Extracellular Matrices Decellularized from Embryonic Stem Cells Maintained Their Structure and Signaling Specificity

Sébastien Sart, PhD, Teng Ma, PhD, and Yan Li, PhD

Embryonic stem cells (ESCs) emerge as a promising tool for tissue engineering and regenerative medicines due to their extensive self-renewal ability and the capacity to give rise to cells from all three-germ layers. ESCs also secrete a large amount of endogenous extracellular matrices (ECMs), which play an important role in regulating ESC self-renewal, lineage commitment, and tissue morphogenesis. ECMs derived from ESCs have a broader signaling capacity compared to somatic ECMs and are predicted to have a lower risk of tumor formation associated with ESCs. In this study, ECMs from undifferentiated ESC monolayers, undifferentiated aggregates, or differentiated embryoid bodies at different developmental stages and lineage specifications were decellularized and their capacities to direct ESC proliferation and differentiation were characterized. The results demonstrate that the ESC-derived ECMs were able to influence ESC proliferation and differentiation by direct interactions with the cells and by influencing the signaling functions of the regulatory macromolecules such as retinoic acid. Such matrices have the potential to present regulatory signals to direct lineage- and development-specific cellular responses for *in vitro* applications or cell delivery.

Introduction

PLURIPOTENT STEM CELLS (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have significant potential in tissue engineering and regenerative medicine due to the unlimited self-renewal ability and the capacity to differentiate into three-germ layers.^{1–3} In addition to PSCs' intrinsic properties, the extracellular PSC microenvironment comprising extracellular matrix (ECM) proteins as well as growth factors/cytokines also plays important roles in PSC development and function. *In vivo*, embryonic development is accompanied by the coordinated synthesis and secretion of ECM proteins, which are known to direct cell growth, survival, differentiation, and morphogenesis.^{4,5} *In vitro* cultured PSCs produce endogenous ECM proteins (i.e., fibronectin [FN], basement membrane proteins) that regulate PSC fate through cell adhesion and/or binding with autocrine factors (i.e., leukemia inhibitory factor [LIF], Wnt, and Activin).^{6–9} Thus, the derivation of ECMs from PSC cultures while preserving their distinct signaling capacities will greatly enhance their potential in cell delivery and tissue repair.^{10,11}

Stem cell-derived ECMs have been used to support *in vitro* cell expansion and differentiation as well as *in vivo* tissue regeneration.^{12–14} Tissue-specific ECMs derived from mesenchymal stem cells (MSCs) directed MSC-lineage specification and augmented tissue regeneration by extending site-specific MSC retention.^{13–17} In addition, microcarriers

made from MSC-derived ECMs promoted MSC adipogenesis and demonstrated *in vivo* compatibility, indicating the feasibility of their use in large-scale cell production and cell/matrix delivery.^{14,15,18,19} The ECMs decellularized from ESC cultures provided a permissive microenvironment for tissue remodeling and fibroblast repopulation.^{11,12} The decellularized ECMs of ESCs have also been shown to contain ESC-secreted factors such as Lefty, which inhibited the growth and migration of tumor cells.^{20,21} Compared to the ECMs derived from adult stem cells or somatic tissues, the decellularized matrices from PSCs may have a broader spectrum of signaling capacity owing to their embryonic origin, serving as novel biomaterials for *in vivo* cell and/or matrix delivery as well as *in vitro* cell expansion and differentiation.^{6,10} The PSC-derived ECMs may have the reduced risk of tumor formation compared to the live cells, significantly improving their prospects in clinical applications.

In vitro, PSCs have been grown as undifferentiated monolayers or 3D aggregates for expansion, or as embryoid bodies (EBs) for differentiation into specific lineages.^{22–25} These organizations have been shown to affect the secretion of ECM proteins and autocrine factors, producing an ECM microenvironment of distinct characteristics.^{20,26,27} For example, deposition of endogenous transforming growth factor-beta inhibitor, Lefty, into ECMs has been reported for undifferentiated ESCs but not EBs, whereas the EBs displayed a unique ECM-shell structure and different cytokine secretion profiles compared to undifferentiated ESCs.^{20,28–30}

PSCs expanded as aggregates upregulated E-cadherin expression and downregulated Wnt signaling upon differentiation compared to PSCs cultured on 2D substrates, suggesting the impact of intercellular interactions on cell signaling.²⁶ Parallel to the organizational dependence, the characteristics of PSC-derived ECMs are also influenced by lineage specifications.^{6,23,29} For example, cerberus, a small antagonist of bone morphogenetic protein (BMP), was detected in the secretome and ECMs of ESCs undergoing cardiac differentiation, but not neural differentiation.^{6,28} Finally, the ECMs derived at the early or late stage of EBs exhibited different signaling capacities, suggesting the influence of the developmental stage on ECM characteristics.^{10,11,28}

The goal of the present study is to evaluate the influence of ESC organizations (i.e., undifferentiated monolayers, undifferentiated 3D aggregates, and differentiated EBs) and lineage commitment on the characteristics of the decellularized ECMs. The ECMs at the early or late stage of EB differentiation were also derived from days 3 or 10 EBs, respectively.^{23,28} To create the ECMs with lineage-specific cues, EBs were treated with retinoic acid (RA) to induce germ-layer differentiation or BMP-4 for mesoderm differentiation.^{31–33} To probe the role of RA signaling in the characteristics of decellularized ECMs, the cellular expression of a RA receptor (RAR) and the effect of a pan-RAR antagonist were investigated. The results suggest that the decellularized matrices derived from ESCs are able to preserve the developmental cues and have a distinct capacity in directing the ESC fate.

Materials and Methods

Undifferentiated ESC culture

Monolayer culture. The murine ES-D3 line (American Type Culture Collection) was maintained on 0.1% gelatin-coated six-well culture plates (Millipore) in a standard 5% CO₂ incubator. The growth medium consists of the Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% ESC-screened fetal bovine serum (FBS; Hyclone), 1 mM sodium pyruvate, 0.1 mM β -mercaptoethanol, penicillin (100 U/mL), streptomycin (100 μ g/mL) (all from Invitrogen), and 1000 U/mL LIF (Millipore). The cells were seeded at 2×10^4 cells/cm² and subcultured every 2–3 days.

Aggregate culture. The aggregates of ES-D3 cells were obtained by seeding the cells from ESC monolayer into Ultra-Low Attachment (ULA) six-well plates (Corning, Inc.) at a seeding density of 2×10^4 cells/cm² in 3 mL of growth media.³⁴ The aggregates were subcultured every 2–4 days

and serially passaged for 5–15 passages. For passaging, the aggregates were trypsinized with 0.05% trypsin-EDTA for 5 min at 37°C, neutralized with the media containing FBS, and resuspended in the fresh media.

Differentiated EB culture

For EB formation, 1×10^6 cells were seeded in the ULA six-well plates. Three types of EBs were cultivated in a differentiation medium for up to 10 days: (1) spontaneously differentiated EBs; (2) EBs treated with 1 μ M all-trans RA (Sigma-Aldrich); (3) EBs treated with 5 ng/mL BMP-4 (R&D Systems). The differentiation medium consists of the DMEM supplemented with 10% FBS, 0.1 mM β -mercaptoethanol, penicillin (100 U/mL), and streptomycin (100 μ g/mL).

Derivation of decellularized ECMs from monolayers, aggregates, and EBs

Four decellularization reagents, including (1) 1% Triton X-100, (2) 20 mM NH₄OH, (3) 1% SDS, and (4) 1% Triton X-100 + 20 mM NH₄OH (all from Sigma), were tested and compared (Table 1).^{11,17,35} All the decellularization reagents were sterilized by membrane filtration before the treatment. Briefly, about 600–1000 EBs (150–300 μ m in diameter) were distributed into each of the 1.5-mL microcentrifuge tubes and treated with different decellularization solutions for 30 min. After the treatment, the samples were spun down at 18,000 g for 2 min, rinsed twice with phosphate-buffered saline (PBS), and incubated with 2000 unit/mL DNase I for 15–30 min. The samples were centrifuged at 18,000 g for 2 min and rinsed twice with PBS before characterization or cell reseeding.

Based on the residual DNA and preservation of the ECM structure, 1% Triton X-100 and 30 min DNase I treatment were selected and used to obtain different decellularized ECMs (DE) from monolayers, aggregates, and EBs (Table 2). The monolayers were decellularized similarly to aggregates, but centrifugation steps were omitted. For undifferentiated ESCs, DEs were obtained from day 3 cultures only because the prolonged culture induces differentiation. For EBs, DEs were obtained after culturing for 3 or 10 days.

DNA assay

The residual DNA after decellularization was measured using a DNA assay.³⁶ A DNA standard was prepared by dissolving salmon testes DNA in TEX (10 mM Tris, 1 mM EDTA, 0.1% Triton X-100 at pH 8) and a standard curve was

TABLE 1. COMPARISON OF VARIOUS DECELLULARIZATION METHODS USED IN THIS STUDY

Condition	Reagents	DNase I treatment	%DNA removal ^a	ECM preservation	Gross appearance
#1	1% Triton X-100	15 min	+	+++	+++
#2	1% Triton X-100	30 min	++	+++	+++
#3	20 mM NH ₄ OH	15 min	–	ND	+++
#4	1% SDS	15 min	+++	+++	–
#5	1% Triton X-100 + 20 mM NH ₄ OH	15 min	++	++	+++

^aFor DNA removal, the scale +++ indicates that 85–100% of DNA is removed (the remaining DNA is 0–15%); ++ indicates 70–85% removal (the remaining DNA is 15–30%); + indicates 30–70% removal (the remaining DNA is 30–70%); – indicates less than 30% removal (the remaining DNA is more than 70%). Please refer to Figure 2 for the detailed DNA expression for each condition.

ECM, extracellular matrices; ND, not determined.

TABLE 2. A LIST OF DECELLULARIZED EXTRACELLULAR MATRICES DERIVED FROM EMBRYONIC STEM CELLS GROWN IN DIFFERENT CONDITIONS

Abbreviation	ESC growth condition	Culture duration	Growth factor treatment	Reseeding
DE-M3	Undifferentiated monolayer	3 days	LIF	Yes
DE-A3	Undifferentiated aggregate	3 days	LIF	Yes
DE-SE3	Spontaneously differentiated EBs	3 days	None	Yes
DE-SE10	Spontaneously differentiated EBs	10 days	None	Yes
DE-RA3	Enhanced germ-layer differentiation	3 days	RA	No ^a
DE-RA10	Enhanced germ-layer differentiation	10 days	RA	Yes
DE-BMP-3	Lineage-specific EBs	3 days	BMP-4	No ^a
DE-BMP-10	Lineage-specific EBs	10 days	BMP-4	Yes

^aReseeding was not performed because the small difference in ECM expression compared to DE-SE. Instead, day 10 samples were reseeded for the comparison with DE-SE scaffolds.

ESC, embryonic stem cell; RA, retinoic acid; LIF, leukemia inhibitory factor; BMP-4, bone morphogenetic protein; DE, decellularized ECMs.

constructed for each assay. The decellularized samples were lysed with 0.1 mg/mL proteinase K (Fisher Scientific) at 50°C overnight. The lysate (100 μ L) were placed in triplicate into a 96-well plate and 100 μ L of Picogreen (Molecular Probes) were added to each well. The plate was incubated for 5 min in the dark, and then read on a fluorescent plate reader (FLX800; Bioinstrument, Inc.).

Scanning electron microscopy and atomic force microscopy

For scanning electron microscopy (SEM), the samples were washed with PBS, fixed in 2.5% glutaraldehyde for 30–60 min, and dehydrated in graded ethanol solutions. The samples were dried by hexamethyldisilazane evaporation, mounted, and sputter-coated with iridium. Observations were made using a Nova 400 Nano SEM (FEI) under low-vacuum conditions. For atomic force microscopy (AFM), the samples were fixed with 4% paraformaldehyde (PFA), dehydrated at 60°C, and then analyzed in contact mode using a Dimension 3000 scanning probe microscope (Digital Instruments). The roughness and topography were assessed from the acquired information.

Immunocytochemistry

For ECM expression, the monolayers, aggregates, or EBs before and after decellularization were fixed with 4% PFA.³⁴ The samples were permeabilized with 0.2–0.5% Triton X-100, blocked, and incubated with primary ECM antibodies, including rabbit polyclonal FN, laminin (LN), collagen IV (Col IV), and vitronectin (VN) (Abcam). For fluorescence staining, the samples were incubated with Alexa Fluor[®] 488 goat anti-Rabbit IgG (Molecular Probes), counterstained with 4',6-diamidino-2-phenylindole (DAPI), and visualized using a fluorescence microscope (Olympus IX70) or a Zeiss 510 confocal microscope (Leica TCS SP2 AOBS). The negative control with a secondary antibody only revealed no signal. For the detection of glycosaminoglycans (GAGs), the samples were incubated in 1% Alcian blue 8GX (Sigma) that was diluted in 3% acetic acid (pH 2.5) for 30 min, washed with water, and analyzed under a light microscope.

To quantify the ECM contents, the samples were incubated with donkey anti-Rabbit IgG conjugated with horseradish peroxidase (HRP; Rockland Immunochemicals, Inc.) after the incubation with primary antibodies. After washing,

1 mL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Thermo Scientific) was added to the samples, incubated for 5–25 min, and stopped by a solution of 0.16 M sulfuric acid. The absorbance units (AU) were measured using a microplate reader (Biorad) at a wavelength of 405 nm with background subtraction at 655 nm. The AU values were also corrected by subtracting the absorbance of negative control stained with HRP-IgG only. The measurements were normalized to the cell number for comparison.

To detect differentiation markers, the cells were incubated with the mouse or rabbit primary antibody against Nestin (Millipore), β -Tubulin III (Sigma), α -Actinin (sarcomeric; Sigma), or FOXA2 (Millipore). After washing, the cells were incubated with the corresponding secondary antibody Alexa Fluor 488 goat anti-Mouse IgG₁ (for Nestin and α -Actinin) or IgG_{2b} (for β -Tubulin III), or Alexa Fluor 488 goat anti-Rabbit IgG (for FOXA2). The localization of RAR- α was also assessed using the goat anti-Rabbit RAR- α antibody (Abcam) detected by Alexa Fluor 594[®] donkey anti-Goat IgG.

Cell reseeded and cultivation on the decellularized ECMs

To assess cell proliferation, ESCs of 0.5×10^5 per mL from the stock culture were seeded on the decellularized ECMs and cultivated for 4 days in expansion media without LIF (Fig. 1). About 30–50% of seeding efficiency was observed for 3D ECMs and 50% for 2D ECMs. During the culture period, the unattached cells died out or self-assembled into aggregates, which may become associated with ECM scaffolds during culture with an estimated additional 20–30% increase in seeding efficiency. Cell numbers were determined at days 0, 2, and 4 using a hemocytometer after trypsin/EDTA dissociation. The specific growth rates were calculated as the slope of the proliferation curve. Cell viability was determined by the incubation with 5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) solution. Afterward, the formazan crystals were hydrolyzed with dimethyl sulfoxide (Sigma) and measured at 500 nm using a microplate reader. For the test of RA signaling, soluble RA (1 μ M) or BMS 493 (1 μ M; Santa Cruz), a pan-RAR antagonist, was supplemented in the media. To assess the differentiation ability after culturing on the decellularized ECMs, ESCs were replated on 0.1% gelatin-coated 12-well plates and cultured for 7 days in the differentiation medium without any inducing growth factors. The cells were evaluated for the expression of

FIG. 1. Schematic diagram of the experimental procedure. ESCs were seeded as monolayer, aggregates, or EBs for 3 to 10 days. The monolayers, aggregates, or EBs were decellularized, and ESCs were reseeded on the ECM scaffolds for 4 days without LIF as short-term expansion. The cells were replated and grown for 7 days in the absence of inducing growth factors for differentiation. Decellularization was also performed for EBs treated with BMP-4 or RA. ESCs, embryonic stem cells; ECM, extracellular matrices; ESCs, embryonic stem cells; EBs, embryoid bodies; RA, retinoic acid. BMP-4, bone morphogenetic protein. Color images available online at www.liebertpub.com/tea

Nestin (ectoderm), β -Tubulin III (ectoderm), α -Actinin (mesoderm), and FOXA2 (endoderm).

Bromodeoxyuridine proliferation assay

The cells were incubated in the media containing 10 μ M bromodeoxyuridine (BrdU; Sigma) for 30 min to allow BrdU, a synthetic thymidine analog, incorporating into the DNA during the S-phase of cell cycle. The cells were then fixed with 70% cold ethanol, followed by a denaturation step using 2N HCl/0.5% Triton X-100 for 30 min in the dark. The samples were reduced with 1 mg/mL sodium borohydride for 5 min and incubated with mouse anti-BrdU (Invitrogen) in the blocking buffer (0.5% Tween 20/1% bovine serum albumin in PBS), followed by the incubation with Alexa Fluor 488 goat anti-Mouse IgG₁. The cells were mounted with DAPI and visualized using a Zeiss 510 confocal microscope.

Flow cytometry

For flow cytometry, 1×10^6 cells per sample were fixed with 4% PFA and washed with a staining buffer (2% FBS in

PBS). The cells were stained with DNA-intercalating dye ethidium monoazide bromide (Sigma) and permeabilized with 100% cold methanol. The samples were incubated with primary antibodies against Oct-4 (Millipore), Nestin, β -Tubulin III (Millipore), α -Actinin, or FOXA2 followed by the corresponding secondary antibody Alexa Fluor 488 goat anti-Mouse IgG₁ (for Oct-4, Nestin, α -Actinin, and β -Tubulin) or Alexa Fluor 488 goat anti-Rabbit IgG (for FOXA2). The cells were acquired with the BD FACSCanto™ II flow cytometer (Becton Dickinson) and analyzed against isotype controls using FlowJo software.

Statistical analysis

Each experiment was carried out at least twice. The average values of two or three independent experiments were presented and the results are expressed as mean $\pm$ mean absolute deviation. In each experiment, triplicate samples were used. To assess the statistical significance, ANOVA followed by Fisher's LSD *post hoc* tests or t-tests were performed. A *p*-value < 0.05 was considered statistically significant.

Results

Derivation and characterization of decellularized ECM from ESCs

Among the various decellularization methods tested, SDS condition displayed the highest efficiency of DNA removal with less than 5% DNA remaining in the acellular structure (Fig. 2A). Treatment with Triton X-100 was also effective when DNase incubation increased from 15 to 30 min (55% vs. 21%). NH_4OH treatment alone did not reduce the DNA content and its combination with Triton X-100 did not add beneficial effects. For ECM proteins, SDS treatment maintained the expression of FN, LN, and Col IV, but reduced VN slightly (Fig. 2B). Treatment with Triton X-100 plus 30 min DNase was comparable to SDS condition, whereas cotreatment with NH_4OH reduced LN and Col IV. Because the SDS-treated ECMs displayed a viscous, semifluidic appearance that was difficult to handle, Triton X-100 plus 30 min DNase treatment was chosen in the following studies (Table 1).

The ultrastructure analysis of the decellularized ECMs revealed contrasting morphologies before and after decel-

ularization, with tightly packed cells becoming indistinguishable after the treatment (Fig. 3A). Compared to the gelatin-coated surface, the roughness (R_a) increased from 3 to 48 nm for the decellularized monolayers. The surface roughness was comparable for the decellularized aggregates and EBs (246 vs. 285 nm) and both were much higher compared with the decellularized monolayers (Fig. 3B), suggesting distinct ultrastructure characteristics of the decellularized ECMs from different ESC organizations.

ECM protein expression before and after decellularization

ECM expression, including FN, LN, Col IV, VN, and GAGs, was preserved after decellularization. The ECMs from aggregates and EBs exhibited denser structures due to the compaction by centrifugation (Fig. 3C and Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea).

The specific ECM expression decreased by three- to five-folds over 3 days of monolayer culture (Fig. 4A and Supplementary Fig. S2). Similarly, for the ESC aggregates, specific ECM expression also significantly decreased (two- to fourfolds) with culture time (Fig. 4B and Supplementary Fig. S2). After decellularization, the expression of ECM proteins in the monolayers (DE-M) was unchanged (Fig. 4C), indicating that decellularization did not modify the ECM composition. For the decellularized aggregates (DE-A), the expressions of FN, LN, and VN were maintained with a slight decrease in Col IV expression (Fig. 4D). Despite the higher specific ECM expression at day 1 compared to day 3, day 1 ECMs of monolayers contained lots of empty surface; thus, the restriction of the reseeded cells to the decellularized ECMs would be more variable compared to day 3 ECMs. So the decellularized ECMs from day 3 monolayers or aggregates were used in the subsequent studies.

For the spontaneously differentiated EBs, extended culture from 3 to 10 days increased the specific ECM expression by two- to fourfolds (Fig. 5A). Following decellularization, the expressions of FN and LN were maintained, whereas the Col IV expression was reduced (Fig. 5B). The EBs were also treated with RA or BMP-4 to favor neural or mesodermal cells (Supplementary Fig. S3). For the growth factor-treated EBs, the differences in ECM expressions among the DE-SE, DE-RA, and DE-BMP were more pronounced for day 10 samples compared to day 3 samples, with the DE-RA10 and the DE-BMP-10 having a higher specific VN and Col IV expression compared to the DE-SE10 (0.8–1.1 vs. 0.2–0.3) (Fig. 5C and Supplementary Fig. S2). For FN and LN, the expression was differentially observed for the DE-SE10, DE-RA10, and DE-BMP-10 (0.4–0.6, 0.7–1.0, and 1.7–1.8, respectively). To ensure sufficient signals, day 10 samples of the DE-BMP and the DE-RA were used to further evaluate cellular responses. Taken together, these results indicated that the decellularized ECMs were dynamically remodeled during ESC expansion and differentiation.

FIG. 2. Comparison of decellularization methods for DNA removal and ECM preservation. EBs after 3-day culture were decellularized with different methods. **(A)** Expression of residual DNA. **(B)** Quantitative immunocytochemistry of different ECM proteins, including FN, LN, Col IV, and VN. * p -value < 0.05. FN, fibronectin; LN, laminin; Col IV, collagen IV; VN, vitronectin.

Effects of the decellularized ECMs on ESC proliferation

ESC proliferation on the decellularized matrices was assessed. The specific growth rate of ESCs on the DE-M3 or the DE-A3 was similar to gelatin control ($4 \times 10^{-2} \text{ h}^{-1}$) (Fig. 6A). Cells on the DE-SE3 had a slightly lower growth rate without

FIG. 3. Characterization of surface topography of decellularized ECMs. Monolayers, aggregates, and EBs were decellularized after 3 days of culture. **(A)** Scanning electron microscopy images of surface morphology of native and acellular EBs. **(B)** Topography and roughness (Ra) of acellular ECMs assessed by atomic force microscopy. **(C)** Expression of ECM proteins pre- and post-decellularization: confocal images of FN, LN, Col IV, and VN expression, and light images of GAGs from EB samples. Scale bar: 100 μm . For native Col IV, scale bar: 50 μm . GAGs, glycosaminoglycans. Color images available online at www.liebertpub.com/tea

statistical significance ($3.1 \times 10^{-2} \text{ h}^{-1}$). The ESCs seeded on the DE-RA10 had a significantly lower specific growth rate compared to the DE-BMP-10 and the DE-SE10 scaffolds (1.6 vs. $3.5 - 4 \times 10^{-2} \text{ h}^{-1}$). The cell viability determined by MTT assay was consistent with the specific growth rates (Fig. 6B). BrdU staining revealed the spatial distribution of the proliferating ESCs after reseeded. Whereas the BrdU⁺ cells evenly distributed in the DE-SE10 and the DE-BMP-10, less BrdU⁺ cells were observed in the central region of the DE-RA10 (Fig. 6C).

The Oct-4 expression of ESCs followed similar trends as observed in the specific growth rates. About 81–86% of the cells seeded on the DE-M3 and the DE-A3 were Oct-4⁺, similar to the gelatin-coated surface (Fig. 6D). The DE-SE3 supported a lower percentage of Oct-4⁺ cells, but the difference was not statistically significant compared to gelatin control. Whereas the DE-SE10 and the DE-BMP-10 maintained 84–91% Oct-4⁺ cells, Oct-4 expression of cells on the DE-RA10 was only 38% (Fig. 6D, E). A similar trend was observed for SSEA-1 (data not shown).

Effects of the decellularized ECMs on ESC differentiation

Cells seeded on various decellularized ECM scaffolds were assessed for the expression of differentiated markers, including Nestin, α -Actinin, β -Tubulin III, and FOXA2 (Fig. 7 and Supplementary Fig. S4). Due to the absence of inducing growth factors, the lineage-specific differentiation for cells on various ECM scaffolds was mainly attributed to the signals from the ECMs. The expression of α -Actinin in this study represented mainly skeletal muscle cells rather than cardiac muscle cells due to the low frequency of beating cells. For the cells grown on the day 10 DEs (DE-SE10, DE-RA10, and DE-BMP-10), higher expressions of FOXA2 (28–52% vs. 14–16%) and α -Actinin (15–32% vs. 4–5%) were observed compared to the cells grown on the day 3 DEs (DE-A3 and DE-SE3). Specifically, after differentiation, the ESCs seeded on the DE-RA10 had the highest expression of Nestin (46%), α -Actinin (32%), β -Tubulin III (36%), and FOXA2 (52%) compared to

FIG. 4. Quantitative analysis of ECM composition pre- and post-decellularization of undifferentiated ESCs. **(A)** ECM expression for the ESC monolayer cultures at days 1 and 3. **(B)** ECM expression for the ESC aggregates at days 1 and 3. **(C)** Comparison of ECM expression pre- and postdecellularization after 3 days of monolayer culture (DE-M3). **(D)** Comparison of ECM expression pre- and postdecellularization after 3 days of aggregate culture (DE-A3). * p -value < 0.05. DE, decellularized ECMs.

the cells seeded on the other ECM scaffolds (Fig. 7C, F, I, L). For the DE-A3 and the DE-SE3, which had low expression of the assessed markers, only about 5–10% of cells expressed Oct-4 (data not shown) indicating the loss of pluripotency. A more detailed analysis with markers to cover the development of lineage specification is warranted in future studies.

Role of RA–RAR interactions

To investigate the role of RA signaling in the ECM scaffolds, RAR- α expression by ESCs cultured on the DE-RA10 was evaluated. First, the translocation of RAR- α from the cytoplasm to the cell nucleus was confirmed for ESCs

FIG. 5. Quantitative analysis of ECM compositions pre- and post-decellularization of EBs. **(A)** ECM expression in the EBs at days 3 and 10. **(B)** ECM expression pre- and post-decellularization of day 3 and 10 EBs (DE-SE3 and DE-SE10, respectively). **(C)** Comparison of decellularized ECM expression for EBs treated by BMP-4 (DE-BMP) or RA (DE-RA) with spontaneously differentiated EBs (DE-SE). * p -value < 0.05. Color images available online at www.liebertpub.com/tea

FIG. 6. Proliferation and Oct-4 expression of the reseeded ESCs on the decellularized ECM scaffolds. **(A)** Specific growth rate of ESCs reseeded on control (gelatin) and different decellularized ECM scaffolds. **(B)** Representative cell viability of reseeded ESCs determined by MTT assay. **(C)** Representative confocal images of BrdU expression in reseeded ESCs. Scale bar: 50 μ m. **(D)** Oct-4 expression of ESCs reseeded on control (gelatin) and different decellularized ECM scaffolds. **(E)** Representative flow cytometry histograms of Oct-4 expression of reseeded ESCs. * p -value < 0.05. Color images available online at www.liebertpub.com/tea

exposed to exogenous RA (Fig. 8A). Then, ESCs grown in DE-RA10 were assessed and the localizations of RAR- α both in the cytoplasm and cell nucleus were observed (Fig. 8B). Because no soluble RA was added in the DE-RA10 culture, the mixed pattern of RAR- α expression suggested active RA signaling presumably due to the presence of the decellularized ECMs.

Addition of a pan-RAR antagonist, BMS 493, in the DE-RA10 culture reduced ESC proliferation but not Oct-4 expression (Fig. 8C). Upon differentiation, blocking RA-RAR signaling significantly reduced the expression of α -Actinin (3% vs. 18%) and FOXA2 (24% vs. 40%) compared to the untreated group (Fig. 8D). Conversely, Nestin expression was increased in the treated group with similar β -tubulin III expression. These results indicated that the effect of the DE-RA10 on the endodermal and mesodermal differentiation of the reseeded ESCs may be a consequence of the active RA signaling.

Discussion

Different from ECMs derived from somatic cells, the decellularized ECMs from PSCs recapitulate the developmentally relevant extracellular microenvironments and have a unique signaling capacity for directing cell fate.^{6,10} The results of the present study demonstrated the organi-

zation- and lineage-specific characteristics of ESC-derived ECMs and their differential signaling capacity in regulating ESC fate.

Characterization of ESC-derived ECM scaffolds

Formation of EBs is an important step in ESC differentiation *in vitro* and the derivation of decellularized matrices from spontaneous EBs has been demonstrated in several studies.^{11,12} However, ESCs are also able to form 3D aggregates while maintaining the undifferentiated phenotype, which facilitates their expansion in suspension.²⁵ The differences of ESC fate in the EB versus the 3D aggregate may be reflected in their ECM microenvironment, but the derivation and properties of the ECMs from the 3D aggregates have not been reported. In the present study, Triton X-100 followed by DNase was effective in maintaining the 3D structures of ESC aggregates similar to those derived from EBs. Despite a slight decrease in Col IV, the main components of ECM proteins in the 3D ESC aggregates were preserved.

Whereas the decellularized matrices derived from EBs and ESC aggregates have similar macroscopic structures, they represent divergent developmental stages and thus may have different ECM compositions and signaling capacities. Indeed, quantitative assessment of the decellularized

FIG. 7. Differentiated marker expression of ESCs grown on decellularized ECM scaffolds. Representative fluorescence images and flow cytometry histograms of ectoderm markers β -Tubulin III (A, B) and Nestin (D, E), mesoderm marker α -Actinin (G, H), and endoderm marker FOXA2 (J, K) for the cells seeded on the DE-A3 and the DE-RA10. Scale bar: 50 μ m. Percentage of positive cells for β -Tubulin III (C), Nestin (F), α -Actinin (I), and FOXA2 (L) in different ECM scaffolds. * p -value < 0.05. Color images available online at www.liebertpub.com/tea

FIG. 8. Role of RA-RAR interactions in ESCs cultured on the decellularized ECM scaffolds. **(A)** Localization of RAR- α in ESC monolayers with or without RA treatment (Scale bar: 50 μm). (i) RAR- α expression without RA treatment; (ii) RAR- α expression with RA treatment; (iii) DAPI image corresponding to (i); (iv) DAPI image corresponding to (ii). **(B)** Confocal images of RAR- α in reseeded ESCs on the DE-RA10 (White scale bar: 100 μm ; yellow scale bar: 50 μm). (i) RAR- α expression; (ii) Enlarged image of region a in (i); (iii) DAPI image corresponding to (i); (iv) Enlarged image of region b in (i). Green arrow: RAR- α expression in the cell nucleus; white arrow: RAR- α expression in the cytoplasm. **(C)** Specific growth rate and Oct-4 expression for cells grown in the DE-RA10 with or without BMS 493 treatment. **(D)** Expression of Nestin, α -Actinin, β -Tubulin III, and FOXA2 for the differentiated cells from ESCs seeded on the DE-RA10 with or without BMS 493 treatment. * p -value < 0.05. RAR, RA receptor. Color images available online at www.liebertpub.com/tea

matrices revealed time- and organization-dependent ECM compositions. The specific expression of ECM proteins increased with time during EB development, in contrast to the decreasing trend for the undifferentiated ESC aggregates and the monolayer cultures. The reduced specific ECM expression in the monolayers and undifferentiated aggregates is thought to be a combined result of accelerated cell proliferation accompanied by limited endogenous ECM secretion by undifferentiated ESCs. Although the endogenous ECM proteins such as FN and LN are required for ESC self-renewal, such ECM proteins are limited in quantity in the undifferentiated ESCs to prevent the loss of self-renewal ability.⁷ In contrast, it is well established that EB formation is closely coupled with ECM secretion, notably the formation of a basement membrane rich in LN and Col IV.^{10,23,37,38} The ECMs in the EBs not only play important roles in directing ESC differentiation, but also form supporting structures that sustain EB development.²³ To further probe whether the decellularized ECMs maintain their lineage-specific properties, the EBs were treated with BMP-4 or RA to induce mesodermal or ectodermal differentiation.^{31,32} The significantly increased ECM expression and the differential expression patterns for both groups suggest the close association of ECM properties with lineage specification of ESCs. For example, RA-induced differentiation of pluripotent cells has been shown to alter the cellular proteoglycan composition.³⁹ Together, these results suggest

that the decellularized ECMs recapitulate the cellular microenvironments associated with ESC development *in vitro* and that the characteristics of ECMs derived from ESC cultures depend on both spatial organization and lineage specification.

ESC proliferation on the decellularized ECM scaffolds

The decellularized ECMs, except the DE-RA10, supported short-term ESC proliferation and Oct-4 expression at a level comparable to that of the gelatin-coated surface. It is interesting to note that no significant difference in cell proliferation was observed between the DE-A and the DE-SE groups, two types of matrices with divergent ECM compositions. This may be explained by the fact that multiple components in the decellularized ECMs influence ESC proliferation. Compared to the purified ECM proteins, the decellularized ECMs contain a mixture of ECM proteins and provide a multitude of binding sites for both cell adhesions and growth factor sequestrations.^{5,6,40} Studies have shown that the combinatorial ECMs supported human ESC expansion more robustly compared to individual ECM proteins, probably due to the increased cell binding and integrin signaling.^{41,42} A limitation of the present study, however, is the short culture duration after reseeded, which may not be indicative of the long-term effects of cellular microenvironments on ESC fate.

The significant reduction in ESC proliferation and Oct-4 expression in the DE-RA10 scaffold indicates its unique ECM microenvironment. Obtained over the same culture period, the DE-SE10, the DE-BMP-10, and the DE-RA10 groups represent the microenvironments of three distinct developmental paths and have different patterns of ECM expressions. However, the differences in ESC proliferation and Oct-4 expression in the DE-RA10 cannot be explained by the differences in ECM composition alone because cell proliferation was not correlated with ECM expressions among these three matrices. Instead, regulatory molecules such as RA retained in the DE-RA10 are thought to play a role. Although unstable and susceptible to oxidative damage in aqueous solutions, RA is intrinsically hydrophobic and its binding to proteins such as serum albumin could significantly prolong its half-life and biological function.^{43,44} Although no soluble RA existed in the DE-RA10 culture, RA may form RA-albumin complexes and are subsequently retained in the ECMs, altering the endogenous retinoid signaling in ESCs grown in DE-RA10.^{44–46} Retinoid signaling is known to reduce ESC growth by increasing p27 accumulation and lowering the Oct-4 level by augmenting the Oct-4 chromatin/transcription factor complex.⁴⁷ Whereas further experiments are required to elucidate RA's bioactivity in the ECMs, the results support the notion that ECMs have the capacity to regulate stem cell fate through their interactions with the regulatory macromolecules.^{4,5}

ESC differentiation on the decellularized ECM scaffolds

Corresponding to the reduction in cell proliferation, the DE-RA10 exhibited the most dramatic effects on ESC differentiation among all scaffolds with increased expression of four differentiated markers. RA is an important signaling molecule in embryonic development, and has been commonly used as an inducer to initiate ESC differentiation into ectoderm, mesoderm, or endoderm through the temporal- and concentration-dependent retinoid signaling.^{32,45,48–50} Besides neural lineage, the increased expression of α -Actinin and FOXA2 was also observed, which was consistent with the effect of RA on the upregulation of markers in three-germ layers.⁴⁵ Although the exact mechanism of RA signaling in ESCs grown in DE-RA10 requires further study, the coexpression of RAR- α in the nucleus and cytoplasm of cells grown in the DE-RA10 suggested the presence of active RA signaling in the absence of soluble RA.⁵¹ The reduced α -Actinin and FOXA2 expression by blocking RA–RAR interactions using BMS 493 in the DE-RA10 provided further evidence that retinoid signaling played an active role in directing ESC differentiation.⁴⁸ The increased Nestin expression may be due to the RAR independent differentiation or the expression from nonectodermal cells.⁵² Together, these results reveal the reciprocal interactions between the ECMs and the stem cells, and the capacity of such a microenvironment to regulate cell fate in the absence of the nonphysiological level of soluble RA.⁵⁰

The effects of decellularized ECMs derived at different developmental stages were also observed. Compared to the day 3 DEs, including both DE-A3 and DE-SE3, cells grown in the day 10 DEs expressed a higher level of α -Actinin and FOXA2. Due to the progression in differentiation, the day 10

DEs may contain cell-secreted signals (e.g., *Cerberus*) that are more inductive for differentiation compared to the day 3 DEs.^{10,28} In addition, the higher content of ECM proteins in day 10 DEs might also contribute to the increased α -Actinin and FOXA2 expression, because the incorporation of biomaterials in ESC aggregates has been shown to promote mesodermal and endodermal differentiation by affecting cell–cell interactions.⁵³

Conclusion

The ECMs derived from ESC cultures maintained their signaling capacities that are specific to cellular organizations, lineage specifications, and developmental stages. Such ECMs regulate ESC fate by providing cell adhesion sites as well as by the interactions with regulatory macromolecules. The ESC-derived ECMs have the potential to direct lineage-specific ESC responses and influence germ-layer differentiation for *in vivo* cell and/or matrix delivery as well as *in vitro* cell expansion and differentiation.

Acknowledgments

The authors would like to thank Dr. Eric Lochner of FSU Condensed Matter and Materials Physics (CMMP) facilities and Dr. Brett Kirkland from the FSU Chemical and Biomedical Engineering department for their help in AFM analysis. The authors also thank Ms. Ruth Didier of the FSU Department of Biomedical Sciences for her help in the confocal microscopy and flow cytometry analysis. This work is supported by the FSU start up fund. Funding support, in part, from the American Heart Association Grant-in-Aid (10GRNT3860040) to T.M. is also acknowledged.

Disclosure Statement

No competing financial interests exist.

References

1. Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., and Jones, J.M. Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145, 1998.
2. Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861, 2007.
3. Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., Slukvin, I.I., and Thomson, J.A. Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917, 2007.
4. Rozario, T., and DeSimone, D.W. The extracellular matrix in development and morphogenesis: a dynamic view. *Dev Biol* **341**, 126, 2010.
5. Hynes, R.O. The extracellular matrix: not just pretty fibrils. *Science* **326**, 1216, 2009.
6. Hughes, C., Radan, L., Chang, W.Y., Stanford, W.L., Betts, D.H., Postovit, L.M., and Lajoie, G.A. Mass spectrometry-based proteomics analysis of the matrix microenvironment in pluripotent stem cell culture. *Mol Cell Proteomics* **11**, 1924, 2012.
7. Hunt, G.C., Singh, P., and Schwarzbauer, J.E. Endogenous production of fibronectin is required for self-renewal of

- cultured mouse embryonic stem cells. *Exp Cell Res* **318**, 1820, 2012.
8. Chen, S.S., Fitzgerald, W., Zimmerberg, J., Kleinman, H.K., and Margolis, L. Cell-cell and cell-extracellular matrix interactions regulate embryonic stem cell differentiation. *Stem Cells* **25**, 553, 2007.
 9. Przybyla, L., and Voldman, J. Probing embryonic stem cell autocrine and paracrine signaling using microfluidics. *Annu Rev Anal Chem (Palo Alto Calif)* **5**, 293, 2012.
 10. Nair, R., Ngangan, A.V., Kemp, M.L., and McDevitt, T.C. Gene expression signatures of extracellular matrix and growth factors during embryonic stem cell differentiation. *PLoS One* **7**, e42580, 2012.
 11. Nair, R., Shukla, S., and McDevitt, T.C. Acellular matrices derived from differentiating embryonic stem cells. *J Biomed Mater Res A* **87**, 1075, 2008.
 12. Ngangan, A.V., and McDevitt, T.C. Acellularization of embryoid bodies via physical disruption methods. *Biomaterials* **30**, 1143, 2009.
 13. Choi, J.S., Yang, H.J., Kim, B.S., Kim, J.D., Kim, J.Y., Yoo, B., Park, K., Lee, H.Y., and Cho, Y.W. Human extracellular matrix (ECM) powders for injectable cell delivery and adipose tissue engineering. *J Control Release* **139**, 2, 2009.
 14. Turner, A.E., and Flynn, L.E. Design and characterization of tissue-specific extracellular matrix-derived microcarriers. *Tissue Eng Part C Methods* **18**, 186, 2012.
 15. Turner, A.E., Yu, C., Bianco, J., Watkins, J.F., and Flynn, L.E. The performance of decellularized adipose tissue microcarriers as an inductive substrate for human adipose-derived stem cells. *Biomaterials* **33**, 4490, 2012.
 16. Lu, H., Hoshiba, T., Kawazoe, N., and Chen, G. Autologous extracellular matrix scaffolds for tissue engineering. *Biomaterials* **32**, 2489, 2011.
 17. Kim, J., and Ma, T. Autocrine fibroblast growth factor 2-mediated interactions between human mesenchymal stem cells and the extracellular matrix under varying oxygen tension. *J Cell Biochem* **114**, 716, 2013.
 18. Sart, S., Errachid, A., Schneider, Y.J., and Agathos, S.N. Modulation of mesenchymal stem cell actin organization on conventional microcarriers for proliferation and differentiation in stirred bioreactors. *J Tissue Eng Regen Med* **7**, 537, 2013.
 19. Lock, L.T., and Tzanakakis, E.S. Expansion and differentiation of human embryonic stem cells to endoderm progeny in a microcarrier stirred-suspension culture. *Tissue Eng Part A* **15**, 2051, 2009.
 20. Postovit, L.M., Margaryan, N.V., Seftor, E.A., Kirschmann, D.A., Lipavsky, A., Wheaton, W.W., Abbott, D.E., Seftor, R.E., and Hendrix, M.J. Human embryonic stem cell microenvironment suppresses the tumorigenic phenotype of aggressive cancer cells. *Proc Natl Acad Sci U S A* **105**, 4329, 2008.
 21. Postovit, L.M., Seftor, E.A., Seftor, R.E., and Hendrix, M.J. A three-dimensional model to study the epigenetic effects induced by the microenvironment of human embryonic stem cells. *Stem Cells* **24**, 501, 2006.
 22. Fok, E.Y., and Zandstra, P.W. Shear-controlled single-step mouse embryonic stem cell expansion and embryoid body-based differentiation. *Stem Cells* **23**, 1333, 2005.
 23. Bratt-Leal, A.M., Carpenedo, R.L., and McDevitt, T.C. Engineering the embryoid body microenvironment to direct embryonic stem cell differentiation. *Biotechnol Prog* **25**, 43, 2009.
 24. Xu, C., Inokuma, M.S., Denham, J., Golds, K., Kundu, P., Gold, J.D., and Carpenter, M.K. Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol* **19**, 971, 2001.
 25. Zweigerdt, R., Olmer, R., Singh, H., Haverich, A., and Martin, U. Scalable expansion of human pluripotent stem cells in suspension culture. *Nat Protoc* **6**, 689, 2011.
 26. Azarin, S.M., Lian, X., Larson, E.A., Popelka, H.M., de Pablo, J.J., and Palecek, S.P. Modulation of Wnt/beta-catenin signaling in human embryonic stem cells using a 3-D microwell array. *Biomaterials* **33**, 2041, 2012.
 27. Kraushaar, D.C., Rai, S., Condac, E., Nairn, A., Zhang, S., Yamaguchi, Y., Moremen, K., Dalton, S., and Wang, L. Heparan sulfate facilitates FGF and BMP signaling to drive mesoderm differentiation of mouse embryonic stem cells. *J Biol Chem* **287**, 22691, 2012.
 28. Farina, A., D'Aniello, C., Severino, V., Hochstrasser, D.F., Parente, A., Minchiotti, G., and Chambery, A. Temporal proteomic profiling of embryonic stem cell secretome during cardiac and neural differentiation. *Proteomics* **11**, 3972, 2011.
 29. Sachlos, E., and Auguste, D.T. Embryoid body morphology influences diffusive transport of inductive biochemicals: a strategy for stem cell differentiation. *Biomaterials* **29**, 4471, 2008.
 30. Ulloa, L., and Tabibzadeh, S. Lefty inhibits receptor-regulated Smad phosphorylation induced by the activated transforming growth factor-beta receptor. *J Biol Chem* **276**, 21397, 2001.
 31. Zhang, P., Li, J., Tan, Z., Wang, C., Liu, T., Chen, L., Yong, J., Jiang, W., Sun, X., Du, L., Ding, M., and Deng, H. Short-term BMP-4 treatment initiates mesoderm induction in human embryonic stem cells. *Blood* **111**, 1933, 2008.
 32. Okada, Y., Shimazaki, T., Sobue, G., and Okano, H. Retinoic-acid-concentration-dependent acquisition of neural cell identity during *in vitro* differentiation of mouse embryonic stem cells. *Dev Biol* **275**, 124, 2004.
 33. Li, Y., Gautam, A., Yang, J., Qiu, L., Melkounian, Z., Weber, J., Telukuntla, L., Srivastava, R., Whiteley, E.M., and Brandenberger, R. Differentiation of oligodendrocyte progenitor cells from human embryonic stem cells on vitronectin-derived synthetic peptide acrylate surface. *Stem Cells Dev* **22**, 1497, 2013.
 34. Sart, S., Ma, T., and Li, Y. Cryopreservation of pluripotent stem cell aggregates in defined protein-free formulation. *Biotechnol Prog* **29**, 143, 2013.
 35. Lu, H., Hoshiba, T., Kawazoe, N., and Chen, G. Comparison of decellularization techniques for preparation of extracellular matrix scaffolds derived from three-dimensional cell culture. *J Biomed Mater Res A* **100**, 2507, 2012.
 36. Grayson, W.L., Ma, T., and Bunnell, B. Human mesenchymal stem cells tissue development in 3D PET matrices. *Biotechnol Prog* **20**, 905, 2004.
 37. Li, X., Chen, Y., Scheele, S., Arman, E., Haffner-Krausz, R., Ekblom, P., and Lonai, P. Fibroblast growth factor signaling and basement membrane assembly are connected during epithelial morphogenesis of the embryoid body. *J Cell Biol* **153**, 811, 2001.
 38. Matsuoka, Y., Kubota, H., Adachi, E., Nagai, N., Marutani, T., Hosokawa, N., and Nagata, K. Insufficient folding of type IV collagen and formation of abnormal basement membrane-like structure in embryoid bodies derived from Hsp47-null embryonic stem cells. *Mol Biol Cell* **15**, 4467, 2004.
 39. Gasimli, L., Stansfield, H.E., Nairn, A.V., Liu, H., Paluh, J.L., Yang, B., Dordick, J.S., Moremen, K.W., and Linhardt, R.J.

- Structural remodeling of proteoglycans upon retinoic acid-induced differentiation of NCCIT cells. *Glycoconj J* **30**, 497, 2013.
40. Badylak, S.F., Freytes, D.O., and Gilbert, T.W. Extracellular matrix as a biological scaffold material: structure and function. *Acta Biomater* **5**, 1, 2009.
 41. Brafman, D.A., Shah, K.D., Fellner, T., Chien, S., and Willert, K. Defining long-term maintenance conditions of human embryonic stem cells with arrayed cellular microenvironment technology. *Stem Cells Dev* **18**, 1141, 2009.
 42. Lee, S.T., Yun, J.I., Jo, Y.S., Mochizuki, M., van der Vlies, A.J., Kontos, S., Ihm, J.E., Lim, J.M., and Hubbell, J.A. Engineering integrin signaling for promoting embryonic stem cell self-renewal in a precisely defined niche. *Biomaterials* **31**, 1219, 2010.
 43. Sharow, K.A., Temkin, B., and Asson-Batres, M.A. Retinoic acid stability in stem cell cultures. *Int J Dev Biol* **56**, 273, 2012.
 44. Belatik, A., Hotchandani, S., Bariyanga, J., and Tajmir-Riahi, H.A. Binding sites of retinol and retinoic acid with serum albumins. *Eur J Med Chem* **48**, 114, 2012.
 45. Simandi, Z., Balint, B.L., Poliska, S., Ruhl, R., and Nagy, L. Activation of retinoic acid receptor signaling coordinates lineage commitment of spontaneously differentiating mouse embryonic stem cells in embryoid bodies. *FEBS Lett* **584**, 3123, 2010.
 46. Nederman, T., Carlsson, J., and Malmqvist, M. Penetration of substances into tumor tissue—a methodological study on cellular spheroids. *In Vitro* **17**, 290, 1981.
 47. Persaud, S.D., Lin, Y.W., Wu, C.Y., Kagechika, H., and Wei, L.N. Cellular retinoic acid binding protein I mediates rapid non-canonical activation of ERK1/2 by all-trans retinoic acid. *Cell Signal* **25**, 19, 2012.
 48. Kennedy, K.A., Porter, T., Mehta, V., Ryan, S.D., Price, F., Peshdary, V., Karamboulas, C., Savage, J., Drysdale, T.A., Li, S.C., Bennett, S.A., and Skerjanc, I.S. Retinoic acid enhances skeletal muscle progenitor formation and bypasses inhibition by bone morphogenetic protein 4 but not dominant negative beta-catenin. *BMC Biol* **7**, 67, 2009.
 49. Micallef, S.J., Janes, M.E., Knezevic, K., Davis, R.P., Elefanty, A.G., and Stanley, E.G. Retinoic acid induces Pdx1-positive endoderm in differentiating mouse embryonic stem cells. *Diabetes* **54**, 301, 2005.
 50. Carpenedo, R.L., Bratt-Leal, A.M., Marklein, R.A., Seaman, S.A., Bowen, N.J., McDonald, J.F., and McDevitt, T.C. Homogeneous and organized differentiation within embryoid bodies induced by microsphere-mediated delivery of small molecules. *Biomaterials* **30**, 2507, 2009.
 51. Chen, N., and Napoli, J.L. All-trans-retinoic acid stimulates translation and induces spine formation in hippocampal neurons through a membrane-associated RARalpha. *FASEB J* **22**, 236, 2008.
 52. Wiese, C., Rolletschek, A., Kania, G., Blyszczuk, P., Tarasov, K.V., Tarasova, Y., Wersto, R.P., Boheler, K.R., and Wobus, A.M. Nestin expression—a property of multi-lineage progenitor cells? *Cell Mol Life Sci* **61**, 2510, 2004.
 53. Bratt-Leal, A.M., Carpenedo, R.L., Ungrin, M.D., Zandstra, P.W., and McDevitt, T.C. Incorporation of biomaterials in multicellular aggregates modulates pluripotent stem cell differentiation. *Biomaterials* **32**, 48, 2011.

Address correspondence to:

Yan Li, PhD

Department of Chemical and Biomedical Engineering
FAMU-FSU College of Engineering
Florida State University
2525 Pottsdamer Street
Tallahassee, FL 32310

E-mail: yli@eng.fsu.edu

Teng Ma, PhD

Department of Chemical and Biomedical Engineering
FAMU-FSU College of Engineering
Florida State University
2525 Pottsdamer Street
Tallahassee, FL 32310

E-mail: teng@eng.fsu.edu

Received: November 20, 2012

Accepted: July 12, 2013

Online Publication Date: August 15, 2013