Heterogeneous Differentiation of Human Mesenchymal Stem Cells in Response to Extended Culture in Extracellular Matrices

Jose A. Santiago, M.S.,¹ Ryan Pogemiller, M.S.,² and Brenda M. Ogle, Ph.D.^{1,2}

Extracellular matrix proteins (ECMs) guide differentiation of adult stem cells, but the temporal distribution of differentiation (i.e., heterogeneity) in a given population has not been investigated. We tested the effect of individual ECM proteins on lineage commitment of human bone marrow–derived mesenchymal stem cells (MSCs) over time. We exposed stem cell populations to ECM proteins representing the primary tissue structures of the body (i.e., collagens type I, III, IV; laminin; and fibronectin) and determined the lineage commitment of the stem cells at 1, 7, and 14 days. We found that collagens that can participate in the formation of fibrils guide differentiation of cardiomyocytes, adipocytes, and osteoblasts. ECMs of the basement membrane initiate differentiation of cardiomyocytes and osteoblasts but not adipocytes, and small facilitator ECMs (e.g., fibronectin) do not significantly affect stem cell differentiation. Differentiation was ECM-dependent because culture on tissue culture polystyrene, with consistent cell morphology, proliferation, and death, initiated differentiation of osteoblasts only. Thus, we show that ECMs independently trigger differentiation of human adult MSCs and that differentiation in this context can be guided down multiple lineages using the same ECM stimulus. This work highlights the importance of more clearly defining progenitor populations, especially those cultured in the presence of ECMs before transplantation.

Introduction

TISSUE ENGINEERING IS based on the premise that engraftment and mechanical stability of many cell-based therapeutics (especially bone, cartilage, and cardiovascular) are enhanced when combined with biocompatible constructs before implantation. One particularly promising group of carrier constructs incorporate extracellular matrices (ECMs).^{1–4} These proteins are frequently used because they provide optimal cell structural support and mimic *in vivo* native tissue architecture. In addition, ECM proteins harbor integrin ligands that when bound promote adhesion and induce cellular signaling, including signals that drive cell fate processes.^{5–7}

The effect of cell–ECM interactions on cell fate processes has been thoroughly described for mature cells⁸ but has only recently been examined for stem cells.⁹ Exploration of these interactions is crucial for directing or avoiding differentiation. For example, embryonic stem cells seeded on collagen type I retain the ability to self-renew,¹⁰ whereas adult mesenchymal stem cells (MSCs) seeded on collagen type I are prompted to undergo osteogenic differentiation.¹¹ Other studies show that MSCs exposed to hydrogels composed of

collagens type I and II undergo chondrogenic differentiation.¹² Collagen type IV and laminin have been found to promote differentiation of neuronal progenitor cells into neurons while inhibiting glial cell differentiation.^{13,14} Collagen type VI constructs induce myofibroblast differentiation, whereas collagens type I and III enhance proliferation of cardiac fibroblasts.¹⁵ In addition to isolated ECM proteins, ECM protein combinations^{16,17} and ECM-derived peptides (e.g., arginine-glycine-aspartic acid and tyrosine-isoleucine-glycine-serine-arginine¹⁸) can guide fate processes of stem cell populations.

Studies of stem cell–ECM interactions have been conducted in a tissue-specific manner. That is, groups interested in bone regeneration study the effect of ECM proteins on osteoblast differentiation, groups interested in cardiac regeneration study the effect of ECM proteins on cardiomyocyte differentiation, and so on. This is a limiting approach because it is well documented that embryonic and adult stem cell populations comprise heterogeneous populations of cells at various stages in a differentiation program.^{19,20} From a tissue engineering perspective, it is therefore crucial to know not only whether exposure to certain ECM constructs will induce differentiation of the cell type(s) of interest but also

¹Material Science Program and ²Department of Biomedical Engineering, University of Wisconsin at Madison, Madison, Wisconsin.

whether that same construct can induce differentiation of other cell types. This point is especially salient in light of a recent study wherein adult bone marrow-derived MSCs were transplanted into the heart with the expectation that the cardiac microenvironment would stimulate differentiation of cardiac cells (i.e., cardiomyocytes, smooth muscle, and endothelium). Several weeks later, functional donor cardiomyocytes were not detected but rather encapsulated structures containing calcifications and ossifications reminiscent of bone formation.²¹

Here we sought to investigate whether and to what extent long-term culture on a broad range of exogenous ECMs affects the differentiation potential of human adult MSCs. Toward this end, we compared the morphology, proliferation, and differentiation of MSCs after extended culture on collagens I, III, and IV; fibronectin; and laminin. We studied the differentiation of MSCs toward somatic cells of a range of tissue types of the mesenchyme (bone, muscle, and adipose tissue). We found that extended culture on all individual ECM proteins facilitated differentiation of osteoblasts. In contrast, initiation of differentiation of adipocytes and cardiomyocytes was limited to collagens type I and III and laminin. Thus, differentiation of mesenchymal cell types was guided in a heterogeneous fashion, with most cultures containing cells of multiple types after 14 days of ECM exposure.

Methods

Cell culture

MSCs were isolated from human bone marrow aspirates of two healthy individuals (35-year-old man, isolate A and 54-year-old woman, isolate C) and from one commercial source (23-year-old woman, isolate B, Cambrex/Lonza, Walkersville, MD). Aspirates were screened to ensure the presence of CD73, CD90, and CD105 and the absence of CD34 and CD45 (see below, Flow cytometry) but were not purified based on level of CD expression, nor were clonal populations procured. Aspirates were obtained with the approval of the University of Wisconsin Institutional Review Board. Mononuclear cells were isolated from aspirates using Ficoll Paque gradient (Sigma, St. Louis, MO) and transferred to six-well plates at a density of 2600 cells/cm² in Mesencult Complete Medium (StemCell Technologies, Vancouver, Canada). Medium was changed every 2 days for 10 days until MSCs were approximately 60% confluent and then passaged. At passages 6 to 8, MSCs were seeded in 24 multiwell plates coated with collagen types I, III, and IV; laminin; or fibronectin (collagen type I, bovine dermis, 5 µg/cm², BD Biosciences, San Jose, CA; collagen type III, bovine placental villi, 1 µg/cm², Southern Biotech, Birmingham, AL; collagen type IV, human placenta, 10 µg/cm², Sigma Aldrich, St. Louis, MO; laminin, murine sarcoma, 1 µg/cm², Sigma Aldrich; fibronectin, bovine plasma, 5 µg/cm², Sigma Aldrich, St. Louis, MO) at a cell density of 250 cells/cm². Collagens were maintained at acidic pH during coating of the wells and thus were maintained primarily in tropocollagen (and not fibril or network) structures upon adherence to the wells. In particular, collagen type I was diluted with 0.01M hydrochloric acid and collagens type III and IV were diluted with 0.05M acetic acid. Fibronectin and laminin were diluted in 1X phosphate buffered saline (PBS, pH 7.2). The concentration of ECM components

reflects the amount needed to ensure complete coating of polystyrene well plates.

Flow cytometry

Before co-culture with ECM proteins, MSCs were probed for markers of multipotency using flow cytometry. Cell suspensions were washed two times with cell suspension buffer containing 1X PBS, 0.2% heparin (v/v), and 0.3% bovine serum albumin (BSA) (w/v) and then incubated for 20 min on ice with the following antibodies (BD Biosciences except CD73): anti-human CD45 fluorescein isothiocyanate (FITC) (HI30), anti-human CD34 phycoerythrin (563), anti-human CD90 allophycocyanin (5E10), anti-human CD105 FITC (46/SH2-B), and anti-human CD73 FITC (V-20, Santa Cruz Biotechnology, Santa Cruz, CA). Cells were washed again after incubation with cell suspension buffer and centrifuged. Fluorescence intensity was assessed using a flow cytometer (FACSCalibur, BD Biosciences; 488nm and 633nm lasers). Debris and dead cells were excluded using forward scatter. Unstained MSCs and NIH-3T3 cells stained with the above antibodies served as negative controls

Micro-bicinchoninic acid protein assay

The amount of protein deposited per unit surface area was determined using the micro-bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL). To each ECM-coated well containing 100 µL of 1X PBS an equal portion of working reagent (micro-BCA reagent A, B, and C in a volume ratio of 25:24:1) was added. The mixture was incubated at 37°C for 2 h and then cooled to room temperature. Absorbance at 562 nm was measured using a fluorescence plate reader (Synergy HT, Biotek Instruments, Winooski, VT). A calibration curve was generated by measuring the absorbance in identical wells containing known concentrations of BSA (0–200 µg/mL). Units were converted from mass/volume to mass/area based on the known surface area of each well. Samples containing diluent only served as negative controls.

Scanning electron microscopy

Glass coverslips were coated with ECM proteins at the concentrations designated above. Samples were fixed using 2% glutaraldehyde in 0.1M sodium-cacodylate buffer for 7 h. After fixation, the samples were washed in distilled water for 10 min and dehydrated using a series of ethanol washes. The samples were immersed in 30%, 50%, 70%, 80%, 90%, 95%, and 100% ethanol for 4 min each. After the ethanol series, the samples were critical point dried (Samdri-780A, Tousimis Research Corp., Rockville, MD). Samples were sputter coated (IBS/TM200S ion beam sputterer, VCR Group Inc., San Clemente, CA) with gold for 15 min to achieve a 2.5-nm coating. Scanning electron microscopy (SEM) imaging was accomplished using a Hitachi, Schaumburg, IL, S-900 scanning electron microscope. Coverslips without ECM coatings served as negative controls.

Proliferation assay

Cell proliferation was studied 1, 7, and 14 days after seeding on coated plates using the carboxy-fluorescein diacetate (CFDA) proliferation assay kit (Vybrant CFDA SE cell

tracer kit, Invitrogen, Carlsbad, CA). CFDA succinimidyl ester (SE) irreversibly couples cell surface proteins, and labeling is evenly distributed between the daughter cells. MSCs were labeled in suspension with the CFDA SE probe according to the manufacturer's protocol. Labeled cells were seeded at a density of 250 cells/cm². The fluorescent CFDA SE product was analyzed at 1, 7, and 14 days after exposure to ECM using a flow cytometer (FACSCalibur, BD Biosciences; 488nm and 633nm lasers). Results were assessed using the proliferation model of ModFit LT 3.1 (Verity Software House, Topsham, MA). MSCs labeled with CFDA SE just before analysis served as negative controls for proliferation.

Reverse transcriptase polymerase chain reaction

Total RNA was isolated 1, 7, and 14 days after incubation with ECM proteins using the RNeasy kit (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was synthesized using the Thermoscript reverse transcriptase polymerase chain reaction (RT-PCR) System (Invitrogen). A 12-μL reaction containing 9 μL of RNA (100–200 ng), 1 μL of oligo dT (10mM), and 2 μL of deoxyribonucleotide triphosphates (dNTPs; 10mM) was denatured by incubation at 65°C for 5 min and then transferred to ice. To the RNA, 4 μL of 5x cDNA synthesis buffer, 1 μL of dithiothreitol (0.1 M), 1 μL of RNaseOUT (40 U/L), 1 μL of ThermoScript RT (15 U/L), and water were added to yield 20 μL. The mixture was incubated for 30 min at 55°C to activate the enzyme and then at 85°C for 5 min to terminate the reaction. PCR reactions were conducted as follows: to a 0.2-mL thin-walled PCR tube, 2 μL of the cDNA product, 1 μL of magnesium chloride (25mM), 2 μL of 10X PCR buffer, 0.4 μL of dNTP mix (10mM), 1 μL of sense primer (5mM), 1 μL of antisense primer (5mM), 0.2 μL of Taq Gold Polymerase, and 12.4 μL of nuclease-free water were added. The mixture was agitated and placed in a thermocycler. The temperature profile for all primer pairs was 95°C for 5 min, followed by 35 cycles of 30 s at 95°C, with a 45-s annealing interval at 58°C followed by a 1-min extension at 72°C. An additional 10-min incubation at 72°C was performed after completion of the last cycle to ensure complete extension of each PCR product. Primers specific for phenotypic markers of cardiomyocytes, osteoblasts, and adipocytes were used to evaluate the *in vitro* differentiation

of cells (Table 1). Primers specific for the housekeeping gene, β-actin, were used to normalize expression of differentiation markers. This primer set spans an intron and so also serves as a means of detecting contaminating genomic DNA (495 bp, RNA; 589 bp, DNA). The PCR products were size fractionated using electrophoresis on 1.5% agarose gels with ethidium bromide. Reactions without cDNA served as controls for cDNA contamination.

Immunofluorescence staining

MSCs were seeded on chamber slides (Becton Dickinson Labware, Bedford, MA) coated with collagen types I, III, and IV; fibronectin; and laminin at the concentrations listed above and a MSC density of 200 cells/cm². Cultures were maintained for 1, 7, or 14 days, and then chambers were removed and slides processed for immunohistochemistry. Slides were placed in acetone for 10 min and then placed in 2% paraformaldehyde for 3 min. Slides were washed with PBS, and primary antibody was applied (SH-2/CD105, 1:100 in 5% BSA/PBS, BD Biosciences); anti-α-sarcomeric actin (1:50, Novus Biologicals, Littleton, CO), anti-peroxisome proliferator-activated receptor gamma (PPARγ)2 (1:50, Novus Biologicals) and anti-osteocalcin (1:50, Millipore, Billerica, MA). MSCs treated with osteogenic, cardiomyogenic, and adipogenic media were used as positive controls. MSCs exposed to secondary antibody only served as negative controls. Slides were again washed, and appropriate secondary antibody was applied. Coverslips were mounted with 4',6-diamidino-2-phenylindole mounting medium and slides were visualized using a 3I/Olympus Deconvolution System with Spherical Aberration Correction (Olympus, Center Valley, PA).

MSC chemical induction

Standard chemical induction regimes were used to stimulate differentiation of MSCs to the adipogenic, osteogenic, and cardiomyogenic lineages (see Supplementary Information).

Alizarin Red staining

Alizarin Red S was used to detect calcium mineralization in MSCs exposed to ECM proteins and induction medium. Cell were washed three times with 1X PBS and then fixed

TABLE 1. REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION PRIMERS

Target gene	Primer sequence (5'-3')	Amplicon size (bp)
α-Cardiac actin	For: GCCTTCCTCATTTAAAGCTC Rev: AACACCACTGCTCTAGCCACG	418
Cardiac troponin T	For: AGGCGCTGATTGAGGCTCAC Rev: ATAGATGCTCTGCCACAGC	416
Osteopontin	For: CATCTCAGAAGCAGAATCTCC Rev: CCATAAACCACACTATCACCTC	354
Osteocalcin	For: ATGAGAGCCCTCACACTCCTC Rev: GCCGTAGAAGCGCCGATAGGC	293
β-actin	For: ATGTTTGAGCCTTCAACAC Rev: CACGTCACACTTCATGATGGA	495
Peroxisome proliferator-activated receptor gamma 2	For: AAGACCACTCCCCTCTTTG Rev: GTCAGCGGACTCTGGATTCA	553
L-type Cav1.2	For: CAAAGGCTACCTGGATTG Rev: CCAGTTGGGCTGGTTGTA	380

with 10% buffered formalin for 20 min. Cells were washed again two times with 1X PBS and one time with distilled water (dH₂O). Slides were incubated with a 40mM Alizarin Red solution (pH 4.1–4.3) for 30 min. Slides were washed with dH₂O to remove the excess and counter-stained with hematoxylin.

Oil Red O staining

Oil Red O staining was used to determine lipid structures in adipogenic differentiated MSCs exposed to ECM proteins or induction medium. Slides were stained using the Oil Red O stain kit (American Master Tech Scientific, Lodi, CA). Slides were placed in propylene glycol for 2 min. Slides were immersed in Oil Red O, incubated at 60°C for 6 min, and then washed with 85% propylene glycol in dH₂O for 1 min. Slides were washed two times with dH₂O and counter-stained with hematoxylin.

In situ cell viability

To assess the viability of cells after exposure to ECM proteins (1, 7, and 14 days), cells were loaded with calcein AM (1μM) and ethidium homodimer-1 (4μM) (Live/Dead Viability/Cytotoxicity kit for mammalian cells, Molecular Probes, Invitrogen, Carlsbad, CA) for 10 min at 37°C. Cells were visualized using a 3I/Olympus Deconvolution System with Spherical Aberration Correction. Live cells stained green (calcein AM), and nuclei of dead cells stained red (ethidium homodimer-1). The number of dead cells per cm² was determined for each ECM type and cells seeded without ECM construct.

Statistics

For comparison of expression levels of various differentiation markers of MSCs with various stimulation, a normal distribution was assumed, and one-way analyses of variance (ANOVA) and Tukey-Kramer honestly significant difference means comparison were used. Data were analyzed with JMP 5.0.1 for Windows (SAS Institute, Inc., Cary, NC).

Results

Characterization of human MSCs and exogenous ECM before co-culture

Stem cells, including MSCs, must be closely monitored and carefully cultured to ensure maintenance of a multipotent phenotype. To confirm that all isolates of MSCs at passages 6 to 8 exhibited MSC phenotype, we conducted flow cytometry for characteristic markers. We found all isolates maintained 98% or greater expression of CD73, CD90, and CD105 and lacked expression of hematopoietic stem cell marker CD34 and hematopoietic lineage marker CD45 (Fig. 1a). MSCs maintained a fibroblast-like morphology (Fig. 1b). Given the high percentage of cells expressing CD73, CD90, and CD105, neither were cell populations purified based on expression level nor were clonal populations of cells generated.

ECM protein concentration was chosen based on previous studies claiming complete coverage of well substrate. To confirm binding of ECM protein at levels consistent with ECM applied, we coated wells of a 96-well plate (0.32 cm²) with ECM proteins and measured total protein content using

a micro-BCA assay. We found that actual total protein did not vary significantly from theoretical values ($P > 0.1$ for all coatings, Fig. 1c). In addition, we conducted SEM on coated wells. ECM contrast is difficult to discern with thin coats because of gold coating (~2.5 nm), although it is clear that plastic of the well is coated and that coating topography varies between ECM proteins (Fig. 1d).

Mesenchyme gene expression in differentiating human MSCs

To determine whether and toward which lineage adult human MSCs differentiate after exposure to ECM proteins, we exposed MSCs to individual ECM components typically used for tissue engineering constructs. In parallel, MSCs were incubated with adipogenic, osteogenic, or cardiogenic induction media. After 2 weeks, genetic expression levels of cardiac-, bone-, and adipose-specific markers, including α -sarcomeric actin, L-type calcium channel isoform (Cav1.2), troponin (cardiac lineage), osteopontin, osteocalcin (bone lineage), and PPAR γ 2 (adipose lineage) were determined. Results were expressed as the intensity of each product relative to the intensity of a housekeeping gene, β -actin. In the presence of collagen type I and III, MSCs expressed transcripts characteristic of mature cells of bone, fat, and cardiac muscle (Fig. 2). A lesser degree of differentiation was observed in response to culture on ECM components of the basement membrane, collagen type IV, and fibronectin (bone cell differentiation only) and laminin (bone and cardiac differentiation only). Somewhat surprisingly, the initiation of differentiation in response to ECM can approximate or exceed that of stimulation media. For example, expression of osteocalcin did not vary significantly between osteogenic stimulation media (arbitrary intensity units, 0.41 ± 0.02) and exposure to laminin (0.36 ± 0.05 , $p < 0.01$) or collagen type I (0.32 ± 0.07 , $p > 0.1$). In addition, expression of PPAR γ was higher with exposure to collagen type III (0.70 ± 0.10) than to adipogenic stimulation medium (0.21 ± 0.04) at 14 days, although cardiogenic medium stimulated expression levels of Cav1.2 and sarcomeric actin 0.2 to 0.3 arbitrary intensity units higher than exposure to any ECM component ($p < 0.001$, comparison of cardiogenic stimulation with each ECM component for Cav1.2 and sarcomeric actin).

The kinetics of differentiation varied somewhat between MSCs of different sources. For example, MSCs from the commercial source (isolate B) expressed osteopontin on day 1 in the presence of all ECM types (isolate B, Supplementary Fig. 1a), whereas MSCs derived from bone marrow aspirates in our laboratory expressed osteopontin on day 1 in the presence of collagen type I and III and laminin (isolate A, Fig. 2) and in the presence of collagen type IV and fibronectin (isolate C, Supplementary Fig. 1b). We postulate that the young age (and possibly sex) of the donor of the cells of the commercial isolate may have played a role because younger individuals are still actively producing bone during development.^{22–24} Despite these early differences, expression of differentiation markers at day 14 was consistent between isolates. In particular, at day 14, expression of osteocalcin and osteopontin was detected after exposure to all ECM types for all isolates; expression of sarcomeric actin and Cav1.2 was restricted to exposure to collagen type I and III and laminin for all isolates; and expression of PPAR γ was

FIG. 1. Characterization of mesenchymal stem cells (MSCs) and extracellular matrix (ECM) before co-culture. MSCs were isolated from the bone marrow of healthy individuals and probed for expression of markers indicative of the MSC phenotype (CD73⁺/CD90⁺/CD105⁺/CD34⁻/CD45⁻). (a) Flow cytometric analysis of MSC phenotype. All MSC isolates were tested and found to express 98% or higher levels of CD73, CD90, and CD105 (data shown from isolate A). (b) Morphology of MSCs. Phase contrast microscopy of MSCs of isolate A before seeding on ECM proteins. (c) ECM content per unit area. ECM proteins were diluted from stock solutions to a concentration previously reported to ensure complete coating of polystyrene well plates (theoretical) and transferred to plates. Twenty-four hours after transfer, the solution was removed, and adherent protein was determined using micro-bicinchoninic acid (BCA) total protein analysis. Actual ECM content per cm² did not vary significantly from the theoretical amount (based on liquid concentration, $p > 0.1$). (d) ECM morphology and coating efficiency. To determine whether the concentration of ECM proteins used resulted in substantial coverage of our well plates, we conducted scanning electron microscopy. We found all regions of the well to be completely and uniformly coated.

restricted to exposure to collagen type I and III for all isolates. Also, all MSC isolates seeded without exogenous ECM expressed osteocalcin at later time points, perhaps as a consequence of cell density^{25,26} or endogenous ECM production. These data indicate that MSCs undergo differentiation after culture with ECM proteins and that MSC differentiation is ECM dependent (Fig. 2).

Mesenchyme protein expression in differentiating MSCs

To determine whether lineage-specific proteins were generated in response to ECM exposure and to observe the relative distribution of maturing cells, immunohistochemical staining for α -sarcomeric actin, osteocalcin, and PPAR γ 2 was performed. In accordance with the gene expression results, histological staining for the three different tissue-specific lineages (bone, fat, and muscle) was positive after ECM exposure (Fig. 3, MSCs exposed to lineage-specific induction medium were used as positive controls). Cells expressing osteocalcin were present in all the ECM proteins studied, with a higher expression level observed after exposure to

fibronectin and collagen type IV. Histochemical staining specific for fat and muscle was observed exclusively in cells seeded on collagens type I and III and laminin.

Functional differentiation of human MSCs in ECM substrates

Genotypic and phenotypic expression indicates initiation of a differentiation program, but terminal differentiation implies acquisition of the functional capacity of the somatic cell of interest. To test whether MSCs on exogenous ECM substrates acquire such functions, we have assessed established endpoints for adipocyte and osteoblast differentiation, namely formation of fat droplets and mineralization (Fig. 4). Formation of fat droplets was determined using Oil Red O staining. Cultures treated with adipogenic induction medium served as positive controls. MSCs cultured on collagen type I and III contained fat droplets at concentrations consistent with MSCs stimulated with adipogenic induction medium. MSCs cultured on collagen type IV, laminin, and fibronectin did not contain fat droplets. Mineralization was determined using Alizarin Red staining. Cultures treated

FIG. 2. Differentiation of MSCs after exposure to ECM proteins. (a) Reverse transcriptase polymerase chain reaction (RT-PCR) for lineage markers was conducted after exposure of three different MSC isolates to ECM proteins (COL I, collagen type I; COL III, collagen type III; COL IV, collagen type IV; LN, laminin; FN, fibronectin) or lineage-specific induction medium (OI, osteogenic induction; CI, cardiogenic induction; AI, adipogenic induction), and products were separated using electrophoresis on a 1.5% agarose gel. OC, osteocalcin; OPN, osteopontin; PPAR, peroxisome proliferator-activated receptor gamma 2; CA, α -sarcomeric actin; Cav1.2, L type calcium channel isoform; NC, no construct (i.e., no ECM proteins coated on the plate before MSC culture). The electrophoresis gel shown is representative of three separate experiments, and each band corresponds to cells from a single well. (b) Densitometry of RT-PCR products. Results are expressed as the intensity of each product relative to the intensity of β -actin (arbitrary intensity units). Results shown correspond to one isolate (isolate A) and are representative of all isolates (complete data set, Supplementary Fig. 1). Levels not connected by the same letter are significantly different, $p < 0.001$.

FIG. 3. Mesenchyme protein expression in differentiating MSCs. (a) Immunohistochemical staining for α -sarcomeric actin, PPAR γ 2, and osteocalcin. Positive staining for PPAR γ 2 was observed exclusively in cultures exposed to collagen type I and III. Positive staining for α -sarcomeric actin was observed in cultures containing collagen type I and III and laminin. Cells expressing osteocalcin were present in all cultures tested (14 days). MSCs cultured in the presence of lineage-specific induction medium (osteogenic stimulation, adipogenic stimulation, and cardiogenic stimulation) served as positive controls. (b) Quantitative protein expression displayed according to ECM type. Numbers of positive cells were counted in eight fields (~100) for each condition and were expressed as a percentage of the total number of cells in each field (black = OC, white = CA, dotted = PPAR) (* $p < 0.05$, ** $p < 0.005$).

FIG. 4. Functional differentiation of MSCs after exposure to ECM proteins. At 14 days after incubation with ECM proteins, MSCs were stained with Oil Red O for fat droplets and Alizarin Red for calcific nodules. MSCs cultured without ECM substrate or induction media were negative for fat droplets (round, red staining) whereas MSC cultures stimulated with adipogenic media and collagen type I and III were positive for fat droplets (black staining is nonspecific hematoxylin debris). All other treatments were negative for fat droplets. MSCs cultured under all conditions (even no construct (NC) and cardiogenic induction medium) formed calcific nodules. Scale bar, 100 μ m.

with osteogenic induction medium served as positive controls. MSCs of all culture conditions (even without construct) contained calcific nodules.

Proliferation of differentiating human MSCs in ECM substrates

MSC differentiation after exposure to ECM proteins could have occurred as a direct consequence of integrin binding or could reflect a secondary consequence of greater cell density. For example, human MSCs seeded at a density of 0.3×10^4 cells/cm² expressed 1.7 times as much aggrecan (indicative of chondrocyte differentiation) as MSCs seeded at a density of 0.05×10^4 cells/cm².²⁵ To determine whether differentiation was a function of changes in cell density through increased proliferation, MSCs were stained with CFDA SE and seeded with ECM proteins. Cells were plated at 250 cells/cm², and flow cytometric analysis was performed the day after (time point 1) and 7 and 14 days after exposure to ECM proteins. Cell distribution in the various generations 1 day after CFDA SE staining revealed that MSCs underwent at least two divisions and by day 14 had completed approximately 10 divisions. Percentage of proliferating cells did not vary significantly between ECM exposure conditions as a function of time ($p = 0.8$; Fig. 5). Thus, multilineage differentiation of MSCs after exposure to ECM proteins does not reflect variations in rate of cell density through greater proliferation.

Morphology and viability of differentiating human MSCs in ECM substrates

To ensure that differences in maturation rate and lineage commitment with ECM exposure did not reflect changes in cell integrity, MSC viability and morphology were assessed. At 1, 7, and 14 days, MSCs seeded with and without ECM proteins were observed using brightfield microscopy and

found to be intact and well spread. Viability of MSCs was assessed using trypan blue 1, 7, and 14 days after exposure to ECM proteins. The average number of live cells at day 1 was $7,000 \pm 108$ cells/mL, whereas the average number of dead cells was $1,100 \pm 84$ cells/mL. At day 14, the average number of live cells was found to be approximately $25,000 \pm 220$ cells/mL, and the average number of dead cells was $3,700 \pm 145$. Because removal of cells from the ECM constructs could induce some cell death, we also assessed viability using an *in situ* live/dead assay (Live/Dead Viability/Cytotoxicity kit for mammalian cells, Molecular Probes). No significant differences were observed between culture conditions, indicating that altered cell integrity did not contribute substantially to changes in MSC differentiation after ECM exposure (Fig. 6).

Discussion

The extracellular microenvironment plays a significant role in the cell fate process of stem cell populations. In this study, we demonstrated that MSC interactions with ECM proteins can guide differentiation of MSCs toward multiple lineages, including bone, fat, and muscle. In addition, differentiation of MSCs exposed to ECM proteins was found to be heterogeneous in all of the ECM proteins studied. That MSCs differentiate in a heterogeneous, ECM-dependent manner could be beneficial or detrimental to tissue engineering efforts. Differentiation before transplantation may be beneficial if a homogenous population of cells can be obtained, but differentiation in this context could also be detrimental if unanticipated, undesirable, or undetected differentiation occurs before transplantation.

Differentiation of stem cells has been driven in a multitude of ways. First, soluble factors have been added to culture medium to induce differentiation of stem cells into different lineages, including osteo, cardiac, and adipogenic.^{27–29}

FIG. 5. Proliferation of MSCs after exposure to ECM proteins. At 1, 7, and 14 days after incubation, MSCs were removed from culture, stained with CFDA-SE, and analyzed using flow cytometry. **(a)** Representative proliferation profiles. Cell distribution 1 day after CFDA SE staining revealed that MSCs underwent at least two divisions, and by 14 days, MSCs underwent approximately 10 divisions, irrespective of culture conditions. **(b)** Quantitative proliferation of MSCs on ECM proteins. The percentage of proliferating cells (relative to all cells in a given condition) were determined and displayed according to ECM type. There were no significant differences in proliferation between cells exposed to ECM proteins and the control group ($p > 0.1$). Color images available online at www.liebertonline.com/ten.

Second, mechanical stimulation and co-culture systems have been used to promote differentiation of MSCs toward bone, vascular, and ligament cell lineages.^{30–32} Finally, electrical stimulation has been used along with soluble factors to induce differentiation of stem cells into the neural lineage.³³ Insoluble factors (e.g., ECM proteins) have also been shown to induce stem cell differentiation, but ours is the first study to our knowledge to investigate the effect of an array of proteins on the maturation of several lineages. Perhaps the most unexpected ECM-induced lineage markers observed in this study were α -sarcomeric actin and L-type calcium channels after exposure of MSCs to collagen type I and III and laminin. Expression of these proteins indicates progression toward (albeit incomplete) cardiomyocyte or smooth muscle cell types. We suspect progression toward the cardiomyocyte cell type because we observed expression of troponin in higher-passage MSCs (passage 10) after exposure to the same ECM proteins (collagen type I and III and laminin; data not shown) and because the protein staining pattern resembled striations of cardiomyocytes (Fig. 3). Induction of stem cells into the cardiac lineage using soluble factors has been done with some success in the recent past. Adult MSCs have been reported to differentiate into cardi-

omyocytes after treatment with 5-azacytidine³⁴ and soluble factors.³⁵ However, fully functional cardiomyocytes with striated cytoskeleton and proper electrical coupling have not been observed. We also fail to observe fully functional cardiomyocytes, but ours is the first study to demonstrate that ECM components alone significantly affect the differentiation of stem cells, even those destined to become cells of the cardiovascular system.

That ECM components can drive heterologous differentiation of stem cells raises cautionary flags for emerging fields, including tissue engineering, drug delivery, and regenerative medicine. One way to address this concern effectively is to develop technologies to identify and purify homogenous stem cell populations before stem cell therapy. The MSC populations used here were “pure” according to published screening guidelines but were almost certainly heterogeneous in many respects because differentiation after ECM exposure varied within and between populations tested. Thus more-rigorous biomarkers are needed to further parcel subpopulations of adult MSCs. If such phenotypic markers are identified, at least two additional hurdles hinder traditional cell-sorting methodologies for purification of stem cells (magnetic separation or flow cytometric separation). First,

FIG. 6. Viability of MSCs after exposure to ECM proteins. At 1, 7, and 14 days after incubation, MSCs were loaded with calcein AM (1 μ M; stains live cells green) and ethidium homodimer-1 (4 μ M; stains nuclei of dead cells red) for 10 min at 37°C. Cells were visualized, and the number of dead cells (red) per cm^2 was determined for each ECM type and cells seeded without ECM construct (NC). **(a)** Dead cell counts. Counts were statistically similar between ECM types (and NC) at each time point ($*p < 0.05$). **(b)** Representative MSC live/dead staining after exposure to ECM proteins. Fluorescence micrographs of MSCs seeded without a construct (NC) or on collagen type I (Col I) at 1, 7, and 14 days. Similar staining and morphology was noted for all other ECM constructs. Color images available online at www.liebertonline.com/ten.

expression levels of identified markers of differentiation are fluid and do not necessarily operate in a binary format. Second, differentiation state varies depending on the surrounding microenvironment, and most separation systems are capable of assessing single cells only, not cells in the context of supporting tissue. To address the first hurdle, efforts are underway to move beyond protein expression, to epigenetic, metabolic, or other intrinsic indicators of maturation. To address the second hurdle, microfluidic systems and flow cytometry systems capable of probing deep within three-dimensional tissues in a high throughput manner are being developed.^{17,36–38}

That ECM components can drive heterologous differentiation of stem cells signals a great opportunity for emerging fields of regenerative medicine. One could envision biomaterial and synthetic constructs tailored with specific ECM-derived peptides to induce lineage-specific differentiation. Cell-matrix interactions through integrin receptors have been found to be crucial for this cellular process. For example, osteogenesis of stem cells cultured on vitronectin has

been found to activate the focal adhesion kinase pathway and diminish the activation of extracellular signal-regulated kinase pathways.¹¹ As another example, loss of β 1-integrin functionality delayed the expression of cardiac-specific genes in developing cardiomyocytes.¹⁶ We are keen to further investigate the mechanism by which stem cells differentiate into a particular lineage when exposed to ECM proteins. Such a task will require more-robust indicators of lineage potential within a given MSC population or the use of clonal populations of MSCs. If successful, we predict that precise spatial and temporal control of ECM-derived peptides will be useful and even critical in generating ample populations of mature, somatic cells.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Brenda Ogle, Ph.D.

University of Wisconsin-Madison

2-114 Engineering Centers Building

1550 Engineering Drive

Madison, WI 53706

E-mail: ogle@wisc.edu

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