Synthetic niches for differentiation of human embryonic stem cells bypassing embryoid body formation

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Abstract: The unique self-renewal and pluripotency features of human embryonic stem cells (hESCs) offer the potential for unlimited development of novel cell therapies. Currently, hESCs are cultured and differentiated using methods, such as monolayer culture and embryoid body (EB) formation. As such, achieving efficient differentiation into higher order structures remains a challenge, as well as maintaining cell viability during differentiation into homogeneous cell populations. Here, we describe the application of highly porous polymer scaffolds as synthetic stem cell niches. Bypassing the EB formation step, these scaffolds are capable of three-dimensional culture of undifferentiated hESCs and subsequent directed differentiation into three primary germ layers. H9 hESCs were successfully maintained and proliferated in biodegradable polymer scaffolds based on poly(lactic-co-glycolic acid) (PLGA). The results showed that cells within PLGA scaffolds retained characteristics of undifferentiated pluripotent stem cells. Moreover, the scaffolds allowed differentiation towards the lineage of interest by the addition of growth factors to the culture system. The in vivo transplantation study revealed that the scaffolds could provide a microenvironment that enabled hESCs to interact with their surroundings, thereby promoting cell differentiation. Therefore, this approach, which provides a unique culture/differentiation system for hESCs, will find its utility in various stem cell-based tissue-engineering applications. © 2013 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 00B:000–000, 2013.

Key Words: human embryonic stem cell, differentiation, polymer scaffold, transplantation, embryoid body


INTRODUCTION
As a basis for cell replacement therapies for restoring lost or damaged cells or stimulating tissue regeneration, pluripotent embryonic stem cells offer enormous potential, particularly given their ability to self-renew and generate mature cells of a desired tissue via differentiation.1–3 When compared with other available therapies, such as organ or tissue transplantation, cell-based therapies based on novel engineering technologies could meet the challenge posed by the limited resource of human donor embryonic stem cells (hESCs).4,5 For example, significant efforts have been made to engineer hESCs for cardiac repair, neuro repair, and the treatment of diabetes.6–9 In vitro culture of hESCs has been well-established using mouse embryonic fibroblast (MEF) feeders or cell-free matrices, such as laminin or matrigel, supplemented with MEF-conditioned medium to promote the proliferation of undifferentiated hESCs in a two-dimensional (2-D) structure.3,10,11 Upon removal of factors that maintain stem cells in the undifferentiated state, hESC differentiation can be induced in a monolayer culture system that does not mimic an in vivo physiological three-dimensional (3-D) environment, thus leading to low level of differentiation into higher order structures.12,13 Another approach involves culturing cells in vitro to aggregate and form 3-D cell colonies known as embryoid bodies (EBs) capable of differentiating into three primary germ layers: endoderm, mesoderm, and ectoderm, respectively.1,4,5 Moreover, EBs can be induced to directly differentiate towards the lineage of interest by introducing a combination of growth factors into the culture system.16–18 However, the formation of EBs from hESCs is inefficient because a large number of cells die during the process.19,20 In addition, the difficulty of controlling the size and morphology of EBs during formation leads to a high degree of heterogeneity of EB populations, subsequently affecting reproducibility and efficiency of stem cell differentiation, even though many efforts have been devoted to optimize the EB-mediated differentiation process.21–23 Moreover, different...
cells within the dense structure of EBs have differential access to nutrients and growth factors, resulting in differentiation of hESCs into a heterogeneous mixture of cell populations. Thus, it would be more desirable to bypass the EB formation step and direct differentiation of undifferentiated hESCs, both in vitro and in vivo, in a confined 3-D environment. Such methods would maximize the developmental potential of hESCs and induce the functional proliferation and differentiation of stem cells into higher order structures, but minimize undesired heterogeneous outcomes. Biomaterials have been widely used as scaffolds for tissue engineering in the treatment of various diseases. Such scaffolds not only serve as a matrix for cell adhesion and growth, but also allow the retention of cell function and promote cell–cell interactions to facilitate tissue regeneration. Maintaining the self-renewal capability of hESCs and control over stem cell fate, such as differentiation, is highly desirable, but it is also challenging from a design and engineering perspective. Many studies have employed the modification of synthetic graft surface properties of scaffolds to modulate the cell–biomaterial interface in a way that mimics the critical features of the extracellular matrix (ECM) environment. Although 2-D surface functions play an important role in cell attachment and growth, the specific effects of many factors governing stem cell fate, including chemical functionalities, hydrophobicity, or stiffness, are too complicated to reproduce. In particular, culturing cells on 2-D surfaces cannot mimic the complexity of the natural ECM and cell–matrix interactions. Biomaterial scaffolds with porous structures have recently been explored for many tissue-engineering applications because they can provide the 3-D physical support of transplanted cells and an environment easily accessible to therapeutic growth factors. In this study, a highly porous biodegradable polymer, poly (lactic-co-glycolic acid) (PLGA), scaffold was generated using a high-pressure CO₂-forming technique to provide a 3-D supportive scaffold for the growth and differentiation of undifferentiated hESCs. We hypothesized that this confined 3-D scaffold with porous structure could (1) be used to support the growth and proliferation of undifferentiated hESCs and (2) provide an environment resembling natural cell–ECM interactions, which would lead to efficient differentiation of hESCs into cell types of particular interest, but without the need for EB formation. Herein, we demonstrated the long-term survival of undifferentiated hESCs on the surface, as well as inside PLGA scaffolds, and their ability to maintain proliferation in the undifferentiated state with the conditioned medium. Moreover, treatment of scaffolded hESCs with various growth factors showed that stem cells could be differentiated into the specific germ layer without forming EBs. In vivo transplantation study further suggested that the porous PLGA scaffold could provide a microenvironment able to maintain the pluripotency of hESCs and efficiently induce direct differentiation.

**EXPERIMENTAL**

**Cell lines, antibodies, and reagents**

hES cells (H9 clone) were grown on MEFs in KSR (knockout serum replacer) medium as described. To passage hESCs, after incubating cells with 1 mg/mL collagenase for 2 min, we used a cutting tool to excavate a healthy undifferentiated colony and dissociate it into the suspension. Fragments from about 20 colonies were transferred onto feeder cells of a new dish. TexasRed- or Alexa488-conjugated goat antimouse IgG antibody and Alexa647-conjugated goat antirabbit IgG antibody were obtained from Invitrogen (Carlsbad, CA). The mouse monoclonal antibodies SSEA-4, GCTM-2, and TRA-1-81 were purchased from Chemicon International, Inc. (Billerica, MA). The mouse monoclonal antibodies OCT-4, Nestin, and AFP were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The rabbit polyclonal antibody Brazyhury was purchased from Abcam (Cambridge, MA). BrdUrd and a mouse monoclonal antibody against BrdUrd were obtained from Sigma-Aldrich (St. Louis, MO). Human transforming growth factor (TGF)−β (final concentration 2 ng/mL) and human activin-A (final concentration 20 ng/mL) were purchased from Pepro Tech, Inc. (Rocky Hill, NJ). Retinoic acid (RA) (final concentration 300 ng/mL) was obtained from Sigma-Aldrich (St. Louis, MO).

**PLGA scaffold preparation**

An 85:15, 109 kDa copolymer of d, l-lactide and glycolide (PLGA) was obtained from Lakeshore Biomaterials (Birmingham, AL). PLGA microspheres were prepared using the standard double emulsion technique. Briefly, 250 μL of PBS was added into 1 g of PLGA dissolved in methyl chloride to form the second emulsion. The resulting double emulsion was poured into 1 L of 1% polyvinyl alcohol (PVA, 88% hydrolyzed, Sigma-Aldrich) saturated with methylene chloride to form the second emulsion. The resulting double emulsion was poured into 1 L of 0.1% PVA and continuously stirred at room temperature overnight to evaporate organic solvents. The microspheres were collected using a Sorvall Legend centrifuge at 10000g for 10 min and further lyophilized overnight into a powder. The microsphere powder was mixed with porogens (NaCl), which were sieved to a particle size between 250 and 425 μm, at a ratio of 2–8. The total mass of PLGA and NaCl was held at 200 mg to form one scaffold in all experiments. The mixture of PLGA and NaCl was compressed at 1500 psi for 1 min to form solid disks. After molding, the disks were exposed to 800 psi CO₂ for 48 h as a gas-forming process. A rapid reduction in gas pressure to the ambient pressure led to the nucleation and growth of CO₂ pores within the polymer disks, allowing for formation of highly porous scaffolds. Subsequently, porogen particles were removed by a water leaching process for 24 h. All processing steps were conducted at room temperature.

For seeding cells on polymer scaffolds, the disks were cut into a rectangular shape of 5 × 4 × 1 mm³. Before cell seeding, the disks were sterilized overnight in 70% ethanol and then washed with PBS three times. Subsequently, the disks were coated with matrigel overnight, followed by washing three times with PBS.
Cell growth and differentiation on scaffolds

For the pluri potency and proliferation study, undifferentiated H9 cells were trypsinized to single cells, and 1 × 10^6 cells were then seeded on a matrigel-coated scaffold and cultured in MEF-conditioned medium for 2 weeks. For the differentiation study, H9 cells were also seeded on scaffolds and cultured in knockout medium supplemented with the following growth factors for 2 weeks: TGF-β (final concentration 2 ng/mL), RA (final concentration 300 ng/mL) and activin-A (final concentration 20 ng/mL). For the transplantation experiment, H9 cells were seeded on matrigel-coated scaffolds with knockout medium for 2 weeks before transplantation.

EB formation

EBs were produced by the enforced aggregation method. Briefly, feeder-free H9 cells were trypsinized and centrifuged at 1000 rpm for 2 min. The pellet was resuspended in conditioned medium at a concentration of 5 × 10^5 cells/mL. 100 μL of 5 × 10^5 cells/mL suspension were added to one well of a V-shaped 96-well plate. After centrifuging the plate for 10 min at 500g and 24 °C, the EBs were formed after 24 h incubation at 37 °C. On day 2, the EBs were cultured in knockout medium supplemented with growth factors, as described above, for 2 weeks. For the quantitative RT-PCR (qRT-PCR) experiment, 20 EBs were transferred to a matrigel-coated dish and cultured for another 3 days.

Tissue processing, immunohistochemical staining, and confocal imaging

PLGA scaffolds were fixed overnight in 10% neutral buffered formalin and embedded in paraffin. Scaffold sections were further stained with hematoxylin and eosin (H&E). Immunohistochemical staining was carried out by using the indicated primary antibodies and secondary antibodies, followed by counterstaining with TO-PRO-3 or DAPI (Invitrogen). For the proliferation experiment, culture medium was supplemented with 10 μM of BrdUrd for 3 h before fixing. After paraffin embedding, scaffold sections were stained with mouse anti-BrdUrd antibodies. Fluorescence images were acquired by a Yokogawa spinning-disk confocal scanner system (Solamere Technology Group, Salt Lake City, UT) using a Nikon Eclipse Ti-E microscope. Illumination powers at 491, 561, and 640 nm solid-state laser lines were provided by an AOTF (acousto-optical tunable filter)-controlled laser-merge system with 50 mW for each laser. All images were analyzed using Nikon NIS-Elements software. For quantification of image sections, an electronic grid (100 × 100 μm^2, width × height) was randomly applied over an area of images. This grid was subdivided into 100 counting boxes (10 × 10 μm^2). Quantification was based on counting the boxes positive for indicated markers and nuclear staining by viewing eight random areas per sample. The volume fraction of differentiation markers was determined by the ratio between numbers of counting boxes positive for indicated differentiation markers and nuclear staining.

qRT-PCR assay

Total RNA was extracted via RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. qRT-PCR was carried out by using Lenti-X qRT-PCR titration kit (Clontech Laboratories, Inc.). The expression levels of genes in three germ layers were detected using the following pairs of primers: MSI1fw (acagcccaagatgggatctc), MSI1bw (ccacgatgtcctcactctca), VIMfw (gggcctctagggaggag), VIMbw (cgacatgtaacatctgctc), AFPfw (agacacctgcaactgctgg), AFPbw (gacacagctgagggatgct), IGF2fw (tctccctgagacaattcga), IGF2bw (agagaacagcacagcatgct), FoxA2fw (gacacagagagagcaagttg), FoxA2bw (agactgtgaaacggagt), Msx1fw (ctctctcttagaggctcct), Msx1bw (cgcattctgctgcttctt). A pair of β-actin primers was used as a control.

Scanning electronic microscopy and mechanical testing

For scanning electronic microscopy (SEM) analysis, the samples were gold-coated using a sputter coater, and the images were then acquired using a scanning electron microscope (JSM/6390LV, JEOL, Tokyo, Japan) at 20 kV. Compression testing of the PLGA disks with and without the gas-forming process, disks were trimmed to dimensions of 14 by 14 mm^2 with a thickness of 1.5 mm and were tested using Instron machines at a loading rate of 1 mm/min.

Transplantation of scaffolds into SCID mice

All mice were maintained in the animal facilities at the University of Southern California (USC) under controlled temperature and a 12 h light/dark cycle, with free access to water and standard laboratory chow. Animal procedures were performed in accordance with the guidelines set by the National Institutes of Health and the animal protocol was approved by the Institutional Animal Care and Use Committee of the USC. Undifferentiated H9 cells were seeded on matrigel-coated scaffolds and cultured in knockout medium for 2 weeks in vitro. The scaffolds were implanted subcutaneously in the dorsal region of 4-week-old SCID mice (CB.17 SCID, Taconic Farms). After 2 weeks of implantation, the scaffolds were retrieved, fixed, paraffin-embedded, and sectioned for histological examination with both undifferentiated and differentiated markers for three germ layers, as described above.

RESULTS

PLGA scaffolds to support the growth and proliferation of hES cells

Our goal was to identify a 3-D supportive environment for the growth of undifferentiated hESCs and the maintenance of cell viability. To accomplish this, a biodegradable macroporous PLGA scaffold was generated using a high-pressure gas forming and particulate leaching process. The PLGA microspheres made using a standard double emulsion method were lyophilized and mixed with sodium chloride (NaCl) as a porogen in an attempt to generate the PLGA scaffold with controlled porous structure. The compressed PLGA scaffold was then exposed to high-pressure CO2 gas (300 psi) for 48 h, leading to the nucleation and growth of pores within the polymer matrix, as well as the enhancement of its mechanical properties. Furthermore, subsequent particulate leaching yielded an interconnected macrostructure in the PLGA scaffold. SEM images...
showed the scaffold’s porous structure with a range of pore size from 200 to 400 μm, indicating that the scaffold could provide sufficient space for the growth of cells [Figure 1(A)].

Next, the stability of the PLGA scaffold was evaluated using the compressive test. After subjecting the PLGA scaffold to high-pressure gas forming and particulate leaching processes, it exhibited a compressive modulus of 47.99 ± 13.6 kPa. In the absence of the gas-forming process, however, no detectable mechanical stability was observed after leaching of the scaffold in water [Figure 1(B)], indicating that the high-pressure CO₂ forming step was responsible for improving the scaffold’s mechanical stiffness. It was previously reported that polymer scaffolds require a cellular compressive stress of approximately 110 Pa to support the growth of cells and resist the force of cell contraction, suggesting that this PLGA scaffold, which was demonstrated to possess mechanical stability and high porosity, could support the growth and proliferation of stem cells.

To examine the ability of the scaffold to support cell adhesion and proliferation, undifferentiated hESCs (clone H9) were seeded on the matrigel-coated polymer scaffolds and further cultured under stem cell-conditioned medium for 2 weeks. SEM analysis showed that cells adhered very well to the internal surface of the scaffold and grew uniformly on the pore structures [Figure 1(D)]; the scaffold without cells was used as the control [Figure 1(C)]. In addition, histological examination cell–polymer matrix cross-sections demonstrated that undifferentiated hESCs were able to grow on both the outer surface and in the center of scaffolds [Figure 1(E)]. Moreover, using immunohistochemical staining of the embedded polymer sections with anti-BrdU antibodies, the cell proliferation study indicated that undifferentiated hESCs seeded and cultured on polymer scaffolds for 2 weeks could maintain high-level cellular proliferation [Figure 1(F)]. These results suggested that the porous biodegradable PLGA scaffold could provide a 3-D supportive environment to promote cell growth and proliferation.

Maintenance of pluripotency of hESCs on PLGA scaffolds

Pluripotent stem cells have the potential to develop into virtually any desirable cell type. Thus, maintenance of stem cell pluripotency is an important parameter for the evaluation of new scaffolds for hESC culture. To investigate whether the hESCs grown on the PLGA scaffold could maintain their pluripotency after culturing for 2 weeks, immunohistochemical staining on the embedded polymer sections was performed using various markers that identify pluripotent stem cells, such as stage-specific embryonic antigen 4 (SSEA-4), octamer-binding transcription factor 4 (OCT-4), protein core of pericellular matrix proteoglycan (GCTM-2), and tumor rejection antigens (TRA-1-81), for undifferentiated hESCs. As shown in Figure 2(A–D), histological analysis indicated high-level expression of GCTM-2, OCT-4, SSEA-4, and TRA-1-81 in scaffolds, suggesting that the pluripotency of hESCs was maintained in polymer and that the PLGA scaffold could provide culture conditions with enough sufficiency to retain the characteristics of undifferentiated pluripotent stem cells.

Directed differentiation of hESCs on PLGA scaffolds with growth factors

When exposed to appropriate and specific culture conditions, hESCs can potentially differentiate into any cell type of three different germ layers: ectoderm, mesoderm, and endoderm. To assess the capability of hESCs maintained on polymer scaffolds to differentiate into functional cell types, undifferentiated H9 cells were seeded on matrigel-coated polymer scaffolds and further cultured in the knockout medium for 2 weeks. After the incubation period, the embedded scaffold sections were stained with antibodies against OCT-4 (undifferentiated marker), AFP (endoderm marker), Nestin (ectoderm marker), or Brachyury (mesoderm marker). As shown in Supporting Information Figure 1, hESCs cultured on polymer scaffolds in the knockout medium could differentiate into three different germ layers, although low levels of differentiation marker expression were observed in scaffolds maintained in the absence of any growth factors.

To enhance differentiation of hESCs towards a specific lineage of interest, the knockout medium was further supplemented with the following growth factors: TGF-β, activin-A, and RA, which are known to induce directed differentiation of hESCs into mesoderm, endoderm, and ectoderm, respectively. Then, using immunohistochemical staining with antibodies that recognize differentiation markers, polymer scaffolds were examined for their ability to direct differentiation of hESCs. Undifferentiated H9 cells were cultured on polymer scaffolds in knockout medium supplemented with representative growth factors for 2 weeks. As shown in Figure 3(A), histological analysis showed that supplementation of TGF-β to the culture medium resulted in a significant increase in the expression of the mesodermal marker (brachyury), as compared to that in unsupplemented control samples and samples supplemented with RA or activin-A. It was also shown that supplementation of RA induced the highest level of Nestin expression, indicating that RA could efficiently direct differentiation of hESCs into ectoderm layer in the 3-D-supportive environment of polymer scaffold. Moreover, the addition of activin-A into the medium was sufficient to form the endoderm layer, as demonstrated by the high level of AFP throughout the polymer matrix sections. The quantification of the immunohistochemical staining results further confirmed the capability of the polymer scaffold to direct the differentiation of hESCs into three germ layers with growth factor supplements [Figure 3(B)].

It has been demonstrated that hESC differentiation can be induced by forming 3-D cell aggregates, that is, EBs in vitro. Indeed, EBs have been shown to differentiate three germ lineages. However, as a consequence of the inefficiency of EB formation from hESCs caused by a large amount of cell death during the process, it has been desirable to create biomaterial scaffolds, such as PLGA discussed
FIGURE 1. Porous PLGA scaffold can support the adhesion, growth, and proliferation of hESCs. (A) Scanning electron microscopy (SEM) of PLGA scaffolds showing macropores and micropores formed within the polymer matrices (scale bar: 200 μm). (B) Compressive testing of scaffolds. (C) SEM of PLGA scaffold without or (D) with undifferentiated hESCs. (E) Histological examination of H&E staining on the stem cell–polymer matrix sections. Undifferentiated hESCs were seeded on matrigel-coated scaffold supplemented with conditioned medium for 2 weeks. (Left: surface of scaffold; middle: 200 μm inner scaffold; right: 350 μm inner scaffold). (F) Histological analysis of proliferation of hESCs on constructs. Undifferentiated hESCs were seeded on matrigel-coated scaffold and cultured in conditioned medium for 2 weeks. Before 3 h of fixing, culture medium was supplemented with BrdUrd. The construct sections were then stained with anti-BrdUrd antibodies (green), followed by nuclear staining (red). Scale bars represent 25 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
in this paper, capable of directing differentiation of hESCs in a way that bypasses EB formation. Therefore, we compared the efficiency of directed differentiation of hESCs cultured on polymer scaffolds supplemented with growth factors to EB-mediated differentiation in the presence of growth factors by evaluating the expression of individual marker genes of three germ layers [Figure 4(A)]. To accomplish this, qRT-PCR was performed using primers for the following genes: MSI1, VIM (ectoderm), FoxA2, AFP (endoderm), and Msx1, IGF2 (mesoderm); β-actin was used as a loading control. Undifferentiated H9 cells were grown and cultured on polymer scaffolds in the presence of TGF-β, activin-A, RA, or unsupplemented control medium for 2 weeks. As a control, EBs were produced and cultured with different growth factors for 2 weeks. RNAs were then extracted from differentiated cells from scaffolds or EBs under the treatment of different growth factors. As shown in Figure 4(B) and Supporting Information Figure 2(A), the addition of TGF-β to

**FIGURE 2.** PLGA scaffold can maintain the pluripotency of hESCs. (A–D) Histological examination of pluripotency of hESCs grown within scaffolds. After 2 weeks culture of hESCs in conditioned medium, the constructs were fixed, paraffin-embedded, and sectioned, followed by immunostaining with undifferentiated markers: (A) GCTM-2, (B) OCT-4, (C) SSEA-4, and (D) TRA 1-81. The green signals represented undifferentiated stem cell markers, and red signals showed nucleus of cells. Scale bar represents 25 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
FIGURE 3. Induction of directed differentiation of hESCs on scaffolds with growth factors. (A) Histological examination of the expression of three germ layer markers (red) in cell–polymer matrices. hESCs seeded on scaffolds were cultured in conditioned medium (control) or in the presence of TGF-β, RA or activin A for 2 weeks. Constructs were then sectioned and examined by H&E staining or stained with Brachyury antibody, Nestin antibody, AFP antibody, OCT-4, and DAPI for nuclear staining (blue) (scale bars: 50 μm). (B) Quantitative analysis of Brachyury⁺, Nestin⁺, and AFP⁺ areas of construct sections, indicating the directed differentiation of hESCs into mesoderm, endoderm, and ectoderm layers induced by growth factors. The volume fraction of differentiation marker was determined by the ratio of differentiation marker-positive counting boxes to nuclear staining-positive counting boxes, as described in “Methods”. The data are presented as the mean ± SD of sample sections (n > 5) obtained in three different experiments performed in duplicate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
hESCs–polymer matrices induced a 2- to 2.5-fold enhancement of Msx1 and IGF2 expression (mesodermal gene), as compared to control samples. RA supplementation resulted in a 2.5-fold increase in the expression level of MSI1 and VIM (ectodermal gene), and activin-A also induced a significant enhancement of differentiation to endodermal layer (FoxA2 and AFP), as compared to control samples. Although a similar pattern of enhancements of marker gene expression by various growth factor treatments was observed in differentiated cells derived from EBs [Figure 4(C) and Supporting Information Figure 2(B)], significantly higher expression of differentiation-associated genes was detected in cells grown on scaffolds as compared to EBs. Therefore, these results demonstrate that the single step of 3-D culture of hESCs on polymer scaffolds appeared to be a successful alternative approach to EB formation in promoting cell growth and directed differentiation of hESCs into different germ layers.

**In vivo transplantation of hESCs on PLGA scaffolds**

It has been reported that the differentiation of hESCs could be affected by the microenvironment at the site of transplantation where communication between host tissues and the scaffold takes place. Thus, the therapeutic potential of hESCs grown in polymer scaffolds was assessed in vivo. To examine whether hESCs seeded on scaffolds can maintain the ability to differentiate into three germ layers in vivo, undifferentiated H9 cells were seeded on scaffolds and

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**FIGURE 4.** Expression of three germ layer-specific genes in PLGA scaffold or embryoid bodies treated by various growth factors. (A) Schematic representation of the experimental procedure for the analysis. (B) RNA was isolated from hESCs seeded on scaffold for 2 weeks in conditioned medium (control) or in the presence of TGF-β, RA, or activin A. Then the isolated RNAs were subjected to qRT-PCR analysis using primers for the following genes: MSI1 (ectoderm), FoxA2 (endoderm), and Msx1 (mesoderm), with β-actin as control. (C) Differential expression of germ layer-specific genes in 14-day embryoid bodies (EBs) using primers for MSI1, FoxA2, Msx1, and β-actin in qRT-PCR. The data are presented as the mean ± SD of sample sections (n > 5) obtained in three different experiments performed in duplicate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
further cultured in knockout medium for 2 weeks in vitro. Scaffolds were then implanted into immunodeficient SCID mice. After 14 days of transplantation, the implants were retrieved, fixed, paraffin-embedded, and sectioned for histological examination with both undifferentiated and differentiated markers, as described above [Figure 5(A)]. During the whole transplantation period, no signs of infection were observed. In fact, the implanted scaffolds were surrounded by host tissues and blood vessels, implying the interaction between the host tissues and the scaffolds. The hESCs appeared to grow and differentiate in defined structures within the scaffold showed in Supporting Information Figure 3. As shown in Figure 5, the results of immunohistochemical staining with brachyury, Nestin, and AFP antibodies indicated that the interaction between stem cells and their surrounding microenvironment in vivo could promote cell

FIGURE 5. Differentiation of hESCs in scaffolds transplanted to SCID mice. (A) Schematic representation of the experimental procedure for the analysis. (B–E) Undifferentiated hESCs were cultured on scaffolds with knockout medium for 2 weeks, and then the scaffolds were transplanted subcutaneously into the dorsal region of SCID mice. After 2 weeks of transplantation, the scaffolds were retrieved, paraffin-embedded, sectioned, and immunostained with antibodies against OCT-4, Brachyury, Nestin, AFP, and DAPI for nuclear staining (scale bar: 25 μm). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
cell growth and high-level cellular proliferation. Furthermore, the highly porous PLGA scaffold allowed for uniform cell growth and high-level cellular proliferation. Furthermore, the high-pressure gas-forming technique could provide the mechanical stiffness necessary for scaffolds to support the hESCs and resist the force of cell contraction.

When combining stem cells with biomaterial scaffolds have proven to be a promising strategy for tissue-engineering applications. Many efforts have been made to investigate a wide variety of natural and synthetic biomaterials as stem cell scaffolds for different applications. Natural protein- or polysaccharide-based materials, such as collagen, fibrin, alginate, chitosan, or hyaluronic acid, have demonstrated potential as a cell scaffold based on their biocompatibility, biodegradability, and limited toxicity. However, the restricted range of mechanical properties of these materials, coupled with their capability of promoting 2-D cell growth, has limited their use in tissue-engineering applications. Encapsulation of stem cells in hydrogel polymers, such as hyaluronic, has also been proposed for the 3-D culture system. However, release of cells from 3-D structures requires enzyme-mediated digestion of hydrogels. For example, the addition of hyaluronidase into growth medium is necessary to release cells from hyaluronic acid hydrogel, which may lead to safety issues arising in long-term cultures of stem cells with enzymatic treatment.

In contrast, synthetic biomaterials, including polymers and peptide-based materials, offer controllable mechanical properties and superior reproducibility by their defined chemical compositions. Particularly, poly(lactic-co-glycolic acid) (PLGA), which is an FDA-approved biomaterial, has been explored as a structural support for many tissue-engineering applications. One study demonstrated that the polymer scaffold composed of 50% PLGA and 50% poly(l-lactic acid) could support the growth and differentiation of hESCs. This study, however, developed a 3-D culture system by seeding differentiating stem cells (8-day-old EBs) on the polymer scaffold rather than undifferentiated hESCs, thereby still necessitating the induction of EB formation before 3-D cell growth. However, as noted above, EB formation can lead to considerable cell death and the heterogeneous mixture of cell types caused by the spontaneous differentiation in EBs, making it more difficult to achieve reproducible culture conditions. Therefore, it would be desirable to develop 3-D supportive structures capable of directly growing and culturing undifferentiated hESCs that potentially possess unlimited capacity of self-renewal and pluripotency.

In this study, we have demonstrated such 3-D culture system for undifferentiated hESCs capable of bypassing EB formation for differentiation. Our data suggested that this porous PLGA scaffold could serve as a synthetic stem cell niche that provides a physical microenvironment in which hESCs naturally reside. The results indicated that the PLGA scaffold could offer culture conditions conducive to maintaining the characteristics of undifferentiated pluripotent stem cells. Pluripotency is the most important property of hESCs, enabling them to differentiate into all cell types of three germ layers. Furthermore, when combined with specific growth factor cues, it was demonstrated that our scaffold was able to direct differentiation of hESCs into ectodermal, endodermal, and mesodermal layers. The results of qRT-PCR and histological examinations further confirmed the directed differentiation into different cell types when treated with appropriate cues and relatively higher expression of differentiation-associated genes from cells grown on PLGA scaffolds compared with that of EBs, suggesting that 3-D culture of hESCs on the scaffolds could be an alternative to EBs in promoting cell growth and directed differentiation. Although PLGA scaffold was coated with matrigel, the growth and differentiation of hESCs in a 3-D organization should be mainly contributed by the scaffold, because it was reported that matrigel alone could not provide enough compressive modulus for hESCs growth.

In addition, in vivo transplantation study indicated that hESC-scaffold matrices could induce normal interaction between stem cells and their surrounding microenvironment, enabling not only promotion of cell differentiation, but also maintenance of differentiation-associated protein expression in vivo. Moreover, this scaffold was made from a PLGA microparticle formed by a standard double emulsion technique that can be used to encapsulate growth factors and/or therapeutic genes of interest into the scaffold, in turn possibly providing a useful platform for growth factor/gene delivery in vivo for controlled and sustained modulation of hESCs.

CONCLUSION

We have demonstrated a highly porous 3-D scaffold that can serve as a new tool to promote 3-D growth and proliferation of undifferentiated hESCs and further differentiation into all three germ layers in vitro and in vivo. This study has provided a practical and efficient means to direct differentiation of hESCs with specific growth factor cues, which enables bypass of EB formation. This biodegradable porous PLGA scaffold system for hESC culture and transplantation can also be readily adapted for use in many different tissue-engineering applications.

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