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Decellularized ECM Effects on Human Mesenchymal Stem Cell Stemness and Differentiation

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Abstract

Microenvironment extracellular matrices (ECMs) influence cell adhesion, proliferation and differentiation. The ECMs of different microenvironments have distinctive compositions and architectures. This investigation addresses effects ECMs deposited by a variety of cell types and decellularized with a cold-EDTA protocol have on multipotent human mesenchymal stromal/stem cell (hMSC) behavior and differentiation. The cold-EDTA protocol removes intact cells from ECM, with minimal ECM damage and contamination. The decellularized ECMs deposited by cultured hMSCs, osteogenic hMSCs, and two smooth muscle cell (SMC) lines were tested for distinctive effects on the behavior and differentiation of early passage ('naïve') hMSC plated and cultured on the decellularized ECMs. Uninduced hMSC decellularized ECM enhanced naïve hMSC proliferation and cell motility while maintaining stemness. Decellularized ECM deposited by osteogenic hMSCs early in the differentiation process stimulated naïve hMSCs osteogenesis and substrate biomineralization in the absence of added dexamethasone, but this osteogenic induction potential was lower in ECMs decellularized later in the osteogenic hMSC differentiation process. Decellularized ECMs deposited by two smooth muscle cell lines induced naïve hMSCs to become smooth muscle cell-like with distinctive phenotypic characteristics of contractile and synthetic smooth muscle cells. This investigation demonstrates a useful approach for obtaining functional cell-deposited ECM and highlights the importance of ECM specificity in influencing stem cell behavior.

Keywords

extracellular matrix; mesenchymal stem cell; osteogenesis; smooth muscle cell; decellularization; differentiation

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1. Introduction

Adult multipotent bone marrow-derived mesenchymal stromal cells, also known as mesenchymal stem cells (MSCs), can differentiate into a variety of lineages including osteoblasts, chondrocytes, myoblasts, and adipocytes (Pittenger et al., 1999). Isolated MSCs that are expanded and banked *ex vivo* have great potential for use in human cell therapies and regenerative medicine (Johnson et al., 2012; Tang et al., 2013). Isolated MSCs currently are used to treat animal arthritis and cardiac problems, despite limited understanding of the biological mechanisms by which local administration of MSCs decreases inflammation and contributes to tissue regeneration. Challenges in stem cell banking and usage include developing protocols to overcome loss of stemness *ex vivo*, without raising the potential for the cells to become malignant, and to induce endogenous stem cell differentiation into specific cell types *in vivo* (Rosland et al., 2009). In addition to potential for clinical usage, isolated MSCs also provide a valuable model system with which to investigate how stem cells could interact with implanted biomaterials *in vivo*.

Important drivers of stem cell behavior and differentiation include the microenvironments or niches to which the cells are exposed. Cell microenvironments play vital roles in regulating cell proliferation (Williams et al., 2008), migration (Hung et al., 2012), and differentiation (Martino et al., 2009; Rowlands et al., 2008). The composition, architecture, and physical properties of microenvironment extracellular matrix (ECM) and its bound growth factors and other ligands provide specific physical and chemical cues that influence distinctive stem cell behaviors (Wipff et al., 2007). Cells respond to microenvironment physical properties such as ECM modulus and topography, for example, through mechanotransduction mechanisms that convert mechanical responses into intracellular biochemical signals (Reilly and Engler, 2010).

To improve biocompatibility and better mimic the protein composition of *in vivo* cell microenvironments, non-biological 2D and 3D *in vitro* culture substrates can be coated with single ECM proteins such as fibronectin (FN), collagen, or laminin or with more complex solubilized ECM protein mixtures such as Matrigel[®]. Although these coated surfaces support proliferation and differentiation of numerous cell types, they lack the specific compositional and architectural complexity of ECMs secreted and assembled by cells. ECMs deposited by cells in culture and then decellularized may better replicate cell-specific features of ECM architectures and presentation of associated bioactive factors and perhaps satisfy the requirement for low immunogenicity if introduced into a body (Badylak and Gilbert, 2008). Several studies have demonstrated that decellularized ECM obtained by cell-lysis protocols is better than standard cell culture substrates and substrates coated with single ECM components for increasing stem cell proliferation while maintaining stem cell multipotency for differentiation into several cell types including osteoblast and adipocytes (Lai et al., 2010; Lin et al., 2012; Ng et al., 2014; Sun et al., 2011).

Most approaches to decellularize cell-deposited ECM have a significant drawback. Enzymatic detachment of intact cells by treatment with proteases such as trypsin and collagenase designed to recover viable cells, for example, may damage the remaining ECM and its bound factors. Cell lysis protocols that include treatment with detergent (Decaris and

Leach, 2011), alkali (Bass et al., 2007), or freeze/thaw cycles (Deutsch and Guldborg, 2010) can contaminate the remaining ECM with intracellular debris that may negatively affect subsequent cell interaction with the ECM or induce immunological reactions if implanted.

The purpose of this investigation was to investigate the effects of decellularized ECMs that were initially assembled by undifferentiated hMSCs, osteogenic hMSCs, and two smooth muscle cell lines on 'naïve' human bone marrow MSCs (hMSCs) growth and differentiation. ECMs from the osteogenic hMSCs and the two smooth muscle cell lines were chosen to determine whether they could influence the behavior of mesenchymal stem cells that might home to and interact with implantable devices such as orthopedic implants and arterial stents, respectively. Our initial attempts to investigate effects of cell-assembled ECM on stem cell proliferation, maintenance of stemness, and differentiation using ECMs decellularized by Triton-X-100 cell lysis yielded poor and highly variable results (results not shown), which spurred us to develop a protease-detergent-free method for removing intact cells from the ECM they secreted and assembled. This method involves incubating cell cultures in EDTA-PBS at 4°C until the cells round up and detach from the underlying ECM. Removal of the detached but intact cells leaves ECM that is largely undamaged by added protease and uncontaminated with the intracellular debris that cells release when lysed with detergent or other lysis protocols. To minimize ECM damage and contamination, the cell-deposited ECMs were decellularized using a simple and effective protease- and detergent-free method involving cold EDTA removal of intact cells.

Our results demonstrate that decellularized ECMs assembled by the different cell types have distinctive effects on naïve hMSCs. ECM deposited by uninduced hMSCs enhances the proliferation and preservation of stemness of naïve hMSCs whereas ECM deposited by osteogenic hMSCs induces naïve hMSC differentiation into osteoblasts, despite absence of added differentiation factors. Additionally, ECMs deposited by the two smooth muscle cell lines induce naïve hMSCs to exhibit distinctive phenotypic characteristics of smooth muscle cells.

2. Materials and methods

2.1. Cell culture and differentiation

The hMSCs used for this investigation were obtained from a 37 year old female donor by the Texas A&M Health Science Center College of Medicine Institute for Regenerative Medicine at Scott & White Hospital, through which hMSC supply is supported by grant P40RR017447 from the NCRN-NIH. The shipped P1 stage cells were immediately subcultured on arrival in the lab and stored frozen at the P2 stage. Before commencing experiments, the multipotentiality of the source hMSCs was confirmed with a CFU formation assay and by differentiation into osteoblasts and adipocytes. All experiments described here were done with P2-P5 stage cells. The hTERT-immortalized myometrial smooth muscle cell line was provided by Dr. James Olcese (Florida State University College of Medicine) and transfected to express mCherry. The A7R5 rat aorta smooth muscle cell line and the U2OS human osteosarcoma cell line originally were purchased from ATCC and then cultured in the lab for several years under recommended conditions. All cells were cultured in standard medium (SM) composed of alpha-MEM modified medium

supplemented with 2.2 g/L NaHCO₃, 10% (16.5% for hMSCs) fetal bovine serum (Atlanta Biologicals), and an antibiotic-antimycotic supplement containing 100 units/ml penicillin G, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, and 10 µg/ml gentamicin (all from Invitrogen/GIBCO, unless otherwise noted) at 37°C in 5% CO₂.

hMSCs assayed for differentiation potential after initial growth for 3 days on decellularized ECM or directly on Tissue Culture Plastic (TCP) were recovered by trypsinization, replated on TCP, and cultured in Bone Differentiation Medium (BDM) or Fat Differentiation Medium (FDM) for an additional 21 days.

Differentiation of hMSCs into osteoblasts was induced 24 hrs after plating by replacing the standard culture medium with BDM, in which the standard culture medium was supplemented with 10 nM Dexamethasone, 20 mM β-glycerophosphate, and 50 µM L-ascorbic acid 2-phosphate (all from Sigma), as described in the Institute for Regenerative Medicine hMSC Manual accompanying the cells. Differentiation of hMSCs into adipocytes was induced 24 hours after plating by replacing the standard culture medium with FDM, in which the standard culture medium was supplemented 0.5 µM Dexamethasone, 0.5 µM isobutylxanthine, and 50 µM indomethacin as described by Sekiya et al (Sekiya et al., 2002).

2.2. ECM decellularization

To obtain decellularized ECM, cell cultures, in which the cells were plated at a density of 1×10^4 cells per well (high density) on coverslips or directly on the TCP in a six-well plate, were cultured under specified conditions for various specified periods of time. To decellularize the deposited ECM, the cultures were washed twice with phosphate-buffered saline (PBS) that was pre-chilled to 4°C and then incubated in 1mM EDTA-PBS at 4°C for 12-24 hrs, which caused cell rounding. The rounded cells were detached by agitating the culture dish and gently rinsing with fresh cold-EDTA. After aspirating the detached cells, the surface was washed once with cold PBS before use for plating fresh cells. For ease of reference, the decellularized ECMs are designated according to the culture condition under which the ECM was deposited. Decellularized ECMs deposited by hMSCs cultured for 3 days in standard growth medium or in BDM medium, for example, are designated 3d-hMSC-ECM and 3d-BDM-hMSC-ECM, respectively. To assay activities of the decellularized ECM, freshly thawed hMSCs ('naïve') were plated onto the decellularized ECMs at 1×10^4 cell per well.

2.3. Atomic Force Microscopy (AFM) imaging of ECM topology

Images of dry ECM surface topographies in ambient air were recorded using the AC mode of an Asylum MFP-3D AFM unit equipped with a 20 nm radius TR400PSA tip (spring constant $\sim 0.02 \text{ N m}^{-1}$), an ARC2 controller (Asylum Research Inc., Santa Barbara, CA), and Igor Pro software. The tip cantilever was tuned to resonate 10% below its resonance frequency.

2.4. Analysis of cell morphology, focal adhesions, cell motility, colony formation, and nuclear shape

Cell morphology was analyzed using phase contrast micrographs that were obtained from triplicate samples of each culture condition. The Freehand Selection tool of ImageJ software was used to encircle each cell, and the cell aspect ratio and area of each cell were measured. Focal adhesions were measured with NIS elements (Nikon) software in images of cells with immunofluorescently stained vinculin obtained from triplicate samples for each culture condition. Cell motility was recorded at 30 sec intervals for 5 hrs at 37°C temperature and 5% CO₂ using a cell incubation chamber (Pathology Devices Inc.) on an inverted microscope (Nikon-Ti series). Motility velocity was determined for 10 min intervals using the Manual Tracker plugin for ImageJ. For colony forming unit analysis, hMSCs were seeded at a density of 100 cells/10cm² in culture dishes and incubated in standard culture medium for 14 days without changing the medium. After 14 days, the cultures were washed twice with PBS and stained with crystal violet. The boundaries of distinguishable colonies were traced with a marker, and the diameter of each tracing was determined with Image J software. The number of colonies with diameters 1mm-2.5mm and 2.5mm (DiGirolamo et al., 1999) were classified into separate groups. For nuclear morphology analysis, the Fit Ellipse Image J plugin was used to measure the major and minor axis of each DAPI-stained nucleus.

2.5. Protein localization by fluorescence microscopy

Cultures on glass coverslips were fixed in 3.7% paraformaldehyde-PBS for 15 minutes, permeabilized with 0.2% of Triton X-100-PBS, and blocked either in 1% goat serum or 1% BSA (for FN immunolocalization) in 0.05% Triton-X-100-PBS solution prior to incubation in Texas red-X phalloidin for total actin filament localization (Invitrogen) or in a primary antibody overnight at 4°C. Incubation in secondary antibodies was done for 1hr at 37°C. A rabbit polyclonal primary antibody to FN (ICN) and mouse monoclonal antibodies (Sigma) were used to localize vinculin and α -smooth muscle actin. The primary antibodies were detected with the appropriate anti-rabbit (Alexa546-labelled, Invitrogen) and anti-mouse (Alexa488-labelled, Invitrogen) secondary antibodies. Minor gamma and contrast adjustments were made to color channels on some Fig. images.

2.6. Western blot analysis of protein expression

A detergent-divalent cation chelator solution (100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% SDS and 0.5% deoxycholate, 10% glycerol, 10 mM Tris-Cl, pH 7.4) was used to extract tissue culture samples for western blot analysis. The samples were fractionated by SDS-PAGE and electroblotted to nitrocellulose membranes (0.45µm pore size). The nitrocellulose blots were blocked in skim milk (5% in PBS) prior to incubation overnight at 4°C in the primary antibodies described above or a mouse monoclonal antibody to SM22 α (Abcam) and a mouse monoclonal GAPDH (Sigma) that was used for loading control. The blots were rinsed with 0.2% Triton X-100-PBS solution and incubated with horseradish peroxidase-tagged goat anti-mouse or goat anti-rabbit secondary antibodies (both from Thermo Scientific) for 1hr at room temperature. Chemiluminescence was developed using an Enhanced ECL western blotting substrate (Pierce).

2.7. Metabolic assay with Alamar blue

To measure metabolic activity, Alamar blue solution was added to the medium up to 10% of the final volume, and the culture was incubated under standard conditions for 1 hr. The medium was removed and its fluorescence intensity at 580 nm was measured with a SpectraMax M5 fluorimeter at an excitation wavelength of 550 nm.

2.8. Alkaline phosphatase assay

Samples were prepared from hMSCs by lysing the cells with two freeze-thaw cycles in 2 ml of 0.5% Triton-X 100 in PBS. The ALP activity in each sample was determined according to Sabokbar et al (Sabokbar et al., 1994). In brief, 50 μ l of each sample was added to 50 μ l of AMP-substrate (0.5 M AMP solution supplemented with $MgCl_2$ and 9 mM p-nitrophenol phosphate, pH 10) in 96-well microtiter plate wells and incubated for 30 min at 37°C. Comparison of absorbance at 405 nm with a 0-0.5 μ mol/ml p-nitrophenol standard curve was used to calculate ALP activity. All samples measurements were normalized using total protein values obtained with a Coomassie plus Bradford assay kit (Thermo Scientific).

2.9. Staining for hMSC Ca^{2+} mineralization and lipid accumulation

Biomaterial deposition by osteoblasts was visualized after 21-24 days of culture on various substrates by fixing cultures with 3.7% paraformaldehyde in PBS, washing twice with diH_2O , and staining with 1% Alizarin red S in diH_2O (ARS, pH 4.2) for 20 min at room temperature. Excess stain was washed away twice with diH_2O . Lipid accumulation by adipocytes was detected by Oil Red staining as described by Sekiya et al (Sekiya et al., 2002). Images were obtained using bright field microscopy. The level of biomaterialization or lipid accumulation was quantified using Image J by converting the microscope images of stained cultures to binary images and determining the total area covered with stain for each image.

2.10. Statistical analysis

The statistical differences between means were calculated using a T-test, assuming one-tail distribution and unequal variance between the two samples.

3. Results

3.1. Decellularization of cell-assembled ECM without proteases or cell lysis

The cold-EDTA decellularization method was tested with two cell types that were transfected to express mCherry as a cytoplasmic protein marker: hTERT-immortalized myometrial smooth muscle cells (myoSMCs) and U2OS osteosarcoma cells. Both cell types were plated at high density and cultured for three days to confluence before the cultures were decellularized. Exposure to cold EDTA caused the myoSMCs (Fig. 1) and the U2OS cells (Fig. S1) to round up and detach. The integrity of the detached cells was evident from retention of the expressed mCherry (Fig. 1B; Fig. S1B) and a general lack of mCherry protein or other visible debris on the remaining surface (Fig. 1C and D). Furthermore, the cold-EDTA detached cells remained viable and survived well when replated on fresh tissue culture plastic plates (TCP) under standard growth conditions (not

shown). The organization of the FN in the cold-EDTA decellularized ECM also appeared to be well preserved after the decellularization process (Fig. 1E and F).

In contrast, removal of the cells by Triton-X-100 detergent cell lysis caused release of the mCherry and contamination of the ECM (Fig. S1C and D). Atomic Force Microscopy imaging revealed clear differences in the extent of ECM contamination after Triton-X-100 decellularization (Fig. 1G) and cold-EDTA (Fig. 1H). Although ECM filaments are evident in the AFM images of both decellularized ECMs, the ECM decellularized by detergent cell lysis appears highly contaminated with unidentified debris (Fig. 1G) not found on the cold-EDTA decellularized ECM (Fig. 1H).

All decellularized ECMs used for further investigation were prepared by the cold-EDTA treatment.

3.2. Decellularized ECM enhances stem cell proliferation

One major question addressed by this investigation is how ECM deposited by hMSCs cultured in standard medium for 3 days and then decellularized (3d-hMSC-ECM) affects naïve hMSCs plated on the ECM. To investigate the effects of 3d-hMSC-ECM on hMSC growth, proliferation, and stemness, hMSCs were plated at high density on TCP or glass coverslips (CSs) and grown in standard growth medium for 3 days. The ECM deposited by these initial hMSCs then was decellularized to yield the 3d-hMSC-ECM. Freshly-thawed, 'naïve', hMSCs then were plated onto control uncoated TCP (Fig. 2A) and the 3d-hMSC-ECM (Fig. 2B) and cultured for another 3 days in standard growth media. Both micrographs (Fig. 2A and B) and Alamar blue metabolic assays (Fig. 2C) revealed that the hMSCs cells proliferated significantly more (by approximately 45%) on the 3d-hMSC-ECM than on the control TCP.

The 3d-hMSC-ECM also enhanced the number and the size of naïve hMSC colonies formed (Fig. 3). When 300 freshly-thawed naïve P2 stage (or P5 stage, not shown) hMSCs from growing cultures were plated at very low density (3 plates of 100 cells/plate for each condition), the naïve hMSCs formed more colonies (Fig. 3 C) and larger colonies (Fig. 3 D) on the 3d-hMSC-ECM than on the control TCP. For this analysis, only the dense circular groups of cells with diameters greater than 1 mm were counted as colonies. After 14 days in culture, 135 (45%) of the initial 300 P2 hMSCs plated on the 3d-hMSC-ECM plates, compared to only 53 (20%) of the hMSCs on the control TCP plates, had formed colonies greater than 1 mm in diameter (Fig. 3C). Furthermore, 39 (28%) of the colonies on the 3d-hMSC-ECM but only 7 (13%) of the colonies on the TCP were ≥ 2.5 mm in diameter, and the largest colonies on the 3d-hMSC-ECM plates were more than twice the diameter of the largest colonies on the TCP plates (Fig. 3D).

The morphologies of the naïve hMSCs plated on the decellularized ECM and TCP also differed. Stem cells can be classified into the following *four morphological categories* based on cell aspect ratio (AR; ≤ 5.0 or >5.0) and spread area (SA; ≤ 5000 or $>5000 \mu\text{m}^2$) (DiGirolamo et al., 1999; Sekiya et al., 2002): *spindle-shaped* (SS; AR >5.0 , SA $\leq 5000 \mu\text{m}^2$; Fig. 4A), *flat spindle-shaped* (FSS; AR >5.0 , SA $>5000 \mu\text{m}^2$; Fig. 4B), *self-renewing round* (RS; AR ≤ 5.0 , SA $\leq 5000 \mu\text{m}^2$; Fig. 4C), and *flat* (FC; AR ≤ 5.0 , SA $>5000 \mu\text{m}^2$; Fig. 4D).

Three days after plating fresh hMSCs, the percentages of SS and FSS cells on control TCP (Fig. 4F; 19.7% SS and 3.4% FSS) were similar to the decellularized 3d-hMSC-ECM (Fig. 4G; 20.1% SS and 1.7% FSS), but the percentage of RS cells was much higher and FC cells was much lower on the decellularized 3d-hMSC-ECM (70.7% RS, 7.5% FC) than on the TCP (57.6% RS, 20.2% FC).

3.3. Stem cell adhesion and motility on decellularized ECM

Decellularized 3d-hMSC-ECM also affected naïve hMSC substrate adhesion and motility. Although the amount of vinculin expressed by naïve hMSCs plated on the 3d-hMSC-ECM and on control TCP was similar (Fig. 5C), the cells formed significantly shorter vinculin-containing FAs on the decellularized 3d-hMSC-ECM (Fig. 5B1-2) than on the control CSs (Fig. 5A1 and 2) and only the cells on the CSs formed supermature focal adhesions (>8 µm long).

Cell motility of the naïve hMSCs plated on 3d-hMSC-ECM and TCP was compared by recording cell locomotion velocities over 10-min intervals for 5 hours for 40 naïve hMSCs each on 3d-hMSC-ECM and control TCP. The velocities, distance moved for each of the recorded 10-min intervals (30 time intervals for each of the 40 cells), were determined using the ImageJ Manual Tracker plugin. A box plot of all the 10-minute intervals revealed that the hMSCs moved significantly faster on the 3d-hMSC-ECM than on the control TCP (Fig. 5D). The median 10-minute interval velocity for the hMSCs on the 3d-hMSC-ECM (1.20 µm/min) was more than twice the median velocity on the control TCP (0.54 µm/min). Furthermore, the range of 10-minute interval velocities for the second fastest quartile of the hMSCs on the 3d-hMSC-ECM (0.54-1.20 µm/min) approximated the velocity range for the third fastest quartile for hMSCs on TCP (0.54-1.32 µm/min). Also, the top 10-minute interval velocity on the 3d-hMSC-ECM (13.38 µm/min) was faster than that on the TCP (11.52 µm/min), and fewer of the cells failed to move at all during any 10-minute interval on the 3d-hMSC-ECM than on the TCP.

3.4. Preservation of hMSC stemness on decellularized ECM

To determine effects on maintenance of stemness, naïve hMSCs cultured on 3d-hMSC-ECM and TCP for 3 days were recovered by trypsinization and plated on TCP and induced with BDM or FDM. After 21 days, the BDM-induced cultures were stained for biomineralization with Alizarin Red, and the FDM-induced cells were stained for lipid accumulation with Oil Red (Fig. 6). The hMSCs precultured on the 3d-hMSC-ECMs biomineralized the surface or accumulated lipid (Fig. 6B and D) as well as or significantly better than did freshly-thawed P2 cells plated directly on TCP in BDM or FDM (Fig. 6A and C), as indicated by the statistically significant difference in the mean of image areas covered with the Alizarin Red stain for biomineralization (Fig. 6E; n=7 images, $p<0.03$) and the strong trend in the difference in means found for the Oil Red stain for lipid accumulation (Fig. 6F; n=6 images, $p<0.059$).

3.5. Decellularized BDM-ECM Induces hMSC Differentiation into Osteoblasts – Differential effects depend on deposition timing

The other major question addressed by this investigation is how ECM deposited by BDM-induced hMSCs and two smooth muscle cell lines affect the differentiation of naïve hMSCs in the absence of added differentiation factors. We first investigated whether decellularized ECM deposited by hMSCs differentiating into osteoblasts in BDM can induce differentiation of naïve hMSCs into osteoblasts in the absence of added dexamethasone. Decellularized ECMs were prepared from cultures in which the ECM-depositing hMSCs were induced for various times in Bone Differentiation Medium (BDM) containing dexamethasone, ascorbic acid, and β -glycerophosphate. The ECMs deposited by hMSCs in standard media for 3 days (3d-hMSC-ECM) as a control and in BDM for 3 days (3d-BDM-hMSC-ECM), 8 days (8d-BDM-hMSC-ECM), and 15 days (15d-BDM-hMSC-ECM) were decellularized and reseeded with naïve hMSCs, which then were cultured for 28 days in media containing ascorbic acid and β -glycerophosphate but lacking dexamethasone. As early as day 3, the naïve hMSCs cultured on the 3d-hMSC-ECM (Fig. 7A1) and the 3d-BDM-hMSC-ECM (Fig. 7A2) in the absence of dexamethasone exhibited differences in cell morphologies. The most extensive differences were in the distribution of cells in the RS morphology (65% on 3d-hMSC-ECM compared to 36.4% on 3d-BDM-hMSC-ECM), and in the FC morphology (17.9% on 3d-hMSC-ECM compared to 48.1% on 3d-BDM-hMSC-ECM).

To determine whether the BDM-hMSC-ECMs osteogenesis potential changes over time of deposition, we investigated effects on alkaline phosphatase activity and biomineralization of naïve hMSCs cultured on 3d-, 8d-, and 15d-BDM-hMSC-ECMs in the absence of dexamethasone. Three days after plating, the alkaline phosphatase (ALP) activity, an early osteoblast differentiation marker, was significantly higher in the naïve hMSCs cultured on the 3d-BDM-hMSC-ECM than in the naïve hMSCs cultured on either the 8d-BDM-hMSC-ECM or 15d-BDM-hMSC-ECM, both of which had ALP activity levels statistically similar to that of uninduced hMSCs plated directly on TCP (Fig. 7B). This difference in osteoblast differentiation induction potential for the decellularized ECMs also is reflected in the eventual biomineralization of the cultures. By 28 days of naïve hMSC culture in the absence of dexamethasone, the mineral deposition was much greater on the 3d-BDM-hMSC-ECM (Fig. 7C2) than on the 8d- or 15d-BDM-hMSC-ECM (Fig. 7C3-C4). The naïve hMSCs on the 8d- and 15d-BDM-hMSC-ECMs deposited little if any more mineral than did control hMSCs cultured in the absence of BDM on TCP (Fig. 7C1).

Differences in the 3d-, 8d-, and 15d-BDM-hMSC-ECMs that might account for the different osteogenesis potentials were investigated by immunofluorescent localization of FN, collagen 1 (COL1), and bone sialoprotein (BSP) (Fig. 8). Representative images revealed some increase from day 3 to 15 but little overall difference in the organization of FN in the 3d-, 8d-, and 15d-BDM ECMs (Fig. 8A, D, G). In contrast, little deposition of Col1 was found in the 3d-BDM-ECM (Fig. 8B), although was evident in the cells prior to decellularization (not shown). By day 8, Col1 was significantly increased (Fig. 8E), and that increase was maintained in the 15d-BDM-ECM, in which individual strands of Col1 were more robust (Fig. 8H). Little to no BSP was found in the 3d- and 8d-BDM-ECM (Fig. 8C and F). By day

15, however, BSP was found localized in the BDM-ECM, and some appeared to be localized along Col1 filaments as expected (Fig. 8I). For comparison, it is interesting to note that the decellularized 15 day ECM deposited by hMSCs induced with FDM instead of BDM contains similar levels and organization of FN (Fig. S2A) as in the 3d-BDM-ECM, but a different organization of Col1 (Fig. S2B), and no BSP (Fig. S2C).

3.6. Decellularized smooth muscle cell ECMs induce hMSC differentiation into smooth muscle cells with distinct phenotypic characteristics

hMSCs also have the potential to differentiate into the smooth muscle cell (SMC) lineage, but even when fully differentiated, SMCs remain phenotypically plastic enough to interconvert along a continuum between ‘synthetic’ and ‘contractile’ phenotypes. We investigated decellularized ECMs deposited by two smooth muscle cell lines not only for induction of hMSC smooth muscle cell characteristics but also for specific effects on induced SMC phenotype.

To generate the decellularized ECMs tested, cultures of rat aortic A7r5 smooth muscle cells, hTERT-immortalized human myometrial smooth muscle cells (myomSMCs), and hMSCs (in standard growth medium) were plated at high density and grown to 100% confluence for 3 days before cold-EDTA decellularization. After decellularization, the organization of FN in the ECMs deposited by the three cell lines was distinctly different (Fig. 9A1-A3). The FN in the A7r5-deposited ECM (A7r5-ECM; Fig. 9A2) was organized mostly into short aligned strands and punctate dots similar to those found in the hMSC-ECM (Fig. 9A1) whereas the FN in myomSMC-ECM (Fig. 9A3, also see Fig. 1F) was organized into long robust branching strands.

When naïve hMSCs were cultured on the A7r5-ECM, myomSMC-ECM, and hMSC-ECM for 7 days in standard growth media lacking added SMC differentiation factors, the hMSCs developed general SMC characteristics and at least some degree of SMC phenotypic specificity. Like SMCs, the naïve hMSCs on both the A7r5-ECM (Fig. 9B4-B6) and myomSMC-ECM (Fig. 9B7-B9) were much more highly elongated than the hMSCs on the hMSC-ECM (Fig. 9B1-B3).

The naïve hMSCs on the three ECM substrates also were distinguishable in the expression levels of the smooth muscle cell marker SM22 α and the expression levels and organization of α -smooth muscle actin (α -SMA), which despite its name, is expressed in a variety of non-smooth muscle cell types including hMSCs. Expression of SM22 α was similar in the naïve hMSCs on the A7r5-ECM and the myomSMC-ECM, but much greater than in the hMSCs on the hMSC-ECM (Fig. 9C).

The hMSCs on the A7r5-ECM and myomSMC-ECM were distinguished, however, by α -SMA expression levels and actin cytoskeletal organization. Although the hMSCs on all three decellularized ECMs expressed α -SMA, the α -SMA expression level was lower in cells grown on myomSMC-ECM compared to those on A7r5-ECM or hMSC-ECM (Fig. 9C). Furthermore, most of the actin filament-containing structures in the cells on the A7r5-ECM (Fig. 9B4-6) contained α -SMA, unlike the actin structures in the cells on myomSMC-ECM (Fig. 9B7-9) and on the hMSC-ECM (Fig. 9B1-3). Intriguingly, the cells on the myomSMC-

ECM formed numerous podocyte-like structures (Fig. 9B8, arrow and inset) that are more characteristic of the SMC synthetic than of the contractile SMC phenotype.

Differences in overall cell elongation and organization on the three decellularized ECMs also were evident in the shape of the cell nuclei, as indicated by the nuclear aspect ratio (major:minor axis lengths, Fig. 9E). Nuclei were significantly more oblong in the naïve hMSCs on the myomSMC-ECM compared to those on A7r5-ECM, the hMSC-ECM, or the uncoated control CSs (Fig. 9D and E), because of the tight packing of highly elongated cells. Greater than 75% of the nuclei in the cells on the CSs, hMSC-ECM, and A7r5-ECM had an aspect ratio below 2.2 whereas greater than 75% of the nuclei in cells on the myomSMC-ECM had an aspect ratio in the 2.2-5.6 range.

4. Discussion

Microenvironment ECMs present structural features and other cues that play major roles in regulating cell proliferation, motility, gene expression, and differentiation *in vivo* (Guilak et al., 2009; Reilly and Engler, 2010; Watt and Huck, 2013). ECMs of adult stem cell niches contribute to maintenance of quiescent stem cells or induction of self-renewal and differentiation into specific cell types. Better replication of the microenvironment ECMs *in vitro* or on surfaces of implantable devices could contribute to a better understanding of cell-microenvironment interactions and to developing more effective regenerative therapies.

A variety of approaches using coatings made of single ECM proteins or solubilized mixtures of ECM proteins such as Matrigel® have provided crucial insight into how many different types of cells including stem cells interact with and respond to ECM components. Such substrates provide excellent support for differentiation factor-induced hMSC differentiation into specific cell lineages (Qian and Saltzman, 2004). Neither single ECM proteins nor mixtures of ECM proteins, however, replicate the specific types of complex ECM architectures found in different cellular microenvironments *in vivo* nor can they specifically induce the variety of cell responses to microenvironmental cues. If well preserved and uncontaminated, decellularized ECMs deposited by cells should better replicate *in vivo* ECM architectures and may induce more complex cell responses.

Decellularizing cell-deposited ECM without significant damage or contamination presents a challenge for obtaining native cell-deposited ECMs. Methods for removing cells from substrates using proteases, which may damage ECM proteins and associated factors, are designed to recover viable cells but not their ECMs for subsequent uses. Other decellularization methods that remove cells with detergents, freeze/thaw cycles, or alkali treatment, lyse cells and release intracellular contents that can contaminate the ECM, as shown in this investigation with detergent lysis of mCherry-expressing cells (Fig. S1). ECM that is contaminated with intracellular debris could interfere with standard cell-ECM interactions and induce immunological responses if implanted *in vivo*. Our initial experiments using ECM decellularized by detergent cell lysis yielded variable results (not shown), which prompted us to develop the cold-EDTA decellularization approach used in this investigation.

The cold-EDTA decellularization method used here preserves the integrity of the removed cells and minimizes ECM damage and contamination caused by added proteases or detergents. Cold-EDTA treatment causes weakening of the cell-cell interactions by EDTA divalent cation chelation and cold-induced cell rounding, presumably caused at least in part by microtubule depolymerization. Although the rounding cells may dislodge some ECM strands from which their receptors fail to release, as possibly indicated by Fig. 1E and F), most of the ECM remains intact and uncontaminated by intracellular debris, as shown in Fig. 1H. Fortuitously, EDTA inhibition of metalloproteases also may contribute to ECM preservation. We found that cold-EDTA caused several cell types including mCherry-expressing U2OS cells, used as a test cell line, and myomSMCs to round up and detach from deposited ECMs without releasing the expressed mCherry used as a marker for intracellular proteins (Fig. 1). AFM imaging showed the remaining ECM to be free of the debris apparent on ECM decellularized with detergent cell lysis (Fig. 1G and H).

The cold-EDTA decellularized ECMs had three distinctive effects on naïve hMSCs. One effect was that decellularized ECM deposited for three days by undifferentiated hMSCs cultured under standard growth conditions (3d-hMSC-ECM) enhanced naïve hMSC proliferation (Fig. 2), colony formation and size (Fig. 3), and induced adhesion-motility and morphological characteristics that are consistent with maintenance of stemness (Figs. 4 and 5) (Saller et al., 2012). On 3d-hMSC-ECM, the naïve hMSCs had a 45% higher proliferation rate and, when plated for clonal analysis, produced 2.25-fold more colonies and had a higher percentage of larger colonies than did hMSCs plated directly on TCP. The significance of colony size is that smaller colonies tend to have more flat cells that may have lost stemness (Sethe et al., 2006). The 3.5-fold higher ratio of RS to FC cells on the 3d-hMSC-ECM than on TCP was consistent with the enhanced capacity of the decellularized ECM to maintain potential to differentiate into osteoblasts and adipocytes (Fig. 6). FCs proliferate less rapidly and have lost much of their multipotentiality, although they retain the ability to differentiate into the osteogenic lineage (DiGirolamo et al., 1999; Sekiya et al., 2002). Cells in the other three categories, especially the RS cells, proliferate more rapidly and retain greater multipotentiality for differentiation. Taken together, these results present the possibility that use of cold-EDTA decellularized hMSC-deposited ECM as a substrate for expansion and storage of hMSCs could improve production of cells for autologous stem cell therapies.

The cold-EDTA decellularized ECMs also promote hMSC differentiation into specific cell lineages in the absence of added differentiation factors such as the dexamethasone in BDM and FDM, which could cause off-target effects if injected or released from dexamethasone-releasing coatings to control stem cell behavior *in vivo* (Zhang et al., 2013). We found that decellularized ECM deposited by BDM-induced hMSCs circumvented the need for exposure to dexamethasone to induce naïve hMSCs to differentiate into osteoblasts (Fig. 7). Our findings are in accord with previous demonstrations that MSCs differentiate into osteoblasts, myoblasts, or nerve cells without addition of differentiation factors depending on the similarity of the surface modulus on which they are cultured with the physiological modulus of the corresponding niches of the differentiating cells *in vivo* (Engler et al., 2006) and that ECM scaffolds on orthopedic implants promote bone mineral formation and vascularization *in vivo* (Datta et al., 2005; Pham et al., 2008).

This investigation yielded an unexpected discovery about the potential for decellularized ECM to induce hMSC osteogenesis. Although we predicted that osteogenic potential might increase over time in correlation with changes in BDM-ECM deposition, we were surprised to find that ECM deposited early in the osteoblast differentiation process (3d-hMSC-ECM) had higher osteogenic induction potential than did ECMs deposited for 8 or 15 days (Fig. 7). These results raise the possibility that as the osteoblast differentiation process progresses, the BDM-induced cells transform the underlying ECM from a composition and architecture that has greatest osteogenic induction potential to one that loses this potential but is better suited for eventual biomineralization. The increase in COL1 and BSP in the later stages of osteogenesis is consistent with this possibility.

The potential for decellularized ECMs to induce hMSCs into osteoblasts was observed previously, but the timing of osteogenesis induction potential remained ambiguous. Previous studies showed that BDM-ECM obtained by detergent decellularization exhibited high potential for osteogenesis early stages (Hoshiba et al., 2009), but BDM-ECM deposited on biodegradable microfibers surface in a flow perfusion bioreactor and decellularized by freeze/thaw cycles had greater osteogenesis potential at later stages (Liao et al., 2010). Other investigations indicate that complex interactions among several parameters, including cell density and specific culture conditions, may affect the timing of ECM deposition with the highest osteogenesis induction potential (Decaris and Leach, 2011). It is possible that varying degrees of ECM contamination during the different decellularization processes also could contribute to the variability in these results.

A third type of ECM deposited by two smooth muscle cell lines - rat aorta smooth muscle A7r5 cells and hTERT-myomSMCs – both induced naïve hMSCs to exhibit smooth muscle cell-like characteristics, as indicated by cell morphologies and increased expression of the smooth muscle cell marker SM-22 α (Fig. 9). It previously was established that ECM can induce hMSC to the smooth muscle cell lineage (Lozito et al., 2009; Suzuki et al., 2010). Our results indicate, however, that the A7r5-ECM and myomSMC-ECMs had different effects on the process of naïve hMSCs smooth muscle cell differentiation. The A7r5-ECM induced a higher level of α -SMA expression and incorporation into long, longitudinal actin structures in the naïve hMSCs, which are consistent with the morphological characteristics of the contractile SMC phenotype. In contrast, the existence of podosomes that lack α -SMA in the highly fusiform cells on the myomSMC-ECM is more consistent with the morphological characteristics of the synthetic SMC phenotype (Beamish et al., 2010; Lener et al., 2006). Moreover, the highly fusiform morphology of the confluent cells on the myomSMC-ECM, not found in the cells on the A7r5-ECM, had a dramatic elongation effect on nuclear morphology, which may in turn affect gene regulation in these cells (Heo et al., 2011).

To what degree these *in vitro* smooth muscle cells decellularized ECM-induced changes in hMSCs would translate into stable physiologically relevant changes in stem cells *in vivo* remains to be determined. Nevertheless, the possibility that decellularized ECMs can influence the phenotypic status of MSC smooth muscle-like cells *in vivo* may be biomedically relevant. Bone marrow-derived MSCs differentiate into synthetic SMCs during arterial stent restenosis (Caplice et al., 2003; Marx et al., 2011). Our results suggest that it

may be possible to direct MSCs toward the more desirable contractile phenotype behavior by pre-coating the stent with decellularized ECM deposited by an appropriate type of SMC. Other types of biomaterials such as wound dressings could be coated with ECM that induces MSC behavior more similar to that of the synthetic SMC phenotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- ECM deposited by several cell types including uninduced hMSCs, hMSCs induced to become osteoblasts with Bone Differentiation Medium (BDM), and two smooth muscle cell lines can be decellularized with a cold-EDTA treatment that preserves cell integrity and minimizes ECM damage and contamination.
- Decellularized ECMs deposited by undifferentiated hMSCs accelerate naïve hMSC proliferation and enhance maintenance of stemness for osteogenesis and adipogenesis.
- ECMs deposited by osteogenic hMSCs induce hMSC differentiation into osteoblasts in the absence of dexamethasone but the osteogenic potential is lost over the course of the BDM-induced ECM deposition.
- ECMs deposited by two smooth muscle cell lines induce hMSC differentiation into cells with distinctive phenotypic characteristics of synthetic and contractile smooth muscle cells.

Fig. 1. Decellularization of ECM deposited by cultured hTERT-myometrial smooth muscle cells. Superimposed DIC and fluorescent images of (A) hTERT-myomSMCs expressing mCherry plated at high density on uncoated glass coverslips and grown to confluence for 3 days and (B) rounded mCherry-expressing hTERT-myomSMCs treated with cold-EDTA. (C) DIC image of culture plate from which cold-EDTA treated cells were washed away with PBS. (D) Fluorescent image of (C). Immunofluorescent localization of ECM fibronectin in hTERT-myomSMC culture (E) prior to and (F) after cold-EDTA decellularization. Scale bars, (A-D) 100 μm ; (E and F) 20 μm . AFM images of ECM in cultures decellularized with (G) Triton-X-100 and (H) cold-EDTA. (G-H) AFM gray scale bars indicate z-axis dimensions.

Fig. 2. Proliferation of hMSCs on TCP and decellularized hMSC-ECM. HMSCs were plated on (A) an uncoated tissue culture plastic (TCP) dish and (B) a TCP plate on which hMSCs had been cultured in standard growth medium for 3 days prior to decellularization with cold-EDTA (ECM). Scale bar, 100 μ m. (C) Alamar blue assay of TCP and ECM cultures (n = triplicate samples from three trials; asterisk indicates significant difference of ECM mean from TCP mean with $p < 0.05$).

Fig. 3.

HMSC colony formation on TCP and decellularized hMSC-ECM. Examples of different size colonies (stained with crystal violet) grown for 14 days from hMSCs plated at low density on (A) TCP and (B) cold-EDTA decellularized ECM deposited by hMSCs cultured for 3 days in standard growth medium on TCP (ECM). The diameter of the small cluster of cells on the left in (A) is below the 1 mm cut off used for colony counting. Scale bar, 2.5 mm. (C) Number of colonies with diameters ≥ 1 mm grown from 300 cells plated on 3 plates (100 cells/plate) of TCP and decellularized hMSC ECM (entire bars) with the colonies in the 1-2.5 mm diameter range (dark gray area of bar) and in the ≥ 2.5 mm diameter range (light gray area of bar). ($P < 0.05$) (D) Colony diameter distribution ≥ 1 mm on TCP and decellularized hMSC ECM plotted with the box representing the 25-75 percentile range, the line in each box representing the median value, and the whiskers on each box representing the range of diameters in the 1-24 and 76-100 percentile ranges.

Fig. 4. Morphology of hMSCs on TCP and decellularized hMSC-ECM. Examples of the SS (A), FSS (B), RS (C), and FC (D) morphological categories scored for hMSCs plated on (F) TCP and on (G) cold-EDTA decellularized ECM deposited by hMSCs cultured for 3 days in standard growth medium on TCP (ECM). Tables in F and G indicate percentages of cells scored in each morphology category (n=200 total number of cells counted from triplicate samples; total cell area between two surfaces is significantly different, $p < 0.01$). Scale bar, 100 μm .

Fig. 5.

Focal adhesion formation and vinculin expression in hMSCs on TCP and decellularized hMSC-ECM. Immunofluorescent staining of vinculin in focal adhesions and focal adhesion length distribution in hMSCs cultured on TCP (A1, A2) and on (B1, B2) cold-EDTA decellularized ECM deposited by hMSCs cultured for 3 days in standard growth medium on TCP (ECM). Number of focal adhesions measured from 12 images of triplicate samples, $n=1500$, $p<0.01$. Scale bar, 20 μm . (C) Western blot of vinculin expression in hMSCs on TCP and ECM compared to GAPDH loading control. (D) Cell motility velocities over 10 minute intervals (300 minutes/cell) for hMSCs plated on TCP and ECM plotted with the box representing the 25-75 percentile range, the line in each box representing the median value, and the whiskers on each box representing the range of velocities in the 1-24 and 76-100 percentile ranges ($n = 40$ cells, asterisk indicates significant difference of ECM mean from TCP mean with $p<0.05$).

Fig. 6. Maintenance of stemness in naïve hMSCs cultured on decellularized 3d-MSC-ECM. (A-D) Naïve hMSCs cultured on TCP (A and C) or on decellularized ECM previously deposited by hMSCs cultured for 3 days in standard medium and decellularized with cold-EDTA (B and D) were recovered by trypsinization, replated on TCP, and induced with BDM (A and B; stained with Alizarin Red for biomineralization) or FDM (C and D; stained with Oil Red for lipid accumulation). Scale bar, 100 μ m. (E and F) Image areas covered by the Alizarin Red stain for biomineralization (E, n=7) as in A and B or by Oil Red stain for lipid accumulation (F, n=6) as in C and D. Asterisks indicate a significant difference between ECM and TCP mean values (p values, * <0.03 and ** <0.059).

Fig. 7.

Cell morphologies, alkaline phosphatase activity, and biomineralization of hMSCs cultured on decellularized ECM deposited by hMSCs cultured in standard medium (ECM) and in bone differentiation medium prior to cold-EDTA decellularization (BDM-ECM). (A1-2) cell morphologies and percentages of cells classified into SS, FSS, RS, and FC categories for hMSCs cultured for three days in media containing with β -glycerophosphate but lacking dexamethasone and ascorbic acid on (A1) ECM and (A2) BDM-ECM deposited by hMSCs induced for 3 days with BDM. (B) Alkaline phosphatase activity in cultures of hMSCs grown for 3 days on TCP, on decellularized ECM deposited by hMSCs induced with bone differentiation medium for 3 days (3d-hMSC-ECM), 8 days (8d-ECM), and 15 days (15d-ECM) prior to decellularization, and for decellularized 3d-hMSC-ECM only without added hMSCs (3d-hMSC-ECM(-)hMSCs). ($p < 0.05$) (C) Alizarin Red staining of biomineralization in hMSCs cultured on: (C1) TCP, (C2) 3d-BDM-hMSC-ECM, (C3) 8d-BDM-hMSC-ECM, and (C4) 15d-BDM-hMSC-ECM. Scale bar, 100 μm .

Fig. 8. Immunofluorescent localization of fibronectin (A, D, and G), collagen 1 (B, E, H), and bone sialoprotein (C, F, and I) in ECMs deposited by hMSCs induced with BDM for 3 days (A-C), 8 days (D-F), and 15 days (G-I) prior to decellularization with cold-EDTA. Scale bar, 10 μ m.

Fig. 9. Morphology and protein expression of hMSCs cultured on decellularized ECM deposited by A7r5 and myometrial smooth muscle cells. (A) Immunofluorescent staining of fibronectin underlying hMSCs cultured for 3 days on cold-EDTA decellularized ECM deposited by: (A1) hMSCs grown in standard growth media, (A2) A7r5 cells, and (A3) hTERT-myomSMCs-immortalized myometrial smooth muscle cells (hTERT-myomSMCs). (B) Staining of (B1, B4, B7) α -smooth muscle actin (α SMA) and (B2, B5, B8) total actin, with merged images (B3, B6, B9) in hMSCs cultured for 3 days on cold-EDTA decellularized ECM deposited by: (B1-3) hMSCs grown in standard growth media, (B4-6) A7r5 cells, (B7-9) hTERT-myomSMCs. Inset in B8 is an enlargement of a podosome-like structure indicated with the arrow. (C) Expression smooth muscle cell markers SM22 α and α SMA compared to GAPDH loading controls. All SM22 α bands were from one blot and α SMA bands from another blot and rearranged for Fig. consistency. (D) Morphologies and (E)

nucleus aspect ratios of DAPI-stained nuclei in hMSCs plated at high density and cultured to confluence on; (D1, CS) an uncoated coverslip, (D2,3d-hMSC-ECM) decellularized 3d-hMSC-ECM, (D3, A7r5-ECM) decellularized A7r5 cell ECM, and (D4, myomSMC-ECM) decellularized hTERT-myomSMC ECM plotted with the box representing the nucleus aspect ratios in the 25-75 percentile range, the line in each box representing the median value, and the whiskers on each box representing the range of ratios in the 1-24 and 76-100 percentile ranges ($n = 314$ cells for each surface; a and b indicate categories of statistical difference of means, $p < 0.01$). Scale bars, (A) 20 μm ; (B, D) 50 μm .