ACS Biomaterials SCIENCE & ENGINEERING

Methods/Protocols

Cite This: ACS Biomater. Sci. Eng. 2018, 4, 836−845

Isolation and Identification of Proteins Secreted by Cells Cultured within Synthetic Hydrogel-Based Matrices

Lisa A. Sawicki,[†] Leila H. Choe,[†,‡] Katherine L. Wiley,[†] Kelvin H. Lee,[†,‡] and April M. Kloxin*[,†,§]

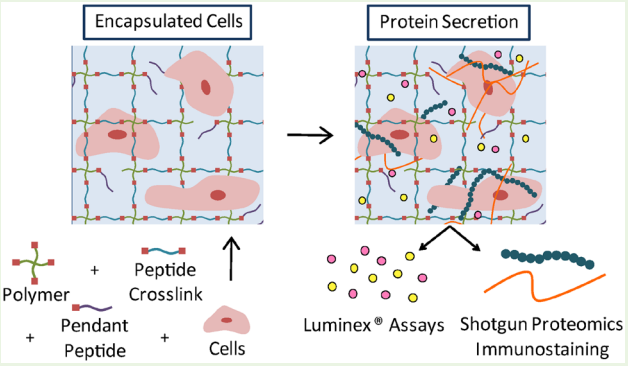
[†]Department of Chemical and Biomolecular Engineering, University of Delaware, Newark, Delaware 19716, United States

[‡]Delaware Biotechnology Institute, Newark, Delaware 19711, United States

[§]Department of Materials Science and Engineering, University of Delaware, Newark, Delaware 19716, United States

Supporting Information

ABSTRACT: Cells interact with and remodel their microenvironment, degrading large extracellular matrix (ECM) proteins (e.g., fibronectin, collagens) and secreting new ECM proteins and small soluble factors (e.g., growth factors, cytokines). Synthetic mimics of the ECM have been developed as controlled cell culture platforms for use in both fundamental and applied studies. However, how cells broadly remodel these initially well-defined matrices remains poorly understood and difficult to probe. In this work, we have established methods for widely examining both large and small proteins that are secreted by cells within synthetic matrices. Specifically, human mesenchymal stem cells (hMSCs), a model primary cell type, were cultured within well-defined poly(ethylene glycol) (PEG)-peptide hydrogels, and these cell-matrix constructs were decellularized and degraded for subsequent isolation and analysis of deposited proteins. Shotgun proteomics using liquid chromatography and mass spectrometry identified a variety of proteins, including the large ECM proteins fibronectin and collagen VI. Immunostaining and confocal imaging confirmed these results and provided visualization of protein organization within the synthetic matrices. Additionally, culture medium was collected from the encapsulated hMSCs, and a Luminex assay was performed to identify secreted soluble factors, including vascular endothelial growth factor (VEGF), endothelial growth factor (EGF), basic fibroblast growth factor (FGF-2), interleukin 8 (IL-8), and tumor necrosis factor alpha (TNF-α). Together, these methods provide a unique approach for studying dynamic reciprocity between cells and synthetic microenvironments and have the potential to provide new biological insights into cell responses during three-dimensional (3D) controlled cell culture.



KEYWORDS: *proteomics, Luminex, hydrogels, controlled cell culture, extracellular matrix*

INTRODUCTION

The extracellular matrix (ECM) of native tissues is complex and contains an ever-changing array of chemical and physical cues that direct cell function and fate.[1] Large insoluble proteins (e.g., collagen, fibronectin, laminin, elastin), proteoglycans (e.g., heparin sulfate), and polysaccharides (e.g., hyaluronic acid) provide structure and binding sites for cell adhesion and migration.[2−4] Cells remodel the ECM by secretion of enzymes (e.g., matrix metalloproteinases (MMPs)) that degrade existing protein structures and by deposition of new insoluble proteins that rebuild the structure. Further, cells secrete small soluble proteins (e.g., growth factors, cytokines, chemokines) that drive additional cellular functions and recruit other cells that further remodel this environment.[5−7] To reduce this complexity and enable hypothesis testing, well-defined, three-dimensional (3D) cell culture environments are of increasing interest to probe key biochemical and biophysical cues within the extracellular environment that play a role in the adhesion, function, and fate of cells, such as the spreading, migration, and differentiation of mesenchymal stem cells in response to different matrix compositions.[8−11] In particular, matrices made from synthetic (e.g., poly(ethylene glycol) (PEG), poly(caprolactone), poly(lactic acid))[12] or hybrid (e.g., chemically functionalized hyaluronic acid or gelatin)[9] materials with tunable mechanical properties (e.g., matrix modulus, or stiffness) may be modified with biochemical factors (e.g., receptor-binding peptides mimicking specific ECM proteins) to offer a high degree of property control. Thus, by independently selecting different biochemical and biophysical properties to incorporate within these matrices, their effects may be more easily decoupled and assessed.[13] While synthetic matrices are initially well-defined, cells remodel these microenvironments, as in native tissues, by depositing insoluble ECM proteins and secreting soluble biochemical factors for matrix remodeling and cell−cell signaling. These cell-secreted proteins may dynamically alter

Received: September 1, 2017
Accepted: February 2, 2018
Published: February 3, 2018

DOI: 10.1021/acsbiomaterials.7b00647
ACS Biomater. Sci. Eng. 2018, 4, 836−845

cell responses observed in synthetic matrices by adding to or masking binding sites presented by the initial matrix, and consequently, their identification is essential in characterizing and understanding cell response.[14−17] We hypothesize that proteomics-based techniques, which allow analysis of complex mixtures of proteins, can be applied to determine a variety of proteins that cells secrete within synthetic extracellular matrices toward ultimately identifying critical secreted factors in processes like wound healing, aging, and disease.[18−22]

Various proteomics techniques have been established to identify proteins secreted by cells in vivo and in vitro. Electrophoresis techniques (e.g., 2D-polyacrylamide gel electrophoresis (PAGE), 2D-difference gel electrophoresis (DIGE)) allow the separation of complex mixtures of proteins by mass and charge for subsequent analyses (e.g., gel imaging software and/or mass spectrometry) and protein identification.[23] In particular, 2D-PAGE has been a foundational tool for proteomics research, described in numerous applications including identification of proteins associated with wound healing and cancer with relevance for drug discovery.[24−26] Other front end separation techniques such as liquid chromatography (e.g., strong cation exchange (SCX), high performance liquid chromatography (HPLC)) have been used in conjunction with mass spectrometry (e.g., electrospray ionization (ESI), matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)) to identify proteins in mixed samples.[25] Specifically, shotgun proteomics is a bottom-up approach to analysis of a proteome: this technique often is performed by tandem liquid chromatography (LC) in conjunction with mass spectrometry on samples containing mixtures of proteins that have been digested with enzymes into their peptide subunits. While some of the complexity of protein structures may be lost as samples are digested prior to separation, this technique allows analysis of particularly complex protein samples where separation of whole proteins with traditional electrophoresis techniques (e.g., 2D-PAGE) would be more difficult.[27] More recently, high-throughput techniques have been developed and utilized for the detection of proteins, including ELISA microarrays and multiplex microbead assays. For example, commercially available Luminex and FirePlex kits have been utilized for the detection of multiple small-protein analytes within a single sample.[28−31]

Despite the range of tools now available to conduct proteomic analyses, analysis of large proteins (e.g., molecular weight >100 kDa) secreted into synthetic 3D microenvironments has focused on immunostaining-based techniques or biochemical assays, partly owing to challenges associated with translating assays from two-dimensional (2D) to 3D culture systems. Although insightful, immunostaining and biochemical assays typically require some initial knowledge of what specific proteins cells may be secreting. For example, the production of the ECM proteins laminin, collagen I, and elastin by human mesenchymal stem cells (hMSCs) and smooth muscle cells (SMCs) has been assessed by immunostaining in a degradable PEG hydrogel-based synthetic matrix for tissue regeneration.[32] Additionally, the synthesis of collagen and elastin by vocal fold fibroblasts within a PEG-diacrylate network has been observed qualitatively with immunohistochemical staining and quantitatively with biochemical assays: quantification of hydroxyproline content as a measure of total collagen with a colorimetric assay, amine content as a measure of elastin with an ninhydrin assay, and elastin by direct measurements with ELISA.[33] These approaches have provided insights into where and how cells are secreting specific proteins. However, immunostaining and ELISA both require the selection of specific antibodies against individual proteins, whereas ninhydrin and hydroxyproline assays provide a quantitative measure of the concentration of total protein in a sample. For identification of smaller secreted proteins (e.g., molecular weight <100 kDa), Luminex assays recently were applied to cells in 3D culture within synthetic or natural matrices, providing insight into a variety of growth factors and cytokines being secreted by human fibroblasts and breast cancer cells within different culture environments.[34] Identification of complex mixtures of both large and small proteins secreted by cells within synthetic matrices remains challenging, particularly for discovering proteins yet unknown to be important in a particular event.

In this work, we aimed to establish an approach for isolating and identifying proteins secreted by cells cultured within synthetic matrices utilizing proteomics-based techniques. hMSCs were encapsulated within a well-defined, MMP-degradable PEG-based hydrogel matrix with tunable mechanical properties and biochemical content.[35,36] After culture, these synthetic matrices were decellularized to remove some cellular structures and leave behind larger proteins for subsequent analysis. Shotgun proteomics was used to identify the presence of large secreted proteins, and immunostaining was used to confirm the presence and location of these proteins within the matrix. Additionally, we investigated soluble factors that were secreted into the culture medium via a Luminex assay. Taken together, the results of these studies demonstrate a promising set of tools that may prove useful in future studies to understand cellular remodeling of synthetic microenvironments and to identify secreted factors that may drive cell responses in conjunction with the initial synthetic matrix. These techniques and assays could be applied and adapted to investigate numerous other cell lines cultured within well-defined hydrogel-based culture models.

MATERIALS

Reagents and Supplies.

- 16% formaldehyde (w/v), methanol-free (Thermo Fisher Scientific, 28908)
- 48-well tissue culture plate with lid, individual, non-treated, sterile (Chemglass Life Sciences, CLS-3501−048)
- α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, 476870)
- Acetonitrile with 0.1% trifluoroacetic acid (v/v), Optima LC/MS grade (Fisher Scientific, LS121)
- Amicon Ultra-0.5 centrifugal filter unit with Ultracel-3 membrane (EMD Millipore, UFC500308)
- Amicon Ultra-0.5 centrifugal filter unit with Ultracel-10 membrane (EMD Millipore, UFC501024)
- ammonium bicarbonate (Fisher Scientific, A643)
- amphotericin B (Thermo Fisher Scientific, 15290018)
- BD syringe with slip (Luer) tips (without needle), 1 mL (Fisher Scientific, 14−823−16H)
- bovine serum albumin, BSA (Sigma-Aldrich, A7906)
- collagenase, type II, powder (Thermo Fisher Scientific, 17101015)
- DAPI (4′, 6-diamidino-2-phenylindole, dihydrochloride) (Thermo Fisher Scientific, D1306)
- dithiothreitol (Bio-Rad, 161−0611)
- Dulbecco's modified Eagle medium, DMEM, low glucose, pyruvate (Thermo Fisher Scientific, 11885092)
- Dulbecco's phosphate buffered saline, no calcium, no magnesium (Thermo Fisher Scientific, 14190250)
- ethylene glycol tetraacetic acid, EGTA (Santa Cruz Biotechnology, SC-3593A)
- fetal bovine serum, certified, US origin (Thermo Fisher Scientific, 16000044)
- goat anti-Mouse IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 488 (Thermo Fisher Scientific, A11001)

DOI: 10.1021/acsbiomaterials.7b00647
ACS Biomater. Sci. Eng. 2018, 4, 836−845

- goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 488 (Thermo Fisher Scientific, A11008)
- Hank's balanced salt solutions (Fisher Scientific, MT21022CV)
- human mesenchymal stem cells (Lonza, PT-2501)
- iodoacetamide (Sigma-Aldrich, I1149)
- magnesium chloride, $MgCl_2$ (Sigma-Aldrich, 208337)
- milliplex map human cytokine/chemokine magnetic bead panel − immunology multiplex assay (EMD Millipore, HCYTOMAG-60K, selected analytes vascular endothelial growth factor (VEGF), endothelial growth factor (EGF), basic fibroblast growth factor (FGF-2), interleukin 8 (IL-8), and tumor necrosis factor alpha (TNF-α))
- mouse anti-collagen type VI, 5C6 (Developmental Studies Hybridoma Bank, 5C6, supernatant)
- mouse anti-fibronectin (Abcam, ab26245)
- NP-40 (Abcam, ab142227)
- OMIX C18 pipet tips (Agilent, A57003100)
- PEG4SH (M_n ~ 20 kDa with >85% thiol functionality per multiarm PEG; synthesized and characterized by published protocols);[36,37] also available from commercial sources such as JenKem and Creative PEGworks
- peptides (K(alloc)GWGRGDS; KK(alloc)GGPQGIWGQGK-(alloc)K) synthesized and characterized by published protocols;[36] also available for purchase commercially from custom peptide synthesis vendors
- penicillin-streptomycin (5,000 U/mL) (Thermo Fisher Scientific, 15070063)
- phalloidin-tetramethylrhodmaine B isothiocyanate (Phalloidin-TRITC) (Sigma-Aldrich, P1951)
- Pierce formic acid, LC-MS grade (Thermo Fisher Scientific, 28905)
- potassium chloride, KCl (Sigma-Aldrich, P9541)
- ProteoMass adrenocorticotropic hormone (ACTH) fragment 18−39 MALDI-MS standard (Sigma-Aldrich, A8346)
- rabbit anti-vimentin (Abcam, ab92547)
- recombinant human FGF-basic (154 a.a.) (FGF-2) (PeproTech, 100−18B)
- sequencing grade modified trypsin (Promega, V5111)
- sodium phosphate dibasic, Na_2HPO_4 (Sigma-Aldrich, S5136)
- Triton X-100 (Fisher Scientific, BP151−100)
- trypsin-EDTA (0.5%), no phenol red (Thermo Fisher Scientific, 15400054)
- water with 0.1% trifluoroacetic acid (v/v), Optima LC/MS grade (Fisher Scientific, LS119)
- general: micropipette tips, microcentrifuge tubes, cell culture plates or flasks, serological pipettes, spatulas, DI water, syringe needles

Equipment.

- Chromolith CapRod RP-18e, 150−0.1 mm (Merck)
- Exfo Omnicure Series 2000 mercury arc lamp with collimating adaptor and 365 nm filter
- hemacytometer (such as Reichert Bright-Line) or cell counter
- Luminex system (Thermo Fisher Scientific, LX 100/200)
- MALDI mass spectrometer (AB Sciex, TOF/TOF 5800)
- Tempo LC-MALDI spotter (AB Sciex)
- Zeiss LSM 800 confocal microscope (or appropriate epifluorescent or confocal microscope)
- General: micropipettes, incubator (37 °C and 5% CO_2), BSL2-rated biosafety cabinet, freezer (−20 °C and −80 °C), lyophilizer (here, Labconco FreeZone 4.5 Plus), Centrifuge (here, Bioexpress GeneMate SpinMate 24R)

PROCEDURE

Overview. Step 1: Hydrogel preparation, cell culture, and encapsulation

Step 2: Collection of culture medium for Luminex assay (Video S1, 00:38)

Step 3: Decellularization and isolation of proteins from hydrogel-based matrices (Video S1, 02:43)

Step 4: Shotgun proteomics (Video S1, 05:58)

Step 5: Shotgun proteomics data processing and analysis

Step 6: Immunostaining proteins in hydrogel matrices

Step 7: Preparation of samples for Luminex assay (Video S1, 01:52)

Step 8: Luminex data processing and analysis

1. Hydrogel Preparation, Cell Culture, and Encapsulation. Cells (here, hMSCs) were encapsulated within hydrogel-based matrices by previously established methods. Below is a brief version of these methods that were previously described.[35,36]

1. Dissolve macromers [4-arm poly(ethylene glycol) thiol (PEG4SH, M_n ~ 20 kDa); enzymatically degradable cross-link Ac−K**K(alloc)**G[GPQG↓IWGQ]G**K(alloc)**K (Pep2Alloc); and pendant peptide **K(alloc)**GWGRGDS (fibronectin/vitronectin mimic, RGDS)] in Dulbecco's phosphate buffered saline (DPBS) supplemented with 1% penicillin/streptomycin (PS) and 0.5 μg/mL Amphotericin-B (AB).
2. Mix macromer stock solutions at 6 wt % (w/v) PEG4SH, 2 mM RGDS, and stoichiometric ratios of Pep2Alloc (final [SH] = [Alloc]) to prepare the hydrogel precursor solution. Add 2.2 mM of the photoinitiator lithium acyl-phosphinate (LAP) to this macromer solution.

   Note: Here, a previously described hydrogel system has been used to establish methods for utilizing proteomics techniques for examining proteins secreted by cells within synthetic matrices. Specifically, a single peptide sequence (RGDS) and matrix density (6 wt %) were selected as a model culture system for the purposes of developing the technique. This particular composition was selected because of frequent and broad use of RGDS for promoting the adhesion and survival of cells within synthetic matrices and the previous observation of cell spreading over days within this matrix density, suggesting some degree of cell-driven remodeling of the initial matrix.[17] With this approach now established, the effects of different peptides or matrix densities and moduli on the profile of secreted proteins may be examined in future studies. Further, if an alternative synthetic matrix is used, materials for cell encapsulation should be prepared per standard procedures for that system.
3. Maintain hMSCs in low glucose (1 g/L) Dulbecco's modified Eagle medium with sodium pyruvate (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% PS, 0.5 μg/mL AB, and basic fibroblast growth factor (FGF-2, 1 ng/mL). Feed every 48 h during culture.

   Note: Here, for Luminex experiments, FGF-2 was selected as a target analyte. If secreted FGF-2 will be assayed as done here, 48 h prior to collecting cells for encapsulation, feed cells with fresh culture medium without FGF-2 (DMEM+FBS+PS+AB only).
4. Apply trypsin-EDTA (0.05%) to hMSC (P6−P7) at 70−80% confluency for approximately 9 min. Check that cells have detached from culture plate under a light microscope.
5. Count cells with a hemacytometer (or similar) per manufacturer's instructions, counting a minimum of 100 cells.
6. Take an aliquot of cell suspension with desired number of cells for encapsulation (here, 5000 cells per μL of gel) and centrifuge (5 min at 94g).
7. Remove supernatant and resuspend cell pellet in hydrogel precursor solution to achieve desired cell density (5000 cells/μL) within the matrix.

DOI: 10.1021/acsbiomaterials.7b00647
ACS Biomater. Sci. Eng. 2018, 4, 836−845

8. Encapsulate cells within hydrogels for subsequent analysis with shotgun proteomics, Luminex, or immunostaining:
   (a) Pipette 10 μL of cell suspension into the tip of a sterile, cut 1 mL syringe. Use pipet tip to evenly spread the cell suspension across the mold.
   (b) Apply collimated light of 10 mW/cm^2 at 365 nm for 1 min using Exfo Omnicure (or similar).
9. Place hydrogels into individual wells of a nontreated 48-well plate and rinse 2× with 0.5 mL of fresh culture medium.
10. Feed cells encapsulated within hydrogels every 48 h with 0.5 mL of fresh culture medium until sample collection.

Note: The time at which samples are collected should be determined by the user. Here, samples were collected at 10 days in culture to ensure sufficient time for matrix remodeling, as indicated by cell spreading, and for protein deposition based on the literature for cells cultured in similar synthetic matrices.[32,34]

2. Collection of Culture Medium for Luminex Assay.

1. Transfer individual hydrogels to wells of a fresh, nontreated 48-well plate 48 h prior to collection of culture medium.
2. Feed cells encapsulated in hydrogels with 0.5 mL of fresh culture medium (DMEM+FBS+PS+AB).
   Note: Here, samples were collected 10 days after encapsulation. Thus, at 8 days post-encapsulation, cells were transferred and fed with fresh culture medium.
3. After 48 h, remove culture medium from each well and place into individual, sterile, 1.5 mL microcentrifuge tubes.
4. Centrifuge media for 5 min at 94*g* to pellet any debris that may have been collected.
5. Collect supernatant with a 1 mL micropipette and place into a sterile microcentrifuge tube, being careful not to transfer any pelleted debris.
6. Store samples at −20 °C until assayed.

3. Decellularization and Isolation of Proteins from Hydrogel-Based Matrices. Decellularize hydrogel matrices using a modified version of a previously described technique[38,39] as detailed below.

1. Prepare wash and lysis buffers for matrix decellularization:
   (a) Wash buffer 1: Dissolve 100 mM Na_2HPO_4 in DI water and adjust to pH 9.6. Add 2 mM $MgCl_2$ and 2 mM EGTA.
   (b) Lysis buffer: Dissolve 8 mM Na_2HPO_4 in DI water and adjust to pH 9.6. Add 1% NP-40.
   (c) Wash buffer 2: Dissolve 300 mM KCl and 10 mM Na_2HPO_4 in DI water and adjust to pH 7.5.
2. Decellularize hydrogels with washing and lysis buffers in preparation for shotgun proteomics (0.5 mL of buffer and 37 °C for each incubation):
   (a) Rinse hydrogels 2 × 15 min with DPBS to remove culture medium.
   (b) Place hydrogels into sterile 1.5 mL microcentrifuge tubes (2 gels per tube).
   (c) Rinse hydrogels 2 × 15 min with wash buffer 1.
   (d) Treat cells 1 × 30 min with lysis buffer.
   (e) Replace buffer with fresh lysis buffer and incubate an additional 1 × 60 min.
   (f) Rinse hydrogels 2 × 15 min with wash buffer 2.
   (g) Rinse hydrogels 4 × 15 min with DI water.
3. Add 0.5 mL of collagenase (50 U/mL in Hank's Balanced Salt Solution, HBSS) to tubes containing decellularized samples and degrade for 1 h at 37 °C to degrade the hydrogels, releasing any proteins that it contains.
   Note: Here, a enzymatically degradable peptide crosslink was used within the hydrogel-based matrices, specifically the sequence GPQG↓IWGQ (a tryptophan variant of the wild type sequence found in collagen I) that degrades in response to a variety of matrix metalloproteinases including MMP-1, MMP-2, and MMP-9;[40] inclusion of this sequence allows both cell-driven degradation of the synthetic matrix during culture and triggered degradation with collagenase for harvesting of proteins. Trypsin or other degradation enzymes may be used if appropriate.
4. Vigorously triturate the sample with a 200 μL micropipette to mix sample and check that the hydrogel is completely degraded. Incubate for an additional 15 min if hydrogel particulate remains, pipetting between incubations to check for complete degradation.
5. Cap the microcentrifuge tube that contains the degraded matrix, and carefully pierce a small hole into the top of the microcentrifuge tube with a syringe needle before placing samples in a freezer at −80 °C.
6. Lyophilize degraded samples and store dried product at −80 °C for subsequent treatments to run proteomic analyses.

Note: Decellularization and degradation may be performed in nonsterile conditions; however, care should be taken not to introduce any contaminants (e.g., dust or debris) into samples as this may interfere with subsequent proteomics analysis.

4. Shotgun Proteomics. Shotgun proteomic analyses were performed as described by Valente et al.[41] with some modifications as described below:

1. Reconstitute lyophilized hydrogel samples in 200 μL of 25 mM ammonium bicarbonate. To the 200 μL sample:
   (a) Add 100 mM dithiothreitol, and incubate for 25 min at 95 °C.
   (b) Add 150 mM iodoacetamide, and incubate for 30 min.
   (c) Add 20 μg of trypsin, and incubate overnight at 37 °C to digest proteins.
2. Acidify digested samples with formic acid to pH <4.
3. Load samples onto 10 kDa MWCO spin columns and separate peptides from PEG, trypsin, and collagenase per manufacturer's instructions (centrifuge at 14,000*g* for 15 min). Retain the bottom fraction, which contains digested peptide fragments.
4. Desalt peptides using C18 OMIX tips (Agilent) per manufacturer's instructions.
5. Perform low pH reverse phase-HPLC (RP-HPLC, pH 2) on a Tempo LC-MALDI Spotter with an acetonitrile gradient in 0.1% trifluoroacetic acid over a 110 min program.
6. Deposit eluate onto a MALDI target plate every 10 s with alpha-cyano-4-hydroxycinnamic acid matrix (HCCA, 7.5 mg/mL) spiked with ACTH (5 nmol/mL).
7. MALDI mass spectrometry data were collected with 1000 laser shots per spot over a mass range of 800−4000 m/z with internal calibration. Up to 15 peaks, above signal/noise 20, per spectrum were selected for tandem mass spectrometry (MS/MS), and acquired with 2000 laser shots per precursor.

Note: Shotgun proteomic analysis can be performed with any standard HPLC, configured for peptide separation, and

DOI: 10.1021/acsbiomaterials.7b00647
ACS Biomater. Sci. Eng. 2018, 4, 836−845

either a MALDI or electrospray platform tandem mass spectrometer.

5. Shotgun Proteomics Data Processing and Analysis.

1. Submit combined MS and MS/MS data to Protein Pilot software (v4.5, ABSciex) for protein database searches against the human taxonomy of NCBInr. Select search processing via Paragon method, and select the following parameters: cys alkylation method (iodoacetamide), enzyme (trypsin), instrument (5800), search effort (Thorough ID).
   Note: Protein Pilot is data analysis software, which when used with ABSciex mass spectrometers can directly access data in the mass spectrometer's Oracle database. Search results contain a list of database matches with various metrics such as rank, sequence coverage %, and number of peptides at 95% confidence interval. For this study, only protein matches with at least one peptide with 95% confidence were considered for protein identification.
2. Input accession numbers of any hypothetical or unnamed proteins to identify additional matches not found in the Protein Pilot software to the Basic Local Alignment Search Tool (BLAST, NIH), using the blastp algorithm.
   Note: BLAST compares protein sequences to known sequence databases and provides statistical significance of matches based on similarity.

6. Immunostaining Proteins in Hydrogel-Based Culture Matrices.

1. Prepare blocking and permeabilization solutions:
   (a) BPSoln1: Dissolve 3% w/v bovine serum albumin (BSA) and 0.05% v/v Triton X-100 in DPBS.
   (b) BPSoln2: Dissolve 5% BSA w/v and 0.1% v/v Triton-X in DPBS.
2. Rinse hMSCs cultured for 10 days in hydrogels 2 × 5 min with DPBS.
3. Dilute 16% paraformaldehyde stock solution to 4% in DPBS and apply to samples for 15 min for fixation.
4. Wash samples 1 × 5 min in DPBS and 2 × 5 min in BPSoln1.
5. Incubate hydrogels for 1 h at room temperature with BPSoln2 to block and permeabilize.
6. Incubate samples with primary antibodies (here, anti-Collagen VI, 10 μg/mL; anti-fibronectin, 10 μg/mL; anti-vimentin, 1 μg/mL in BPSoln2) overnight at 4 °C with primary antibodies.
   Note: Optimal antibody dilution should be determined by the user, typically via a titration, for visualization of protein(s) of interest.
7. Rinse hydrogels 3 × 1 h in BPSoln1.
8. Incubate samples with secondary antibodies overnight at 4 °C in BPSoln2 (here, phalloidin-TRITC, 1:250 dilution; goat-antimouse Alexa Fluor 488, 1:300 dilution; goat-antirabbit Alexa Fluor 488, 1:300 dilution).
9. Rinse hydrogels 3 × 45 min in BPSoln1.
10. Incubate hydrogels for 1 h with DAPI (700 nM in DPBS).
11. Rinse hydrogels 3 × 30 min in DPBS.
12. Store samples at 4 °C, protected from light until imaging.
13. Image on an epifluorescence or confocal microscope. Here, a Zeiss LSM 800 confocal microscope was used (z-stacks, 100 images per stack, 2 μm spacing).

7. Preparation of Samples for Luminex Assay.

1. Thaw stored culture media samples to room temperature.
   Note: If desired, concentrate analytes with 3 kDa spin columns. Add 400 μL of sample to spin columns and concentrate analytes per manufacturer's protocol, retaining the concentrate.
2. Perform Luminex assay per manufacturer's instructions. Here, an EMD Millipore kit containing VEGF, EGF, FGF-2, IL-8, and TNF-α was selected as a diverse array of factors relevant for various hMSC functions.

8. Luminex Data Processing and Analysis.

1. Analyze data using the xPONENT Software with a 5-parameter logistic.
2. Invalidate standard curve points that do not fit according to parameters described in the Luminex manual as deemed appropriate by the user, for example:
   (a) Visually inspect the curve and invalidate points where the curve plateaus (i.e., signal saturation) or any abnormal curve fits.
   (b) Invalidate standard points with net MFI values that are too close to those of the background (i.e., inadequate signal).
3. Analyte concentrations are calculated with the xPONENT software after validating the standard curves for each analyte.

TIMING

Step 1: Hydrogel preparation, cell culture, and encapsulation

- encapsulation = 4 h
- culture = days until time point(s) of interest

Step 2: Collection of culture medium for Luminex assay

- 15 min

Step 3: Decellularization and isolation of proteins from hydrogel-based matrices

- buffer preparation = 30 min
- decellularization = 4 h
- degradation/isolation = 1 h
- lyophilization = 1 day

Step 4: shotgun proteomics

- digestion = overnight
- sample preparation = 30 min
- RP-HPLC Gradient = 2 h

Step 5: Shotgun proteomics data processing and analysis

- 1 h

Step 6: Immunostaining proteins in hydrogel matrices

- day 1 = 2 h
- day 2 = 3 h
- day 3 = 5 h

Step 7: Preparation of samples for Luminex assay

- Thawing samples and concentrating via spin column = 1 h
- Luminex = 2 days (overnight incubation)

Step 8: Luminex data processing and analysis

- 1 h

Note: The procedure is for a multiday experiment. Steps may be performed out of order if samples are stored properly as described in the protocol.

TROUBLESHOOTING

Step 3: Decellularization and degradation process

DOI: 10.1021/acsbiomaterials.7b00647
ACS Biomater. Sci. Eng. 2018, 4, 836−845

If samples are larger than 20 μL in volume, incubation times may need to be increased for the decellularization process. Inspect these samples after incubation with decellularization buffer. If encapsulated cells appear intact under a light microscope, incubate with fresh lysis buffer for additional 60 min increments.

Step 4: Shotgun proteomics

If protein concentrations are too low to detect, increasing cell density or pooling samples may help with detection by increasing total protein content.

Step 6: Immunostaining proteins within hydrogel-based matrices

Here, collagen VI, fibronectin, and vimentin were stained. Different primary antibodies may be selected for different proteins identified via proteomics analyses.

Step 7: Preparation of samples for Luminex assay

If desired, samples with low concentrations of analytes may be concentrated using spin columns. Preliminary measurements with Luminex should be run by the user to determine if concentrating samples is necessary to detect analytes secreted at low concentrations.

Here, 3D cell culture samples were cultured without FGF-2 since FGF-2 secretion was assayed with the Luminex kit. While FGF-2 often is included within growth medium for hMSCs to maintain stem-ness, hMSCs can be cultured and propagated without it. More broadly, care must be taken in changing media compositions for cell types of interest, as cell response may change. As a result, not all available Luminex analytes may be appropriate to include in the assay if media components are critical in driving or maintaining phenotype.

■ ANTICIPATED RESULTS

Shotgun proteomics identified large ECM, membrane, and intracellular proteins from cells cultured within hydrogel matrices (abbreviated results, Table 1; full results, Table S1). Protein identification was determined by high confidence matches (>95% confidence, >1.85 contribution) reported from a database search. In these cultures that begin as single cell suspensions within a synthetic matrix rich in the integrin-binding peptide RGDS, we expected to observe hMSCs producing ECM proteins associated with matrix remodeling and potentially the early stages of differentiation, such as fibronectin that natively is produced by hMSCs during the early stages of cartilage development.[42−44] Notably, collagen VI and fibronectin, which have previously been identified as secreted by hMSCs,[45−48] were observed during culture within these synthetic matrices, indicating that the techniques described allowed isolation and identification of relevant large ECM proteins. Although we are primarily interested in the identification of large secreted ECM proteins, vimentin, and several other intracellular proteins also were identified. We can take advantage of the identification of both extra- and intracellular proteins to make additional observations about cell phenotype and function within these matrices. Specifically, proteins associated with a mesenchymal phenotype (vimentin) and several associated with hMSC matrix remodeling activities and potential differentiation (collagen VI, actin, fibronectin, tubulin) were identified.[42,49,50]

Immunostaining was used to confirm the presence of the proteins identified using the shotgun proteomics approach and verify their location within 3D culture. Strong staining was observed for each protein (Figure 1A) when compared to

Table 1. Abbreviated Table of Results for Proteins Identified by Shotgun Proteomics

accession number	protein name	confidence (%)
gil4502027	serum albumin preproprotein	99
gil31874109	hypothetical protein*	99
	*fibronectin (BLAST search)	
gil62896523	vimentin variant	99
gil55743106	collagen alpha-3(VI) chain isoform 5 precursor	99
gil49457374	HIST1H4F	99
gil62897625	beta actin variant	99
gil73909156	annexin A2	99
gil62087582	H2A histone family, member V isoform 1 variant	99
gil73762521	delta-globin Troodos variant	99
gil87196339	collagen alpha-1(VI) chain precursor	99
gil49456871	TUBB	99
gil189053217	unnamed protein product*	99
	*peroxiredoxin (BLAST search)	
gil156104889	protein AF-9 isoform a	99
gil21739834	hypothetical protein*	99
	*AP-5 complex subunit beta-1 (BLAST search)	
gil258690785	estrogen receptor alpha 3,4,5,6,7,8/1068 isoform, partial	99
gil68533107	MYH10 variant protein	99
gil2330597	MHC class I antigen	99
gil74099694	sulfite oxidase, mitochondrial	99

negative control (secondary antibody only, Figure 1B). Immunostaining previously has been described to identify proteins secreted by hMSCs cultured within hydrogels, as well as protein localization within networks.[32,33] Here, we observed localization of fibronectin and collagen VI in the pericellular matrix while vimentin was localized within the cell body as previously reported. Local erosion of MMP-cleavable peptides around the pericellular region may provide space for accumulation of secreted large ECM proteins, whereas the cross-link density within the bulk of the hydrogel remains high enough to hinder elaboration of these large biomolecules through the hydrogel mesh.[17,51] Some amount of large secreted proteins also may diffuse out of the synthetic matrix over time owing to lack of specific interactions with the hydrogel network. The combination of shotgun proteomics followed by immunostaining presented here provides broad insight into the types of proteins present and their distribution within the matrix, which will vary for based on the culture system used (e.g., synthetic matrix design, cell type and density, culture conditions).

Soluble factors secreted by hMSCs have been implicated in wound healing and disease processes (e.g., tissue repair and regeneration,[52,53] cancer progression and tumor growth[54]). Here, we used a Luminex multiplex bead assay to identify the presence of soluble factors and their relative concentrations secreted by hMSCs into culture medium. A 6-plex panel targeting VEGF, EGF, FGF-2, IL-8, and TNF-α was selected for the identification of factors associated with inflammation and wounding healing conditions.[55−57] Growth medium collected from hMSCs and growth medium alone (control) were assayed, and concentrations of analytes identified (Table 2). Low concentrations of FGF-2, with no detectable concentrations of EGF and TNF-α, were present in the media collected from hMSCs cultured in the hydrogel-based matrices. IL-8, a pro-inflammatory cytokine that has been shown to promote cell migration in wounding and cancer metastasis,[58,59] was found to be secreted at high

DOI: 10.1021/acsbiomaterials.7b00647
ACS Biomater. Sci. Eng. 2018, 4, 836−845

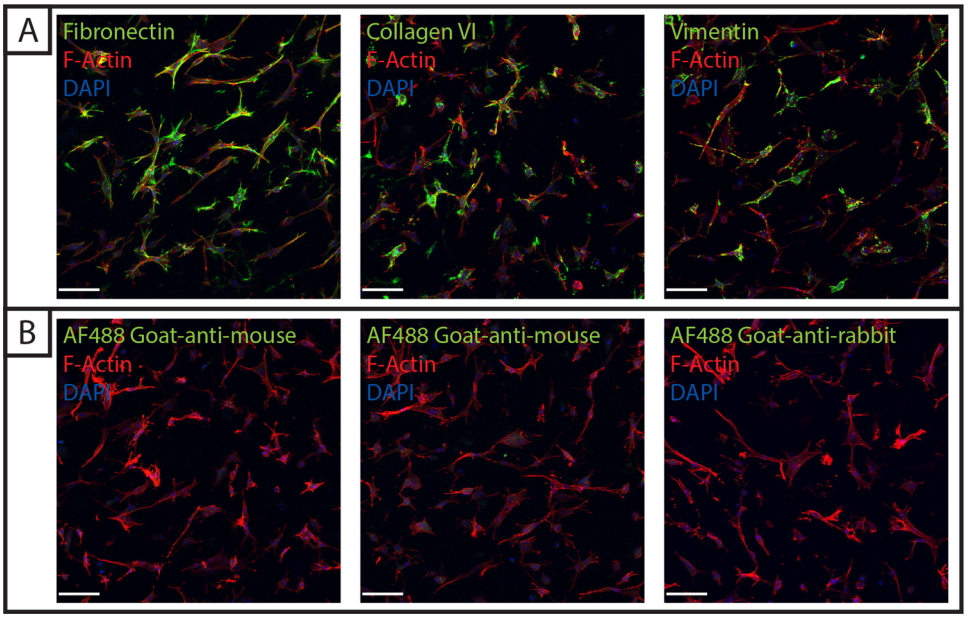


Figure 1. Immunostaining to confirm presence and location of proteins identified by shotgun proteomics. (A) Samples were stained for fibronectin, collagen VI, and vimentin, which were identified by shotgun proteomics. (B) Negative controls with only secondary antibodies applied were used to confirm specific positive staining for each protein in A. Z-stack projections of confocal microscopy images, 100 μm scale bar.

Table 2. Concentration of Analytes (pg/mL) Secreted by hMSCs in 3D Culture[a]

sample	EGF	FGF-2	IL-8	TNF-α	VEGF
hMSC	<16	516.89 ± 41.98	7773.18 ± 1231.20	<3.2	6118.68 ± 259.21
background, culture medium	<16	<400	<3.2	<3.2	<400

[a]Note, readouts reported as less than a specific value indicate that negligible concentrations of factors were present in the sample, so the minimum value accepted for the standard curve concentration is reported. Standard error values for sample concentrations are reported (n > 5).

concentrations. Additionally, high levels of VEGF secretion were observed, which promotes tissue regeneration and wound healing particularly angiogenesis.[60−62] These data indicate that hMSCs are secreting soluble factors involved in regulation of wound healing and tissue repair, matching those reported in the literature.

SUMMARY/DISCUSSION

In the human body, cells remodel their microenvironments in response to stimuli experienced during wound healing, aging, and disease, secreting an array of proteins both small and large that ultimately regulate cell functions and phenotype. The use of 3D polymer-based scaffolds and matrices for cell culture applications has expanded our ability to test hypotheses about specific cell−microenvironment interactions. However, how cells remodel these initially well-defined synthetic matrices is less understood and may be an important part of observed cell responses. Numerous proteomic techniques to identify proteins secreted by cells are available; however, application of these techniques to cells cultured within synthetic matrices can prove challenging due to sample type and complexity.[24,63] Shotgun proteomics permits the analysis of complex mixtures of proteins without information a priori about the proteins present within the sample. We selected this technique for analyses of proteins secreted within our hydrogel-based culture system because of the broad insight that it can provide and the ease of protein isolation with the application of enzymes for facile integration within existing proteomic workflows.

Whole proteins entrapped within synthetic matrices are difficult to isolate due to the high abundance of polymers relative to proteins and potential entanglements of polymers with proteins. Additionally, the removal of the polymer often is required to prevent potential interference with mass readings (e.g., increased molecular weight of proteins due to PEG-protein interactions). We have incorporated a MMP-degradable peptide cross-link to permit cell-driven degradation within our hydrogel networks. Importantly, we also capitalize on its degradability to selectively digest hydrogels by cleavage of this peptide upon the exogenous application of enzymes (here, collagenase). With this, hydrogel degradation and PEG removal was easily incorporated into the workflow for shotgun proteomics. Hydrogel-based matrices containing large cell-secreted proteins were digested with collagenase prior to further degradation of both secreted proteins and any remaining PEG-peptide matrix oligomers by trypsin. The resulting digested-protein fragments (peptides) were separated from large synthetic matrix fragments using a spin column in preparation for analysis by column chromatography and mass spectroscopy.[41] A notable benefit to using shotgun proteomics for this analysis is that preservation of protein structure is not necessary as peptides are used to identify whole proteins from analysis. However, if alternate modes of degradation that preserve protein structure (e.g., photodegradation, hydrolysis, reversible chemistries)[64,65] are used, this protocol can be easily adjusted accordingly.

Although we were most interested in identifying large ECM proteins secreted within our hydrogel networks, intracellular proteins also were identified. We hypothesize that intracellular proteins remain within the hydrogel after decellularization owing to the strong binding of hMSCs to presented ligands (RGDS), as indicated by robust cell spreading and F-actin stress fiber

DOI: 10.1021/acsbiomaterials.7b00647
ACS Biomater. Sci. Eng. 2018, 4, 836−845

formation, and the small pore size of the matrix ($\xi \sim 10$ nm) relative to the size of these large proteins. Here, the detergent NP-40 was used to decellularize matrices and allow removal of nuclear components.[39] Alternative techniques for decellularization[66] to remove cytoskeletal components (e.g., actin, vimentin), or whole cells completely via centrifugation after gel degradation, could be investigated in future studies if intracellular protein removal is desired. However, the current approach allows sample preparation (decellularization and degradation) with relative ease and straightforward incorporation into the shotgun proteomics workflow. This method is promising for the identification of proteins secreted by other cell types or in response to extracellular stimulus (e.g., peptides that promote specific binding, addition of cytokines) within synthetic microenvironments.

Traditionally, immunostaining has been used to identify large proteins secreted within synthetic 3D culture matrices due to challenges associated with the isolation of proteins from within a cross-linked material.[32,33,45,48] However, this technique is limited in that the user must select a panel of antibodies targeting proteins of interest. Here, we pair shotgun proteomics and immunostaining to circumvent the need for a priori knowledge of what proteins the cells may be secreting. Following shotgun proteomics with immunostaining provides both validation of protein identifications and insight into how identified proteins are presented within the cell microenvironment. With this approach, we observed localization of fibronectin and collagen VI within the pericellular matrix, suggesting that local binding sites provided by the synthetic matrix initially (e.g., RGDS) may be masked by these secreted proteins at late culture times.

To gain a more complete picture of proteins secreted by cells within synthetic networks, soluble factors secreted into culture medium during 3D culture were identified by a Luminex assay. A major benefit to Luminex is that sample volumes required to assay multiple analytes are small. Consequently, factors secreted by multiple cell lines may be evaluated with a single kit and small volumes of collected media. Additionally, kits are commercially available for a number of analytes or may be developed to target analytes of interest that are not currently available in predesigned kits. Here, we investigated factors secreted into culture medium and identified the presence of FGF-2, IL-8, and VEGF. A recent study also demonstrated the use of Luminex assays for identifying cytokines secreted by cells cultured within PEG-peptide hydrogel matrices that can be degraded upon the exogenous application of Sortase A, a bacterial (S. Aureus) transpeptidase that cleaves between threonine and glycine in a LPXTG recognition motif upon the addition of a triglycine peptide.[65] Specifically, cytokines and other small secreted factors entrapped within matrices were identified by Luminex after release from synthetic matrices following Sortase A-mediated hydrogel degradation. Incorporation of sites for Sortase-A-mediated cleavage reactions within other synthetic matrices could be used to identify entrapped secreted factors in tandem with the technique presented in this protocol to identify factors secreted into culture medium, allowing researchers to complete the "mass balance" on small cell-secreted factors within synthetic culture systems.

In conclusion, we have established techniques to identify both large and small proteins secreted by cells within synthetic hydrogel-based matrices. A shotgun proteomics approach permitted the identification of several large proteins isolated from decellularized hydrogel samples, and their presence confirmed by immunostaining. A Luminex multiplex bead assay identified hMSC secretion of factors associated with wound healing and disease. Together these techniques can be used for the identification of unknown soluble and insoluble proteins secreted by cells within synthetic matrices. This approach utilizing proteomic assays for probing cell responses within well-defined culture systems is promising for future application in identifying proteins secreted by other cell types, understanding cell response to applied stimuli, and the rational design of new matrix materials for tissue engineering and cell culture applications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsbiomaterials.7b00647.

Complete list of proteins identified by shotgun proteomics, list of unnamed or hypothetical proteins identified by BLAST search (PDF)

Video S1, techniques for the isolation and identification of proteins secreted by cells cultured within synthetic matrices (MPG)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: akloxin@udel.edu.

ORCID

Kelvin H. Lee: 0000-0003-0908-2981

April M. Kloxin: 0000-0002-4594-2953

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank the NSF SBE2 IGERT program at the University of Delaware (fellowship awarded to Lisa Sawicki), the Institutional Development Award from NIH for COBREs (P20GM104316 and P30GM110758-01), the Susan G. Komen Foundation Career Catalyst Research Grant (CCR16377327), and the Pew Charitable Trusts (00026178) for funding. Additionally, the authors thank Prof. Justin Cooper-White for helpful discussions related to diffusion of large secreted proteins out of hydrogel-based synthetic matrices. The monoclonal antibody mouse anti-collagen type VI (5C6) developed by E.S. Engvall was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242.

■ REFERENCES

(1) Alberts, B.; Johnson, A.; Lewis, J.; Raff, M.; Roberts, K.; Walter, P. Cell Junctions, Cell Adhesion, and the Extracellular Matrix. In *Molecular Biology of the Cell*, 4th ed.; Garland Science: New York, 2002; pp 1065−1125.

(2) Hynes, R. O. The Extracellular Matrix: Not Just Pretty Fibrils. *Science* **2009**, *326* (5957), 1216−1219.

(3) Bosman, F. T.; Stamenkovic, I. Functional Structure and Composition of the Extracellular Matrix. *J. Pathol.* **2003**, *200*, 423−428.

(4) Theocharis, A. D.; Skandalis, S. S.; Gialeli, C.; Karamanos, N. K. Extracellular Matrix Structure. *Adv. Drug Delivery Rev.* **2016**, *97*, 4−27.

(5) Bonnans, C.; Chou, J.; Werb, Z. Remodelling the Extracellular Matrix in Development and Disease. *Nat. Rev. Mol. Cell Biol.* **2014**, *15* (12), 786−801.

(6) Birkedal-Hansen, H. Proteolytic Remodeling of Extracellular Matrix. *Curr. Opin. Cell Biol.* **1995**, *7*, 728−735.

DOI: 10.1021/acsbiomaterials.7b00647
ACS Biomater. Sci. Eng. 2018, 4, 836−845

(7) Larsen, M.; Artym, V. V.; Green, J. A.; Yamada, K. M. The Matrix Reorganized: Extracellular Matrix Remodeling and Integrin Signaling. *Curr. Opin. Cell Biol.* **2006**, *18*, 463−471.
(8) Lutolf, M. P.; Gilbert, P. M.; Blau, H. M. Designing Materials to Direct Stem-Cell Fate. *Nature* **2009**, *462*, 433−441.
(9) Guvendiren, M.; Burdick, J. A. Engineering Synthetic Hydrogel Microenvironments to Instruct Stem Cells. *Curr. Opin. Biotechnol.* **2013**, *24* (5), 841−846.
(10) DeForest, C. A.; Tirrell, D. A. A Photoreversible Protein-Patterning Approach for Guiding Stem Cell Fate in Three-Dimensional Gels. *Nat. Mater.* **2015**, *14*, 523.
(11) Caiazzo, M.; Okawa, Y.; Ranga, A.; Piersigilli, A.; Tabata, Y.; Lutolf, M. P. Boost Induction of Pluripotency. *Nat. Mater.* **2016**, *15*, 344−352.
(12) Hoffman, A. S. Hydrogels for Biomedical Applications. *Adv. Drug Delivery Rev.* **2012**, *64*, 18−23.
(13) Tibbitt, M. W.; Anseth, K. S. Hydrogels as Extracellular Matrix Mimics for 3D Cell Culture. *Biotechnol. Bioeng.* **2009**, *103* (4), 655−663.
(14) Benton, J. A.; Fairbanks, B. D.; Anseth, K. S. Characterization of Valvular Interstitial Cell Function in Three Dimensional Matrix Metalloproteinase Degradable PEG Hydrogels. *Biomaterials* **2009**, *30* (34), 6593−6603.
(15) Raeber, G. P.; Lutolf, M. P.; Hubbell, J. A. Mechanisms of 3-D Migration and Matrix Remodeling of Fibroblasts within Artificial ECMs. *Acta Biomater.* **2007**, *3*, 615−629.
(16) Nuttelman, C. R.; Tripodi, M. C.; Anseth, K. S. Synthetic Hydrogel Niches That Promote hMSC Viability. *Matrix Biol.* **2005**, *24* (3), 208−218.
(17) Schultz, K. M.; Kyburz, K. A.; Anseth, K. S. Measuring Dynamic Cell−material Interactions and Remodeling during 3D Human Mesenchymal Stem Cell Migration in Hydrogels. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112*, E3757−64.
(18) Didangelos, A.; Yin, X.; Mandal, K.; Saje, A.; Smith, A.; Xu, Q.; Jahangiri, M.; Mayr, M. Extracellular Matrix Composition and Remodeling in Human Abdominal Aortic Aneurysms: A Proteomics Approach. *Mol. Cell. Proteomics* **2011**, *10* (8), M111.008128.
(19) Delany, J. P.; Floyd, Z. E.; Zvonic, S.; Smith, A.; Gravois, A.; Reiners, E.; Wu, X.; Kilroy, G.; Lefevre, M.; Gimble, J. M. Proteomic Analysis of Primary Cultures of Human Adipose-Derived Stem Cells: Modulation by Adipogenesis. *Mol. Cell. Proteomics* **2005**, *4* (6), 731−740.
(20) Eustace, B. K.; Sakurai, T.; Stewart, J. K.; Yimlamai, D.; Unger, C.; Zehetmeier, C.; Lain, B.; Torella, C.; Henning, S. W.; Beste, G.; Scroggins, B. T.; Neckers, L.; Ilag, L. L.; Jay, D. G. Functional Proteomic Screens Reveal an Essential Extracellular Role for hsp90α in Cancer Cell Invasiveness. *Nat. Cell Biol.* **2004**, *6* (6), 507−514.
(21) Kim, W.; Park, B.; Sung, J.; Yang, J.; Park, S.-B.; Kwak, S.-J.; Park, J.-S. Wound Healing Effect of Adipose-Derived Stem Cells: A Critical Role of Secretory Factors on Human Dermal Fibroblasts. *J. Dermatol. Sci.* **2007**, *48*, 15−24.
(22) Lee, M. J.; Kim, J.; Kim, M. Y.; Bae, Y.; Ryu, S. H.; Lee, T. G.; Kim, J. H. Proteomic Analysis of Tumor Necrosis Factor-a-Induced Secretome of Human Adipose Tissue-Derived Mesenchymal Stem Cells. *J. Proteome Res.* **2010**, *9*, 1754−1762.
(23) Timms, J. F.; Cramer, R. Difference Gel Electrophoresis. *Proteomics* **2008**, *8*, 4886−4897.
(24) Hirsch, J.; Hansen, K. C.; Burlingame, A. L.; Matthay, M. A. Proteomics: Current Techniques and Potential Applications to Lung Disease. *Am. J. Physiol. - Lung Cell. Mol. Physiol* **2004**, *287* (1), L1−L23.
(25) Issaq, H. J. The Role of Separation Science in Proteomics. *Electrophoresis* **2001**, *22*, 3629−3638.
(26) Reymond, M. A.; Schlegel, W. Proteomics in Cancer. *Adv. Clin. Chem.* **2007**, *44* (7), 103−142.
(27) Baggerman, G.; Vierstraete, E.; De Loof, A.; Schoofs, L. Gel-Based Versus Gel-Free Proteomics: A Review. *Comb. Chem. High Throughput Screening* **2005**, *8*, 669−677.
(28) Gonzalez-Gonzalez, M.; Jara-Acevedo, R.; Matarraz, S.; Jara-Acevedo, M.; Paradinas, S.; Sayagües, J. M.; Orfao, A.; Fuentes, M. Nanotechniques in Proteomics: Protein Microarrays and Novel Detection Platforms. *Eur. J. Pharm. Sci.* **2012**, *45*, 499−506.
(29) Espina, V.; Woodhouse, E. C.; Wulfkuhle, J.; Asmussen, H. D.; Petricoin, E. F.; Liotta, L. A. Protein Microarray Detection Strategies: Focus on Direct Detection Technologies. *J. Immunol. Methods* **2004**, *290*, 121−133.
(30) Macbeath, G. Protein Microarrays and Proteomics. *Nat. Genet.* **2002**, *32*, 526−532.
(31) Krishhan, V. V.; Khan, I. H.; Luciw, P. A. Multiplexed Microbead Immunoassays by Flow Cytometry for Molecular Profiling: Basic Concepts and Proteomics Applications. *Crit. Rev. Biotechnol.* **2009**, *29* (1), 29−43.
(32) Adelow, C.; Segura, T.; Hubbell, J. A.; Frey, P. The Effect of Enzymatically Degradable Poly(ethylene Glycol) Hydrogels on Smooth Muscle Cell Phenotype. *Biomaterials* **2008**, *29*, 314−326.
(33) Liao, H.; Munoz-pinto, D.; Qu, X.; Hou, Y.; Grunlan, M. A.; Hahn, M. S. Influence of Hydrogel Mechanical Properties and Mesh Size on Vocal Fold Fibroblast Extracellular Matrix Production and Phenotype. *Acta Biomater.* **2008**, *4*, 1161−1171.
(34) Regier, M. C.; Montanez-sauri, S. I.; Schwartz, M. P.; Murphy, W. L.; Beebe, D. J.; Sung, K. E. The Influence of Biomaterials on Cytokine Production in 3D Cultures. *Biomacromolecules* **2017**, *18*, 709−718.
(35) Sawicki, L. A.; Kloxin, A. M. Light-Mediated Formation and Patterning of Hydrogels for Cell Culture Applications. *J. Visualized Exp.* **2016**, No. 115, e54462.
(36) Sawicki, L. A.; Kloxin, A. M. Design of Thiol−ene Photoclick Hydrogels Using Facile Techniques for Cell Culture Applications. *Biomater. Sci.* **2014**, *2* (11), 1612−1626.
(37) Fairbanks, B. D.; Singh, S. P.; Bowman, C. N.; Anseth, K. S. Photodegradable, Photoadaptable Hydrogels via Radical-Mediated Disulfide Fragmentation Reaction. *Macromolecules* **2011**, *44* (8), 2444−2450.
(38) Chen, L. B.; Segal, R.; Murray, A.; Bushnell, A.; Walsh, M. L. Studies on Intercellular LETS Glycoprotein Matrices Studies on Intercellular Matrices. *Cell* **1978**, *14*, 377−391.
(39) Mao, Y.; Schwarzbauer, J. E. Stimulatory Effects of a Three-Dimensional Microenvironment on Cell-Mediated Fibronectin Fibrillogenesis. *J. Cell Sci.* **2005**, *118* (19), 4427−4436.
(40) Patterson, J.; Hubbell, J. A. Enhanced Proteolytic Degradation of Molecularly Engineered PEG Hydrogels in Response to MMP-1 and MMP-2. *Biomaterials* **2010**, *31* (30), 7836−7845.
(41) Valente, K. N.; Schaefer, A. K.; Kempton, H. R.; Lenhoff, A. M.; Lee, K. H. Recovery of Chinese Hamster Ovary Host Cell Proteins for Proteomic Analysis. *Biotechnol. J.* **2014**, *9*, 87−99.
(42) Kloxin, A. M.; Kasko, A. M.; Salinas, C. N.; Anseth, K. S. Photodegradable Hydrogels for Dynamic Tuning of Physical and Chemical Properties. *Science* **2009**, *324* (5923), 59−63.
(43) Delise, A. M.; Fischer, L.; Tuan, R. S. Cellular Interactions and Signaling in Cartilage Development. *Osteoarthritis and Cartilage* **2000**, *8*, 309−334.
(44) Tavella, S.; Bellese, G.; Castagnola, P.; Martin, I.; Piccini, D.; Doliana, R.; Colombatti, A.; Cancedda, R.; Tacchetti, C. Regulated Expression of Fibronectin, Laminin and Related Integrin Receptors during the Early Chondrocyte Differentiation. *J. Cell Sci.* **1997**, *2270*, 2261−2270.
(45) Grayson, W. L.; Ma, T.; Bunnell, B. Human Mesenchymal Stem Cells Tissue Development in 3D PET Matrices. *Biotechnol. Prog.* **2004**, *20*, 905−912.
(46) Twomey, J. D.; Thakore, P. I.; Hartman, D. A.; Hsieh, A. H. Roles of Type VI Collagen and Decorin in Human Mesenchymal Stem Cell Biophysics during Chondrogenic Differentiation. *Eur. Cells Mater.* **2014**, *27*, 237−250.
(47) Xu, J.; Wang, W.; Ludeman, M.; Cheng, K.; Hayami, T.; Lotz, J. C.; Kapila, S. Chondrogenic Differentiation of Human Mesenchymal Stem Cells in Three-Dimensional Alginate Gels. *Tissue Eng., Part A* **2008**, *14* (5), 667−680.

DOI: 10.1021/acsbiomaterials.7b00647
ACS Biomater. Sci. Eng. 2018, 4, 836−845

(48) Kollmer, M.; Keskar, V.; Hauk, T. G.; Collins, J. M.; Russell, B.; Gemeinhart, R. A. Stem Cell-Derived Extracellular Matrix Enables Survival and Multilineage Differentiation within Superporous Hydrogels. *Biomacromolecules* **2012**, *13*, 963−973.
(49) Bobick, B. E.; Chen, F. H.; Le, A. M.; Tuan, R. S. Regulation of the Chondrogenic Phenotype in Culture. *Birth Defects Res., Part C* **2009**, *87*, 351−371.
(50) Park, H. W.; Shin, J.; Kim, C. Proteome of Mesenchymal Stem Cells. *Proteomics* **2007**, *7*, 2881−2894.
(51) Rice, M. A.; Waters, K. R.; Anseth, K. S. Ultrasound Monitoring of Cartilaginous Matrix Evolution in Degradable PEG Hydrogels. *Acta Biomater.* **2009**, *5*, 152−161.
(52) Discher, D. E.; Mooney, D. J.; Zandstra, P. Growth Factors, Matrices, and Forces Combine and Control Stem Cells. *Science* **2009**, *324*, 1673−1678.
(53) Aggarwal, S.; Pittenger, M. F. Human Mesenchymal Stem Cells Modulate Allogeneic Immune Cell Responses. *Blood* **2005**, *105* (4), 1815−1822.
(54) Derose, Y. S.; Wang, G.; Lin, Y.; Bernard, P. S.; Buys, S. S.; Ebbert, M. T. W.; Factor, R.; Matsen, C.; Milash, B. A.; Nelson, E.; Neumayer, L.; Randall, R. L.; Stijleman, I. J.; Welm, B. E.; Welm, A. L. Tumor Grafts Derived from Women with Breast Cancer Authentically Reflect Tumor Pathology, Growth, Metastasis and Disease Outcomes. *Nat. Med.* **2011**, *17* (11), 1514−1520.
(55) Motaln, H.; Schichor, C.; Lah, T. T. Human Mesenchymal Stem Cells and Their Use in Cell-Based Therapies. *Cancer* **2010**, *116* (11), 2519−2530.
(56) Kyurkchiev, D.; Bochev, I.; Ivanova-todorova, E.; Mourdjeva, M.; Oreshkova, T.; Belemezova, K.; Kyurkchiev, S.; Kyurkchiev, D.; Ivanova-todorova, E. Secretion of Immunoregulatory Cytokines by Mesenchymal Stem Cells. *World J. Stem Cells* **2014**, *6* (5), 552−570.
(57) Ranganath, S. H.; Levy, O.; Inamdar, M. S.; Karp, J. M. Harnessing the Mesenchymal Stem Cell Secretome for the Treatment of Cardiovascular Disease. *Cell Stem Cell* **2012**, *10* (3), 244−258.
(58) Scherzed, A.; Hackenberg, S.; Radeloff, A.; Froelich, K.; Rak, K.; Hagen, R.; Kleinsasser, N. Human Mesenchymal Stem Cells Promote Cancer Motility and Cytokine Secretion in Vitro. *Cells Tissues Organs* **2013**, *198*, 327−337.
(59) Walter, M. N. M.; Wright, K. T.; Fuller, H. R.; Macneil, S.; Johnson, W. E. B. Mesenchymal Stem Cell-Conditioned Medium Accelerates Skin Wound Healing: An in Vitro Study of Fibroblast and Keratinocyte Scratch Assays. *Exp. Cell Res.* **2010**, *316* (7), 1271−1281.
(60) Kagiwada, H.; Yashiki, T.; Ohshima, A.; Tadokoro, M.; Nagaya, N.; Ohgushi, H. Human Mesenchymal Stem Cells as a Stable Source of VEGF-Producing Cells. *J. Tissue Eng. Regener. Med.* **2008**, *2*, 184−189.
(61) Zisa, D.; Shabbir, A.; Suzuki, G.; Lee, T. Vascular Endothelial Growth Factor (VEGF) as a Key Therapeutic Trophic Factor in Bone Marrow Mesenchymal Stem Cell-Mediated Cardiac Repair. *Biochem. Biophys. Res. Commun.* **2009**, *390* (3), 834−838.
(62) Wu, Y.; Chen, L.; Scott, P. G.; Tredget, E. E. Mesenchymal Stem Cells Enhance Wound Healing Through Differentiation and Angiogenesis. *Stem Cells* **2007**, *25*, 2648−2659.
(63) Domon, B.; Aebersold, R. Options and Considerations When Selecting a Quantitative Proteomics Strategy. *Nat. Biotechnol.* **2010**, *28* (7), 710−721.
(64) Kharkar, P. M.; Kiick, K. L.; Kloxin, A. M. Designing Degradable Hydrogels for Orthogonal Control of Cell Microenvironments. *Chem. Soc. Rev.* **2013**, *42* (17), 7335−7372.
(65) Valdez, J.; Cook, C. D.; Ahrens, C. C.; Wang, A. J.; Brown, A.; Kumar, M.; Stockdale, L.; Rothenberg, D.; Renggli, K.; Gordon, E.; Lauffenburger, D.; White, F.; Griffith, L. On-Demand Dissolution of Modular, Synthetic Extracellular Matrix Reveals Local Epithelial-Stromal Communication Networks. *Biomaterials* **2017**, *130*, 90−103.
(66) Keane, T. J.; Swinehart, I. T.; Badylak, S. F. Methods of Tissue Decellularization Used for Preparation of Biologic Scaffolds and in Vivo Relevance. *Methods* **2015**, *84*, 25−34.

DOI: 10.1021/acsbiomaterials.7b00647
ACS Biomater. Sci. Eng. 2018, 4, 836−845