were then collected and lysed by lysis buffer. 20 μg of EB lysate (from 5–10 EBs) was then analysed by western blot analysis using antibodies specific for PCNA and NF 68 with GAPDH as control (C). The expression levels of PCNA and NF68 were quantified and then normalized by the level of GAPDH. Data are mean ± standard deviation of four independent western blot analyses (D).

L15 medium with 10 μM RA (L15 + RA/L15 + RA) and the EB under the laminar flow of complete L15 medium and complete L15 medium with 10 μM RA (L15/L15 + RA). Most importantly, the level of PCNA for the EBs cultivated under the laminar flow of complete L15 medium and complete L15 medium with 10 μM RA (L15/L15 + RA) is significantly higher than those cultivated in complete L15 medium (L15/L15) and complete L15 medium with 10 μM RA (L15 + RA/L15 + RA) (The two-tailed P value equals 0.0014) (Fig. 4C and 4D), suggesting a portion of the EB remains un-induced and proliferative.

Conclusions

In conclusion, we have fabricated a microfluidic device and cultured EB derived from mouse ES cells in this device. Furthermore, we have applied the microfluidic device in EB differentiation, resulting in differentiated and un-induced cells in different areas of the same EB simultaneously. Specific differentiation into different specialized cell lineages on the same EB can be achieved by applying microfluidic technology.

Acknowledgements

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