Prechondrogenic ATDC5 Cell Attachment and Differentiation on Graphene Foam; Modulation by Surface Functionalization with Fibronectin

Stephanie M. Frahs,† Jonathon C. Reeck,† Katie M. Yocham,‡§ Anders Frederiksen,‖ Kiyo Fujimoto,§ Crystal M. Scott,† Richard S. Beard, Jr.,† Raquel J. Brown,† Trevor J. Lujan,‡ Ilia A. Solovyov,†∥ Richard S. Beard, Jr.,† Trevor J. Lujan,‡ Ilia A. Solovyov,†∥ and Julia Thom Oxford‡⊥

†Center of Biomedical Research Excellence in Matrix Biology, Biomolecular Research Center, Boise State University, Boise, Idaho 83725, United States
‡Department of Mechanical and Biomedical Engineering, Boise State University, Boise, Idaho 83725, United States
§Micron School of Materials Science and Engineering, Boise State University, Boise, Idaho 83725, United States
‖University of Southern Denmark, Department of Physics, Chemistry and Pharmacy, Campusvej 55, 5230 Odense M, Denmark
♯Department of Physics, Carl von Ossietzky Universität Oldenburg, Carl-von-Ossietzky-Straße 9-11, 26129 Oldenburg, Germany
∥Department of Biological Sciences, Boise State University, Boise, Idaho 83725, United States

ABSTRACT: Graphene foam holds promise for tissue engineering applications. In this study, graphene foam was used as a three-dimension scaffold to evaluate cell attachment, cell morphology, and molecular markers of early differentiation. The aim of this study was to determine if cell attachment and elaboration of an extracellular matrix would be modulated by functionalization of graphene foam with fibronectin, an extracellular matrix protein that cells adhere well to, prior to the establishment of three-dimensional cell culture. The molecular dynamic simulation demonstrated that the fibronectin–graphene interaction was stabilized predominantly through interaction between the graphene and arginine side chains of the protein. Quasi-static and dynamic mechanical testing indicated that fibronectin functionalization of graphene altered the mechanical properties of graphene foam. The elastic strength of the scaffold increased due to fibronectin, but the viscoelastic mechanical behavior remained unchanged. An additive effect was observed in the mechanical stiffness when the graphene foam was both coated with fibronectin and cultured with cells for 28 days. Cytoskeletal organization assessed by fluorescence microscopy demonstrated a fibronectin-dependent reorganization of the actin cytoskeleton and an increase in actin stress fibers. Gene expression assessed by quantitative real-time polymerase chain reaction of 9 genes encoding cell attachment proteins (Col44, Ctnma1, Ctnnb1, Iga3, Iga5, Igav, Igbl1, Niam1, Sgc6), 16 genes encoding extracellular matrix proteins (Col1a1, Col2a1, Col3a1, Col5a1, Col6a1, Ecm1, Emlin1, Fn1, Hapl1, Lamb3, Postn, Sparc, Spp1, Thbs1, Thbs2, Tnc), and 9 genes encoding modulators of remodeling (Adams1, Adams2, Ctgf, Mmp14, Mmp2, Tgfb1, Timp1, Timp2, Timp3) indicated that graphene foam provided a microenvironment conducive to expression of genes that are important in early chondrogenesis. Functionalization of graphene foam with fibronectin modified the cellular response to graphene foam, demonstrated by decreases in relative gene expression levels. These findings illustrate the combinatorial factors of microscale materials properties and nanoscale molecular features to consider in the design of three-dimensional graphene scaffolds for tissue engineering applications.

KEYWORDS: ATDCS, chondroprogenitor cells, graphene foam, fibronectin, extracellular matrix, differentiation, tissue engineering, three-dimensional cell culture, bioscaffold, molecular dynamic simulation, dynamic mechanical analysis

1. INTRODUCTION

Biophysical, biochemical, and biomechanical cues from the extracellular environment have a significant effect on cellular response. Synthetic materials can be tailored to mimic the extracellular matrix in a context-specific manner to allow an investigation into fundamental mechanisms that govern how cells sense and respond to their environment, which will aid in the design and development of biomaterials for tissue repair and regeneration. In this study, our goal was to investigate the cellular attachment and response to a graphene foam (GF) scaffold functionalized with the extracellular matrix molecule fibronectin. Our results highlight the suitability of GF as a scaffold for chondrogenesis and the influence of fibronectin in combination with a GF scaffold on such processes. Extracellular
matrix functionalization can influence the measurable cellular response including cellular morphology, gene expression, and progress toward cellular differentiation outcomes.

Chondroprogenitor cells arise from several mesenchymal sources during vertebrate development, including the neural crest and the somites. Chondrogenesis is initiated as mesenchymal cells aggregate into condensations during skeletal development. During the process of condensation, the interactions among cells and between cells and matrix molecules are critical to the process. Cell–cell interactions are driven by cell surface receptors, and cell–matrix interactions are driven by extracellular matrix molecules, including collagens, proteoglycans, thrombospondins, laminins, and fibronectin. Prechondrogenic condensation is facilitated by extracellular matrix molecules, cell surface receptors and adhesion molecules. Fibronectin is essential in early embryogenesis and is upregulated in association with prechondrogenic condensations.

Fibronectin, a ubiquitous extracellular matrix protein, is assembled into a fibrillar matrix through a cell-mediated process and links cells with other extracellular matrix proteins, including collagens. Fibronectin matrix assembly is essential for cell condensation during chondrogenesis. In the chondroprogenitor ATDC5 cell line, cell condensation, and induction of chondrogenesis are dependent on the assembly of the fibronectin matrix. During condensation, cells produce a unique transitional extracellular matrix that is rich in specific proteins including collagen type I and fibronectin. In contrast to the early matrix composition, the extracellular matrix molecules produced by mature differentiated chondrocytes is rich in collagen type II.

Biomimetic scaffolds mimic the properties of a specific tissue environment. Three-dimensional scaffolds provide space in which cells can be trapped to foster cell–cell interaction, the establishment of a pericellular matrix and cell–matrix interactions, which together subsequently support cellular differentiation in response to local cues. In regenerative medicine, the biomimetic scaffold may be designed to recreate the native stem cell environment rather than that of the mature tissue. Novel scaffolds have been used to mimic the characteristics of the extracellular matrix of specific tissues, providing binding sites for ligands, timed-release of specific cytokines, and also the mechanical properties that are intrinsic to specific tissue types. GF as a biomimetic scaffold may provide a versatile platform upon which to design niche environments for stem cells and supply specific cues to drive differentiation of the cell and regeneration of the tissue.

The wide use of graphene scaffolds for stem cell investigation demonstrates the potential of graphene-based materials for the study of stem cell self-renewal, proliferation, and specific differentiation that will ultimately enable biomedical and regenerative medicine applications. To date, graphene-based materials have been used for the study of specific differentiation pathways including osteogenesis, neurogenesis, myogenesis, adipogenesis, chondrogenesis, and oligodendrogenesis. Although promising, what is not known currently are the conditions under which stem cell attachment can be fostered while also providing the specific molecular and biomechanical cues to promote differentiation along specific lineages to ultimately regenerate a functional tissue. This is particularly challenging in the field of cartilage tissue engineering due to cellular senescence, hypertrophy, and the dual potential for cells to convert to an osteoblast phenotype, resulting in mineralized tissue rather than cartilage.

Previous studies have revealed the importance of surface roughness on cell-substrate interactions as well as the surface functionalization on cell attachment and behavior. Additionally, the effect of interfaces on cell attachment and differentiation have been extensively investigated, specifically the effect of surface rigidity and viscoelasticity. However, a need exists to increase our understanding of the interaction between stem cells and three-dimensional bioscaffolds and how the interaction influences cell morphology and gene expression patterns. Studies testing the combinatorial effects of graphene foam as a scaffold material plus a biological molecule such as fibronectin on both attachment and differentiation have not been performed for chondroprogenitor cells. A more in-depth and fundamental understanding will support future therapeutic applications in regenerative medicine.

The objective of this study was to identify differences between GF and fibronectin-derivatized GF with respect to cell attachment, cell morphology, and expression of genes encoding early indicators of differentiation. Here we show that chondroprogenitor ATDC5 cells adhere to fibronectin and GF. In response, cells adopt a distinct cellular morphology dependent on the presence or absence of fibronectin. Fibronectin on GF changed the elastic mechanical properties of the GF, yet no significant changes in the dynamic mechanical properties were detected. An additive effect was observed in the mechanical stiffness when the graphene foam was both coated with fibronectin and cultured with cells for 28 days. The fibronectin protein adhered to the GF surface via interactions involving arginine amino acid side groups. Cells responded to their environment by expressing specific genes in a differential manner that was dependent upon both the scaffold and the fibronectin. The results of this study indicate that GF in combination with ECM molecules to serve as a transitional matrix may provide the cellular niche to drive differentiation. An ECM molecule other than fibronectin will be required for the productive regeneration of challenging tissues such as cartilage.

2. MATERIALS AND METHODS

2.1. Materials. Three dimensional GF was obtained from Graphene Laboratories (Graphene Laboratories Inc., Calverton, NY, U.S.A.). The scaffold used in these experiments comprised 7–10 atomic layers of graphene. The foam construct was two mm thick with a density of 4 mg/cm³, and a pore size of 580 μm. ATDC5 cells were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). The ECM Select Array was obtained from Advanced BioMatrix (San Diego, CA). The prechondrogenic cell line ATDC5 was originally derived from the differentiating teratocarcinoma stem cell line AT805. ATDC5 cells undergo a sequential transition of phenotype in vitro, including stages from mesenchymal condensation to calcification. Bovine fibronectin protein solution was obtained from R & D Systems (Biotechne Corporation, Minneapolis, MN, U.S.A.) and diluted to a concentration of 100 μg/ml in Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS). Paraformaldehyde and Triton X-100 were obtained from Sigma-Aldrich (St. Louis, MO). Block-Aid, Alexa Fluor 488 conjugated to phalloidin, and ProLong Gold Antifade with DAPI were obtained from Life Technologies (Carlsbad, CA). Glass bottom cell culture dishes were obtained from MatTek Corporation (Ashland, MA). Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12) and fetal bovine serum (FBS) were obtained from Gibco by Life Technologies (Grand Island, NY). TRizol reagent was obtained from Thermo Fisher Scientific (Hampton, NH).

2.2. Methods. 2.2.1. ECM Protein Cell Attachment Assay. ECM Select Array was obtained from Advanced BioMatrix (San Diego, CA). DOI: 10.1021/acsami.9b14670
The extracellular matrix screening array was composed of nine printed replicates of 400 μm diameter areas on glass functionalized with hydrogel printed with the extracellular matrix proteins at a concentration of 250 μg/mL. The following extracellular matrix proteins were screened for attachment: collagen I (COL I), collagen III (COL III), collagen IV (COL IV), collagen V (COL V), collagen VI (COL VI), fibronectin (FN), vitronectin (VTN), laminin (LMN), and tropoelastin (TE). Bovine serum albumin (BSA) was used as a negative control for attachment assays.

ATDC5 cells were seeded (5 × 10^4 cells/mL) to screen for cell adhesion to extracellular matrix proteins. After the ECM array was rinsed with PBS and conditioned for 5 min in the culture medium, five mL of cells suspended in culture medium was evenly distributed across the slide and incubated at 37 °C in 5% CO2. Attached cells were counted at 12 and 30 h for each extracellular matrix protein and each of nine replicates for each protein. Cell morphology and attachment were visualized using bright field microscopy. Cell counts were determined at 30 h and mean ± standard deviation was determined.

2.2.2. Molecular Dynamic Simulation of Fibronectin-Graphene Interaction. The PDB file for fibronectin type III domains 8–10 was obtained from the Protein Data Bank (PDB ID 1FNP). The fibronectin structure was placed atop of three 100 × 200 Å^2 graphene sheets and neutralized in water using NaCl by assuming the height of the simulation box equal to 8 Å. The protocol was adopted from earlier molecular dynamics (MD) simulation on similar systems. Two different orientations of fibronectin on graphene were considered, resulting in two independent simulations. Each configuration was simulated for a total of 400 ns with an integrator time step of 2 fs under 1 bar pressure control, 310 K temperature control and using periodic boundaries using NAMD. Particle Mesh Ewald method was used to treat the long-range electrostatics with a cutoff distance of 1.2 Å. CHARMM 36 force field was used to model the interatomic interactions in both the protein and in the graphene sheet.

2.2.3. Culture Conditions for Seeding and Maintenance of ATDC5 Cells. GF coated with fibronectin was prepared by applying 700 μL of 100 μg/mL fibronectin solution to 1 cm^2 200 Å graphene specimens (GF, GF + T2, GF + Wr). Following the functionalization, GF scaffolds were conditioned for 24 h in cell culture medium.

The GF scaffolds were seeded with 1.5 × 10^5 ATDC5 cells cultured for 24 h in DMEM/F-12 supplemented with 5% FBS, 100 U/mL penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere, 5% CO2. During the seeding process, approximately 30% of the cells adhered to the GF or GF—fibronectin surface. Cells were maintained in parallel under 2-D culture conditions on glass-bottom tissue culture wells for comparison. At day 11 of the proliferation phase, the growth medium was supplemented with 50 μg/mL ascorbate 2-phosphate, 10 mg/mL insulin, 5.5 mg/mL transferrin, and 6.7 μg/mL sodium selenite to induce chondrogenic differentiation. Samples for RNA extraction were collected 0, 3, and 7 days after initiation of differentiation from cells maintained in 2-D culture conditions and 17 days later for both 2-D and 3-D GF samples. Samples were collected on day 28 for the measurement of elastic and viscoelastic conditions and 17 days later for both 2-D and 3-D GF scaffolds. Samples for RNA extraction were collected 0, 3, and 7 days after induction of differentiation. Samples were collected on day 28 for the measurement of elastic and viscoelastic conditions and 17 days later for both 2-D and 3-D GF scaffolds. Samples were collected on day 28 for the measurement of elastic and viscoelastic conditions and 17 days later for both 2-D and 3-D GF scaffolds.

2.2.4. Confocal and Fluorescence Microscopy. Samples were fixed in 2.5% glutaraldehyde. After rinsing in deionized water, samples underwent dehydration using 50%, 70%, 90%, and 100% ethanol sequentially. After dehydration, the sample was taped to a silicon wafer for sputtering. The dehydrated GF with cells were sputter-coated with chromium using a CRC-150 (Torr Laboratories). A 12 nm coat was achieved after 75 s of exposure at 9.6 × 10^-6 Torr and 50W. An FEI-Teneo scanning electron microscope set at 3.00 kV was used to collect images while utilizing the T2 detector by the Boise State Center for Materials Characterization.

2.2.5. Scanning Electron Microscopy. Samples were fixed in 2.5% glutaraldehyde. After rinsing in deionized water, samples underwent dehydration using 50%, 70%, 90%, and 100% ethanol sequentially. After dehydration, the sample was taped to a silicon wafer for sputtering. The dehydrated GF with cells were sputter-coated with chromium using a CRC-150 (Torr Laboratories). A 12 nm coat was achieved after 75 s of exposure at 9.6 × 10^-6 Torr and 50W. An FEI-Teneo scanning electron microscope set at 3.00 kV was used to collect images while utilizing the T2 detector by the Boise State Center for Materials Characterization.

2.2.6. Mechanical Testing of GF with Fibronectin and Cells. The dynamic mechanical analysis was carried out using the Instron ElectroPuls E-10000 mechanical test system (Instron, Norwood, MA) using previously described methods. In brief, at day 28, GF specimens (GF, GF + fibronectin, GF + fibronectin + cells) were subjected to cyclic preconditioning to 14% compression, quasi-static loading to 12% compression, 2 min of relaxation, and then 1 Hz cyclic compression at 1% amplitude, where compressive strain was calculated as the ratio of change in thickness to original thickness. The compressive elastic modulus, equilibrium modulus, stress relaxation, dynamic modulus, and phase shift were then calculated from the corresponding stress—strain waveform.

2.2.7. Quantitative Real Time Polymerase Chain Reaction (qRT-PCR). RNA from each sample was extracted following the TRIzol protocol for RNA extraction (Thermo Fisher Scientific). Samples were flash-frozen with liquid nitrogen and then pulverized within the TRIzol reagent with an OMNI International TH homogenizer (Thomas Scientific). The RNA concentration was determined by measuring the absorbance at 260 and 280 nm. The RT2 First Strand synthesis method (Qigen) was used to generate cDNA. Expression levels were measured by qRT-PCR using a Roche Lightcycler 96 (Roche). Genes analyzed included extracellular matrix proteins, matrix remodeling enzymes, and cell adhesion molecules. Relative gene expression levels, mean plus/minus standard deviation, were expressed with respect to housekeeping genes determined empirically for this study.

2.2.8. Selection of Housekeeping Genes. Actb and Hsp90ab1 were selected as the housekeeping gene for normalization in these experiments based on comparison to three other candidate housekeeping genes (Gapdh, B2m, and Gusb) and were found to be stably expressed independent of experimental conditions based on minimal variance. Relative abundance values were calculated and reported here as mean plus/minus standard deviation.

2.2.9. Statistical Analysis. Cell attachment to extracellular matrix molecules was analyzed using the mean plus/minus standard deviation. The effect of culture time on the mechanical properties (compressive modulus, equilibrium modulus, stress relaxation, dynamic modulus, and phase shift) of the cellular graphene composites was analyzed using a one-way MANOVA in SPSS (p = 0.05) using the Least Significant Difference (LSD) correction for multiple comparisons. Selection of housekeeping genes for qRT-PCR was based on pairwise analysis of variance for differences between cycle threshold values for five candidate housekeeping genes from 15 samples within this study. Additionally, correlation analysis was carried out and data were fit to a trend line and R^2 was determined. Relative expression of genes of interest was analyzed relative to average values for Actb and Hsp90ab1 and expressed as mean plus/minus standard deviation. Log transformed gene expression data was subject to a paired t test to determine if the differences in mean values for relative gene expression were statistically significant, setting significance at p < 0.05.

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3. RESULTS

3.1. ECM Protein–Cell Attachment Assay. The chondroprogenitor cell line ATDC5 was derived from a mouse teratocarcinoma cell line. An extracellular matrix molecule array was utilized to screen specific extracellular matrix proteins for the ability of ATDC5 cells to adhere. Bright-field images were collected from each of the nine replicates of specific ECM proteins. Cell counts were determined at 12 and 30 h after initial cell plating. ATDC5 cells were found to adhere to collagen types I and IV, and fibronectin more extensively than other ECM molecules screened. The moderate affinity of ATDC5 to collagen types V and VI and little to no adherence of cells was observed for collagen III, vitronectin, tropoelastin, and laminin (Figure 1).

On the basis of this assay and information from published literature that indicates that fibronectin is essential for condensation during chondrogenesis, while in contrast, collagen type I is prevalent in dedifferentiated chondrocytes, bone, and other noncartilaginous tissues, and that collagen type IV, while present at low levels around chondrocytes, is a key marker for basement membranes, we chose fibronectin as a coating for GF to increase cellular adhesion of ATDC5 cells to the GF scaffold.

3.2. Fibronectin–graphene Interaction by Molecular Dynamic Simulation. Prior to coating GF with fibronectin, we used molecular dynamics simulations to better understand the interaction of fibronectin with our GF scaffolds. The binding of fibronectin was investigated in silico in two different independent molecular dynamics simulations of 400 ns each. One simulation studied a random configuration, while the other simulation was set up to investigate the effects of the arginine-glycine-aspartic acid (RGD) tripeptide to the binding energy, as it has been theorized to be a major contributor to binding with integrin. The simulations revealed that in both simulated cases, fibronectin interacted with the graphene sheets; characteristic renderings of the binding motifs are shown in Figure 2A and B.

The computations demonstrated that in both cases, arginine significantly stabilized the binding as demonstrated in the plots directly beneath the graphical representations in Figure 2. Several arginine residues were identified to bind directly to the surface in both configurations with approximate binding energy of 15 kcal/mol. The binding of fibronectin to the graphene
surface was due to a contribution from all amino acids, as summarized in Figure 2C and D. These plots stress that although arginine residues are only a small fraction of the total interacting amino acids, they provide the largest contribution to the fibronectin–graphene interaction, approximately 20–30% of the total binding energy as shown in Figure 2E and F.

The total fibronectin–graphene interaction energy was lower for the second configuration considered, approximately ~400 kcal/mol, however, other binding configurations with even lower binding energies may be possible and should not be excluded based on the data presented here. Figure 2D also demonstrates that the contribution from the RGD tripeptide to the total binding energy of fibronectin to the graphene surface is ~26.2 kcal/mol as the tripeptide consists of the residues Arg1493, Gly1494, and Asp1495. The RGD tripeptide therefore provides about 7% of the total binding energy. Note that in the

Figure 2. Fibronectin interaction with graphene is stabilized by arginine residues. (A) Graphical rendering of the stabilized fibronectin atop the three graphene sheets with the four best arginine binders highlighted (Arg1166, Arg1369, Arg1374, Arg1403). The time evolution of the binding energy of these arginine residues with graphene is shown in the lower panel, color-coded for the amino acid residues. (B) Analogous to A but showing the data for the second studied configuration. This configuration features five arginine residue binders (Arg1166, Arg1351, Arg1379, Arg1445, Arg1493). (C) Binding energy with graphene computed for every amino acid with average binding energy above 1 kcal/mol, averaged over the 400 ns simulation. (D) Analogous to C, for the second studied configuration. The residue numbers are indicated, while the corresponding amino acid types are color-coded for both panels (C and D). (E and F) Time evolution of the fibronectin and arginine interaction energy with graphene for the two configurations. The lower plots in both panels show the fraction of arginine residue binding energy with respect to the total fibronectin-binding energy as a function of simulation time.

Figure 3. Cell seeding on GF overview.
performed analysis, the graphene sheet atoms were assumed neutral and no induced charge effects were considered. The binding energies are therefore, purely van der Waals in nature, and are expected to be even lower if polarization effects are accounted for.

3.3. Cellular Response to GF. Cells were seeded on GF according to the timeline shown in Figure 3. Cells seeded on GF were able to adhere to the surface of the GF as well as to other cells during an initial 24 h incubation period, forming small clusters of cells in and between the cavities of the foam scaffold during the subsequent growth and differentiation period (Figure 4).

Using a combination of transmitted and fluorescence microscopy, DAPI was used to determine the location of cellular nuclei and phalloidin to label the cytoskeleton. Images shown in Figure 4 represent cells in culture on bare three-dimensional GF for 28 days. GF imaged by transmitted light microscopy images are shown in Figure 4A and D. Fluorescence microscopy of DAPI stained cells on GF to show nuclei are shown in Figure 4B and F. Fluorescence microscopy was also used to demonstrate the organization of the F-actin of the cytoskeleton using phalloidin labeled with Alexa Fluor 488 (Figure 4C and G). An overlay of transmitted light, DAPI, and phalloidin staining provides information about the relative location of the scaffold and the cells and is shown in Figure 4D and H.

3.4. Mechanical Properties of GF−FN+cells. The effect of fibronectin and cells on the mechanical properties (compressive modulus, equilibrium modulus, stress relaxation, dynamic modulus, and phase shift) of the cellular graphene composites was analyzed using a one-way MANOVA in SPSS ($p = 0.05$) using the LSD correction for multiple comparisons. The elastic properties of GF were enhanced by the addition of fibronectin (Figure 5A and B) and when fibronectin was used in cell culture, an additive effect was observed. The viscoelastic mechanical properties, phase shift and stress relaxation (Figure 5C and D) were also measured.
5C and E), of GF were unaffected by the addition of fibronectin to the GF scaffold. The ratio of dynamic modulus (Figure 5D) to equilibrium modulus (Figure 5B) remained consistent between groups (∼4×). The effects of cells and fibronectin on the mechanical properties (compressive modulus, equilibrium modulus, stress relaxation, dynamic modulus, and phase shift) of the cellular graphene composites were analyzed using a one-way MANOVA in SPSS (p = 0.05) using the LSD correction for multiple comparisons. These results indicate a significant change due to the addition of fibronectin, even at this early stage in culture and may provide new insights on the structure—function relationships of GF.

Cytoskeletal organization within cells on GF was dependent on the presence or absence of fibronectin coating (Figure 6). Comparison of actin cytoskeletal arrangement on GF compared to control cultures grown on glass-bottom tissue culture wells confirmed that the cytoskeletal morphology was a function of the presence of fibronectin rather than the scaffold. Fluorescence micrographs demonstrate that cell growth on a surface in the presence of fibronectin resulted in an enhancement of stress fibers within the cytoskeleton accompanied by an absence of globular puncta of F-actin that were prevalent in control cultures without fibronectin (compare green Alexa Fluor 488 staining in Figure 6A, B, and E, F). ATDC5 cells grown on GF in the absence and presence of fibronectin demonstrate similar findings to cells grown on the glass surface. Fibronectin coating resulted in alteration in the cytoskeletal organization in a manner that supported the formation of stress fibers on GF (compare Figure 6C, D, and G, H). Globular puncta of F-actin is more prevalent in the absence of fibronectin on glass-bottomed tissue culture wells as well as on GF. The cytoskeletal arrangement is a key aspect of cellular phenotype during chondrocyte differentiation and has been shown to correlate to gene expression of chondrogenic markers.57–65

3.5. Visualization of GF and Cell—GF Associations Using Scanning Electron Microscopy. Cell—GF interactions were visualized using scanning electron microscopy. Figure 7 illustrates the 3D spaces available for cells to colonize (Figure 7A) and surface roughness characteristics of the GF (Figure 7B).

The GF had a density of 4 mg/cm³, and pore size of 580 μm. Cells were able to adhere to bare GF (Figure 7C) as well as fibronectin-coated GF (Figure 7D). Cell adhesion may be supported by both surface roughness as well as the presence of fibronectin, consistent with studies from other laboratories that have investigated adhesion of nonchondrogenic cells as a function of surface roughness and fibronectin.28–68

3.6. Gene Expression Analysis. 3.6.1. Housekeeping Gene Selection. We used qRT-PCR gene expression analysis for the selection of housekeeping genes (HKGs). ActB, B2m, Gapdh, GusB, and Hsp90ab1 were analyzed for all samples in this study. ActB and Hsp90ab1 cycle threshold levels were most consistent among all samples analyzed for candidate HKGs.
considered, based on pairwise analysis of variance for differences between threshold values (Figure 8A). Correlation analysis resulted in a trend line with a slope close to 1 (1.177) and R² close to 1 (0.9699) (n = 15) (Figure 8B).

| gene symbol | protein name | function in chondrogenesis | reference |
|-------------|--------------|----------------------------|-----------|
| Cd44        | Hyaluronate receptor | cell–matrix interactions during chondrogenesis and matrix assembly | Knudson 2003\(^{39}\) |
| Ctnna1      | Catenin, alpha 1  | mediates functional mesenchymal cell condensation | Delise 2002\(^2\) |
| Ctnnb1      | Catenin, beta 1  | mediates functional mesenchymal cell condensation | Delise 2002\(^2\) |
| Igα3        | Integrin alpha 3 | mediates the connection between the cell and its external environment | Kim 2005\(^{38}\) |
| Igα5        | Integrin alpha 5 | mediates chondrocyte adhesion to cartilage | Kurtis 2003\(^{71}\) |
| Igαv        | Integrin alpha V | mediates the connection between the cell and its external environment | Kurtis 2003\(^{71}\) |
| Igβ1        | Integrin beta 1  | maintains the chondrocyte phenotype, prevents chondrocyte apoptosis, regulates chondrocyte-specific gene expression; mediates cell–matrix interactions; involved in chondrocyte mechanoreception | Kurtis 2003\(^{71}\); Shokiba 2008\(^{72}\) |
| Ncam1       | Neural cell adhesion molecule | present in mesenchymal cell condensations; abundance increases during cell aggregation | Tavella 1994\(^{73}\) |
| Sgce        | Sarcoglycan epsilon | transmembrane protein linking cytoplasm to extracellular matrix | Rouillard 2016\(^{24}\) |
Table 2. ECM Genes Expressed during Chondroprogenitor Cell Differentiation on GF

| gene symbol | protein name | functional classification: ECM | function in chondrogenesis | reference |
|-------------|--------------|---------------------------------|-----------------------------|-----------|
| Col1a1      | Collagen α1(I) | major fibrillar collagen         | Treilleux 1992              |
| Col2a1      | Collagen α1(II) | major fibrillar collagen         | Liu 2013, Atsumi 1990       |
| Col3a1      | Collagen α1(III) | fibrillar collagen               | Lodewyckx 2012              |
| Col5a1      | Collagen α1(V)  | fibrillar collagen               | Lodewyckx 2012              |
| Col10a1     | Collagen α1(VI) | pericellular collagen            | Zelenksi 2015               |
| Ecm1        | Extracellular matrix protein-1 | interacts with perlecain; regulates chondrogenesis | Kong 2016, Mongiat 2003 |
| Emn1        | Elastin microfibril interface-located protein 1 | integrin binding activity; tissue remodeling in noncartilaginous tissues | This paper for chondrocyte differentiation |
| Fn1         | Fibronectin    | essential for early chondrocyte differentiation | White 2003, Singh 2014      |
| Hapl1       | Hyaluronan and proteoglycan link protein 1 | organizes extracellular matrix; links proteoglycan to hyaluronan | Xu 2008    |
| Lamk3       | Laminin subunit beta-3 | basement membrane protein; promotes chondrogenesis | Sun 2017 |
| Postn       | Periostin      | basement membrane protein; promotes chondrogenesis | Inaki 2018 |
| Sarc        | Secreted protein acidic and rich in cysteine; Osteonectin | matricellular protein with calcium-binding properties; osteonectin, functions in growth and remodeling | Sage 1989 |
| Spn1        | Secreted phosphoprotein; Osteopontin Bone sialoprotein 1 | small integrin-binding ligand, N-linked glycoprotein | Shibata 2002 |
| Thbs1       | Thrombospondin 1 | matricellular protein; modulates cell−matrix interactions; Cartilage protection; | Miller 1988, DjCesare 1994, Pfander 2000, Maumus 2017 |
| Thbs2       | Thrombospondin 2 | matricellular protein; interacts with cell surface; regulates the bioavailability of proteases and growth factors in the pericellular environment | Jeong 2015 |
| Tnc         | Tenascin       | hexameric extracellular matrix glycoprotein prevalent in development; modulates cellular adhesion and interaction with fibronectin among other proteins | Ghubak 1996, Unno 2019, Mackie 1987 |

Table 3. Matrix Remodeling Genes Expressed during Chondroprogenitor Cell Differentiation Supported by Growth on GF

| gene symbol | protein name | functional classification: remodeling | function in chondrogenesis | reference |
|-------------|--------------|----------------------------------------|-----------------------------|-----------|
| Adams1      | A disintegrin and metalloproteinasin with thrombospondin motifs 1 | Aggrecanase and proteoglycanase; matrix rearrangement during chondrogenesis and cartilage regeneration | Boeuf 2012, Kelwick 2015 |
| Adams2      | A disintegrin and metalloproteinasin with thrombospondin motifs 2 | Procollagen N-propeptidase; regulates structure and function of extracellular matrix collagen fibril assembly | Kelwick 2015 |
| Ctgf        | Connective tissue growth factor (CCN2) | Cysteine-rich secreted protein with adhesive and chemostatic activities modulates matrix remodeling during skeletal development | Nakashishi 2000, Ivkovic 2003 |
| Mmp14       | Matrix metalloproteinase 14 | Matrix turnover during early chondrogenesis | Sekiya 2002 |
| Mmp2        | Matrix metalloproteinase 2 | Gelatinaise; required for matrix remodeling during fracture repair and skeletal and craniofacial development | Arai 2016, Liu 2011, Mosig 2007 |
| Tgfb        | TGF-beta-induced 68 kDa protein | Binds to collagen type I fibrils, inhibits mineralization and maintains chondrocyte phenotype | Hashimoto 1997, Huang 2010 |
| Timp1       | Tissue inhibitor of matrix metalloproteinase 1 | Inhibitor of MMPs and ADAMTSs | Peterson 2000 |
| Timp2       | Tissue inhibitor of matrix metalloproteinase 2 | Inhibitor of MMPs and ADAMTSs | Lin 2008 |
| Timp3       | Tissue inhibitor of matrix metalloproteinase 3 | Inhibitor of MMPs and ADAMTSs | Lin 2008 |

decreased initially during early chondrogenic differentiation (Figure 10C). Cells grown on GF expressed levels of Cd44, Sgc3, and Ncam1 meeting the threshold established in our control conditions (Figure 10D). Iiga3, Iiga5, and Iigav decreased during early chondrogenic differentiation followed by a gradual increase under our experimental conditions (Figure 10E). Growth on GF supported or enhanced gene expression levels for Iiga3, Iiga5, and Iigav, while the presence of fibronectin reduced this expression level (Figure 10F). Igbf1 expression increased initially during chondrogenic differentiation and plateaued between days 7 and 17 of our experiment (Figure 10G). Growth on GF supported the expression of Igbf1 at a level consistent with control conditions for chondrogenic differentiation (Figure 10H). These results suggest that 3D-GF supports chondrogenic differentiation and expression of adhesion molecules that serve as biomarkers for chondrocyte cells. Further, fibronectin alone or in combination with 3D-GF does not provide an advantage to 3D-GF alone.

3.6.2.2. Extracellular Matrix Molecules. Sixteen genes encoding extracellular matrix molecules were analyzed for differential expression over time under 2-D and 3-D culture conditions on GF with and without fibronectin (see Table 2 for list of genes and description). Col1a1 and Col3a1 increased over time during early chondrogenic differentiation (Figure 11A). Cells grown on GF expressed levels of Col1a1 and Col3a1 similar to control cultures (Figure 11B), indicating that growth on GF supported gene expression patterns consistent with chondrogenic differentiation. The effect of fibronectin coating on GF was...
a reduction in the expression level of these markers to below the levels observed in our controls. Col2a1 and Col5a1 increased throughout chondrogenic differentiation, while Col6a1 increased during early chondrogenesis and then plateaued at later time points (Figure 11C). Cells grown on GF expressed levels of Col2a1, Col5a1, and Col6a1 similar to control conditions with an enhancement of Col2a1 expression by cells seeded onto bare GF (Figure 11D). Enhancement of Col2a1 expression was diminished in the presence of fibronectin coating in contrast to the enhancement observed on bare GF.

Ecm1, Emilin1, Sparc, Spp1, Thbs1, and Postn increased at early chondrogenic time points and then plateaued, while Tnc, Fn, and Thbs1 increased throughout the time course of the experiment (Figure 11E, G, and I). Growth on GF supported gene expression levels for Ecm1, Emilin1, Tnc, Fn, Sparc, Spp1, Thbs1, Thbs2, and Postn consistent with or enhanced compared to levels observed under control conditions for chondrogenesis, and these levels were slightly reduced in the presence of fibronectin (Figure 11F, H, and J). Thbs1 and Postn expression were enhanced when cells were grown on bare GF. Hapln1 and Lamb3 mRNA levels initially dropped significantly during chondrogenic differentiation, followed by an increase in the case of Hapln1 and relative plateau for Lamb3 (Figure 11K). Cells seeded on GF expressed Hapln1 and Lamb3 at enhanced levels compared to control conditions, while the presence of fibronectin diminished the observed enhancement (Figure 11L). Taken together, these results suggest that 3D-GF supports chondrogenic differentiation and the expression of genes encoding extracellular matrix molecules that serve as biomarkers for chondrocyte cells. Further, fibronectin alone or in combination with 3D-GF does not provide an advantage to 3D-GF alone.

3.6.2.3. Matrix Remodeling Genes. Nine genes encoding remodeling enzymes their endogenous inhibitors, and mediators of remodeling were analyzed for differential expression over time under 2-D and 3-D culture conditions, and on GF with and without fibronectin (see Table 3 for a description of genes encoding matrix remodeling molecules). Adams1 and Adams2...
increased over time during early chondrogenic differentiation and then plateaued or decreased later in our time course of chondrogenic differentiation (Figure 12A). Cells grown on GF expressed levels of Adamts1 and Adamts2 similar to control cultures (Figure 12B), indicating that growth on GF supported gene expression patterns consistent with chondrogenic differentiation. The effect of fibronectin coating on GF was a reduction in the expression level of these markers to below the levels observed in our controls.

Mmp2 increased early in chondrogenesis and then plateaued while Mmp14 increased throughout chondrogenic differentiation (Figure 12C). Cells grown on GF expressed levels of Mmp2 and Mmp14 similar to control conditions (Figure 12D). Expression levels were decreased in the presence of fibronectin.

Timp1 expression levels remained constant over time during chondrogenesis while Timp2 and Timp3 expression levels increased throughout the time course (Figure 12E). Cells grown on GF expressed mRNA for Timp2 and reduced levels, Timp3 at enhanced levels, and Timp1 and a level consistent with expression levels observed under control conditions (Figure 12F). Reduced expression levels were observed in the presence of fibronectin coating. Expression levels of Ctgf and Tgfb1 increased during chondrogenic differentiation followed by a decrease or plateau level (Figure 12G). While cells seeded on GF expressed Ctgf and Tgfb1 at levels consistent with chondrogenesis, they were not influenced by the presence of fibronectin (Figure 12H), unlike other genes investigated in this study. These results suggest that 3D-GF supports chondrogenic
Figure 11. Expression of genes encoding extracellular matrix proteins by ATDC5 cells on glass-bottom tissue culture wells, GF, and fibronectin-GF. (A) Time course of gene expression during chondrogenic differentiation for Col1a1 (circle) and Col3a1 (triangle). (B) Relative gene expression levels of Col1a1 (gray) and Col3a1 (black) at day 17 of chondrogenic differentiation in control 2D culture, 2D culture in the presence of fibronectin, 3D-GF, and 3D-GF coated with fibronectin. (C) Time course of gene expression during chondrogenic differentiation for Col2a1 (circle), Col5a1 (triangle), and Col6a1 (square). (D) Relative gene expression levels of Col2a1 (gray), Col5a1 (black), and Col6a1 (white) at day 17 in control 2D culture, 2D culture in the presence of fibronectin, 3D-GF, and 3D-GF coated with fibronectin. (E) Time course of gene expression during chondrogenic differentiation for Ecm1 (circle), Emilin1 (triangle), and Tnc (square). (F) Relative gene expression levels of Ecm1 (gray), Emilin1 (black), and Tnc (white) at day 17 in control 2D culture, 2D culture in the presence of fibronectin, 3D-GF, and 3D-GF coated with fibronectin. (G) Time course of gene
expression during chondrogenic differentiation for Fn (circle), Sparc (triangle), and Spp1 (square). (H) Relative gene expression levels of Fn (gray), Sparc (black), and Spp1 (white) at day 17 in control 2D culture, 2D culture in the presence of fibronectin, 3D-GF, and 3D-GF coated with fibronectin. (I) Time course of gene expression during chondrogenic differentiation for Thbs1 (circle), Thbs2 (triangle), and Postn (square). (J) Relative gene expression levels of Thbs1 (black), Thbs2 (white), and Postn (gray) at day 17 in control 2D culture, 2D culture in the presence of fibronectin, 3D-GF, and 3D-GF coated with fibronectin. (K) Time course of gene expression during chondrogenic differentiation for Hapln1 (circle) and Lamb3 (triangle). (L) Relative gene expression levels of Hapln1 (gray) and Lamb3 (black) at day 17 in control 2D culture, 2D culture in the presence of fibronectin, 3D-GF, and 3D-GF coated with fibronectin. Error bars = Mean ± SD Table 2 lists extracellular matrix genes with description, function, and literature citations that corroborate an upregulation during early chondrogenic differentiation.

Figure 12. Expression of genes encoding matrix remodeling proteins and their endogenous inhibitors by ATDC5 cells on glass-bottom tissue culture wells, GF, and fibronectin-GF. (A) Time course of gene expression during chondrogenic differentiation for Adamts1 (circle) and Adamts2 (triangle). (B) Relative gene expression levels of Adamts1 (gray) and Adamts2 (black) at day 17 of chondrogenic differentiation in control 2D culture, 2D culture in the presence of fibronectin, 3D-GF, and 3D-GF coated with fibronectin. (C) Time course of gene expression during chondrogenic differentiation for Mmp2 (triangle) and Mmp14 (circle). (D) Relative gene expression levels of Mmp2 (black) and Mmp14 (gray) at day 17 in control 2D culture, 2D culture in the presence of fibronectin, 3D-GF, and 3D-GF coated with fibronectin. (E) Time course of gene expression during chondrogenic differentiation for Timp1 (circle), Timp2 (triangle), and Timp3 (square). (F) Relative gene expression levels of Timp1 (gray), Timp2 (black), and Timp3 (white) at day 17 in control 2D culture, 2D culture in the presence of fibronectin, 3D-GF, and 3D-GF coated with fibronectin. (G) Time course of gene expression during chondrogenic differentiation for Ctgf (circle) and Tgfb1 (triangle). (H) Relative gene expression levels of Ctgf (gray) and Tgfb1 (black) at day 17 in control 2D culture, 2D culture in the presence of fibronectin, 3D-GF, and 3D-GF coated with fibronectin. Error bars = Mean ± SD Table 3 lists matrix remodeling genes analyzed in this study with descriptions and literature citations that have demonstrated a link between increases in gene expression and chondrogenic differentiation.

4. DISCUSSION

In this study, we used GF as a three-dimension scaffold to support chondroprogenitor cell attachment and differentiation. Our results indicate that cell morphology can be modified by the
functionalization of GF with fibronectin. The molecular dynamic simulation demonstrated that arginine residue side chains play a stabilizing role in the graphene–fibronectin interaction. Cells adhered to GF and GF functionalized with fibronectin. GF provided a microenvironment compatible with chondroprogenitor gene expression as indicated in Tables 1–3 and in Figures 9–12, and in some cases, enhanced the expression of key chondrogenic markers. However, fibronectin influenced the cellular morphology as well as the gene expression patterns, resulting in decreased gene expression levels for the majority of genes analyzed.

We note that previous studies have revealed the importance of surface roughness on cell—substrate interactions.28–32 Our graphene foams exhibit wrinkles on the order of several nanometers to 10s of nanometers in good agreement with previous studies.17,49,107 Furthermore, previous studies have shown the importance of surface functionalization on cell culture.108–113 While we have focused on the impact of protein functionalization of graphene–cell interfaces, further investigations are needed to better understand the time-dependent biochemical nature of such interfaces.

We analyzed only the early cellular responses in the differentiation pathway rather than later events and the formation of mature cartilage tissue. The GF used in this study was made by chemical vapor deposition processing on a nickel foam template, which was removed prior to use. Although our results were consistent among all GF used, it is possible that lot-to-lot variability may exist and therefore precaution should be taken to confirm lot or batch effects. The manufacturing process used to prepare the scaffold may influence the outcome of cell-based investigations and may alter both cytoskeletal organization as well as gene expression profiles.

The interactions between fibronectin and the graphene surface may be stabilized by the π electron cloud in graphene, which is capable of interacting with the hydrophobic protein core. Alternatively, as we investigated here, the interaction may be stabilized by arginine side chains. Note that in the performed analysis, the graphene sheet atoms were assumed neutral and no induced charge effects were considered. The binding energies are therefore, purely van der Waals in nature, and are expected to be even lower if polarization effects are accounted for.

Prior testing by Yocham and colleagues demonstrated an increase in the GF’s elastic modulus after 28 days of cell culture without the use of fibronectin. When compared to this study, the compressive elastic modulus measured previously and the compressive elastic modulus reported here is not significantly different.49 This suggests that the elastic strength contributed to the GF scaffold by the fibronectin coating is similar to the strength contributed to the scaffold after 28 days of cell growth. The compressive modulus of GF coated with fibronectin and then cultured with ATDC5 cells for 28 days was significantly higher than either one individually, suggesting that fibronectin coating and cell growth contribute to the resulting elastic strength of the GF additively. Neither cell growth nor fibronectin coating affected the viscoelastic properties phase shift and stress relaxation. This may be due to insufficient chondrogenic differentiation to maintain an increase in interstitial fluid pressure. As in the prior study of GF without fibronectin, the ratio of dynamic modulus to equilibrium modulus of this study remains consistent between groups, reinforcing the conclusion that the time-dependent mechanisms are unchanged by cell culture and fibronectin coating, and these factors primarily affect the elastic strength of the scaffold. One potential time-dependent mechanism is protein adsorption, as a study by Lee and colleagues observed that GF absorbed 8% of serum proteins after 24 h in tissue culture media24 which may contribute to the greater load dissipation by ripple effect as described by Nautiyal and colleagues.111

Cells exist in unique microenvironments in vivo that influence their survival and differentiation and gene expression patterns. Here, we took measures to provide extracellular matrix cues to support prechondrogenic cells. While the optimal in vitro matrix environment is not known for ATDC5 cells, it is known that both materials properties as well as biochemical signals play critical roles. Here we used fibronectin to promote cell attachment and early condensation. Col I and Col IV were not used for this study because Col I is associated with non-chondrogenic tissues as well as dedifferentiated chondrocytes and Col IV is a marker for basement membranes.34,55

The clonal mouse embryonic cell line ATDC5 was used in this study as a chondroprogenitor cell line. Originally isolated from an embryonal carcinoma, ATDC5 cells demonstrate all phases of chondrocyte differentiation from early cell attachment and condensation, through a proliferative phase, a chondrogenic differentiation phase marked by increased levels of cartilage matrix constituent production, and finally, differentiation into hypertrophic chondrocytes that produce an extracellular matrix suitable for mineralization.35 Differentiation of chondroprogenitor cells depends upon fibronectin for the early stages of condensation and differentiation.41 In addition, epithelial to mesenchymal transitions depend on fibronectin.112 Enhancement of chondrogenesis of ATDC5 cells has been demonstrated by using an RGD-functionalized scaffold.113

Because of the importance of cell adhesion in the condensation and differentiation process, cell adhesion molecules involved in chondrogenic differentiation including Cd44,59 Ctnnα1,6 Col1α1,77 Col3α1,7 Col5α1,7 Col6α1,7 Ecm1,79,80 Emilin1,6 Fn1,6,81 Hapln1,2,63 Lamb3,83 Postn,64 Sparc,65 Spp1,66 Thbs1,87–90 Thbs2,91 and Tnc.92–94 The upregulation of these genes supports our conclusion that 3D-GF provides an environment supporting of chondrogenic differentiation and that fibronectin did not provide an advantage over 3D-GF alone.

Fibronectin is an extracellular matrix protein that plays an important role in bringing cells together at the earliest stage of mesenchymal cell differentiation in chondrocytes. Fibronectin matrix also acts as a platform for type I collagen deposition, and may also serve this role for type II collagen. ECM molecules that play a role in chondrogenic differentiation analyzed in this study included Col1α1,77 Col2α1,33,76 Col3α1,7 Col5α1,7 Col6α1,7 Ecm1,79,80 Emilin1,6 Fn1,6,81 Hapln1,2,83 Lamb3,83 Postn,84 Sparc,85 Spp1,66 Thbs1,87–90 Thbs2,91 and Tnc.92–94
genic differentiation of ATDC5 cells can be supported by 3D-GF compared to control conditions. We found that pretreatment of 3D-GF with fibronectin did not improve the gene expression of matrix remodeling molecules during chondrogenic differentiation.

Graphene-based scaffolds have been widely investigated for numerous applications including their effect on cell stem commitment. Graphene coated with laminin was shown to support neural stem cell attachment and differentiation, as well as accelerate myogenesis of C2C12 cells on GF.17,114–116 Chondrogenic differentiation of placenta-derived and tonsil-derived mesenchymal stem cells on graphene-based scaffold/hydrogel was reported by Park and colleagues117 Differentiation and long-term survival of neural and mesenchymal stem cells in an undifferentiated state has been accomplished using graphene foam.118,119 These examples from current literature demonstrate the use of GF to enhance osteogenesis and facilitate neurogenesis and astrocytogenesis of neuronal stem cells.

GF in conjunction with extracellular matrix proteins may provide tissue functionality during the transient regeneration phase of cartilage healing and repair. Additionally, the electrical conductivity may provide the advantage of stimulating cells to produce more matrix. GF in combination with a hydrogel scaffold may be ideally suited for bone/cartilage repair in the case of osteochondral defects. With an improved understanding of the influences of scaffold and biochemical factors, an ideal microenvironment can be designed.

5. CONCLUSIONS

Future studies are warranted to investigate the role of other extracellular matrix molecules and three-dimensional scaffolds to determine cell fate in tissue engineering and regenerative medicine applications. Damaged articular cartilage repair is a challenging issue in regenerative medicine, due in part to the limited ability for cartilage to heal. According to the World Health Organization, the United Nations has categorized OA as a priority disease in need of research on potential therapies. Given that between 2015 and 2050, the proportion of the world’s population over 60 years will nearly double from 12% to 22%, an estimated 130 million people will suffer from OA worldwide (WHO, 2018). Existing methodologies to treat OA are palliative, nonreparative, nonrestorative, reparative, restorative, and transplantation strategies. Autologous chondrocyte transplantation shows promise in clinical treatment, however the process involves the harvest, culture, and transplantation of cells grown in a monolayer (2-D culture). Under these culture conditions, the risk of dedifferentiation of the chondrocyte phenotype before use is a major concern of tissue engineering.120 Unlike chondrocyte cells, mesenchymal stem cells may maintain their chondrogenic potential if provided the proper biochemical, biophysical, and mechanical cues during proliferation and subsequent differentiation to regenerate cartilage tissue.

Cartilage engineering approaches need to consider the cell source, biomaterial scaffold, and a conducive environment to promote the formation of functional tissues, promoting the very early stages of chondrogenic commitment to the later differentiation stages of chondrocytes during which they produce high levels of cartilage biomarkers, while providing a scaffold that can provide functionality during the various stages of the regeneration process. GF in combination with a transitional extracellular matrix may provide the necessary niche environment in which to support all phases of mesenchymal stem cell differentiation, chondrocyte differentiation, and cartilage production by mature chondrocytes. On the basis of the findings of this investigation, we conclude that because cell differentiation is regulated by a combination of molecular and materials properties of the underlying scaffold, both the characteristics of the scaffold and the nature of the ECM protein used for functionalization must be considered carefully to align with the tissue-specific goals of the application.

■ AUTHOR INFORMATION

Corresponding Author
*E-mail: joxford@boisestate.edu (J.T.O.).

ORCID
Ilia A. Solovyov: 0000-0002-8626-145X
David Estrada: 0000-0001-5894-0773
Julia Thom Oxford: 0000-0002-4850-3569

Author Contributions
Substantial contributions to the conception and design of the work (JTO, DE, TJL, IAS, CMS, RJB); acquisition, analysis, and interpretation of data (JCR, SMF, AF, KF, RSB, Jr, CMS); drafting the work and revising it critically for intellectual content (JTO, DE, TJL, SMF, RJB, IAS). All authors have approved the final approval of the version to be published and agree to be accountable for all aspects of the work.

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Notes
The authors declare no competing financial interest.

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■ ABBREVIATIONS

Cd44, Hyaluronate receptor
Ctna1, catenin, alpha 1
Ctnnb1, catenin, beta 1
Itga3, integrin alpha 3
Itga5, Integrin alpha 5
Itgav, integrin alpha V
Itgb1, Integrin beta 1
nCam1, neural cell adhesion molecule
Sgcε, Sarcoglycan epsilon
Col1α1, Collagen α1(I)
Col2α1, Collagen α1(II)
Col3α1, Collagen α1(III)
Col5α1, Collagen α1(V)
Col6α1, Collagen α1(VI)
DAPI, 4′,6-Diamidino-2-phenylindole
Ecm1, Extracellular matrix protein-1
Emilin1, Elastin microfibril interface-located protein 1
Fn, Fibronectin
GF, Graphene foam
Hapln1, Hyaluronan and proteoglycan link protein 1
Lamb3, Lamin subunit beta-3
Postn, Periostin
Sparc, Secreted protein acidic and rich in cysteine; Osteonectin
Spp1, Secreted phoprotein; Osteopontin; Bone sialoprotein 1
Thbs1, Thrombospondin 1
Thbs2, Thrombospondin 2
Tnc, Tenascin
Adams1, A distintegral and metalloproteinase with thrombospondin motifs 1
Adams2, A distintegral and metalloproteinase with thrombospondin motifs 2
Ctgf, Connective tissue growth factor (CCN2)
Mmp14, Matrix metalloproteinase 14
Mmp2, Matrix metalloproteinase 2
Tgb1, TGF-beta-induced 68 kDa protein
Timp1, Tissue inhibitor of matrix metalloproteinase 1
Timp2, Tissue inhibitor of matrix metalloproteinase 2
Timp3, Tissue inhibitor of matrix metalloproteinase 3
COL I, Collagen type I protein
COL III, Collagen type III protein
COL IV, Collagen type IV protein
COLV, Collagen type V protein
COL VI, Collagen type VI protein
FN, Fibronectin protein
VTN, Vitronectin protein
LMN, Laminin protein
TE, Tropoelastin protein
MANOVA, Multivariate analysis of variance
LSD, Least significant difference

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