Nanofibrous substrates support colony formation and maintain stemness of human embryonic stem cells

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Abstract

Inadequate cell numbers in culture is one of the hurdles currently delaying the application of human embryonic stem cells (hESCs) for transplantation therapy. Nanofibrous scaffolds have been effectively used to expand and differentiate non-colony forming multipotent mesenchymal stem cells (MSC) for the repair of tissues or organs. In the present study, we evaluated the influence of nanofibrous scaffolds for hESC proliferation, increase in colony formation, self-renewal properties, undifferentiation and retention of 'stemness'. Polycaprolactone/collagen (PCL/collagen) and PCL/gelatin nanofibrous scaffolds were fabricated using electrospinning technology. The hESCs were seeded on the nanofibrous scaffolds in the presence or absence of mitomycin-C treated mouse embryonic fibroblasts (MEFs). The hESCs grown on both scaffolds in the presence of the MEFs produced an increase in cell growth of 47.58% ($P \leq 0.006$) and 40.18% ($P \leq 0.005$), respectively, over conventional controls of hESCs on MEFs alone. The hESC colonies were also larger in diameter on the scaffolds compared to controls (PCL/collagen, 156.25 $\pm$ 7 μM and PCL/gelatin, 135.42 $\pm$ 5 μM). Immunohistochemistry of the hESCs grown on the nanofibrous scaffolds with MEFs, demonstrated positive staining for the various stemness-related markers (octamer 4 [OCT-4], tumour rejection antigen-1–60, GCTM-2 and TG-30), and semi-quantitative RT-PCR for the pluripotent stemness genomic markers (NANOG, SOX-2, OCT-4) showed that they were also highly expressed. Continued successful propagation of hESC colonies from nanofibrous scaffolds back to conventional culture on MEFs was also possible. Nanofibrous scaffolds support hESC expansion in an undifferentiated state with retention of stemness characteristics thus having tremendous potential in scaling up cell numbers for transplantation therapy.

Keywords: stem cells • nanofibres • cell proliferation • pluripotency • colony formation

Introduction

Human embryonic stem cells (hESCs) were first isolated in 1994 from the inner cell mass of human blastocysts [1] and later in 1998, the first hESC line was established [2]. Even though it is a decade now since the first derivation of a hESC line, the clinical applications of hESCs still face major challenges. Various strategies at controlled differentiation of hESCs to desirable tissues have been attempted, including spontaneous differentiation into the three primordial lineages and mechanical separation of the desired lineage, the use of growth factors and agents that direct the cells into a single desired lineage, the use of companion cells as coculture to help coax the hESCs into the same companion cell lineage and the use of gene constructs to control differentiation along a specific lineage. The above approaches have resulted in tremendous success to date by several groups in directing these pluripotent cells towards a desired lineage to make hESC-derived tissues clinically useful. For example, hESCs have been differentiated into cardiomyocytes [3, 4], endothelial cells [5, 6], bone cells [7, 8], neuronal cells [9, 10] and pancreatic islets [11] *in vitro* and when transplanted into diseased animal models successful engraftment has been accomplished. However, three major hurdles still exist that need to be overcome before such hESC-derived tissues enter

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human clinical trials. Although hESCs are very versatile being able to produce all 210 tissues of the human body, they possess certain inherent properties that delay their application. Their pluripotent nature poses them as a risk of teratoma formation induced by rogue undifferentiated hESCs residing in the differentiated hESC-derived tissue. Attempts are being made to destroy or eliminate these undifferentiated hESCs using antibodies or agents that specifically target the cells, labelled flow sorting methods for separation of these cells and the use of suicide genes for self destruction. The second hurdle of immunorejection of hESC-derived tissues was recently overcome by personalizing the tissues to patients by transfecting the patient's somatic cells with two to four pluripotent gene constructs that yield induced pluripotent stem cells (iPSCs) from which desirable tissues containing the patient's genome could be prepared [12, 13]. The third hurdle is the problem of inadequate hESC numbers for derivation of desirable tissues. The population doubling time is long for hESCs (36–48 hrs) and it is estimated that at least 1–5 million hESC-derived cells may be required for each injection site when it comes to human application. Current culture protocols using feeder and feeder-free methods do not yield large cell numbers for research and application. Additionally, hESC grow as small colonies further limiting cell numbers and the use of animal feeders pose a contaminating viral risk to the hESCs.

The interactions of various non-biological biomaterials with hESCs for differentiation have been studied. A highly degradable porous polymer scaffold of poly-L-lactic acid (PLLA) and polylactic-co-glycolic acid (PLGA) with a pore size of 250–500 μM seeded with differentiated hESCs and various growth factors was shown to support attachment, growth and differentiation into capillary and neuroectodermal-like structures [5, 14]. Acrylate-based polymers in the form of microarray spots enabled large scale screening of different polymers and their ability to support the growth and differentiation by cells isolated from embryoid bodies [15]. Synchronously contracting engineered cardiac tissue was developed using porous sponges composed of 50% PLLA and 50% PLGA seeded with triple cell-based culture of hESC derived cardiomyocytes, endothelial cells and embryonic fibroblasts [16].

Nanofibre technology is a useful and very recent tool in stem cell biology as the special properties of nanofibres make them suitable for a wide range of biomedical applications. Nanofibres represent fibres in the scale of 1–1000 nm, which can be used to develop safe non-biological matrices to serve as substrates for cells to attach and grow for tissue regeneration. Polymer-based nanofibres belong to a new class of biomaterials that could be produced using electrospinning technology. Using this technique nanofibres with diameters as small as 5 nm could be generated to fabricate scaffolds that would mimic the human extracellular matrix (ECM) offering both physical and biological support to the cells depending upon the polymer used [17]. Being non-biological, they serve as useful and safe substitutes to feeder cells to derive and propagate hESCs eliminating the cross-contaminating risk posed by mouse feeder cells. The role of nanofibres to expand hESC numbers *in vitro* and encourage differentiation along specific lineages using different topographic profiles created by electrospinning has not as yet been exploited. For example, nanofibres electrospun in a non-random aligned topographic pattern may encourage the differentiation of hESCs along a neuronal lineage because neurons produce thin long cytoplasmic processes. Thus, the field of nanotechnology opens up new avenues to study hESC expansion, growth and differentiation for transplantation therapy.

In the present study, we evaluated the effects of polycaprolactone (PCL)/collagen and PCL/gelatin nanofibrous scaffolds electrospun in a randomized topography as support systems for hESC cell morphology, proliferation, cell surface marker expression and stemness-related gene expression to find out whether nanofibrous scaffolds could help in scaling up hESC numbers for use in regenerative medicine.

Materials and methods

Cell culture

A karyotypically normal hESC line (HES3) was obtained from ES Cell International, Singapore. Ethical approval for its use was given by the Institutional Review Board of the National University of Singapore. HES3 cells were maintained in an undifferentiated state for the respective experiments in the present study by culturing them on mitomycin-C treated mouse embryonic fibroblasts (MEFs) in 80% DMEM culture medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 20% knockout serum replacement, 1% non-essential amino acids, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 16 ng/ml basic fibroblast growth factor and 1% insulin-transferrin-selenium. This complex culture medium was supplemented with an antibiotic mixture of penicillin (50 IU) and streptomycin (50 μg/ml). Early passage hESCs were used for this study.

Fabrication of electrospun PCL/collagen and PCL/gelatin nanofibres

PCL (mol wt, 80,000), gelatin, 1,1,1,3,3,3-hexafluoropropanol (HFP), and 2,2,2-trifluroethanol (TFE) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and collagen type I was purchased from Koken Company Ltd. (Tokushima-ku, Tokyo, Japan). PCL/collagen type I (1:7% w/v) was dissolved in HFP by stirring for even distribution up to 24 hrs. The ratio of PCL to gelatin was (1:9% w/v) dissolved in TFE for 24 hrs. For electrospinning, the polymer solutions of PCL/collagen and PCL/gelatin samples were fed into a 3 ml standard syringe attached to a 22G blunt stainless steel needle using a syringe pump (KD-100, KD Scientific Inc., Holliston, MA, USA) at a flow rate of 1.0 ml/hr with an applied voltage of 12.5 and 9.5 kV, respectively, using a high-voltage power supply (Gamma High Voltage Research, Ormond Beach, FL, USA). The ground collection plate of aluminium foil was located around 12 to 13 cm from the needle tip. A positively charged jet was formed from the syringe needle and nanofibres were sprayed onto the grounded aluminium foil target. The ambient conditions of the spinning apparatus were controlled to 23°C and 45% humidity. Cover slips of different sizes were spread on the aluminium foil target to collect nanofibres to investigate biocompatibility with cultured cells as well as to observe the structure and properties of nanofibres. The nanofibres were dried under

vacuum at room temperature overnight to remove the residual solvent present in the nanofibrous scaffolds. Electrospun nanofibres were sputter-coated with gold (JEOL JFC-1600 Auto Fine Coater, Tokyo, Japan) and visualized by field emission scanning electron microscopy (SEM; FEI – QUANTA 200 F, Czech Republic; Eindhoven, The Netherlands) at an accelerating voltage of 10 kV for characterization.

Cell morphology – phase contrast microscopy

The HES3 cells were cultured in four experimental *in vitro* systems using the nanofibres and one conventional culture system (control). For the experimental arms, HES3 cells were grown on (*i*) PCL/gelatin nanofibrous scaffolds alone (*ii*) PCL/collagen nanofibrous scaffolds alone (*iii*) PCL/gelatin nanofibrous scaffold with mitomycin-C treated MEFs and (*iv*) PCL/collagen nanofibrous scaffold with mitomycin-C treated MEFs. For the conventional control, the same batch of HES3 cells were grown on mitomycin-C treated MEFs only. The same complex culture medium described above was used for all five arms. During the culture period, the cell morphological characteristics were monitored and imaged and the colony size determined using Nikon Digital sight DS-Fi1 and NIS elements software version BR3 (Nikon Instruments, Tokyo, Japan) on fixed days after culture for all arms.

Cell proliferation assay

The HES3 cells were cultured on glass cover slips in 24-well plates with (*i*) mouse embryonic feeders as in standard hESC culture, (*ii*) feeder-free culture condition using Geltrex™, (*iii*) PCL/gelatin and (*iv*) PCL/collagen nanofibrous scaffolds. Nanofibre scaffolds (0.02–0.03 mm thickness) electrospun on 15 mm glass cover slips (0.02–0.03 mm thickness) were initially treated with ultraviolet light for 3 hrs, followed by sterilization with 70% ethanol (30 min.). The scaffolds were then washed with phosphate buffered saline (PBS) thrice (15 min. each) and soaked in complete medium overnight. The hESCs were seeded on the scaffolds and maintained under standard culture conditions at 37°C in a 5% CO_2 atmosphere with daily changes of respective culture medium for up to 5 days. Following the culture period, the cell proliferation assay was performed with MTT reagent [3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide] (Sigma) according to the manufacturer's instructions. Absorbance was spectrophotometrically measured using a microplate ELISA reader (μQuant, BioTek, Winooski, VT, USA).

SEM

The HES3 cells were cultured on (*i*) mouse embryonic feeders, (*ii*) feeder free culture condition using Geltrex™, (*iii*) PCL/gelatin and (*iv*) PCL/collagen nanofibrous scaffolds. After 7 days of culture, the hESCs were washed with PBS to remove non-adherent cells and then fixed in 3% glutaraldehyde for 3 hrs at room temperature, dehydrated through a series of graded alcohol solutions and finally dried in hexamethyldisilazane overnight. Dried cellular constructs were sputter-coated with gold and observed under FESEM at an accelerating voltage of 10 kV.

Marker analysis of hESCs

Briefly, the HES3 cells were cultured as above on (*i*) mouse embryonic feeders, (*ii*) feeder free culture condition using Geltrex™, (*iii*) PCL/gelatin and (*iv*) PCL/collagen nanofibrous scaffolds for 7 days. The cells were then fixed with 4% buffered paraformaldehyde solution, washed with PBS and treated with 10% normal goat serum. The cells were incubated with primary antibody (tumour rejection antigen [*TRA-1–60*, 5 μg/ml]; transcription factor octamer 4 [*OCT-4*, 4 μg/ml]; TG-30 [undiluted supernatant]; GCTM-2 [undiluted supernatant]) for 1 hr and goat antimouse FITC (fluorescein isothiocyanate) secondary antibody (at 5 μg/ml [Sigma, IgM]; 2 μg/ml [Alexa Fluor 488, Invitrogen Life Technologies, Carlsbad, CA, USA]) for 1 hr. Following PBS washes, the cells were treated with 4′, 6-diamidino-2-phenylindole (DAPI; 0.5 μg/ml) (Molecular Probes, Invitrogen) and incubated for 5 min. at room temperature, washed with PBS and analysed using fluorescence microscopy.

RT-PCR

The HES3 cells were cultured as above on (*i*) mouse embryonic feeders, (*ii*) feeder free culture condition using Geltrex™, (*iii*) PCL/gelatin and (*iv*) PCL/collagen nanofibrous scaffolds for 7 days. Total RNA was isolated using TRIzol™ reagent (Invitrogen Life Technologies) and RNA quality and quantity was measured with a Nanodrop™ spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). All samples were treated with DNase-I prior to first strand synthesis. First strand cDNA synthesis was carried out with random hexamers using the SuperScript™ first strand synthesis system (Invitrogen Life Technologies) according to the manufacturer's protocol. The cDNA was amplified using Peltier Thermal Cycler 200 (MJ Research, Groton, CT, USA) with following sets of primer, annealing temperature and cycles. GAPDH – (F): 5′-GAGTCAACGGATTTGGTCGT-3′, (R): 5′-TTGATTTTGGAGGGATCTCG-3′, 55°C, 30 cycles; OCT-4 – (F): 5′-CGACCATCTGCCGCTTTGAG-3′, (R): 5′-CCCCCTGTCCCCCATTCCTA-3′, 55°C, 30 cycles; NANOG (F); 5′-AATACCTCAGCCTCCAGCAGATG-3′, (R): 5′-CAAAGCAGCCTCCAAGTCACTG-3′, 55°C, 30 cycles; SOX-2 – (F): 5′-CCCCCGGCGGCAATAGCA-3′, (R): 5′-TCGGCGCCGGGGAGATACAT-3′, 60°C, 30 cycles. The amplified products were separated by electrophoresis on a 1.2% agarose gel and stained with ethidium bromide for visualization.

Statistical analysis

The differences in HES3 numbers for the different nano-scaffolds studied were compared and analysed using Student's t-test with statistical package for social sciences (SPSS 13) and the results were expressed as mean ± S.E.M. for three different replicates and the level of significance for comparisons set at $P < 0.05$.

Results

SEM

The electrospun PCL/gelatin and PCL/collagen nanofibres of random design were imaged using SEM and the diameter of the fibre was measured using Image J software (National Institutes of Health, USA). The diameter of PCL/collagen, and PCL/gelatin nanofibre was 275 ± 65 nm, 283 ± 87 nm (Fig. 1A and B), respectively. The HES3 cells cultured on plain PCL/gelatin and PCL/collagen nanofibrous scaffolds alone did not exhibit colony

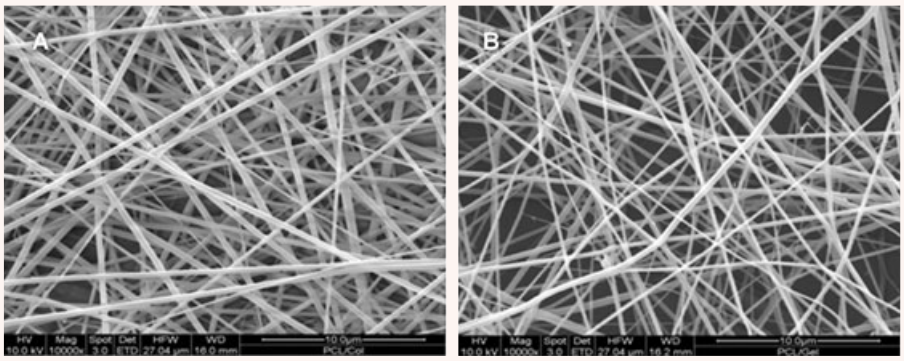

Fig. 1 Electrospun nanofibrous scaffolds: (**A**) The polycaprolactone/collagen *i.e.* PCL/collagen (1:7%w/v) and (**B**) PCL/gelatin (1:9%w/v) electrospun nanofibres of random topography were sputter coated with gold (JEOL JFC-1600 Auto Fine Coater) and visualized by field emission SEM (FEI – QUANTA 200 F) at an accelerating voltage of 10 kV for characterization. The diameter of PCL/collagen and PCL/gelatin nanofibre is 275 ± 65 nm, 283 ± 87 nm, respectively.

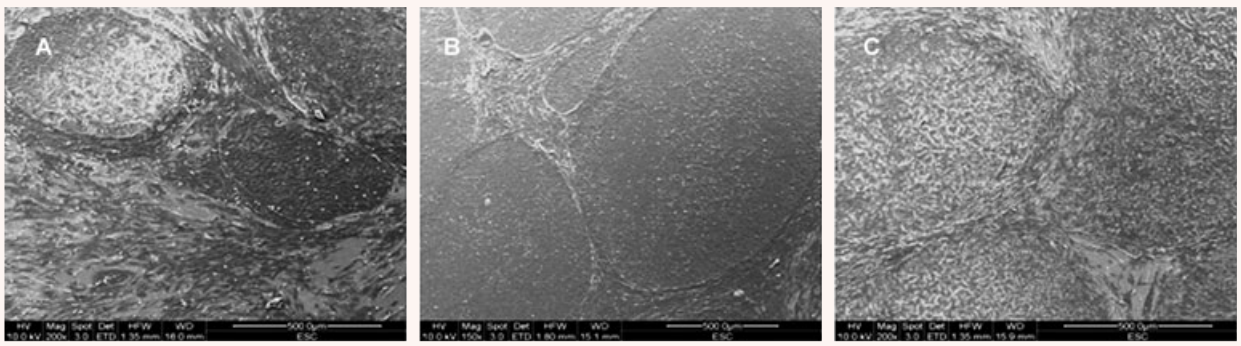

Fig. 2 Scanning electron microscopic images of human embryonic stem cell (hESC) colonies on nanofibrous scaffolds: (**A**) hESCs cultured on mouse embryonic fibroblasts (MEFs) in conventional culture (control). (**B**) hESCs cultured on PCL/gelatin nanofibrous scaffolds and MEFs (experimental) (**C**) hESCs cultured on PCL/collagen nanofibrous scaffolds and MEFs (experimental). The nanofibres could not be visualized by SEM even at higher magnification but the typical hESC colonies could be seen at low magnification.

formation. The HES3 cell clumps remained viable but underwent spontaneous random differentiation. In contrast, the HES3 cells cultured on PCL/gelatin and PCL/collagen nanofibres together with MEFs developed well formed colonies comparable to the standard conventional HES3 cell culture with MEFs (controls) (Fig. 2A, B and C). However, the colonies were thin and sparse on gelatin nanofibrous scaffolds with MEF compared to those on PCL/collagen nanofibrous scaffolds with MEF which were thicker, had increased numbers and exhibited more confluent cultures.

Cell culture and morphology

Plain PCL/gelatin and PCL/collagen nanofibrous scaffolds without the MEF did not favour HES3 cell growth. Upon initial seeding of HES3 cells, a few colony-like structures were observed with both PCL/gelatin and PCL/collagen without MEF. These colony-like structures did not attach firmly to either of the scaffolds and by day 3 they began to undergo spontaneous differentiation unlike the HES3 cells grown on the nanofibrous scaffolds with MEFs and in conventional culture conditions.

The HES3 cells cultured on mitomycin-C treated MEFs alone (controls) had the typical circular undifferentiated colonies usually seen in conventional culture (Fig. 3A). The HES3 cells that were cultured on PCL/gelatin and PCL/collagen nanofibrous scaffolds in the presence of mitomycin-C treated MEFs had an increased number of nicer large compact circular undifferentiated colonies compared to the conventional controls. Such a nice compacted circular undifferentiated colony is shown in Fig. 3C and E (low magnification) and Fig. 3D and F (high magnification). The colonies on PCL/collagen nanofibrous scaffolds with MEF were significantly larger in size (156.25 ± 7 μM) compared to those on PCL/gelatin nanofibres with MEF (135.42 ± 5 μM) (Fig. 4) and the HES3 cells maintained their typical 'stemness' characteristic of high nuclear-cytoplasmic ratios.

We observed in our pilot experiments that when plain gelatin and collagen nanofibrous scaffolds without PCL were used, the nanofibres were clearly visible through the culture medium when observed under the inverted phase contrast microscope on day 1. Subsequently, as the culture progressed it was not possible to visualize either of the nanofibrous scaffolds under the inverted microscope. To ensure that the nanofibrous scaffolds were still present and acting as support for the cells in culture, we modified

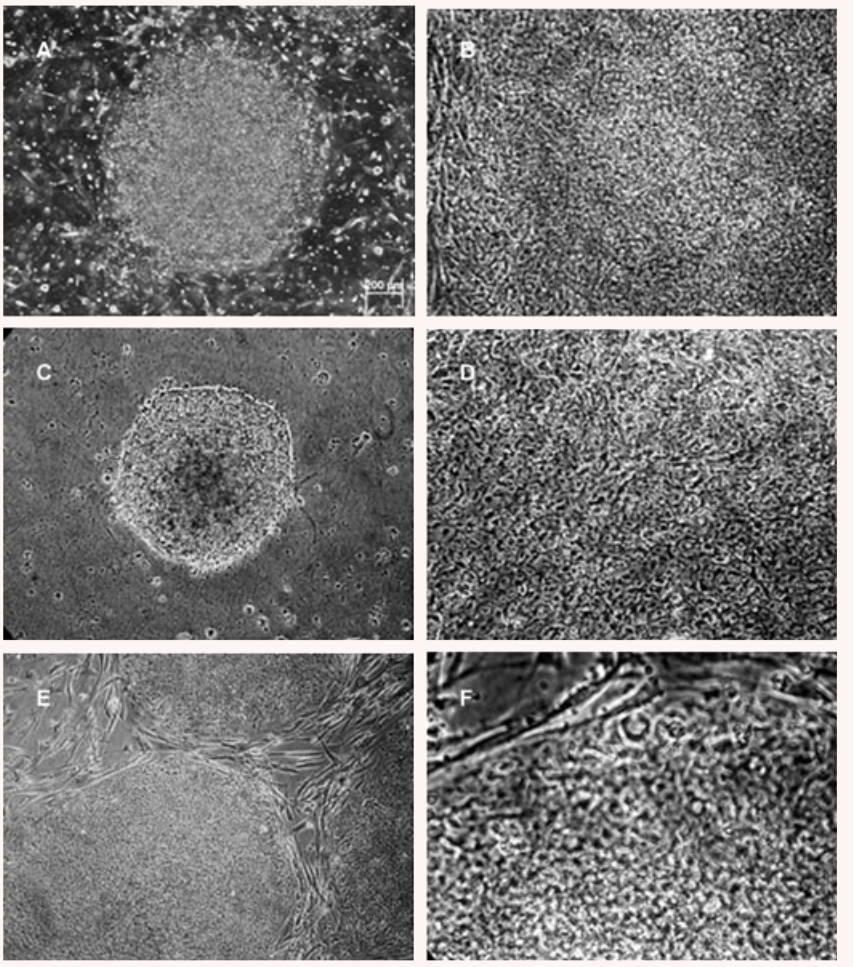

Fig. 3 Phase contrast images of human embryonic stem cell (hESC) colonies on nanofibrous scaffolds: (**A**, **B**) Low (10×) and high (40×) magnification of hESCs cultured on mouse embryonic feeders (MEFs) in conventional culture (controls); (**C**, **D**) Low (10×) and high (40×) magnification of hESCs cultured on PCL/gelatin nanofibrous scaffolds and MEFs (experimental); (**E**, **F**) Low (10×) and high (40×) magnification of hESCs cultured on PCL/collagen nanofibrous scaffolds and MEFs (experimental). The hESC colonies were well formed and there were more colonies on PCL/collagen nanofibrous scaffolds compared to PCL/gelatin nanofibrous scaffolds and controls.

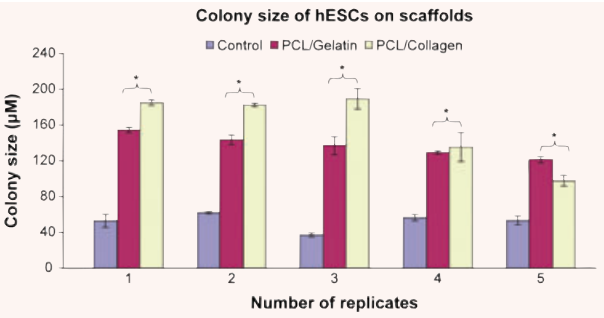

Fig. 4 Mean ± S.E.M. diameters from five replicates of human embryonic stem cell (hESC) colonies following culture on (*i*) mouse embryonic feeders (MEFs) (controls), (*ii*) PCL/gelatin nanofibrous scaffolds + MEFs (experimental) and (*iii*) PCL/collagen nanofibrous scaffolds + MEFs (experimental). Colony size were determined using Nikon Digital sight DS-Fi1 and NIS elements software version BR3 (Nikon Instruments) and compared between the control and experimental groups. *Indicates statistical significance of $P < 0.05$.

the scaffolds with the addition of PCL to obtain better stability in all the actual experiments and replicates of the present study (Fig. 5A and B). Even with this modification the scaffolds were not clearly visible as culture progressed and the colonies became larger. However, the existence of these scaffolds was confirmed because they were again clearly visible upon trypsinization of the HES3 cell colonies (Fig. 5C and D). The trypsinized HES3 cells upon re-plating on MEFs formed circular colonies once again (Fig. 5E and F).

Cell proliferation – MTT assay

The HES3 cells grown in conventional culture conditions in the presence of MEFs (controls) had good colonies with minimal differentiation at the periphery of the colonies. Similarly, the HES3 colonies cultured on both types of nanofibrous scaffolds with MEFs showed nice circular colonies with minimal differentiation. A significant increase in undifferentiated HES3 cell numbers was observed in both PCL/gelatin and PCL/collagen nanofibrous scaffold

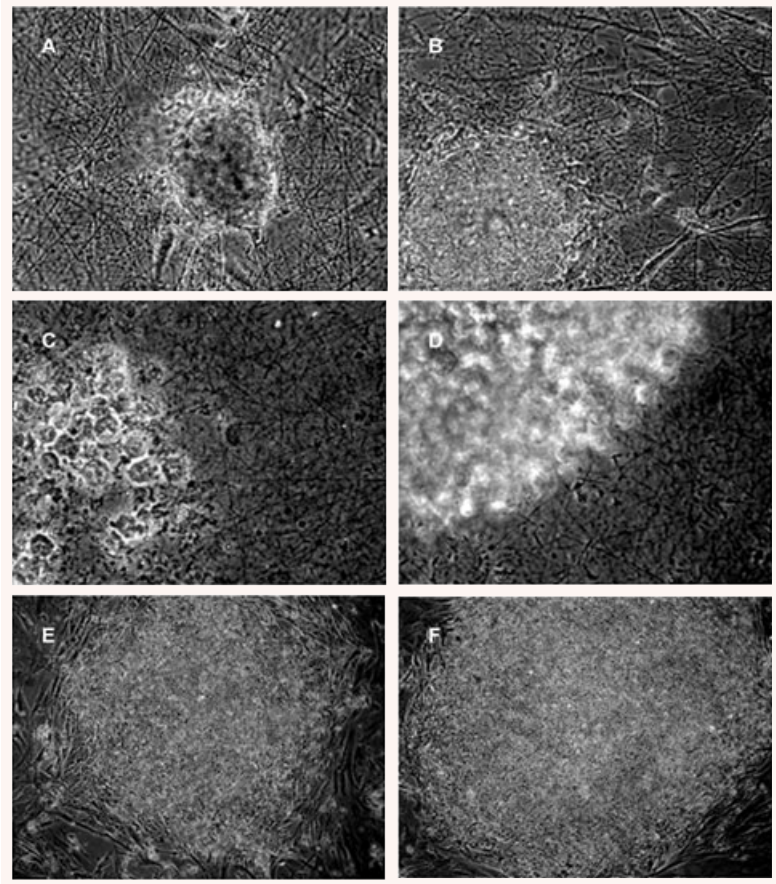

Fig. 5 Passaged hESCs following culture on nanofibrous scaffolds: **A**, **B**: Day-3 hESC colonies on the PCL/gelatin (**A**) and PCL/collagen (**B**) nanofibrous scaffolds, respectively. **C**, **D**: The PCL/gelatin (**C**) and PCL/collagen (**B**) nanofibrous scaffolds which usually become covered by matrix secreted by cells (day 5) could be clearly visualized again following trypsinization. **E**, **F**: hESCs previously grown on the nanofibrous scaffolds when replated on fresh mouse embryonic feeders reproduced typical hESC colonies. **E**; PCL/gelatin; **F**: PCL/collagen (40× magnification).

experimental arms compared to control HES3 cells on MEFs. The increases in cell proliferation were 40.18% ($P \leq 0.005$) and 47.58% ($P \leq 0.006$) for PCL/gelatin with MEF and PCL/collagen with MEF, respectively, and these increases in values were statistically significant (Fig. 6).

Marker analysis of hESCs

Immunophenotyping of HES3 cells grown in conventional culture on MEFs and on PCL/gelatin and PCL/collagen nanofibrous scaffolds with MEFs (experimental) demonstrated strong positive staining for OCT-4, TRA-1–60, TG-30 and GCTM-2 (Fig. 7).

RT-PCR

Semi-quantitative RT-PCR analysis revealed expression of the hESC stemness-related genes, namely NANOG, OCT-4 and SOX-2 for HES3 cells grown on the nanofibrous scaffolds with MEFs and also the controls (Fig. 8) (The internal control is the GAPDH and last lane was the negative control). The 'stemness' markers were

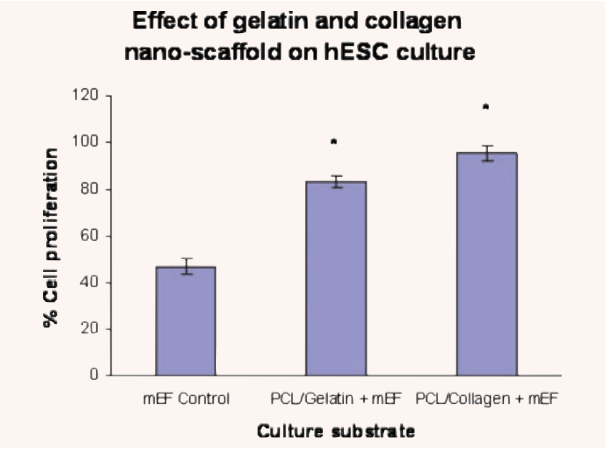

Fig. 6 Human embryonic stem cell (hESC) proliferation following culture on nanofibrous scaffolds: The hESCs were cultured on (*i*) mouse embryonic feeders (MEFs) (controls), (*ii*) PCL/gelatin nanofibrous scaffolds + MEFs (experimental) and (*iii*) PCL/collagen nanofibrous scaffolds + MEFs (experimental). The cell proliferation was determined by MTT assay following culture for 5 days. All values are expressed as mean ± S.E.M. from three different replicates. *Indicates statistical significance of $P < 0.05$ from control.

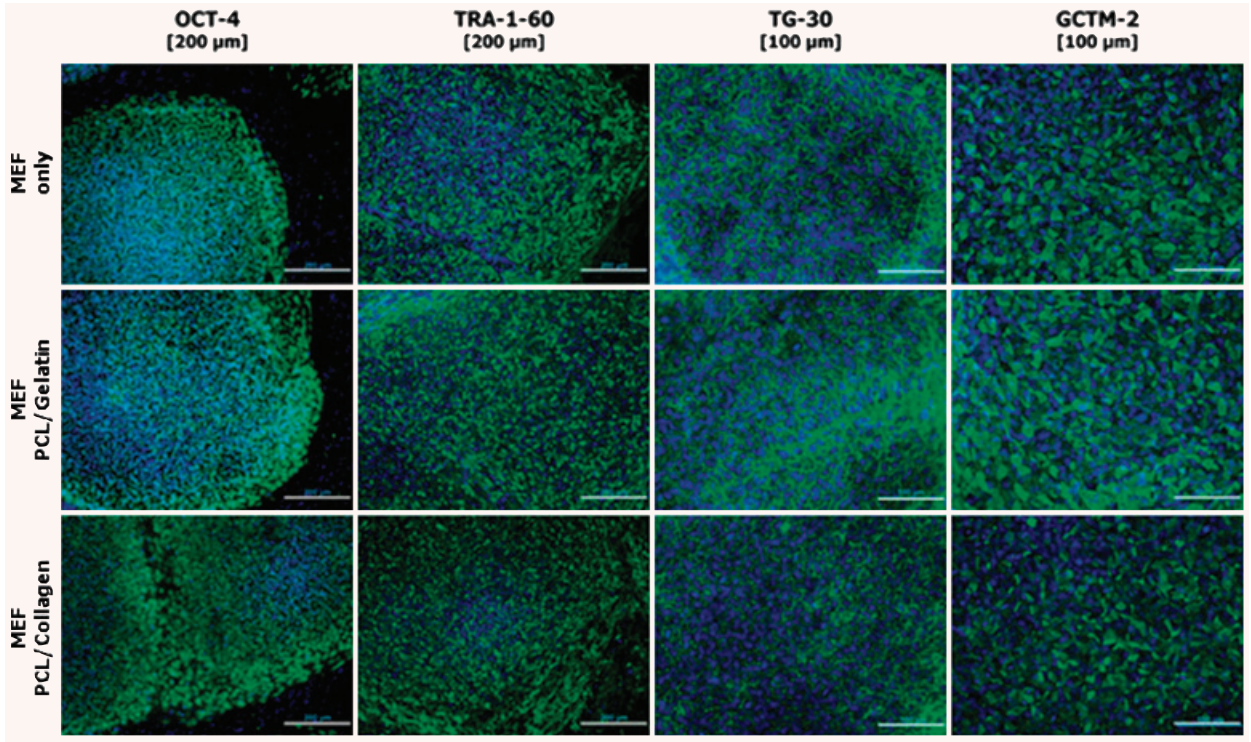

Fig. 7 Immunohistochemistry images of human embryonic stem cells (hESCs) following culture on nanofibrous scaffolds. The hESCs were cultured on (*i*) mouse embryonic feeders (control) (MEFs; top panel), (*ii*) PCL/gelatin nanofibrous scaffolds + MEFs (experimental) (middle panel) and (*iii*) PCL/collagen nanofibrous scaffolds + MEFs (experimental) (bottom panel). The stem cell marker antigen transcription factor octamer 4 (OCT-4, 4 μg/ml); tumour rejection antigen (TRA-1–60, 5 μg/ml); TG-30 (undiluted supernatant); GCTM-2 (undiluted supernatant) were analysed following hESC culture after 5 days. The secondary antibodies used were the goat antimouse fluorescein isothiocyanate (5 μg/ml; IgM) and Alexa Flour 488 (2 μg/ml). The cell nucleus was stained using 4′, 6-diamidino-2-phenylindole (DAPI).

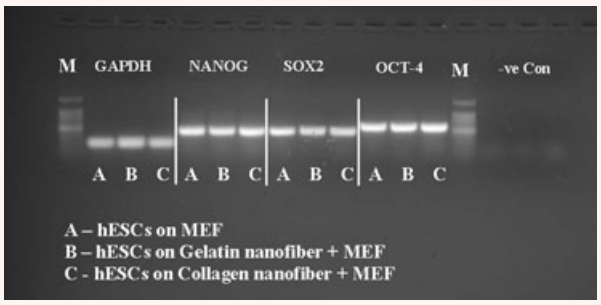

Fig. 8 Stemness related gene expression by RT-PCR reaction: Human embryonic stem cells (hESCs) were cultured on (**A**) mouse embryonic feeders (MEFs) (controls), (**B**) PCL/gelatin nanofibrous scaffolds + MEFs (experimental) and (**C**) PCL/collagen nanofibrous scaffolds + MEFs (experimental). Semi-quantitative gene expression of stemness genes NANOG, SOX-2 and OCT-4 were highly expressed following hESC culture on nanofibrous scaffolds. The amplified products were separated by electrophoresis on a 1.2% agarose gel and stained with ethidium bromide for visualization. M – marker standard; GAPDH was the internal control and the last lane was the negative control.

highly expressed in the HES3 cells cultured on PCL/gelatin and PCL/collagen with MEFs (experimental) and these expression levels were almost similar in intensity to the controls (HES3 cells cultures on MEFs).

Discussion

The use of nanotechnologies to manipulate and track stem cells offers new opportunities in stem cell biology. It has tremendous potential in advancing our understanding of various cellular events happening at the lower order of the microscopic scale and has important applications in stem cell tracking, differentiation and transplantation. It is quite difficult to comprehend the biological events that might happen within the *in vivo* system at nanoscale levels, but the fact is that the living cells would react with other objects even at 5 nm which are few thousand times less than the cell itself and continue to exert its functions [18]. Variations in surface topography, surface chemistry and conformations have

enabled enhanced cell adhesion, growth and differentiation towards an osteogenic lineage [19, 20]. Three-dimensional matrices to mimic the ECM have been used to culture hESCs that expressed markers for all three embryonic germ layers [21] and also direct them towards a neuronal phenotype [22]. The use of nanofibre scaffolds to enhance proliferation and self-renewal of mouse embryonic stem cells [23] and to direct mesenchymal stem cells towards osteogenic differentiation [24] have also been reported previously. Mesenchymal stem cells grown on 500–1000 nm nanofibres had greater cell viability than tissue culture polystyrene controls [24].

Although nanofibrous scaffolds have gained interest in adult or mesenchymal stem cell biology their use in hESC biology has been limited. Their use to expand hESC numbers and differentiate them into desirable tissues would be a tremendous boost to regenerative medicine and this specific area remains an unexplored territory. In fact the present study is the first report on the use of nanofibrous scaffolds to expand hESC numbers for research and application for cell-based therapies. Other potential healthcare applications using nanotechnology include diagnostic, therapeutic and disease state monitoring, developments of new drug delivery systems, cell tracking and imaging and the development of better and durable prosthetics for different disciplines of medicine [25]. An ideal scaffold should be biocompatible, biodegradable, bioactive, impart necessary support, be highly porous with a large surface area to volume ratio, non-toxic and minimally antigenic [26, 27]. In the present study, we have designed nanofibrous scaffolds using a combination of PCL/collagen and PCL/gelatin that have the above criteria and hence their positive support of the growth of hESCs. Similar nanofibrous scaffolds have supported other cell types [28–30].

In the present study, the hESC colonies on both nanofibrous scaffolds were larger and had increased cell proliferation compared to controls but the PCL/collagen scaffold was superior in terms of cell number, size of colonies and attachment to the culture substrate (Figs 2–4 and 6). Collagen is a natural ECM component to which most living cells form focal adhesions easily, whereas gelatin is a thermally denatured form of collagen and possibly renders less support for cells to attach and grow on the substrate. Additionally, the porous nature of the scaffolds with large surface to volume ratio perhaps provided more cell and matrix interaction favouring cell attachment and proliferation of the MEFs and not the direct attachment of the hESCs because the nature, behaviour and properties of these two cell types are very different; the MEFs exhibiting fibroblast-like cell growth and the hESCs exhibiting colony formation *in vitro*. The ECM is necessary for both physical and chemical cues for the living cells and hence the various cellular responses. The benefits provided by the nanofibrous scaffolds with the MEFs for expanded growth of the hESCs in the present study would have been due to (1) stable 3D support from a combination of scaffold and MEFs, instead of 2D support from MEFs alone (controls) (2) the creation of stem cell niches provided by the 3D scaffolds and (3) the provision of a variety of conducive proteins and growth factors released by the MEFs into the conditioned culture medium for prolonged undifferentiated hESC growth. The stem cell niche which is the microenvironment of the stem cells helps to interact with the stem cells to regulate their fate. Stem cell niches actively signal the stem cells to promote self-renewal. Within the stem cell niche created by the 3D nanofibrous scaffold, the interaction between stem cells and adhesion molecules, ECM component, cytokines, oxygen tension, growth factors from the culture medium and the physiochemical nature of the microenvironment encourage and support undifferentiated hESC growth. The chemical, mechanical and 3D features of these scaffolds can influence the activation of different signalling pathways, resulting in stem cell proliferation and self-renewal [30]. Additionally, stem cell adhesion to substrates or scaffolds with nanoscale resolution can cause clustering of cell integrins into focal adhesion complexes and the concomitant activation of intracellular signalling cascades and guidance of stem cell behaviour [31]. The combined effect of various growth factors available in the internal milieu, the scaffold material and its associated properties would direct cell attachment and proliferation [32].

The PCL used in the present study had a MW of 80,000. Following electrospinning the same MW may not be retained thus possibly changing the degradation properties of the polymer when applied to *in vivo* conditions. However, in the present study we used a low concentration of 1% of PCL to provide only mechanical stability to the collagen and gelatin nanofibres. In most *in vivo* situations much higher concentrations of PCL (up to 20%) are used and average molecular weights of PCL alone remained unchanged over 5 weeks of degradation [33]. Such low concentrations of PCL (1%) may not result in major changes in molecular weight after electrospinning thus affecting degradation properties during *in vivo* conditions. Also, PCL is a Food and Drug Authority approved semi-crystalline, bioresorbable polymer belonging to the aliphatic polyester family. One big advantage of such bioresorbable materials is their degradability and they can be assimilated by a biological system by elimination of the initial foreign material with no residual side effects. Hence even if its MW changes slightly, this inherent bioresorbable property would help in the elimination or metabolization of degraded by-products from the body. Lam *et al*. [33] emphasized that the concept of bioresorption should encompass the degradation of the polymer system and the elimination or metabolization of the degraded by-products from the body. Furthermore, the high surface area of the nanofibres may accelerate degradation rate.

In the present study, we successfully passaged further the hESCs that were grown on nanofibrous scaffolds confirming that the nanofibrous scaffolds were non-toxic to the cells. This would help in further amplification and downstream applications (Fig. 5E and F).

The nanofibrous scaffolds did not appear to affect the 'stemness' characteristics of the hESCs because they demonstrated positive staining for some of the stemness related surface markers namely OCT-4, TRA-1–60, TG-30 and GCTM-2 (Fig. 7). This was further confirmed with the expression of the stemness-related genes namely, NANOG, SOX-2 and OCT-4 (Fig. 8).

Various approaches are in progress to boost hESC numbers for clinical application. These include automated platforms and bioreactor-based systems. Rotary suspension culture enhanced to

some extent the efficiency, yield and homogeneity of hESC-derived embryoid bodies for differentiation [34]. All these have their advantages and disadvantages as reviewed by Thomson [35]. The use of nanofibrous scaffolds to enhance hESC numbers as shown in the present study will complement all these approaches to boost hESC numbers for clinical application.

It would be desirable to move away from the use of xenosupports like the MEFs because of the concern of transmission of adventitious agents. We are currently attempting to refine our protocols to eliminate MEF support to derive and propagate pluripotent hESCs and hESC-derived tissues on nanofibrous scaffolds using different topographic profiles for downstream applications. Further, refinement in the nanofibrous scaffold fabrication so as to obtain the desired nanotopography, variations in thickness, the incorporation of various growth factors, the biodegradation time of the polymers and defining optimal porosities would go a long way in not only providing the much needed mechanical support but would also provide optimal cues for deriving specific tissues. The successful culturing of hESCs on nanofibrous scaffolds with retention of stemness, enables us to pursue directed differentiation of hESCs along specific lineages such as neurons, hepatocytes, cartilage, vascular structures, etc. It is hoped that the marriage of nanotechnology and stem cell biology would expedite taking hESCs from the laboratory to the clinic.

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