Original Research *Highlight article*

Screening a chemically defined extracellular matrix mimetic substrate library to identify substrates that enhance substrate-mediated transfection

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Impact statement

Substrate-mediated gene delivery (SMD) approaches have potential for modification of cells in applications where a cell-material interface exists. Conventional SMD uses ill-defined serum or protein coatings to facilitate immobilization of nucleic acid complexes, cell attachment, and subsequent transfection, which limits reproducibility and clinical utility. As an alternative, we screened a defined library of extracellular matrix mimetic substrates containing combinations of different glycosaminoglycans and bioactive peptides to identify optimal substrates for SMD transfection of fibroblasts and human mesenchymal stem cells. This strategy could be utilized to develop substrates for specific SMD applications in which variability exists between different cell types and patient samples.

Abstract

Nonviral gene delivery, though limited by inefficiency, has extensive utility in cell therapy, tissue engineering, and diagnostics. Substrate-mediated gene delivery (SMD) increases efficiency and allows transfection at a cell-biomaterial interface, by immobilizing and concentrating nucleic acid complexes on a surface. Efficient SMD generally requires substrates to be coated with serum or other protein coatings to mediate nucleic acid complex immobilization, as well as cell adhesion and growth; however, this strategy limits reproducibility and may be difficult to translate for clinical applications. As an alternative, we screened a chemically defined combinatorial library of 20 different extracellular matrix mimetic substrates containing combinations of (1) different sulfated polysaccharides that are essential extracellular matrix glycosaminoglycans (GAGs), with (2) mimetic peptides derived from adhesion proteins, growth factors, and cell-penetrating domains, for use as SMD coatings. We identified optimal substrates for DNA lipoplex and polyplex SMD transfection of fibroblasts and human mesenchymal stem cells. Optimal extracellular matrix mimetic substrates varied between cell type, donor source, and transfection reagent, but typically contained Heparin GAG and an adhesion peptide. Multiple substrates significantly increased transgene expression (i.e. 2- to 20-fold) over standard protein coatings. Considering previous research of similar ligands, we hypothesize extracellular matrix mimetic substrates modulate cell adhesion, proliferation, and survival, as well as plasmid internalization and trafficking. Our results demonstrate the utility of screening combinatorial extracellular matrix mimetic substrates for optimal SMD transfection towards application- and patient-specific technologies.

Keywords: Mimetic peptides, glycosaminoglycans, extracellular matrix, substrate-mediated gene delivery, transfection, human mesenchymal stem cells

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Introduction

Gene expression within a cell population can be directly altered through gene delivery approaches, which have tremendous potential for clinical applications, such as gene and cell therapy, tissue engineering, diagnostics, and bioactive medical devices. However, inefficient nonviral gene delivery is a critical factor limiting the development of these applications. Nucleic acid delivery to cells can be limited by mass transport, or deactivation processes such as

degradation and aggregation.[1] Alternative delivery methods, including substrate-mediated gene delivery (SMD), can be used to overcome these limitations in applications where a cell-material interface exists.[2] SMD, also termed solid phase delivery or reverse transfection, is the immobilization of nucleic acids, typically complexed with cationic lipid or polymer nonviral transfection reagents, to a biomaterial or substrate through specific[3–5] or nonspecific[6–9] interactions. SMD transfection allows for reduced complex aggregation and elevated nucleic acid concentrations within the microenvironment of cells adhered to surfaces, which in turn has been shown to increase total transgene expression and transfection efficiency (i.e. the proportion of cells expressing transgene), relative to complexes delivered by bolus to culture media.[8–16] SMD has potential applications for *ex vivo* modifications of cells for cell therapies and tissue engineering strategies, as well as *in vivo* for bioactive devices and implants.[17–19]

SMD has been investigated with nucleic acid complexes immobilized to 2D substrates and 3D scaffolds made from a wide variety of biomaterials, such as collagen,[20,21] hyaluronic acid,[4,22] natural[7] and synthetic[6,23–25] polymers, and hydrogels, but has been most successful with nonspecific adsorption of complexes mediated by serum (e.g. fetal bovine serum (FBS)[10,14,26]) or other protein coatings such as albumin,[14] fibronectin,[14,19,27–30] collagen,[14,19] and laminin[14,19] applied to substrates prior to addition of the nucleic acid complexes. These protein coatings facilitate complex immobilization by non-specific electrostatic interactions,[8] cell adhesion and spreading,[31] activation of endocytic and trafficking pathways,[32] and promote subsequent transgene expression.[14,27,33] While these studies have demonstrated enhanced SMD transfection when compared to uncoated substrates or traditional bolus delivery of complexes, the use of an ill-defined serum or other protein coating to support nucleic acid complex adsorption, and subsequent cellular adhesion and transfection, limits reproducibility (due to batch variability), and thus clinical utility.[34] In addition, cell type, tissue source, human donor variability, and specific applications will dictate what bioactive signals are most appropriate for a given substrate to support functional transgene expression and desired cellular phenotype. Therefore, the ability to screen a library of substrates containing different combinations of bioactive ligands for optimal complex immobilization, cell adhesion and growth, activation of endocytic and trafficking pathways, subsequent transgene expression, as well as desired cellular phenotypes,[14] would aid the development of materials tailored for specific SMD applications. For translatable therapeutic applications that utilize SMD transfection, including spatially patterned gene delivery devices[4,8,23,35] for drug discovery,[36] diagnostics,[37] cell therapies, tissue engineering,[7] regenerative medicine,[20] and bioactive implants,[6] libraries of chemically-defined, scalably produced, and tunable bioactive substrates, which mediate efficient SMD transfection, would be valuable.

Towards developing clinically translatable substrates for optimal cell adhesion and SMD transfection, we have generated a chemically defined substrate library by combining sulfated polysaccharides, which are essential glycosaminoglycans (GAGs) present in the extracellular matrix (ECM), with different bioactive mimetic peptides. Specifically, we combined heparin (Hep), heparan sulfate (Hepa), chondroitin sulfate (Chon), dermatan sulfate (Derm), or dextran sulfate (Dext), with four-arm polyethylene glycol (starPEG) conjugated to a GAG-binding peptide containing a repetitive Lys-Ala sequence (KA7).[38,39] GAGs and KA7-starPEGs interact to form coacervation-mediated biomatrix films on cell culture ware[38] (Figure 1). We linked KA7 with different peptide motifs that mimic the ligands of proteins found in the ECM that promote cell adhesion (i.e. RGD[40] and AG73[41]), proliferation and endocytosis (i.e. fibroblast growth factor mimetic peptide F2A[42]), and cell membrane penetration (i.e. R9[43]) (Table 1). These combinatorial ECM mimetic substrate libraries have been screened previously to identify biomatrices for optimal adhesion, growth, and differentiation of different cell types.[38] The objective of this current work is to screen the combinatorial library of ECM mimetic substrates for their ability to facilitate nucleic acid complex immobilization, cell adhesion and growth, and subsequent SMD transfection of NIH/3T3 murine fibroblasts and clinically relevant human adipose-derived

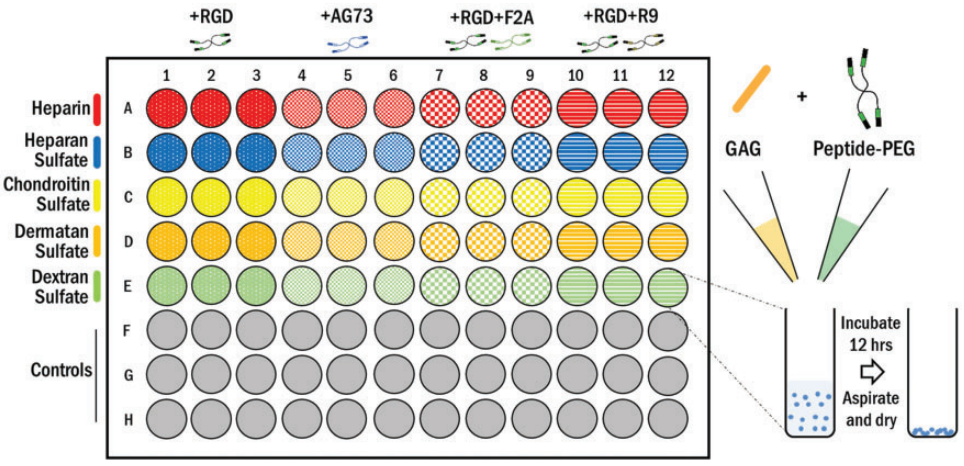

Figure 1. ECM mimetic substrate library formulation. One of five different glycosaminoglycans (GAGs) was combined with one or more peptide conjugates in well plates and incubated for 12 h before drying to form biomatrices for SMD transfection experiments. (A color version of this figure is available in the online journal.)

Table 1. List of glycosaminoglycans and peptides in ECM mimetic substrates.

Glycosaminoglycans (GAGs)		
Heparin (Hep)		
Heparan Sulfate (Hepa)		
Chondroitin Sulfate (Chon)		
Dermatan Sulfate (Derm)		
Dextran Sulfate (Dext)		
Peptide	**Description**	**Amino acid sequence**
KA7	GAG-binding motif (Lys-Ala repeats) with cysteine residue for starPEG conjugation (40)	CWGGKAKAKAKAKAKAKA
RGD	Arg-Gly-Asp (RGD) is a cell adhesion sequence in many ECM proteins (42)	(KA7)-RGDSP
AG73	ECM protein laminin-derived cell adhesion peptide (43)	(KA7)-RKRLQVQLSIRT
F2A	Fibroblast growth factor mimetic peptide (44)	(KA7)-HYRSRKYSSWYVALKR
R9	Polyarginine cell penetrating peptide (45)	(KA7)-RRRRRRRRR

mesenchymal stem cells (hAMSCs) from multiple donors, to demonstrate the utility of such screens to rapidly identify optimal biomaterials for a wide variety of clinical applications that will vary between cell types, as well as between patients.

Material and methods

Materials for ECM mimetic substrate synthesis

For peptide synthesis, all required chemicals were purchased from IRIS Biotech GmbH (Marktredwitz, Germany) unless otherwise specified. Four-arm polyethylene glycol (pentaerythritol) maleimide (maleimide functionalized starPEG) 10 kDa was purchased from JenKem Technology (Beijing, China). Phosphate-buffered saline (PBS) powder was purchased from AppliChem GmbH (Darmstadt, Germany). Spectra/Por dialysis membrane, molecular weight cut off 8 kDa, was purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA, USA). TentaGel S RAM Fmoc (fluorenylmethyloxycarbonyl) rink amide resin was purchased from Rapp Polymere GmbH (Tuebingen, Germany). Peptide synthesis 6 mL columns and 5 mL syringes with included filters were bought from Intavis AG (Cologne, Germany). Polytetrafluoroethylene filter, polyvinylidenefluoride (PVDF) syringe filter, and filter holder were purchased from Sartorius Stedtim (Aubagne, France); 14 kDa heparin sodium salt from porcine intestinal mucosa was purchased from Millipore (MERCK KGaA (Cat. No. 375095-500KU, Lot: DO0162382, Darmstadt, Germany). Chondroitin sulfate A sodium salt from bovine trachea, dermatan sulfate from porcine intestinal mucosa, heparan sulfate sodium salt from bovine kidney, and 5 kDa dextran sulfate were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Peptide synthesis

ResPep SL automated solid-phase peptide synthesizer was purchased from Intavis (Cologne, Germany). ProStar preparative high pressure liquid chromatography (HPLC) machine was purchased from Agilent Technologies (Santa Clara, USA) and the AXIA 100 A preparative C18 column (bead size 10 μm, 250 × 30 mm) from Phenomenex (Torrance, CA, USA). ACQUITY analytical ultra HPLC (UPLC) with ultra violet light detector, the ACQUITY UPLC BEH analytical reverse phase C18 column (bead size 1.7 μm, 50 × 2.1 mm), and ACQUITY TQ electrospray ionization mass spectroscope (ESI-MS) are from Waters (Milford MA, USA). The ALPHA 2–4 LD plus lyophilizer was purchased from Martin Christ Gefriertrocknungsanlagen GmbH (Osterode am Harz, Germany) and the vacuum pump RZ6 from VACUUBRAND GmbH + Co KG (Wertheim, Germany). MR Hei-Standard stirring plate was purchased from Heidolph (Schwabach, Germany).

The peptides were synthesized using standard solid phase fluorenylmethyloxycarbonyl (Fmoc) chemistry with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorphosphate (HBTU) activation on an automated solid-phase peptide synthesizer. To ensure peptide quality, the coupling of each amino acid was performed twice with five times molar excess. All the non-reacted amino groups were capped with 5% acetic anhydride. The peptide was cleaved from the resin with TFA/TIS/water/DTT (90(v/v):5(v/v):2.5 (v/v):2.5(m/v)) for 2 h. The product was precipitated and washed with ice-cold diethyl ether. Peptide purification was performed via reverse-phase HPLC on a preparative HPLC equipped with a preparative reverse-phase C18 column. The peptide was eluted from the column by applying a graded mixture of two solvents over 30 min at 20 mL/min, where solvent A is 0.1% TFA in water and solvent B is 0.1% TFA and 5% water in acetonitrile. Purity was confirmed by analytical reverse phase UPLC using an analytical reverse phase C18 column and electrospray ionization mass spectrometry (MS) (Supplementary Figure 1). The peptide was lyophilized and stored at −20°C under dry conditions until it was coupled to the starPEG maleimide.

Synthesis of peptide-starPEG

The synthesis of the peptide-starPEG conjugates utilized for the coating of the cell culture ware was conducted via Michael-type addition reactions between maleimide-terminated starPEG and cysteine-terminated peptides. The peptides were dissolved in PBS (pH 7.4) and mixed in a molar ratio of 5:1 (peptide:starPEG) with a total concentration of 80 mg/mL. The reaction mixture was quickly sealed and stirred on a stirring plate at 750 r/min at room

temperature for 2 h. The crude product was dialyzed to remove uncoupled peptides using salt in a dialysis tube with an 8 kDa cut off against 10 L water under constant water exchange for five days. The dialysis purified product was then lyophilized for two days, for storage. The peptide-starPEG was lyophilized and stored at 4°C under dry conditions until it was dissolved in PBS for cell culture ware coating. The peptide-starPEG conjugates will henceforth be referred to with the name of the respective peptide.

The conjugation efficiency was calculated using NMR (Nuclear Magnetic Resonance) spectroscopy and found to be 99%. The samples were prepared by dissolving the peptide-PEG conjugate in deuterium oxide, the NMR solvent at a concentration of 5 mg/mL. NMR data were recorded on a Bruker AV-III 600 spectrometer operating at 600 MHz for 1H. The chemical shifts δ are given in ppm relative to tetramethylsilane. The proton NMR spectra were measured without rotation with a longer delay time between the scans to make sure the spectrum will be quantitative. Those spectra were measured with 64 scans to have a better signal-to-noise rate. As an example the spectra of the starPEG maleimide and KA7-starPEG are shown in Supplementary Figures 2 and 3.

Formulation of ECM mimetic substrate library and coating of cell culture ware

Automated pipettes were purchased from INTEGRA Biosciences (Biebertal, Germany). Black-walled polystyrene 96-well cell culture microplates, F-Bottom (chimney well) together with a lid having condensation rings were purchased from Greiner Bio-One (Kremsmünster, Austria). Polystyrene 48-well cell culture microplates together with a lid were purchased from VWR (Pennsylvania, PA).

Quantitative quality control and analysis of stock material of the substrates were performed using HPLC, MS, and NMR, as discussed above, prior to the formulation of the cell culture coating. MS was used to characterize the synthetic peptides and HPLC was used to quantify the peptide purity. After conjugation of a peptide to PEG polymer, NMR was used to determine the conjugation yield. In addition, light scattering assays were performed to confirm that different formulations of sulfated polysaccharides and peptide-starPEGs undergo similar coacervation-mediated film formation (Supplementary Figure 4). A library of 20 different ECM mimetic substrates was produced by combining one of five different sulfated polysaccharides, which are essential ECM glycosaminoglycans (GAGs), together with one or more of the peptide-starPEG conjugates[38] (Table 1). After sterile filtering, 5 μM peptide-starPEG conjugates (mixed at ratios optimized for cell adhesion and growth in preliminary experiments (data not shown)) in PBS and 5 μM GAGs in PBS were mixed in uncoated polystyrene wells of 48- or 96-well plates (total volume scaled to well surface area), and incubated for 12 h (Figure 1). After removing the supernatant, plates were dried in the laminar flow hood to form stable ECM mimetic substrate coatings (Supplementary Figure 5(a)). The deposited ECM mimetic substrates are stable and not affected by addition of culture media or buffers. Extensive mechanical characterization of the ECM mimetic substrates is described in our previous work.[38] The dry plates were then vacuum sealed until the cell culture and transfection experiments were conducted. As controls, 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY) or 10 μg/mL human fibronectin (Sigma-Aldrich, St. Louis, MO) diluted in PBS was adsorbed to uncoated polystyrene wells by adding 210 μL/cm^2 of cell growth area, to each well, and incubating at room temperature for 90 min. All wells were rinsed with PBS prior to addition of DNA complexes.

Cell culture

Murine fibroblast NIH/3T3 cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's media (DMEM) (ATCC) completed with 10% calf serum (Colorado Serum Co., Denver, CO) and 1% penicillin/streptomycin (Fisher Scientific, Hampton, NH). Fibroblasts were grown at 37°C and 5% CO_2 and passaged every two days by removing cell media, washing with 1 × PBS, and dissociating cells with 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco). For transfection experiments, 300 μL of NIH/3T3 cells suspended at 50,000 cells/mL (15,000 cells/well) were seeded onto the substrates prepared in 48-well plates described above.

Adipose-derived human mesenchymal stem cells (hAMSCs) from four different human donors were purchased at passage 1 from Lonza (Walkersville, MD) and were positive for CD13, CD29, CD44, CD73, CD90, CD105, CD166, and negative for CD14, CD31, CD45 cell surface markers. All human cells were acquired with informed consent using established ethical methods approved by appropriate authorities. All experiments and methods were performed in accordance with relevant guidelines and regulations. All experimental protocols were approved by the University of Nebraska-Lincoln Institutional Biosafety Committee (see Supplementary Table 1 for hAMSC donor information). hAMSCs were cultured in Minimum Essential Medium Alpha (MEM Alpha) (Gibco) supplemented with 10% FBS, 6 mM L-Glutamine (Gibco), and 1% Penicillin–Streptomycin (Pen-Strep) (10,000 U/mL) (Gibco) and incubated at 37°C with 5% CO_2. At 80% confluence, cell media was removed and cells were washed with PBS and dissociated with 0.25% trypsin-EDTA (Gibco), then an equal volume of growth medium was added and cells were pelleted to remove trypsin-EDTA, resuspended and counted with trypan blue staining and a hemocytometer before diluting in growth medium. For transfection experiments, 100 μL of AMSCs suspended at 30,000 cells/mL (3,000 cells/well) were seeded onto the substrates prepared in 96-well plates described above.

SMD transfections

pEGFP-Luc plasmid DNA (pDNA) (Clontech, Mountain View, CA) encodes a fusion protein of enhanced green fluorescent protein (EGFP) and firefly luciferase under the direction of a cytomegalovirus (CMV) promoter and containing simian virus 40 (SV40) enhancer. Plasmids were purified from *E. coli* bacteria using Qiagen (Valencia, CA)

reagents and stored in Tris-EDTA (TE) buffer solution (10 mM Tris, 1 mM EDTA, pH 7.4) at −20°C. Lipoplexes were formed with Lipofectamine 2000 (LF2K; Invitrogen, Carlsbad, CA) or Lipofectamine 3000 (LF3K; Invitrogen) in serum-free Opti-MEM media (Invitrogen) at a pDNA:lipid ratio of 1:1.75 or 1:1, respectively, following the manufacturer's instructions and as noted in the text. Polyplexes were prepared with 25 kDa branched polyethylenimine (PEI, Sigma-Aldrich) at a nitrogen/phosphorus (N/P) ratio of 15 in serum-free Opti-MEM media.[10] Immediately after complex formation, 210 μL lipoplexes per cm^2 of cell growth area, or 158 μL polyplexes per cm^2 of cell growth area were added to appropriate ECM mimetic substrate- and control protein-coated wells (2.1 μg pDNA per cm^2 of cell growth area) and allowed to immobilize for 2 h at room temperature followed by a 210 μL/cm^2 rinse with serum-free Opti-MEM of all wells to remove unbound complexes. Cells were then seeded into wells containing DNA complexes adsorbed to ECM mimetic substrates or control coatings, as described above. NIH/3T3s were cultured for 24 h, and AMSCs cultured for 48 h, before performing assays described below.

Metabolic activity assay

Metabolic activity of cells adhered to ECM mimetic substrates with adsorbed pDNA complexes was assessed using a water soluble tetrazolium (WST-8) salt cell proliferation assay kit (Dojindo Molecular Technologies, Rockville, MD), according to the manufacturer's protocol. Briefly, cells were washed with PBS and cultured in WST-8 solution (10% WST-8 reagent in phenol-free DMEM (Gibco)) for 1 h. After incubation, WST-8 solution was removed and the absorbance values of the solution were measured on an Epoch UV-Vis microplate spectrophotometer (Biotek, Winooski, VT) at 450 nm and corrected for pathlength. Plotted "Metabolic Activity (Relative to FBS)" for each experimental condition was calculated by dividing each treatment condition replicate value by the average of the values from cells cultured on FBS-coated control substrates.

Transfection assessment

Microscopy. After removing media containing WST-8 reagent described above, transfection in cells adhered to ECM mimetic substrates and controls was assessed by fluorescent microscopy. Cell nuclei were stained with 1 μg/mL Hoechst 33342 (Sigma-Aldrich), then cells were imaged using a Cytation 1 Cell Imaging System (Biotek, Winooski, VT) configured with a 4× objective and light cubes for Hoechst (nuclei stain) and GFP (transfection reporter). After image preprocessing and deconvolution to subtract background fluorescence from captured digital images (Supplementary Figure 5(b)), Gen5 software (Biotek) object analysis was used to determine the number of cells from Hoechst images and to determine the number and signal intensities of GFP-positive cells. Analysis identified objects in both channels by their fluorescence with a minimum and maximum size selection of 10 μm and 100 μm, respectively. Hoechst and GFP intensity thresholds of 4000 and 1500 relative fluorescent units (RFU) were selected to accurately identify nuclei and transfected cells, respectively. Transfection efficiency was calculated by dividing the number of GFP objects by the number of Hoechst objects. Plotted "Transfection Efficiency (Relative to FBS)" for each experimental condition was calculated by dividing each treatment condition replicate value by the average of the values of transfected cells cultured on FBS-coated control substrates. The GFP mean fluorescence of GFP+ cells was also calculated and analyzed, but not reported, as no conditions significantly increased the GFP mean fluorescence, relative to FBS controls.

Luciferase expression quantification. After microscopy, described above, cells adhered to ECM mimetic substrates and controls were lysed with 1× reporter lysis buffer (Promega, Madison, WI) and stored at −80°C. Transgenic luciferase activity levels were quantified by measuring luciferase luminescence in relative light units (RLUs) with a Luciferase Assay kit (Promega) and a luminometer (Turner Designs, Sunnyvale, CA). RLUs were normalized to total protein amount determined with a Pierce BCA protein colorimetric assay (Pierce, Rockford, IL) using an Epoch UV-Vis microplate spectrophotometer (Biotek) to measure absorbance at 562 nm. Plotted "RLU/mg Protein (Relative to FBS)" for each experimental condition was calculated by dividing each treatment condition replicate value by the average of the values from cells cultured on FBS-coated control substrates.

pDNA complex immobilization measurements

pDNA radiolabeled with [α-^{32}P]dATP (Perkin Elmer, Akron, OH) was used to measure the immobilization of pDNA complexes on ECM mimetic substrates and control substrates. To label the pDNA, a nick translation kit (Invitrogen, Waltham, MA) was used following the manufacturer's protocol. The radiolabeled pDNA was diluted with unlabeled pDNA to a final concentration (0.806 μg/μL) and used to form pDNA complexes with LF2K, LF3K, and PEI, as described above. Complexes were immobilized, as described above, in 48-well plates (2.0 μg pDNA per well) by incubation on substrates and controls for 2 h, as described above. After complex immobilization, the complex solution was removed and the quantity of pDNA in the removed solution was determined by adding the solution to a scintillation cocktail (5 mL, Thomas Scientific, Swedesboro, NJ) for measurement with a Packard Tri-Carb 1900 TR Liquid Scintillation Counter. An Opti-MEM wash was added to each well, removed, and also added to scintillation cocktail for pDNA quantification to include loosely bound complexes. Counts per minute were correlated to the pDNA amount using a standard curve. The amount of pDNA immobilized to each substrate was calculated by subtracting the sum of the amount of pDNA in the removed complex solution and the amount of pDNA in the Opti-MEM wash, from the original amount of pDNA added to a respective well.

Statistical analysis

All plotted values were normalized to values from FBS-coated control substrate conditions. For each experimental condition, values were calculated by dividing each treatment condition replicate value by the average of the values from FBS-coated control substrate conditions. All data are plotted as mean ± standard error. All transfection experiments were performed in triplicate wells on two or three different days, as specified in legends. All hAMSC experiments were performed on cells derived from four different human donors. Heat maps were generated by mapping means to double gradient color maps in which baseline is equal to one, and shaded white. Mapped means larger or smaller than baseline are shaded increasingly green or red, respectively. Comparative analyses were completed using one-way analysis of variance (ANOVA) with Dunnett's *post hoc* test. All data collected and analyzed were assumed to be normally distributed. Statistical difference was considered at $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***), and $P \leq 0.0001$ (****). Statistics and fold changes within figures are between experimental treatments versus FBS control treatment. Statistics were calculated using Prism GraphPad software (GraphPad Software, La Jolla, CA).

Results

Screen of substrate library identifies formulations that enable SMD transfection

A library of 20 different extracellular matrix (ECM) mimetic substrates was formulated by combining one of five different glycosaminoglycans (GAGs) with one or more mimetic peptide-starPEG conjugates[38] (Table 1). Coating cell culture ware with these formulations (Figure 1) forms stable substrates presenting bioactive ligands on surfaces (Supplementary Figure 5(a)). In order to determine if ECM mimetic substrates could be used in SMD applications, plasmid DNA (pDNA) encoding for a fusion protein of luciferase and EGFP (pEGFP-Luc) was complexed with commonly used transfection reagents: either lipid-based Lipofectamine 2000 (LF2K) or polymer polyethylenimine (PEI), and adsorbed to the library of ECM mimetic substrates, as well as to the commonly used coatings of fetal bovine serum (FBS) and fibronectin as controls, prior to seeding NIH/3T3 murine fibroblasts and measuring transgenic luciferase expression after 24 h. In general, ECM mimetic substrates containing the heparin (Hep) or dextran sulfate (Dext) GAGs supported the highest transgenic luciferase expression, mediated by either immobilized lipid- or polymer-pDNA complexes (Supplementary Figure 6(a) and (b)). Based on the results of this initial screen, the library was refined to 12 ECM mimetic substrates, containing Hep or Dext along with different combinations of peptides, to be characterized further in SMD transfection experiments. All ECM mimetic substrates included both a GAG and an adhesion peptide (i.e. RGD or AG73) for optimal cell attachment and spreading.[38]

ECM mimetic substrates enhance lipofectamine 2000 (LF2K) SMD transfection of NIH/3T3 murine fibroblasts

We continued our characterization of ECM mimetic substrates by next SMD transfecting NIH/3T3s with pEGFP-Luc complexed with LF2K, adsorbed to the 12 different ECM mimetic substrates selected from our initial screen, as well as to the commonly used coatings of FBS and fibronectin as controls. After 24 h, cells cultured on Hep + RGD and Hep+RGD+R9 substrates with immobilized lipoplexes had statistically significant ($P \leq 0.05$) increases in transgenic luciferase expression by over 2-fold, relative to cells cultured on complexes immobilized to FBS-coated control substrates (i.e. FBS adsorbed to polystyrene) (Figure 2(a)). NIH/3T3s cultured on Hep+RGD substrates with immobilized lipoplexes also displayed a statistically significant ($P \leq 0.01$) increase in transfection efficiency (i.e. the proportion of cells expressing EGFP) by over 5-fold, relative to cells cultured on lipoplexes immobilized to FBS control substrates (Figure 2(b)). NIH/3T3s cultured on different ECM mimetic substrates with immobilized lipoplexes displayed no statistically significant changes in cell count (Figure 2(c)) or cellular metabolic activity, relative to FBS-coated control substrates with immobilized lipoplexes (Figure 2(d)).

ECM mimetic substrates enhance polyethylenimine SMD transfection of NIH/3T3 murine fibroblasts

After demonstrating that lipoplex-mediated SMD transfection of NIH/3T3 murine fibroblasts could be enhanced by ECM mimetic substrates, we next aimed to determine if ECM mimetic substrates could enhance polyplex-mediated SMD transfection. We SMD transfected NIH/3T3s on the 12 ECM mimetic substrates selected from our initial screen, as well as on the commonly used coatings of FBS and fibronectin as controls, with adsorbed pEGFP-Luc complexed with 25 kDa branched PEI. After 24 h, cells cultured on Hep+RGD+F2A and Hep+AG73+F2A substrates with immobilized polyplexes had statistically significant ($P \leq 0.0001$ and $P \leq 0.05$, respectively) increases in transgenic luciferase expression by about 3- and 2-fold, respectively, relative to cells cultured on FBS-coated control substrates with immobilized polyplexes (Figure 3(a)). NIH/3T3s cultured on Hep+RGD+F2A substrates with immobilized polyplexes also had a statistically significant ($P \leq 0.0001$) increase in transfection efficiency (i.e. the proportion of cells expressing EGFP) by over 4-fold, relative to cells cultured on FBS-coated control substrates with immobilized polyplexes (Figure 3(b)). Furthermore, cells cultured on seven different ECM mimetic substrates with immobilized polyplexes (i.e. Hep+RGD, Dext+RGD+R9, Dext+RGD+F2A, Hep+AG73, Hep+AG73+R9, and Hep+AG73+F2A) had statistically significant increases in cell counts, ranging from 1.5- to 2-fold, relative to FBS-coated control substrates with immobilized polyplexes (all $P \leq 0.05$) (Figure 3(c)), and cells cultured on Dext+RGD substrates with immobilized polyplexes had a statistically significant increase in cellular metabolic activity (1.3-fold), relative to FBS coated control substrates with immobilized polyplexes ($P \leq 0.001$) (Figure 3(d)).

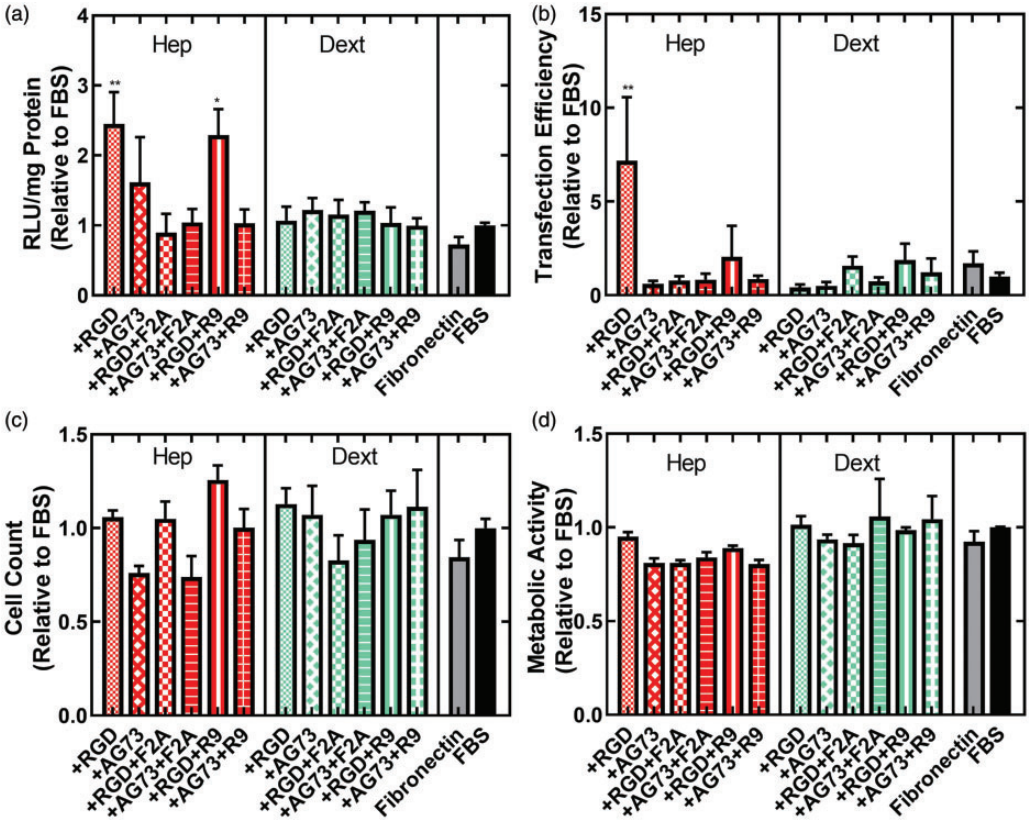

Figure 2. ECM mimetic substrates enhance LF2K SMD transfection of NIH/3T3 murine fibroblasts. pEGFP-Luc was complexed with LF2K and adsorbed to substrates prior to seeding of NIH/3T3 cells. Cells were assayed 24 h after SMD transfection. (a) Hep + RGD and Hep + RGD + R9 substrates significantly increased cell luciferase transgene expression, as measured by relative light units per mg of protein (RLU/mg Protein), and (b) Hep + RGD significantly increased the proportion of cells positive for EGFP fluorescence (transfection efficiency). (c) Cell count and (d) cellular metabolic activity was not significantly affected by substrate. All data from three independent experiments run on different days in triplicate was normalized to FBS conditions and graphed ($n = 9$). Asterisks (*) denote significance to FBS conditions (*$P \leq 0.05$; **$P \leq 0.01$). (A color version of this figure is available in the online journal.)

Enhancement of SMD transfection on ECM mimetic substrates is not due to increased complex immobilization

Nucleic acid complexes have been shown to be immobilized on substrates through non-specific electrostatic interactions between cationic complexes and anionic substrates,[10] and this non-specific immobilization mechanism is presumably similar for our ECM mimetic substrates, which contain anionic sulfated polysaccharides. In order to determine if enhanced transfection on ECM mimetic substrates was mediated by increased immobilization of pDNA complexes, and thus increased pDNA doses administered to cells, we quantified complex adsorption with radiolabeled pDNA. pDNA complex immobilization was not significantly affected by any substrate, relative to FBS-coated control substrates, for LF2K (Figure 4(a)), PEI (Figure 4(b)), or Lipofectamine 3000 (LF3K) (Figure 4(c)) ($P > 0.05$), suggesting enhanced SMD transfection on ECM mimetic substrates was not the result of increased pDNA complex doses administered to cells seeded on substrates.

ECM mimetic substrates enhance SMD transfection of hAMSCs in a donor-dependent manner

After identifying ECM mimetic substrates that support optimal LF2K- and PEI-mediated SMD transfection of NIH/3T3 murine fibroblasts, and determining that enhanced transgene expression was not due to increased immobilization of pDNA complexes, we next aimed to determine if ECM mimetic substrates could enhance SMD transfection of clinically relevant adipose-derived primary human mesenchymal stem cells (hAMSCs). Eight different ECM mimetic substrates were selected for use in hAMSC experiments and again compared to the commonly used coatings of FBS and fibronectin as controls. We adsorbed pEGFP-Luc complexed with lipid-based transfection reagent, Lipofectamine 3000 (LF3K), to substrates prior to seeding hAMSCs derived from four different human donors. Similar to NIH/3T3 experiments, hAMSC transfection was assessed by luciferase assay, high-content imaging to count total cells and transfected cells, and metabolic activity assay. The effect of different ECM mimetic substrates with immobilized lipoplexes on hAMSC SMD transfection was highly variable between cells derived from different human donors.

We chose to use hAMSCs from four different donors in our SMD transfection experiments because many properties, including transfection efficiency[44] of hMSCs are known to significantly vary between donors.[45–47] Therefore, we made use of heat maps to visualize the differing results in our SMD transfection experiments between hAMSC donors (Figure 5). hAMSC experiment results for

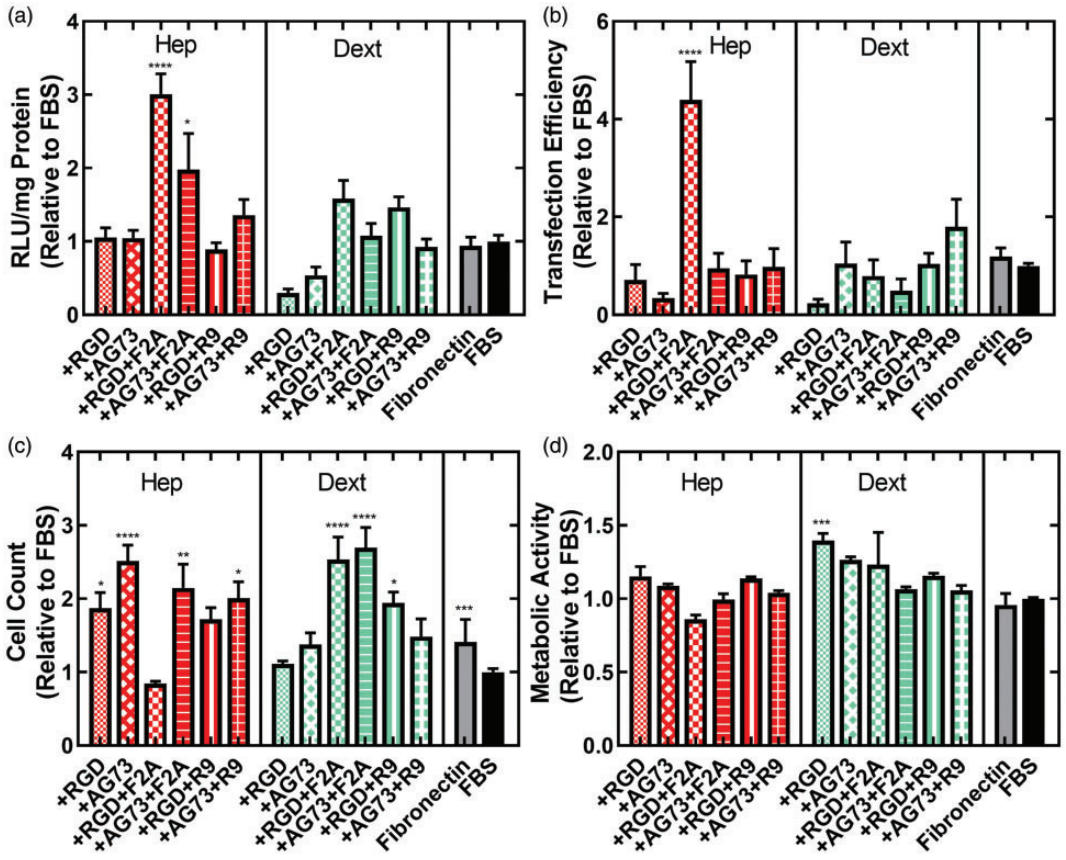

Figure 3. ECM mimetic substrates enhance PEI SMD transfection of NIH/3T3s. pEGFP-Luc was complexed with PEI and adsorbed to substrates prior to seeding of NIH/3T3 cells. Cells were assayed 24 h after SMD transfection. (a) Hep + RGD + F2A and Hep + AG73 + F2A substrates significantly increased cell luciferase transgene expression, as measured by relative light units per mg of protein (RLU/mg Protein), and (b) Hep + RGD + F2A significantly increased the proportion of cells positive for EGFP fluorescence (transfection efficiency). (c) Cell count was significantly increased by seven different ECM mimetic substrates, and (d) cellular metabolic activity was significantly increased by Dext+RGD substrates. All data from three independent experiments run on different days in triplicate were normalized to FBS condition and graphed ($n = 9$). Asterisks (*) denote significance to FBS conditions (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$). (A color version of this figure is available in the online journal.)

the four individual donors can be found in Supplementary Figures 7 to 10. The degree to which different ECM mimetic substrates with immobilized lipoplexes increased transgenic luciferase expression in hAMSCs over FBS coated control substrates with immobilized lipoplexes varied widely between the four donors tested, but multiple ECM mimetic substrates increased luciferase expression relative to FBS-coated control substrates, in hAMSCs derived from all donors except Donor 3 (Figure 5(a)). All donors except for Donor 3 also displayed increased cell counts on multiple ECM mimetic substrates with immobilized lipoplexes, relative to FBS-coated control substrates with immobilized lipoplexes (Figure 5(b)). Additionally, all donors displayed dramatic decreases in luciferase expression and cell count on substrates containing AG73 peptide with immobilized lipoplexes, relative to FBS-coated control substrates with immobilized lipoplexes (Figure 5(a) and (b)). Excluding Donor 3, all ECM mimetic substrates that did not contain AG73 (i.e. Hep + RGD, Dext + RGD, Hep + RGD + F2A, Dext + RGD + F2A, Hep + RGD + R9, and Dext + RGD + R9) with immobilized lipoplexes resulted in increased luciferase transgene expression and did not decrease cell count, relative to FBS-coated control substrates with immobilized lipoplexes (Figure 5(a) and (b)).

Discussion

Substrate-mediated gene delivery (SMD) involves the immobilization of nucleic acid complexes to a biomaterial prior to seeding cells for transfection on complex-coated surfaces. SMD transfection allows for reduced complex aggregation and elevated nucleic acid concentrations within the local microenvironment of adhered cells to increase transfection efficiency over bolus delivery of complexes.[8–16] SMD has potential applications for *ex vivo* modifications of cells for cell therapies and tissue engineering strategies, as well as *in vivo* for bioactive devices and implants.[17–19] Traditionally, SMD substrates have required coatings of natural materials like serum or isolated ECM proteins, to promote nucleic acid complex immobilization,[9] cell adhesion,[48] and subsequent transfection. Specifically, coatings of fetal bovine serum (FBS) and fibronectin enable SMD transfection by facilitating complex adsorption through non-specific electrostatic interactions,[10] promoting cell adhesion, and inducing nucleic acid complex endocytosis and trafficking, to increase luciferase transgene expression in fibroblasts by over 10- and 100-fold, respectively, compared to uncoated tissue culture polystyrene substrates with immobilized complexes.[10,28] While natural

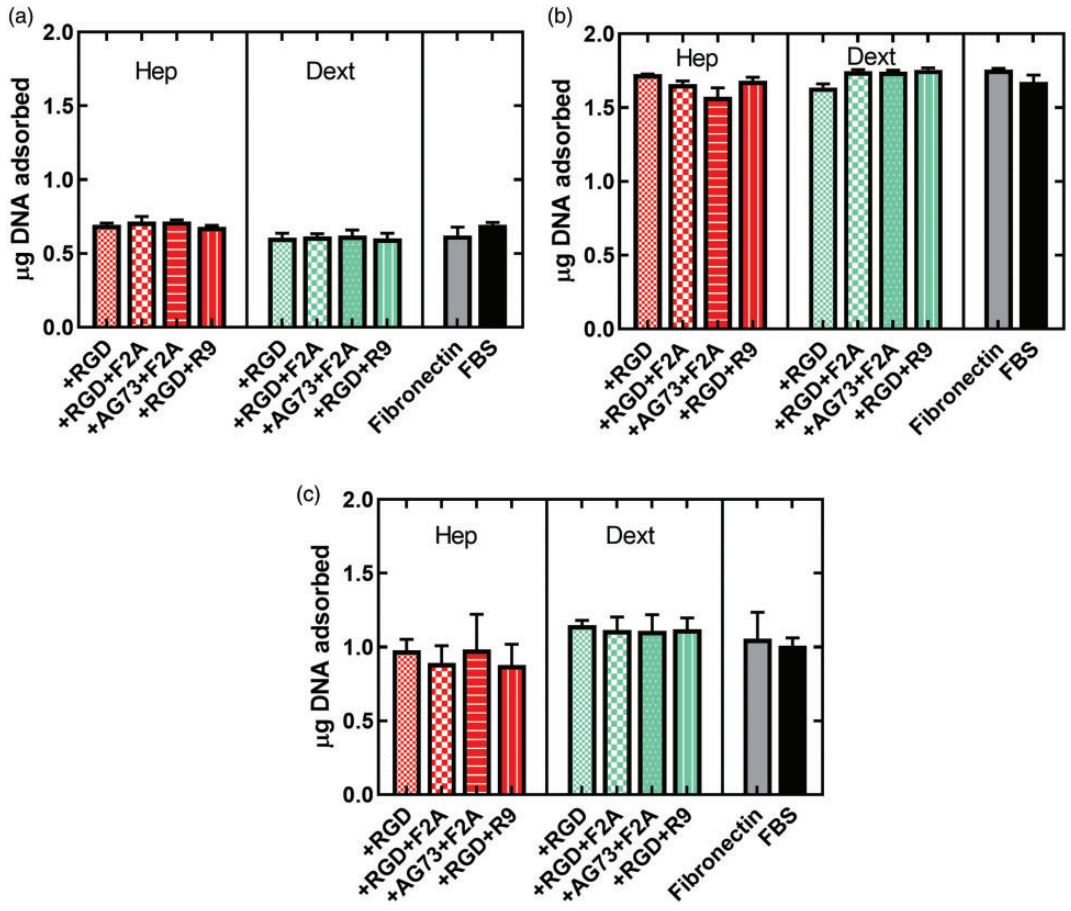

Figure 4. ECM mimetic substrates do not increase pDNA complex immobilization. pDNA complex adsorption to substrates was quantified by measuring radiolabeled pDNA. Substrates did not significantly affect the amount of pDNA complexes adsorbed, relative to FBS controls, for (a) LF2K complexes, (b) PEI complexes, or (c) LF3K complexes ($P > 0.05$) ($n = 3$). A total of 2.0 μg of complexed pDNA was added to each well, in 48-well plates. (A color version of this figure is available in the online journal.)

coatings like FBS and fibronectin have demonstrated enhancement of both bolus and SMD transfection by presenting bioactive signals similar to those found in ECM,[28,30] natural materials also display high batch variability based on source, which limits reproducibility and potential clinical translation of SMD transfection applications.[34] Therefore, developing chemically defined synthetic SMD substrates with peptides that mimic those found in natural materials is an attractive strategy to replace the current materials that have limited clinical utility.

We first designed a library of 20 different ECM mimetic substrates by combining GAGs and mimetic peptides (Figure 1), derived from proteins shown to influence cell adhesion, growth, and transfection mechanisms (Table 1), to be screened for their ability to promote SMD transfection of plasmid DNA (pDNA) encoding for a fusion reporter protein of luciferase and enhanced green fluorescent protein (EGFP). We show that screening the novel, chemically defined, and scalably produced, combinatorial library of substrates can be used to identify surface parameters optimal for different SMD transfection protocols. We initially screened the library of 20 different substrates for their ability to promote luciferase transgene expression in NIH/3T3s SMD transfected with common transfection reagents, either the lipid-based Lipofectamine 2000 (LF2K) or the polymer polyethylenimine (PEI) (Supplementary Figure 6) before selecting a refined set of substrates to characterize in further transfection experiments. The results of LF2K and PEI SMD transfection of NIH/3T3s on ECM mimetic substrates, relative to FBS controls, differed between preliminary screens (Supplementary Figure 6(a) and (b)) and subsequent experiments (Figures 2 and 3), which may be due to batch-to-batch variability in FBS, and motivates the development of more defined materials for SMD transfection.

In SMD transfection of NIH/3T3s (Figure 2) using lipoplexes, no ECM mimetic substrates with immobilized lipoplexes resulted in significantly decreased luciferase transgene expression, relative to FBS-coated control substrates with immobilized lipoplexes, suggesting our chemically defined ECM mimetic substrates could replace conventional protein coatings in SMD applications. Additionally, Hep + RGD and Hep + RGD + R9 substrates with immobilized lipoplexes increased luciferase transgene expression by over 2-fold, relative to FBS-coated control substrates with immobilized lipoplexes (Figure 2(a)). RGD is a natural motif within fibronectin and other ECM proteins found in serum, which is bound by ubiquitously

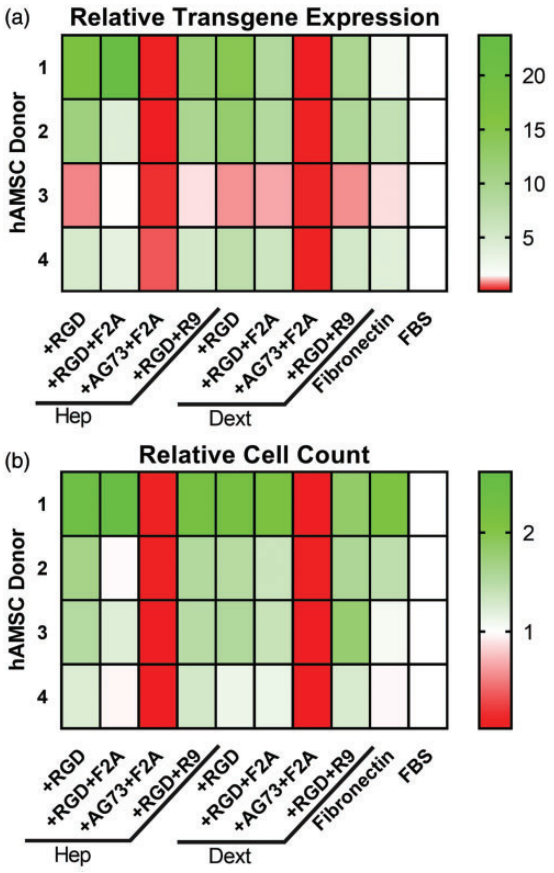

Figure 5. Normalized results of all hAMSC donors SMD transfected on different ECM mimetic substrates. Heat map represents means of (a) transgenic luciferase expression, as measured by relative light units per mg of protein (RLU/mg Protein), and (b) total cell count, for each hAMSC donor on all ECM mimetic substrates with immobilized pDNA LF3K complexes, normalized to FBS-coated control substrates, 48 h after seeding cells. (A color version of this figure is available in the online journal.)

expressed transmembrane receptors known as integrins,[49–51] to facilitate cell adhesion, motility, as well as endocytosis.[52,53] RGD peptides in ECM mimetic substrates likely promoted NIH/3T3 LF2K SMD transfection by inducing endocytosis of immobilized complexes, as RGD peptides have been incorporated within gene delivery vectors to increase endocytic internalization[54–57] and nuclear translocation,[58,59] and coating substrates with integrin-binding antibodies has been shown to provide equal or greater LF2K SMD internalization and transgene expression than fibronectin or collagen coatings, in six different cell types.[60] In addition to the RGD peptide, the R9 peptide also affected NIH/3T3 LF2K SMD transfection. Like many cell-penetrating peptides (CPPs), R9 can interact with cell membranes and induce endocytosis.[61] CPPs have been used to deliver a wide variety of biomolecules to cells, including nucleic acid complexes, and it is possible that R9 peptide in ECM mimetic substrates promoted transfection by inducing endocytosis and trafficking of immobilized lipoplexes. With respect to the different GAGs in the ECM mimetic substrates affecting LF2K SMD transfection of NIH/3T3s, substrates containing Hep promoted more efficient transfection than substrate coatings containing Dext, as Dext + RGD and Dext + RGD + R9 substrates with immobilized lipoplexes did not enhance NIH/3T3 luciferase expression over FBS-coated control substrates with immobilized lipoplexes (Figure 2(a) and (b)). Regardless of peptide or GAG type in the coating, enhanced LF2K SMD transfection in NIH/3T3s on ECM mimetic substrates does not appear to be due to increased cell adhesion, proliferation, or survival, as cells transfected on ECM mimetic substrates with immobilized lipoplexes displayed no statistically significant changes in cell count or metabolic activity, relative to FBS-coated control substrates with immobilized lipoplexes (Figure 2(c) and (d)). Furthermore, no ECM mimetic substrates significantly affected pDNA lipoplex immobilization relative to FBS-coated control substrates (Figure 4(a)), suggesting that combinatorial cues from heparin, along with either RGD or R9, induced endocytic and trafficking pathways to promote transgene internalization and trafficking.

Because mechanisms of transfection can differ between nonviral gene delivery vectors, we next screened our ECM mimetic substrate library for SMD transfection of NIH/3T3s using a different nucleic acid complexing reagent. Similar to the LF2K results, no ECM mimetic substrates significantly decreased luciferase transgene expression after PEI SMD transfection of NIH/3T3s, but luciferase expression was increased by 3- and 2-fold on Hep + RGD + F2A and Hep + AG73 + F2A substrates with immobilized polyplexes, respectively, all relative to FBS-coated control substrates with immobilized polyplexes (Figure 3(a)). In contrast to LF2K results, several ECM mimetic substrates with immobilized polyplexes significantly increased NIH/3T3 total cell counts, relative to FBS control conditions with immobilized polyplexes (Figure 3 (c)), indicating improved cell growth and/or survival, potentially through induction of adhesion, proliferation, and/or survival pathways. As expected, due to transfection mechanisms differing by vector, the highest performing substrates with immobilized LF2K lipoplexes contain different peptide combinations than the highest performing substrates with immobilized PEI polyplexes. Both of the substrates that enhanced PEI SMD transfection of NIH/3T3s contain F2A (i.e. Hep + RGD + F2A and Hep + AG73 + F2A), a mimetic peptide of fibroblast growth factor, a signaling protein which, upon binding of cell surface receptors, activates pathways to promote survival and proliferation, and induces receptor endocytosis.[62] Addition of F2A peptide may support PEI SMD transfection of NIH/3T3s by possibly promoting survival and proliferation to ameliorate PEI toxicity[63] and/or by activating endocytic and trafficking pathways that increase uptake of immobilized polyplexes. The highest performing substrates for PEI SMD also contained either RGD or AG73 adhesion peptides. In contrast to RGD, which enhanced LF2K SMD transfection discussed above, AG73 is a peptide derived from laminin, a protein found in basement membrane ECM that facilitates adhesion of some cell types. AG73 may provide signals to NIH/3T3s that modulate SMD transfection with PEI, but do not affect LF2K transfections. Similar to LF2K results, Hep GAG substrates displayed improved polyplex-mediated transfection over

Dext substrates, as Dext + RGD + F2A and Dext + AG73 + F2A substrates with immobilized polyplexes did not significantly increase NIH/3T3 luciferase expression or transfection efficiency, relative to FBS-coated control substrates with immobilized polyplexes. Similar to LF2K, ECM mimetic substrates did not affect PEI-pDNA complex immobilization (Figure 4(b)), suggesting that SMD PEI transfection of NIH/3T3s was enhanced by combinatorial cues from heparin, along with RGD or AG73, and F2A, to presumably influence cell adhesion, survival, and proliferation, and/or transgene internalization and trafficking.

After demonstrating that NIH/3T3 SMD transfection with both LF2K and PEI can be enhanced by ECM mimetic substrates, we next aimed to determine if ECM mimetic substrates could enhance SMD transfection of clinically relevant hAMSCs, which are intensely investigated for use in cell and gene therapies.[64] Safe and efficient gene delivery to hMSCs could advance applications in tissue engineering and regenerative medicine by enabling expression of transgenes for differentiation, enhanced survival, directed tissue homing, or secretion of therapeutic biomolecules.[65] While viral gene delivery to hMSCs is efficient, viral methods can be immunogenic and mutagenic,[66] and viruses are limited by small transgene capacity and difficult design and scale-up.[67] Nonviral methods overcome many of these shortcomings, but suffer from low efficiency, especially in hMSCs. For example, optimized transfection of hMSCs with LF2K results in only 10–30% efficiency,[68–71] and PEI is only able to achieve about 20% transfection efficiency.[71–73] Furthermore, both lipid- and polymer-based transfection is associated with significant toxicity in hMSCs,[74,75] limiting both transgene expression levels and downstream therapeutic efficacy. Therefore, more efficient and less toxic nonviral gene delivery methods are needed to advance clinical applications of transfected hMSCs. hAMSCs were transfected with LF3K because it has displayed superior hMSC transfection and viability in our unpublished experiments, relative to LF2K and PEI.

In addition to low transfection efficiency, hMSC therapies are limited by large variability between donors, with respect to gene expression, proliferative capacity, differentiation potential, and immunomodulatory potency.[45–47] hMSC transfection efficiency and luciferase transgene expression have also been shown to vary by up to 4-fold and 10-fold, between donors, respectively.[44,69] Because of such variability, we screened selected ECM mimetic substrates for SMD transfection using hAMSCs from four different donors, and hAMSC SMD transfection with LF3K complexes on ECM mimetic substrates varied remarkably among cells from the four different human donors tested (Figure 5, Supplementary Figures 7 to 10), presumably due in part to the large variability in properties referenced above. The large variability in transfection seen between donors on defined substrates in this study highlights the challenge of reproducibility that must be met when developing future cell therapies. Multiple ECM mimetic substrates with immobilized lipoplexes promoted significantly increased luciferase transgene expression over FBS-coated control substrates with immobilized lipoplexes, in hAMSCs derived from all donors, except Donor 3 (Figures 5). For example, five or more different ECM mimetic substrates with immobilized lipoplexes increased luciferase expression by at least 5-fold in Donors 1, 2, and 4 hAMSCs, and Donors 1 and 2 displayed over 10-fold luciferase expression increases on multiple ECM mimetic substrates with immobilized lipoplexes, all relative to FBS-coated control substrates with immobilized lipoplexes (Figure 5(a)). All donors displayed dramatic decreases in luciferase expression and cell count on substrates containing AG73 peptide with immobilized lipoplexes, presumably due to poor adhesion and proliferation of hAMSCs on AG73 containing substrates (Figure 5). Excluding Donor 3, all ECM mimetic substrates with immobilized lipoplexes that did not contain AG73 (i.e. Hep + RGD, Dext + RGD, Hep + RGD + F2A, Dext + RGD + F2A, Hep + RGD + R9, and Dext + RGD + R9) resulted in increased luciferase transgene expression in hAMSCs (Figure 5(a)) without negative effects on cell adhesion and/or growth (Figure 5(b)), all relative to FBS-coated control substrates with immobilized lipoplexes.

Enhanced hAMSC transgene expression induced by the above ECM mimetic substrates with immobilized lipoplexes, over FBS-coated control substrates with immobilized lipoplexes, is likely due to increased cell adhesion, proliferation, or survival, and/or possibly increased lipoplex internalization and trafficking induced by the presented peptides. Previous studies have demonstrated that, in the absence of protein coatings, RGD peptides presented on surfaces promote hMSC adhesion, proliferation, and viability,[76–78] and simply treating hMSCs with soluble RGD significantly increases adenoviral endocytosis and subsequent transgene expression.[79] Furthermore, fibroblast growth factor has been shown to promote proliferation, inhibit apoptosis, and reduce oxidative stress in hAMSCs,[80] and fibroblast growth factor-derived peptides have been conjugated to nucleic acid-complexing polymers[81] as well as viral vectors[82] to facilitate receptor-mediated endocytosis and subsequent transgene expression. Moreover, the cell penetrating R9 peptide on ECM mimetic substrates likely activates endocytic pathways in hMSCs to increase SMD transfection. Based on our results, considered along with the studies discussed above involving transfection mechanisms and ligands similar to those found in ECM mimetic substrates, we hypothesize that combinations of GAGs and mimetic peptides in our ECM mimetic substrates enhanced SMD transfection by promoting hAMSC adhesion, proliferation, and/or survival, as suggested by increased cell counts relative to FBS controls (Figure 5(b)), while possibly also activating endocytic and trafficking pathways to promote transgene uptake and subsequent expression. Given our results, future SMD studies in hAMSCs should make use of ECM mimetic substrates containing Heparin or Dextran sulfate GAGs along with RGD adhesion peptide.

Most critically, our results showing variability between cell type, human donor source, and transfection reagent demonstrate the utility of a combinatorial library to identify optimal parameters for the development of application- and patient-specific transfection technologies. The

challenges of large variability and unpredictability between different cell types and patient samples could be overcome by a personalized medicine approach making use of screenable biomaterials. In addition to screening other peptides for promotion of cell adhesion, proliferation, and survival, as well as transgene uptake and expression, the strategy described in this work could also be utilized to identify substrates that promote other cellular phenotypes important for specific applications, such as genetic modification of cells for tissue engineering,[7] regenerative medicine,[20] and bioactive implants.[6] Furthermore, the hydrogel system used to produce the ECM mimetic substrate library in this work has previously shown high biocompatibility, and did not cause acute immune response after injection into immunocompetent mice for up to 10 months,[83] suggesting the ECM mimetic substrates with immobilized nucleic acid complexes could be used for coated bioactive implants or tissue engineering scaffolds, to facilitate SMD transfection *in vivo*.

Finally, we acknowledge some limitations and future directions for this study. The goal of this paper was to compare defined ECM mimetic substrates against ill-defined FBS coatings, which can produce variable outcomes due to batch-to-batch variability. Normalizing values to a standard control with high variability (i.e. FBS), allows for comparisons of ECM mimetic substrates against FBS, in experiments run on different days, and in cells from different donors, but makes the variability of results on ECM mimetic substrates difficult to assess. Future studies to further optimize ECM mimetic substrates for SMD transfection will focus on direct comparisons of different ECM mimetic substrate formulations, without the use of ill-defined and variable coatings. In addition, all ECM mimetic substrates used 5 μM peptide-starPEG to form films, meaning the absolute concentration of specific peptide conjugates (i.e. RGD or AG73) changed when other conjugates were added to a formulation (i.e. F2A or R9). These ratios of the component peptides were used, as they have been optimized for cell adhesion and growth without transfection in preliminary experiments (data not shown), but future experiments will optimize all parameters in the context of SMD transfection and/or specific applications.

Conclusions

We screened a chemically defined combinatorial library of self-assembling ECM mimetic substrates, containing GAGs and mimetic peptides derived from adhesion proteins, growth factors, and cell penetrating proteins (Table 1), for pDNA lipoplex and polyplex SMD transfection of fibroblasts and human mesenchymal stem cells. Optimal ECM mimetic substrates varied between cell type, donor source, and transfection reagent, but multiple substrates significantly improved transgene expression (i.e. 2- to 20-fold) over standard protein coatings. Our results suggest that future SMD studies in hMSCs should make use of substrates containing Heparin or Dextran sulfate GAGs along with RGD adhesion peptide. Considering previous research on similar ligands, we hypothesize ECM mimetic substrates modulate cell adhesion, proliferation, and survival, as well as transgene internalization and trafficking. This study demonstrated the utility of screening libraries of chemically defined ECM mimetic substrates, which could replace conventional protein coatings, for clinically translatable SMD transfection technologies that may require formulations unique to specific applications, cell types, or patients for personalized medicine.

Authors' contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. AH, AKT, TK, EF, and SL conducted experiments AH, AKT, TK, YZ, and AKP wrote the manuscript.

DECLARATION OF CONFLICTING INTERESTS

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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