The Effect of Topography on Differentiation Fates of Matrigel-Coated Mouse Embryonic Stem Cells Cultured on PLGA Nanofibrous Scaffolds

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Due to pluripotency of embryonic stem (ES) cells, these cells are an invaluable *in vitro* model that investigates the influence of different physical and chemical cues on differentiation/development pathway of specialized cells. We sought the effect of roughness and alignment, as topomorphological properties of scaffolds on differentiation of green fluorescent protein-expressing ES (GFP-ES) cells into three germ layers derivatives simultaneously. Furthermore, the effect of Matrigel as a natural extracellular matrix in combination with poly(lactic-co-glycolic acid) (PLGA) nanofibrous scaffolds on differentiation of mouse ES cells has been investigated. The PLGA nanofibrous scaffolds with different height and distribution of roughness and alignments were fabricated. Then, the different cell differentiation fates of GFP-ES cells plated on PLGA and PLGA/Matrigel scaffolds were analyzed by gene expression profiling. The findings demonstrated that distinct ranges of roughness, height, and distribution can support/promote a specific cell differentiation fate on scaffolds. Coating of scaffolds with Matrigel has a synergistic effect in differentiation of mesoderm-derived cells and germ cells from ES cells, whereas it inhibits the derivation of endodermal cell lineages. It was concluded that the topomorphological cues such as roughness and alignment should be considered in addition to other scaffolds properties to design an efficient electrospun scaffold for specific tissue engineering.

Introduction

EMBRYONIC STEM (ES) cells are pluripotent cells derived from the inner mass of pre-implantation embryos, and they can differentiate into all cell lineages derived from three germ layers.¹ This capacity makes them an invaluable *in vitro* model that investigates the influence of different physical^{2,3} and chemical cues⁴ on differentiation/development of specialized cells.^{5–7} Mostly, the differentiation process is begun by embryoid body (EB) formation, which ensures the existence of ectodermal, mesodermal, and endodermal precursors for further differentiation.

Electrospinning is a simple and reproducible method of producing nanofibrous mats with diameters sized from micron to sub-micron ranges, which can be applied for various research and biomedical applications.^{8–11} Recently, differentiation of ES and mesenchymal stem cells cultured on electrospun nanofibrous scaffolds, which mimic the extracellular matrix (ECM), into specialized cells such as neural and epi-

dermal cell lineages and cardiomyocytes has received a lot of attention for tissue engineering.^{2,12,13} To enhance the differentiation-promoting effect of electrospun nanofibrous mats, they can be functionalized by blending, encapsulation, or immobilization of bioactive materials such as growth factors, for instance, epidermal growth factor (EGF) or ECM proteins such as Laminin.^{14,17–20}

The different physical and chemical properties such as diameter and alignment of nanofibrous mats in scaffolds, pore size, porosity of scaffolds, and chemistry of polymer and solvent can promote or inhibit a specific differentiation/programming pathway. For instance, several investigations proved the promoting effect of aligned architecture of nanofibrous mats in neurite outgrowth and neural differentiation of ES, nerve stem cells, and dorsal root ganglion cells.^{2,15,16} Xie *et al.* demonstrated the efficient differentiation of EBs derived from murine CE3 and RW4 ES cells into neural lineages when they are differentiated on aligned polycaprolactone (PCL) nanofibrous scaffolds.² Similarly,

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Ghasemi-Mobarakeh *et al.* also confirmed the positive effect of alignment in neural differentiation of C17.2 and showed that the effect can even be augmented by incorporation of gelatin in the PCL nanofibrous scaffolds by blending.¹⁵ Matrigel as a natural ECM, which is mainly composed from laminin and collagen type IV, is used for *in vivo* angiogenesis,²¹ improvement of graft survival,^{22,23} proliferation, and differentiation of stem cells.^{22,24} Interestingly, different studies showed that Matrigel can support/promote the differentiation of stem cells into different cell lineages, such as neural, hepatic, and cardiac cell lineages.^{22,25–27} Furthermore, several investigations showed that the coating of culture surface with Matrigel bypassed the necessity of ES and induced pluripotent stem (iPS) cell cultures to the feeder and provided a niche for maintaining the undifferentiated status of the pluripotent cells.^{28,29}

Porosity, pore size, and chemical components of nanofibrous scaffolds and grafting materials have significant impacts on infiltration, proliferation, and differentiation of stem cells.^{30–33} To the best of our knowledge, so far there is no report that reveals the effect of roughness and alignment as topomorphological properties on differentiation of mouse ES (mES) to three germ layers and their derivatives simultaneously. In most differentiation studies, the investigators only trace a specific cell programming in the differentiated cell population, and eventually, they exclude only the presence of other related cells, which are derived from the same progenitors as interested cells in development^{34,35}; whereas the ES cells are pluripotent and have the potential to differentiate to all three germ layers cell derivatives. Therefore, the presence of other cell lineages should be studied to estimate the purity of differentiated cell population.

This study aims first at comparing the efficiency of different programming of murine ES cells seeded on electrospun PLGA scaffolds with different roughness topographies, confirmed by atomic force microscopy (AFM), and second, the combinatory effect of Matrigel and PLGA scaffolds on the differentiation efficiency of EB-differentiating cells.

Materials and Methods

Electrospinning

PLGA (50:50, Lactic acid to glycolic acid) with a 48,000 Da average molecular weight was purchased from Sigma-Aldrich. To fabricate PLGA-4, 5, 6, and 7 scaffolds, PLGA was dissolved in chloroform:methanol (3:1) as 12.25% (W/V) solution, and the PLGA nanofiber mats were fabricated by the electrospinning method using different parameters (Table 1). To generate PLGA-50 scaffold, PLGA was dissolved in 1,1,1,3,3,3-hexa-fluoroisopropanol as 12.25% (W/V) solution.

Characterization of electrospun nanofibrous scaffolds

To study nanofibrous scaffolds morphology, the different scaffolds were coated with gold using sputter coater and then observed by a scanning electron microscope (SEM; Tescan vega-II). SEM micrographs were analyzed by Image J (National Institutes of Health).

For determination of roughness of nanofibrous scaffolds and alignments of fiber mats in scaffolds, Dual AFM (Scope C26 DME) was used. Ten AFM micrographs for each scaffold were analyzed by DualScope(tm)/Rasterscope(tm) SPM, version 2.1.1.2. software, and the roughness values were reported as mean $\pm$ standard deviation (SD).

To assess the alignment of nanofibrous mats in each scaffold, the angle of all fibers in ten AFM micrographs for each scaffold were measured relative to horizon line using Image J.

In order to chemical analysis of PLGA and PLGA/Matrigel scaffolds, the attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectrophotometry over a range of 4000–400 cm^{-1} was performed. ATR-FTIR spectra of scaffolds were obtained by BOMEM Model BM-102 spectrometer with a DTGS detector.

Generation of GFP-ES cells and differentiation on nanofibrous scaffold

The undifferentiated CGR8 (P7 [passage 7]), a feeder-free mES cell line (a gift of A. Skoudy, IMIM, Barcelona), were routinely cultured on gelatin-coated dishes (0.1%) in Glasgow's Modified Eagle's Medium (GMEM) supplemented with 10% fetal bovine serum (FBS)/10% serum replacement and 1000 U/mL human recombinant leukemia inhibitory factor (LIF). To generate GFP-expressing mES cells (GFP-ES), CGR8 cells were infected by GFP-harboring lentivirus with multiple of infection 20–100. The GFP-expressing cells were picked up 72 h after infection and sub-cloned in 96-well plates. To induce differentiation, GFP-ES cells (passage three after transduction) were subjected to EB formation in six-well suspension-culture plates for 7 days at a density of 50×10^4 cells/mL in GMEM supplemented with 5% FBS/5% serum replacement without LIF. To seed the ES-derived differentiating EBs on scaffolds, 75–100 EBs were resuspended in 150 μL of GMEM supplemented with 10% FBS/10% Serum Replacement, dotted on scaffolds in the 24-well plates, and incubated at 37°C for 4 h. Then, 150 μL of Matrigel (1%; BD Bioscience)-containing GMEM medium without serum was added as on-top coating to plated EBs and incubated for the next 20 h. The differentiating EBs were maintained for 10 days, and the medium was changed every other day with GMEM supplemented with 5% FBS/5% serum replacement. To investigate attachment and morphology of cells on

TABLE 1. PARAMETERS USED FOR ELECTROSPINNING OF DIFFERENT ELECTROSPUN PLGA NANOFIBROUS SCAFFOLDS

Nanofibrous scaffolds	Injection velocity (mL/h)	Potential differences (kV)	Needle-collector distance (mm)	Collector type	Velocity of collector (rpm)	Collection duration (h)
PLGA-4	0.5	25	150	Drum	2500	3.5
PLGA-5	0.7	20	120	Drum	2500	0.25
PLGA-6	0.5	20	180	Drum	2500	0.25
PLGA-7	0.5	25	150	Plate	—	0.5
PLGA-50	2	24	155	Drum	250	1.5

PLGA, poly(lactic-co-glycolic acid).

scaffolds after differentiation, the cell-containing scaffolds were fixed by glutaraldehyde 2.5%, dehydrated by a gradient of alcohol concentration, and eventually observed by SEM.

Quantitative mRNA expression analysis

The mRNA expression patterns of different genes were analyzed 10 days after plating on different prepared scaffolds using real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted by *QIAzol* lysis reagent, and 1–2 µg of RNA was reverse transcribed using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas). Quantitative PCR reactions were performed in the 48-well optical reaction plate on StepOne™ Real-Time PCR System using primers listed in Table 2. The threshold cycle (Ct) of each target gene obtained from StepOne software (Applied Biosystems) was normalized by HPRT expression as housekeeping genes. The relative quantity of target gene expression in each sample to control (EBs before plating) was calculated by the comparative $2^{-\Delta\Delta C_t}$ method. Measurements were performed in two identical samples in each experiment, and the relative gene expression values were presented as a mean of three independent experiments.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 30 min and permeabilized with 0.1% TX-100 in tris buffered saline (TBS). Then, the cells were blocked with 5% BSA for 45 min and incubated with primary antibodies against OCT4 (Abcam; ab27985, 1:300) and Nanog (Abcam; ab21624, 1:200) overnight. After washing, cells were incubated with secondary antibodies: Alexa Fluor 594 donkey anti-goat (Invitrogen; A-11058, 1/400) and Alexa Fluor 594 donkey anti-rabbit (Invitrogen; A-21207, 1/400) for 45 min. Cells were washed again and incubated with 4',6-diamidino-2-phenylindole (DAPI; 1:2000) for nuclear staining.

Statistical analysis

Mann–Withney *U* test type from two independent samples test was used for statistical analysis, and significant differences were expressed by *p*-value.

Results

Morphology, topography, and chemical properties of different prepared PLGA nanofibrous scaffolds

The electrospun PLGA nanofibrous scaffolds were synthesized in different conditions (Table 1). As can be seen in Figure 1, all the different PLGA scaffolds have highly porous structures that make them suitable for cell feeding and infiltration; however, the alignment and the diameter range of fibers were different. The fibers' diameter were analyzed by Image J and summarized in Table 3. PLGA-5 (Fig. 1B) nanofiber mats have the smallest and narrowest diameter size range (124–380 nm) compared with all scaffolds, which resulted from high injection velocity (0.7 mL/h) as well as shortest needle-collector distance.

To check whether roughness of scaffolds can influence the attachment and differentiation of mES cells, an AFM study was accomplished in micro/nano scales. As shown in Figure 2, the different conditions of PLGA scaffold fabrication have

resulted in different roughness. The AFM micrographs (Fig. 2A–E) were analyzed by SPM software, and the average (S_a) and standard deviation of roughness (S_d) were determined and graphed (Fig. 2F). The PLGA-4 scaffold showed highest average (438 nm) and standard deviation (± 536 nm) of roughness compared with other scaffolds, implying the highest eminence and a deep hollow in the surface of PLGA-4, which may enable this scaffold to maintain and trap cells more efficiently than other scaffolds (Fig. 2F).

Since in this study we suppose that the frequency of roughness height distribution may play an important role in the differentiation and homogeneity of differentiation during differentiation of ES cells on scaffolds, the frequency of different roughness distribution was measured in 10 different fields of each PLGA scaffold and graphed in Figure 3. PLGA-4 scaffolds possess the roughness sizes in a wide range from nano- to micro-scale (10 nm–3.25 µm) in a relatively high frequency, whereas other scaffolds show the roughness height no more than 2500 nm. Therefore, PLGA-4 scaffolds may recapitulate the roughness of a variety of niches in different tissues in comparison to other scaffolds.

To understand the effect of alignment of nanofibrous mats on differentiation fates of cells, the alignments were measured relative to the horizon line and reported as standard deviation (Fig. 4). More standard deviation indicates less alignment and vice versa. As seen in AFM micrographs in Figure 2 and has been quantified in Figure 4, PLGA-6 (7.64) and PLGA-5 (15.76) showed a higher degree of alignment compared with PLGA-4 and 7 scaffolds, which can be due to low electrical potential of differences and more collection duration in fabrication (Table 1). Using of the drum as a collector in the preparation of PLGA-4 instead of a plate, which is used in PLGA-7, resulted in more alignment in PLGA-4, despite similarity in other parameters (Table 1). The assessment of pore size and distribution by capillary flow porometry showed no significant differences between pores of different PLGA nanofibrous scaffolds. In this study, in combination with synthetic PLGA nanofibrous scaffolds, the Matrigel, a natural ECM, was used as on-top coating. To characterize the surface of different prepared PLGA scaffolds after coating by Matrigel, ATR-FTIR analysis was performed. Infrared spectra for PLGA-related stretching modes were observed for all different prepared PLGA and PLGA/Matrigel scaffolds (Fig. 5). These include 2922 cm^{-1} (asymmetric CH₂ stretching), 2853 cm^{-1} (symmetric CH₂ stretching), 1743 cm^{-1} (carbonyl stretching), 1168 cm^{-1} (C–O–C stretching), and 1089 cm^{-1} (C–O stretching). Covering of scaffolds with Matrigel resulted in two additional peaks (as shown by arrow in Fig. 5) at 1650 cm^{-1} (amide I) corresponding to the stretching vibrations of C=O bond and 1548 cm^{-1} (amide II) corresponding to the coupling of bending of N–H bond and stretching of C–N bonds. The amid I band (1650 cm^{-1}) was attributed to both a random coil and α -helix conformation of collagen and laminin as main components of Matrigel. For other scaffolds, the results were the same as PLGA-4 and 7 (data not shown).

Generation of pluripotent GFP-ES cells to monitor the behavior of cells on three-dimensional scaffolding materials

In order to study all the possible cell differentiation programs, CGR8 (P7), a feeder-free mES, cells were used. To

TABLE 2. PRIMERS USED FOR REAL-TIME REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

Gene	Accession no.	Primer sequence (5'-3')	Size (bp)	Annealing (°C)	Recognition tissue(s)
OCT4	NM_013633	F:GTTCTCTTTGGAAAGGTTTC R:GCATATCTCCTGAAGGTTCT	146	55	ESC
Nanog	NM_028016	F:TGATTTGGTTGGTGTCTTG R:TGTGATGGCGAGGGGAAG	175	55	ESC
Sox2	NM_011443	F:CTGGAGAAGGGGAGAGATTTT R:CGTTAATTTGGATGGGATTGG	157	55	ESC
ALP	NM_007433	F:GTGTTGTGTTATTGCAGCTTCT R:CATCCCATCTCCCATGAGGAT	248	58	ESC
THY1	NM_009382	F:TCAAGTGTGGCGGCATAAG R:GAGGAGGGAGAGGGAAAGC	77	58	T-cells/MSC
Sox1	NM_009233	F:GCTACATGAGCGCGTCGCCT R:AGAGATCCGAGGGCGCCAG	160	58	Neuroectoderm
gFAP	NM_010277	F:AGGGGCCTCGGTCTAGT R:CGCCCGTGTCTCCTTGAAG	137	60	Glial/Asterocyte
TH	NM_009377	F:GCCACAGCCCCAAGGGCTTCA R:TGAGACTCTGCCCGCTCCA	101	58	Neuron/Dopaminergic
Nestin	NM_016701	F:AGCAGGGTCTACAGAGTCAG R:GTCCTGTATGTAGCCACTTCC	100	58	Neuronal precursors
GSC	NM_010351	F:GCTGGCCAGGAAGGTGCACC R:CGGCGAGGCTTTTGAGGACGT	148	58	Mesendoderm/endoderm
Albumin	NM_009654	F:CAGGATTGCAGACAGATTATGC R:GCTACGGCACAGTGGCTTG	265	60	Hepatocyte
TTR	NM_013697	F:GGCTGAGTCTCTCAATTC R:CTCACCACAGATGAGAAG	223	58	Hepatocyte
TAT	NM_146214	F:ACCTTCAATCCCATCCGA R:TCCCGACTGGATAGGTAG	205	55	Hepatocyte
Foxa2	NM_010446	F:CCAGCGAGTTAAAGTATGC R:TGTGTTTCATGCCCATTATC	150	55	Definitive endoderm
HNF4 α	NM_008261	F:ACA CGT CCC CAT CTG AAG R:CTT CCT TCT TCA TGC CAG	269	55	Hepatocyte
Brachuruy	NM_009309	F:CCCTGCACATTACACACCAC R:AGGTGTCCACGAGGCTATGA	154	58	Mesendoderm/Mesoderm
MyoD2	NM_010866	F:CTGATGGCATGATGGATTAC R:GACACAGCCGCACTTTC	120	58	Muscle
Myf5	NM_008656	F:CGCTGGTCGCTGGAGAG R:GAGGGAACAGGTGGAGAACTA	292	60	Muscle
α -MHC	NM_010856	F:CCAGATTATCCAGGCTAACC R:CCAGAAGGTAGGTCTCTATGT	151	60	Cardiomyocyte
β -MHC	NM_080728	F:GCTGTTTCCTTACTTGCTACC R:GGATTCTCAAACGTGTCTAGT	116	60	Cardiomyocyte
Troponin-T	NM_011619	F:CGAGCAGCAGCGTATTC R:CCTCATCCTCAGCCTTCC	110	58	Cardiomyocyte
Adipsin	NM_013459	F:ATGGTATGATGTGCAGAGTGT R:CACACATCATGTTAATGGTGAC	448	60	Adipocyte
Osteocalcin	NM_007541	F:GACCATCTTTCTGCTCACTCTG R:GTGATACCATAGATGCGTTTGT	230	55	Adipocyte
Osteopontin	NM_009263	F:CAGTGATTTGCTTTTGCTGTG R:GGTCTCATCAGACTCATCCGA	362	60	Adipocyte
VegFR2	NM_010612	F:GCCATCAACAAAGCGGGACGA R:GATTGCGCCATGTGGACCGAT	200	60	Endothelium
VWF	NM_011708	F:GACAGACGCCATCTCCAG R:ATGTCTCATCTTGCTTCAGG	175	58	Endotelium
Dazl	NM_010021	F:ATCAGCAACCACAAGTCAAG R:CAAATCCATAGCCCTTCG	187	55	Germ cells
Sycp3	NM_011517	F:AAAGCATTCTGGGAAATCTG R:GTACTTCACCTCCAACATCTTC	187	58	Germ cells
Ddx4	NM_010029	F:CGGAGAGGAACCTGAAGC R:CGC CAATATCTGATGAAGC	160	58	Germ cells
HPRT	NM_013556	F:GGCCAGACTTTGTTGGATTTG R:TGCGCTCATCTTAGGCTTTGT	144	55-60	Internal standard

ESC, embryonic stem cell; MSC, mesenchymal stem cell.

FIG. 1. SEM images of (A) PLGA-4 (B) PLGA-5, (C) PLGA-6, (D) PLGA-7, and (E) PLGA-50, nanofibrous scaffolds. SEM, scanning electron microscope; PLGA, poly(lactic-co-glycolic acid).

monitor the behavior of the cells such as attachment and morphological events, by fluorescent microscopy, a GFP-ES cell line was established by lentiviral transduction of GFP to CGR8 (P7) cells (Fig. 6A, B). To avoid the effect of a clone on further differentiation analyses, two GFP-ES clones were selected and characterized. The karyotype analysis showed normal numerical and structural chromosome profiles (40;XY) for both the clones (data not shown). To evaluate the pluripotency of generated GFP-ES clones, they were spontaneously differentiated *in vitro*. Two weeks after EB plating, the beating cardiomyocytes were observed in the center of EBs with many neural-like cells with long neurite outgrowths on the border of EBs. The immunocytochemistry (ICC) confirmed expression of Gossoid (GSC) and Foxa2 as early markers of endoderm in a subset of spontaneously differentiated cells (data not shown). Furthermore, the expression of pluripotency markers including OCT4 and Nanog was analyzed by ICC. The result showed a homogeneous and high expression of Nanog (Fig. 6C–E) and OCT4 (Fig. 6F–H) in GFP-ES cells. The appropriate nuclear localization of these pluripotency markers were confirmed after merging by DAPI (Fig. 6E, H). All results together confirmed that the established GFP-ES clones are pluripotent with normal genotypes. To induce the differentiation, the EBs were formed from two GFP-ES clones (P3 after

transduction), as mentioned in Materials and Methods. The further differentiation analyses showed that both GFP-ES clones behave the same with insignificant differences. Thus, the result of one clone has been reported here as representative of two GFP-ES clones.

In vitro assay of attachment and interaction of cells with PLGA nanofibrous scaffolds with different roughness

To reveal the effect of different roughness, distribution thereof, and alignment as different topomorphological properties on cell-scaffold interaction, the *in vitro* study was performed. To elucidate the influence of roughness in differentiation of different cell lineages derived from all three germ layers (ectoderm, mesoderm, and endoderm), the pluripotent ES cells that express GFP (GFP-ES) were used. Furthermore, in the next step, the GFP-expressing embryoid bodies (GFP-EBs) were formed from GFP-ES cells. The formation of EBs can ensure the existence of all germ layers precursors in the plating time. The number of attached GFP-EBs was counted under fluorescent microscopy in 20 fields, 10 days after plating (Fig. 7A). The attachment of EBs on scaffold has been improved two and threefolds in PLGA-6 and PLGA-7, respectively, after covering of EBs by Matrigel, which can be due to physical trapping of EBs on scaffold and maintaining them during differentiation. PLGA-4 and 7, which possess a high frequency of roughness distribution in the wide range of roughness height, showed more attachment capacity before and after coating by Matrigel, respectively (Fig. 7A). SEM micrographs also confirmed the fluorescent microscopy data when we monitored attached EBs in 20 different fields by SEM. The morphological features and behavior of EBs on PLGA-4, 7, and 50 scaffolds were similar, as they were the same for PLGA-5 and 6 scaffolds. As representatives, the SEM of differentiated EBs on PLGA-4 (Fig. 7B, C) and PLGA-5 scaffolds (Fig. 7D, E) has been shown. The morphologies of differentiated EBs on scaffolds

TABLE 3. DIAMETER SIZE OF PLGA NANOFIBROUS SCAFFOLDS SYNTHESIZED IN DIFFERENT CONDITIONS

Nanofibrous scaffolds	Minimum diameter (nm)	Maximum diameter (nm)	Mean $\pm$ standard deviation diameter (nm)
PLGA-4	197	393	294.5 $\pm$ 59.0
PLGA-5	124	380	253 $\pm$ 62.2
PLGA-6	153	446	299.5 $\pm$ 95.6
PLGA-7	153	422	286.2 $\pm$ 83
PLGA-50	93	606	256.2 $\pm$ 118.2

PLGA, poly(lactic-co-glycolic acid).

FIG. 2. Atomic forced microscopy images of (A) PLGA-4, (B) PLGA-5, (C) PLGA-6, (D) PLGA-7, (E) PLGA-50, PLGA nanofibrous scaffolds, and (F) quantification of the scaffolds' roughness and their standard deviations. Color images available online at www.liebertonline.com/tea

(Fig. 7B–E) showed that covering of EBs after plating by Matrigel resulted in more expansion and infiltration of differentiated cells on/into the scaffolds (compare Fig. 7B with Fig. 7C, and Fig. 7D with Fig. 7E). Overall, the infiltration of differentiated cells into PLGA-4, 7, and 50 with the highest frequency of roughness distribution in the most range of roughness heights was more than PLGA-5 and 6, which have low roughness size with less frequency of

roughness distribution (compare Fig. 7B with Fig. 7D and Fig. 7C with Fig. 7E).

The effect of roughness and alignment of nanofibrous mats as well as Matrigel on differentiation of pluripotent mES cells

To evaluate the topography effect of nanofibrous scaffolds, as well as coating by Matrigel on differentiation fates of ES cells, GFP-ES-derived EBs were seeded on different scaffolds with different surface roughness, then coated by

FIG. 3. Distribution frequency of roughness with different height sizes in different prepared scaffolds.

FIG. 4. Histogram showing the alignment in different PLGA scaffolds.

FIG. 5. FTIR spectra of PLGA (PLGA-4 and 7) and PLGA/Matrigel nanofibers. FTIR, Fourier transform infrared.

Matrigel, and differentiated without any other inducing factors. The mRNA expression profile of differentiated cells was analyzed by quantitative RT-PCR using 29 specific primers for three germ layers, their fully differentiated derivatives, and pluripotency and germ cell markers (Table 2).

The differentiated cells were analyzed for expression of definitive (GSC) and foregut (*Foxa2*) endoderm markers (Fig. 8A). The visceral tissues such as lung, pancreas, liver, stomach, and intestine are originated from definitive endoderm; therefore, the efficiency of definitive endoderm formation will determine the efficacy of ES differentiation to endoderm-derived tissues. The coating of plated cells on scaffolds with Matrigel has significantly decreased the expression of GSC and *Foxa2* genes in all PLGA scaffolds (Fig. 8A). The mRNA expression pattern of *HNF4 α* and albumin as early markers of hepatic development and liver-specific *TAT* and *TTR* as mature hepatic markers showed a significant reduction after coating by Matrigel (Fig. 8B). As shown in Figure 8, the differentiated cells on PLGA-4, 7, and 50 expressed more endoderm- and endodermal-derived cell lineage-specific genes compared with other scaffolds, regardless of coating by Matrigel. We already showed (Fig. 3) that the PLGA-4, 7, and 50 have a higher frequency of roughness distribution at roughness sized from 1750 to 2250 nm on the scaffolds. Therefore, these ranges of roughness may support/promote the differentiation of mES cells to endoderm-derived lineages.

To analyze the mesoderm-originated tissues differentiation, the expression of specific markers (Table 2) for primary mesoderm and mesoderm-derived cells including T-cells, muscle, cardiomyocytes, adipocytes, osteocytes, and endothelial cells was investigated by quantitative RT-PCR (Fig. 9). The coating of plated cells on scaffolds with Matrigel has

FIG. 6. (A) Fluorescent microscopy, and (B) light microscopy of generated GFP-ES cells. Immunocytochemistry performed on GFP-ES cells for Nanog (C) and OCT4 (F) as pluripotency markers, (D and G) staining of nuclei by DAPI and (E and H) merged images. GFP, green fluorescent protein; ES, embryonic stem; DAPI, 4',6'-diamidino-2-phenylindole. Color images available online at www.liebertonline.com/tea

FIG. 7. (A) The percentage of GFP-EB attachment to different prepared PLGA scaffolds without (w/o Mtg) and with Matrigel (Mtg) covering (* $p < 0.05$; ** $p < 0.01$). The SEM images of plated/differentiated GFP-EB cells on/into (B) PLGA-4, (C) PLGA-4/Matrigel, (D) PLGA-5, and (E) PLGA-5/Matrigel, 10 days after seeding. EB, embryoid body.

FIG. 8. Quantitative expression analysis of (A) definitive endoderm Goss-coid (GSC), foregut endoderm Foxa2, and (B) hepatic-specific markers in differentiated GFP-EB cells on PLGA scaffolds, using real-time RT-PCR. RT-PCR, reverse transcriptase-polymerase chain reaction. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

FIG. 9. Real-time RT-PCR analyses of mRNA levels for specific genes for mesoderm-derived cells including (A) early mesoderm marker *Brachyury*, T-cell marker *THY1*, cardiac markers, and (B) *Adipsin* for adipocyte, *Osteocalcin* and *Osteopontin* for osteocyte, and *VegFR2* and *VWF* for endothelial, 10 days after differentiation on scaffolds. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

significantly increased the expression of all mesoderm-derived cell lineage-specific markers, and it was independent of scaffold preparation (Fig. 9). The results were in accordance with two-dimensional (2D) culture investigations showing the mesodermal promotion effect of Matrigel in the differentiation of ES cells⁷. The mRNA expression pattern of *Brachyury*, which is expressed in mesendoderm very early in development and then limited to mesoderm, showed a higher expression in cells differentiated on PLGA-4 and 5 scaffolds than other scaffolds. *THY1* gene, which is expressed in mature T lymphocyte³⁶ and involved in the adhesion of signal molecules, was up-regulated more on cells plated on PLGA-4, 7, and 50 (Fig. 9A). The expression of muscle-specific transcription factors *MyoD2* and *Myf5*, and *Adipsin* as adipocyte-specific adipokine shows no significant change between differentiated cells plated on scaffolds with different surface roughness (Fig. 9A). The culture and differentiation of ES cells on PLGA-4, 5, and 50 compared with other PLGA nanofibrous scaffolds promoted more cardiac differentiation, as it was proved by analyzing the expression of myocardial-specific genes such as α - and β -myosin heavy chains (α - and β -MHCs) and *Troponin T* (Fig. 9A). The high frequency of roughness distribution for the myocardial promoting/supporting-scaffolds, including PLGA-4, 5, and 50, was at roughness sized from 1500 to 1750 nm on the

scaffolds, implying that this range of roughness height may support/promote the myocardial differentiation of mES cells. The analysis of osteocyte-specific markers including *osteocalcin* and *osteopontin* as bone-specific matrix proteins and endothelial-specific markers such as *VegFR2* and *VWF* revealed an up-regulation in differentiated cells plated on PLGA-4 and 7 scaffolds. Since PLGA-4 and 7 have more frequency of roughness distribution compared with others in roughness sized from 1500 to 2250 nm (Fig. 3), it may argue the suitability of this roughness range to promote/support endothelial differentiation of mES cells.

The expression of specific markers (Table 2) for neuroectoderm (*Sox1*), glial (*GFAP*), neurons (*TH* and *Nestin*), germ cells (*Dazl*, *Sycp3*, and *Ddx4*), and pluripotency (alkaline phosphatase [ALP] specific for ES cells and transcription factors such as *OCT4*, *Nanog*, and *Sox2*) was analyzed in the level of mRNA (Fig. 10).

The coating of plated/differentiating cells on scaffolds with Matrigel has significantly increased the expression of germ cell and pluripotency markers (Fig. 10C, D), and this effect was independent of scaffold surface roughness. However, the expression of neuroectoderm and neural cell-lineage markers was not regulated after coating by Matrigel (Fig. 10A). So, the combination of PLGA and Matrigel has a synergistic effect in derivation of germ cells from ES cells and

FIG. 10. Quantitative expression analysis of genes specific for (A) neuroectoderm-derived cell lineages, (B) germ cells, and (C) pluripotency markers using real-time RT-PCR in differentiated GFP-EB cells, 10 days after seeding. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

maintenance of pluripotency properties in stem cells (Fig. 10B, C). These results confirmed the stemness-preserving effect of Matrigel, which has been used in several studies for culturing of ES and iPS cells^{28–29}. The differentiated cells on PLGA-4, 7, and 50 scaffolds expressed more level of transcripts specific for neuroectoderm and neural differentiation among all PLGA scaffolds. The EB-differentiating cells plated on PLGA-4 scaffold with more aligned oriented mats (less SD) than PLGA-7 and -50 (Fig. 4) expressed more ectoderm/neural markers than cells plated on PLGA-7 and 50. The result demonstrated that aligned orientation of scaffold mats can promote the neural differentiation. Since PLGA-4, 7, and 50 have a higher frequency of roughness distribution at roughness sized from 1750 to 2250 nm (Fig. 3), we suggested that this range of roughness may support/promote the differentiation of mES cells to neuroectoderm and neuroectoderm-derived cells. The expression of *Dazl*, *Sycp3*, and *Dax4* as germ-cell-specific markers increased more in Matrigel-coated cells, which were differentiated in PLGA-4, 6 scaffolds, indicating the suitability of these scaffolds to promote/support germ cell differentiation (Fig. 10B). The gene expression analysis for pluripotency master genes showed no significant changes between Matrigel-coated cells in different scaffolds (Fig. 10C).

Discussion

ES cells are pluripotent cells that can differentiate to a specific cell lineage by chemical and physical cues.^{15,37} However, coaxing of ES cells to differentiate to a specific lineage with high purity and functionality is an important challenging issue in the therapeutic application of stem cells.^{7,38} Culture and differentiation of ES cells on electrospun nanofibrous scaffolds has been attended more recently for

tissue engineering.^{2,8,9} It has been demonstrated that external physical and chemical properties of electrospun scaffolds, including the porosity, pore size, alignment of nanofiber's mats, and chemistry of polymers and grafted material, can strongly influence the specific differentiation of ES cells,^{2,15,30} however there is no report that reveals the effect of the scaffold's roughness in differentiation fates of ES cells. In the present study, the effect of surface roughness and its distribution in scaffolds on the differentiation fate of pluripotent GFP-ES cells was studied by mRNA expression profiling of 29 genes specific for three germ layers and their derivatives cell lineages. In parallel for the first time, we analyzed the effect of Matrigel as a natural ECM, on different cell differentiation fates, when it was used as on-top coating. The on-top coating of plated cells with Matrigel will not mask the effect of electrospun PLGA scaffolds from the bottom, whereas it can still influence the ES differentiation from the top, concomitantly. SEM and AFM results showed that different parameters in the fabrication of PLGA nanofibers have resulted in (1) a different range of diameter size (124–606 nm); however, there were no significant differences between means of diameter sizes in different scaffolds, (2) different roughness heights (Fig. 2), (3) different roughness distribution (Fig. 3), and (4) different alignment of nanofibrous mats. The capillary flow porometry showed no obvious significant differences between pore size and distribution thereof between different PLGA nanofibrous scaffolds. Infrared spectra for PLGA-related stretching modes were observed for all different prepared PLGA and PLGA/Matrigel scaffolds (Fig. 5). However, the coating of scaffolds with Matrigel resulted in two additional peaks at 1650 cm^{-1} (amide I) and 1548 cm^{-1} (amide II), which amid I band (1650 cm^{-1}) was attributed to both a random coil and α -helix conformation of collagen and laminin as main components of

Matrigel. The mRNA expression analyses of differentiated GFP-ES cells on different scaffolds coated by Matrigel showed a decrease in expression of the definitive endoderm-specific marker and its derivatives. The result implies the inhibitory effect of Matrigel in differentiation of ES cells into endodermal cell lineages. Furthermore, it demonstrated that PLGA-4, 7, and 50 scaffolds that have a higher frequency of roughness distribution at roughness sized from 1750 to 2250 nm could promote/support the endodermal cell lineages differentiation in comparison to other scaffolds. Therefore, this range of roughness may provide a better topomorphology for endodermal differentiation.

The result of gene expression for mesodermal and its derivatives markers confirmed the mesodermal-promoting effect of Matrigel, which has been already demonstrated in 2D culture⁷. PLGA-4, 5, and 50 promoted more cardiac differentiation than other PLGA nanofibrous scaffolds. The high frequency of roughness distribution for the myocardogenesis promoting/supporting scaffolds was at a roughness sized from 1500 to 1750 nm, implying that this range of roughness height may support/promote the myocardial differentiation from mES cells. The expression analysis of osteocyte- and endothelial-specific genes revealed an up-regulation in differentiated cells plated on PLGA-4 and 7 scaffolds with a high frequency of distribution of roughness sized from 1500 to 2250 nm, which may suggest the suitability of this roughness range in promoting/supporting osteocytes and endothelial differentiation programs.

The result demonstrated that the combination of PLGA and Matrigel has a synergistic effect in derivation of germ cells from ES cells, and maintenance of pluripotency properties in stem cells. The findings confirmed the stemness-preserving effect of Matrigel, which has been used in several studies for the culturing of ES and iPS cells.^{28–29} The expression of neuroectoderm and neural cell lineage markers was not regulated after coating of the scaffold by Matrigel.

The differentiated cells on PLGA-4, 7, and 50 scaffolds expressed more level of mRNAs specific for neuroectoderm and neural differentiation in comparison to other PLGA scaffolds. The aligned orientation of nanofibrous mats in PLGA-4 scaffolds resulted in a higher expression of ectoderm/neural markers than PLGA-7 and 50, indicating the neural differentiation-promoting effect of scaffold's alignment. These findings were in accordance with previous investigations showing the increase until 40% in neuronal differentiation efficiency after differentiation of cells on aligned scaffolds compared with random-oriented scaffolds.^{2,15,30} Despite high alignment topography of PLGA-6 and 5, our result demonstrated the least neurogenic-promoting effect of these scaffolds. We argued that although the alignment of nanofibrous scaffolds is necessary for efficient neural differentiation, it can affect in the context of appropriate roughness, as we compared aligned orientated PLGA-4 scaffold with random-oriented PLGA-7 scaffold. It was shown that scaffolds with a higher frequency of roughness distribution at roughness sized from 1750 to 2250 nm can support/promote more neural differentiation. The result showed that different roughness size and distribution have no impact on the maintenance of pluripotency property.

In conclusion, we demonstrated that roughness height and its distribution on scaffolds should be considered influential properties in the differentiation of stem cells to different cell

lineages. However, in this study, we did not investigate the mechanisms underlying these effects, but we are suggesting that different roughness may provide different niches, albeit in combination with other scaffold properties, and support/promote different cell differentiation fates. It was demonstrated that, although the scaffolds with a wide range of roughness height and distribution can support/promote a variety of cell differentiation programs, such as hepatic, cardiac, endothelial, and so on, but to design an efficient electrospun scaffold for specific tissue engineering with high purity, it needs to fabricate scaffolds with desirable roughness height and a homogeneous distribution. So, the result of this study may have an impact on the design of future scaffolds for special tissue engineering.

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Disclosure Statement

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