Influence of 3D Printed Porous Architecture on Mesenchymal Stem Cell Enrichment and Differentiation

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Abstract

The interactions between cells and an underlying biomaterial are important for the promotion of cell adhesion, proliferation, and function. Mesenchymal stem cells (MSCs) have great clinical potential as they are an adult stem cell population capable of multilineage differentiation. The relationship between MSC behavior and several material properties including substrate stiffness and pore size are well investigated, but there has been little research on the influence of porous architecture in a three-dimensional scaffold with a well-controlled architecture. Here, we investigate the impact two different three-dimensionally printed, pore geometries on the enrichment and differentiation of MSCs. 3D printed scaffolds with ordered cubic pore geometry were supportive of MSC enrichment from unprocessed bone marrow, resulting in cell surface marker expression that was comparable to typical adhesion to tissue culture polystyrene, the gold standard for MSC culture. Results also show that scaffolds fabricated with ordered cubic pores significantly increase the gene expression of MSCs undergoing adipogenesis and chondrogenesis, when compared to scaffolds with ordered cylindrical pores. However, at the protein expression level, these differences were modest. For MSCs undergoing osteogenesis, gene expression results suggest that cylindrical pores may initially increase early osteogenic marker expression, while protein level expression at later timepoints is increased for scaffolds with ordered cubic pores. Taken together, these results suggest that 3D printed scaffolds with ordered cubic pores could be a suitable culture system for single-step MSC enrichment and differentiation.

1. Introduction

Mesenchymal stem cells (MSCs) are an adult stem cell population found in multiple tissues throughout the body including bone marrow, adipose tissue, the synovial membrane, and trabecular bone. MSCs are of particular interest for therapeutic applications because the cells can be differentiated into a number of lineages including chondrocytes, adipocytes, and osteoblasts [1]. MSCs exist in very low numbers in the highly cellular and heterogeneous bone marrow, and lack unique identifying markers necessary for definitive isolation. Isolation and characterization of MSCs is currently based on a set of established properties set forth by the International Society for Cellular Therapy [2]. Based on these properties, MSCs must show: adherence to tissue culture polystyrene (TCPS), the expression of a panel of positive cell surface markers, the lack of expression for a panel of negative surface markers, and the ability to undergo trilineage differentiation into osteoblasts, adipocytes, and chondrocytes [2, 3]. Standard protocols for the isolation of MSCs from the bone marrow include extraction of whole bone marrow, density gradient centrifugation, and expansion of the mononuclear cell fraction on TCPS. While this protocol is well accepted for the use of MSCs, there is evidence to suggest that this expansion phase may result in phenotypic changes in MSCs [4, 5], including a loss of differentiation potential and cell senescence. The use of biomaterials for the enrichment and expansion of MSCs in a more physiologically relevant three dimensional (3D) environment is of growing interest in the field, with several successful attempts to expand MSCs in 3D with the use of microcarriers under dynamic conditions [6-8]. However, these strategies do not eliminate the need for initial culture on plastic.

Although the use of MSCs as a therapeutic agent is promising, there is still much to learn about the native MSC niche and how these properties can be exploited for regenerative medicine applications. The MSC niche is a complex and dynamic structure consisting of cellular components, secreted factors, extracellular matrix, and physical factors among others [9]. The influence of physical parameters such as substrate elasticity and geometry on MSCs is of rising interest in the field of tissue engineering, especially with the development of novel micropatterning techniques [10]. With the rise of new technologies, substrates can be controlled such that studies can be completed on the single cell level. Substrate stiffness has been shown to play a large role in stem cell differentiation, with softer substrates resulting in neurogenic or

adipogenic differentiation while more rigid substrates are favorable for osteogenic differentiation [11]. The investigation of substrate shape and its relationship to cell contraction and differentiation has been well characterized [10, 12, 13] using micropatterning techniques. These studies have shown that culturing cells in geometries that permit cell spreading and therefore higher levels of contractility promote osteogenic phenotypes, even when cells are exposed to a mixture of chemical cues. Likewise, under an identical chemical environment, rounded geometries encouraged low cell tension and an adipogenic phenotype [10].

The discovery of the link between cell shape and differentiation has been critical in the design of biomaterials that can be used for cell therapy applications. However, many of these fundamental studies have been conducted in two dimensional (2D) or “pseudo”- 3D [12] substrates, which is dissimilar to the native environment where cells may be implanted for regenerative therapy. Here, we aim to elucidate the relationship between 3D geometry and MSC enrichment and differentiation through the use of 3D printed biodegradable scaffolds. 3D printing technology provides a means to fabricate highly ordered and complex scaffold structures with great reproducibility. The design criteria for scaffolds capable of supporting MSC enrichment and differentiation includes a structure that consists of an interconnected porous network with pore sizes on the range of hundreds of microns [14-16], mechanical properties, such as modulus (0.03-1600 MPa), in the range of mesenchymal tissues, and mechanical stability during *in vivo* degradation [17]. Previous studies, from our laboratory and others, have investigated the impact of various aspects of 3D printed scaffolds on MSC behavior, including substrate stiffness, pore size, and substrate composition [18-20]. This has been especially advantageous for applications involving interfacial tissue engineering, where 3D printing can be used to recapitulate the complex architecture of the tissue interface [21, 22].

Here, our first objective was to study the impact of 3D pore structure using two pore architectures printed with the synthetic polymer poly(propylene fumarate) (PPF). PPF is a biodegradable polymer that can form crosslinked networks through its carbon-carbon double bond, and has been used extensively in tissue engineering applications [19, 23-25]. Scaffolds were designed to have an interconnected porous network with pores that exhibited cubic or cylindrical geometry.

Using 3D printed PPF scaffolds, we hypothesized that MSC enrichment in 3D can equal or surpass that of traditional TCPS due to the ability of the scaffold to encourage 3D interactions native to the cell population. Further, we hypothesized that the response of MSCs to chemical cues known to induce differentiation will be influenced by the pore architecture of the 3D printed scaffold.

2. Materials and Methods

2.1 Scaffold Design

Three-dimensional scaffolds composed of cubic or cylindrical pore geometries were designed using SolidWorks® (Waltham, MA). All scaffolds were designed to have an ordered pattern of continuous pores, resulting in an interconnected porous network. Scaffolds were constructed to have a theoretical porosity of 80% and a pore volume of 1mm³. Several iterations of the each design were tested until an interconnected porous network and high reproducibility were achieved.

2.2 Poly(Propylene Fumarate) Synthesis and 3D Printing

All scaffolds were fabricated out of a polymer resin consisting primarily of PPF. PPF was synthesized using a previously established protocol [26], and the number average molecular weight (M_n) and polydispersity index (PDI) were determined by gel permeation chromatography. The M_n of PPF used to synthesize all scaffolds in this study was 1133-1987 Da with a PDI of 1.8-2.1. Scaffolds were fabricated using an EnvisionTEC Perfactory® P4 with an exposure to UV light of 350mW/dm² for 100s per layer. Scaffolds were printed out of polymer resin that was composed of PPF and its monomer diethyl fumarate (DEF) in a 1:0.8 ratio as well as the photoinitiator bis (2,4,6-trimethylbenzoyl) phenylphosphine oxide (BAPO, 1%), and photoinhibiting dyes HMB (1%) and α -tocopherol (0.1%). Following printing, several post-processing methods were investigated to achieve the desired scaffold properties, including optimization of solvent, length of exposure to solvent, and post-curing. Following optimization, it was determined that the most effective post-processing method was for scaffolds to be immediately placed in a 30 min wash of isopropyl alcohol, followed by a 30 min wash of 100% acetone to remove any uncured resin from within the porous network. After these washes,

scaffolds were post-cured in a 3D systems UV box at a total energy of 110J. Following post-curing, scaffolds were rinsed overnight in a solution of 70% ethanol to remove any remaining unreacted resin or debris. For characterization studies, scaffolds were vacuum dried overnight prior to testing. For cellular studies, scaffolds were sterilized in 70% ethanol for 4 hours followed by 4, 30min washes in sterile phosphate-buffered saline (PBS) to remove any traces of ethanol.

2.3 Soluble Fraction Determination

To evaluate PPF crosslinking and the effectiveness of the post processing methods, the cured network soluble fraction, or sol fraction was determined using previously established methods [23]. The sol fraction is the percent of soluble polymer remaining after post processing. Scaffolds were weighed (W_i) prior to immersion in acetone, which served as the extraction solvent. The samples were incubated in acetone for 24 hours. Following incubation, samples were completely dried and weighed again (W_d) ($n = 5$). The sol fraction was calculated using the equation:

$$\text{sol fraction} = \frac{W_i - W_d}{W_i} \times 100\%$$

2.4 Compressive Mechanical Testing

Compressive mechanical testing of scaffolds was conducted using a mechanical testing system (Model 858, MTS System Corporation, Eden Prairie, MN). Force and displacement were zeroed prior to compression, with the top plate slightly above the surface of the sample. Samples ($n = 5$) were compressed at a crosshead speed of 2 mm/min while force and displacement were monitored throughout the experiment and converted to stress and strain following completion. The experiment was halted after sample fracture. The elastic modulus for each sample was calculated using MATLAB to determine the slope of the linear region of the stress-strain curve as previously described [27]. Briefly, the linear region was calculated by using the linear fit command starting with the first 10 data points, considering a preload of 1N. The program continued to systematically add points in steps of 8 until the R^2 value dropped below 0.95. The slope of this region is representative of the elastic modulus of the sample.

2.5 Micro-Computed Tomography

Micro-computed tomography (μ CT) was used as a means to noninvasively image and characterize scaffold parameters. Scanning was performed using a μ CT 100 (SCANCO Medical, Brüttisellen, Switzerland) operated at 70kVp, 9mm isotropic voxels, and 200mA. The 3D datasets that resulted from each scan were segmented using predetermined thresholds (lower: 35, upper: 188, maximum: 1000), with gauss sigma (0.8) and support (1) values to distinguish pores from polymer [18]. All resulting 3D images were evaluated using the Image Processing Language (IPL) from Scanco to determine pore size and porosity. Three samples of each scaffold type were scanned.

2.6 Unprocessed Human Bone Marrow Culture

Whole unprocessed human bone marrow (Lonza, Walkersville, MD) was seeded onto cubic pore scaffolds that were either unmodified or modified to contain type I collagen (Corning, Corning, NY), a mixture of type I collagen and hyaluronic acid (HA, Sigma), or TGF- β 3 (R&D Systems, Minneapolis, MN) on the surface of the scaffold. The surface of PPF scaffolds were modified using EDC/NHS chemistry. Briefly, scaffolds were immersed in 50mM MES buffer (pH = 5.5, Sigma) containing 0.5M N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, Sigma) and 0.5M N-hydroxysuccinimide (NHS, Sigma) for 14 hours at room temperature, followed by a wash in PBS to remove any excess byproducts. For protein modification, scaffolds were modified with type I collagen or a mixture of type I collagen and HA at 1mg/mL. For growth factor modification, scaffolds were modified with a solution of 10 μ g/mL of TGF- β 3. All solutions were made up in PBS and sterile filtered prior to incubation with the EDC/NHS activated scaffolds for 1 hour at room temperature. Following modification, scaffolds were rinsed 3 times in sterile PBS to remove any unbound protein or growth factor. Tissue culture plastic (TCPS) served as a control. Groups were incubated at 37°C, 5% CO₂, and 80% humidity until experimental analysis. Media (growth media described below) was changed every 2 days.

2.7 Evaluation of Mesenchymal Stem Cells Isolated from Whole Bone Marrow

To analyze the cell population that was captured from unprocessed bone marrow, cells were subjected to flow cytometry analysis using a Human MSC (hMSC) Analysis Kit (BD Stemflow, BD, Franklin Lakes, NJ). At days 1 and 7, cells were lifted from the surface of the scaffold or

TCPS and resuspended at a concentration of 5×10^6 cells/mL. Cells were stained following the manufacturer's protocol. Briefly, 100 μ L of each cell suspension was mixed with 20 μ L of the hMSC Positive Cocktail (CD90 FITC, CD105 PerCP-Cy5.5, and CD 73 APC) and 20 μ L of the hMSC Negative Cocktail (CD34 PE, CD11b PE, CD19 PE, CD45 PE, and HLA-DR PE) and incubated at 4°C in the dark for 30 minutes. Following incubation, the cell suspensions were washed 2x with 500 μ L of PBS containing 1% BSA. After washing, cells were resuspended in 500 μ L of PBS containing 1% BSA and analyzed using a BD FACSCanto II to quantify the amount of each marker present. Human MSCs at P4 (Lonza) were used as a control, and to set up an analysis template for all samples.

2.8 Human Mesenchymal Stem Cell Culture and Scaffold Seeding

Human MSCs (Lonza) were expanded prior to the study on tissue culture polystyrene flasks in media consisting of DMEM (Life Technologies, Frederick, MD) supplemented with 10% fetal bovine serum (Life Technologies), 1.0% v/v penicillin/streptomycin (Life Technologies), 0.1mM non-essential amino acids (Life Technologies), and 4mM L-glutamine (Life Technologies) following manufacturer's protocols and previously described methods [28]. Media was changed every 3 days according to the manufacturer's specifications, and cell cultures were incubated at 37°C, 5% CO₂, and 80% humidity. Cells at passage 4 were used for all experiments. Prior to cell seeding, adherent MSCs were lifted with trypsin/EDTA (Life Technologies) and counted using trypan blue uptake to determine viability. MSCs were seeded onto sterilized scaffolds in a 48 well plate. Each scaffold was seeded with approximately 42,000 cells (11,000 cells/cm²) in a concentrated solution of 20 μ L and allowed to adhere for 1 hour at 37°C before additional media was added.

2.9 XTT Assay

To quantitatively evaluate cell metabolic activity on the scaffolds surface, a Cell Proliferation Kit II (XTT) (Roche, Mannheim, Germany) was used. XTT [2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide] was used following the manufacturer's protocols. The electron coupling and XTT labeling reagents were thawed and immediately combined in a 1:50 ratio. Scaffolds were moved into new cell culture wells to avoid evaluating cells that did not attach to the scaffold surface. The XTT solution was added to cell culture wells and

incubated at 37°C for 4 hours. Following incubation, absorbance was measured with an M5 SpectraMax plate reader (Molecular Devices, Sunnyvale, CA). Net absorbance was calculated (A450-A650) for each sample and reported. Cells grown on tissue culture polystyrene at the same density served as a control. Samples were evaluated at day 1, 4, and 7.

2.10 Fluorescence Imaging

The viability of cells seeded onto scaffolds was qualitatively evaluated using live/dead fluorescent staining. At days 1, 4, and 7, scaffolds were soaked in PBS containing 1mM CaCl₂ and 0.5mM MgCl₂ for 30 min to remove non-adherent cells as well as any remaining medium. Following standard protocols, scaffolds were incubated with 2mM ethidium homodimer and 4mM calcein AM (Life Technologies) for 30min. Images were taken using a fluorescent microscope (Axiovert 40 CFL with filter set 23; Zeiss, Thornwood, NY) equipped with a digital camera (Diagnostic Instruments 11.2 Color Mosaic, Sterling Heights, MI).

2.11 Induction of MSC Differentiation

To evaluate the impact of pore geometry on human MSC differentiation, MSCs were seeded onto each scaffold type and induced down the three main mesenchymal lineages (osteogenesis, chondrogenesis, and adipogenesis). Cells were seeded onto scaffolds as described above. Following incubation for 24 hours in growth medium, cells were cultured for 21 days in osteogenic (DMEM with 10% FBS, 1mM sodium pyruvate (Life Technologies), 100U/100µg penicillin-streptomycin, 10⁻⁷M dexamethasone (Sigma-Aldrich), 50µg/mL ascorbic acid (Sigma-Aldrich), and 10mM β-glycerophosphate), chondrogenic (high glucose DMEM supplemented with ITS (BD), 4mM L-proline (Sigma-Aldrich), 50µg/mL ascorbic acid, 1% sodium pyruvate, 10⁻⁷M dexamethasone, and 100U/100µg penicillin-streptomycin, and 10ng/µL TGF-β3 (R&D Systems)), or adipogenic (DMEM with 10% FBS, 1mM sodium pyruvate, 100U/100µg penicillin-streptomycin, 10⁻⁶M dexamethasone, 10µg/mL insulin (Sigma-Aldrich), 0.5mM IBMX (Life Technologies), and 200µM indomethacin (Life Technologies)) media as described previously [29]. MSCs cultured in growth media served as a control. All media were changed every 3 days. At days 7, 14, and 21, samples were collected for RNA isolation and histological analysis, by lifting the cells from the surface of the scaffolds. The number of cells remaining on

the scaffolds was not quantified, though it is not anticipated that a significant cell population remained after exposure to trypsin.

2.12 Quantitative Reverse Transcriptase-Polymerase Chain Reaction

RNA was isolated using an RNeasy Mini Plus Kit (Qiagen, Valencia, CA) following standard protocols. Isolated RNA was then reverse transcribed to cDNA using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). The sequences of the gene expression assays used (Applied Biosystems) are proprietary. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Hs99999905_m1) served as an endogenous control gene for all samples. Alkaline phosphatase (ALP, Hs00758162_m1) and osteopontin (OPN, Hs00960641_m1) were assayed as markers of early and late osteogenesis, respectively. Adiponectin (Hs00605917_m1) and type II collagen (Hs00264051_m1) were assayed as markers for adipogenesis and chondrogenesis, respectively. Gene expression assays were combined with the cDNA to be analyzed and TaqMan PCR Master Mix (Applied Biosystems). The reaction was performed on a 7900HT real-time PCR System (Applied Biosystems) using thermal conditions of 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The relative gene expression level of each target gene was then normalized to the mean of the GAPDH in each group. Fold change was calculated using the $\Delta\Delta CT$ relative comparative method as described previously [28]. Samples were completed in triplicate and standard deviations are reported (n = 3).

2.13 Histological Analysis

At days 7, 14, and 21, cells were lifted from the surface of the scaffolds and seeded onto 96 well plates for 24 hours. At this time cells were fixed with 10% formalin for 10 minutes, and stored at 4°C in PBS. Histological staining was completed to show calcification, glycosaminoglycan production, and the presence of lipid droplets. Mineralization was visualized using alizarin red (Sigma) staining following standard protocols. Briefly, freshly prepared 2% w/v alizarin red solution was added to cell monolayers for 5 minutes. Cells were washed 3 times in DI water and imaged immediately. Glycosaminoglycan production was visualized by staining with 0.5% Alcian Blue solution (Poly Scientific, Bay Shore, NY) followed by a nucleic counter stain with Nuclear Fast Red (Poly Scientific). For the visualization of lipid vacuoles in adipogenic

samples, a working solution of Oil Red O (Poly Scientific) was prepared and applied to fixed monolayers, followed by counterstaining with hematoxylin. For quantification, 5 x 5 tiled images (total tiled image area = 3.177 mm²) were captured of each well (n = 4) using an inverted microscope (Zeiss AxioVert, equipped with an AxioCam MRm digital camera; Zeiss, Inc., Thornwood, NY) and AxioVision Software (Version 4.8.2; Zeiss, Inc.). A routine was created in Axiovision for each stain to capture and calculate regions positively stained for calcification, lipid deposits, or proteoglycans.

2.14 Statistical Analysis

All data was analyzed using one-way analysis of variance and Tukey's multiple-comparison test to determine statistical differences between scaffolds. A confidence interval of 95% ($\alpha=0.05$) was used for all analyses, and means and standard deviations are shown in each figure.

3. Results

Scaffolds were designed to have an interconnected cubic or cylindrical porous network with 1000 μm pore size and 80% porosity. In order to achieve an interconnected porous network that could be reproducibly printed, several design changes were made including altering the design of the pores within the scaffold backbone, the pore side length or diameter, and the wall thickness between pores. Using μCT , we were able to evaluate the actual scaffold parameters that resulted following fabrication and post-processing (Figure 1). The optimized printed scaffolds showed good correlation to the designed parameters, with cubic pore and cylindrical pore scaffolds having an actual porosity of $78.8 \pm 1.8\%$ and $70.4 \pm 11.8\%$ respectively. Pore size also printed close to the theoretical values with cubic pore scaffolds resulting in a pore size of $882.0 \pm 68.0 \mu\text{m}$ and cylindrical pore scaffolds a pore size of $735.0 \pm 91.0 \mu\text{m}$. By using μCT to generate a pore map of each scaffold, it was apparent that the pores regardless of architecture were continuous and interconnected throughout the scaffold.

Imaging with μCT also showed that the scaffold integrity was not compromised by the use of chemical washes in post processing, as there are few areas of the scaffold surface that are roughened by surface defects. Similarly, there is little to no remaining debris around the scaffold surface. Sol fraction testing revealed that all methods tested had a sol fraction of less than 5%.

The post processing methods used were effective at removing any remaining uncured polymer resin which could be potentially damaging to cell viability (Figure 3A).

Compressive mechanical testing (Figure 2A) revealed that there was an increase in the elastic modulus of the scaffolds with an increasing amount of total energy delivered to the scaffold using post printing UV crosslinking. For both acetone wash conditions, 110 J resulted in significantly lower modulus, followed by 550 J. There were no statistical differences between 1100 and 2200 J for either acetone wash. When comparing batches of PPF, it was found that there is a correlation between the molecular weight of the polymer and the elastic modulus, with higher molecular weights resulting in increased moduli of the printed scaffolds. Comparing geometries, cubic pore scaffolds had a statistically higher elastic modulus of 25.9 MPa, when compared to 17.5 MPa for cylindrical pore scaffolds (Figure 2B). However, this difference, though statistically significant, is modest compared to those thought to influence stem cell fate [11].

Flow cytometry analysis for the panel of positive surface markers for human MSCs was completed on days 1 and 7 following bone marrow seeding. Results show that as early as day 1, scaffolds modified with biomolecules and unmodified scaffolds demonstrate expression levels equivalent to the TCPS control (see Table 1). Scaffolds with surface modification have a marginally increased expression of positive surface markers. Scaffolds modified with a mixture of collagen and HA, as well as scaffolds modified to contain the growth factor TGF- β 3, showed the highest level of stem cell marker expression at day 1, with levels above that of the TCPS control. On day 7, all experimental groups again show stem cell marker expression levels similar to the control. Here, both the unmodified scaffold group and the collagen modified scaffold group exhibit stem cell marker expression levels that are increased over the control, with the collagen modified scaffold group having the highest overall expression compared to all groups.

The results of the XTT assay show that MSCs cultured on scaffolds over a period of 7 days exhibit levels of metabolic activity that are not statistically different from cells cultured on tissue culture polystyrene (Figure 3A). Quantitative results were confirmed using qualitative imaging

techniques. In Figure 3B, the majority of the cells stain positively for calcein AM (green) indicating that they are viable on the scaffold surface and within the pores of the scaffold. An increased level of fluorescence within the pores suggests that MSCs have begun to fill the pores. Taken together, these results confirm that the post processing methods used to remove unreacted polymer following fabrication is effective and promotes cell function on and within the scaffold architecture. We did not distinguish between cells cultured on the interior or exterior of the scaffold, but cells on the scaffold as a whole. As the pore sizes of our scaffolds are on the range of hundreds of microns, we do not anticipate that there should be oxygen or diffusional differences between the interior and exterior of the scaffold.

Human MSC differentiation was assessed over 21 days using qRT-PCR and histological staining for markers of each mesenchymal tissue. Gene expression analysis shows that cylindrical pore scaffolds appear to promote an early increase in osteogenic markers (see Figure 4A and B) following exposure of the cells to osteogenic media. Both ALP and OPN show a statistically greater expression of the respective marker in the cylindrical pore group at day 7 when compared to the cubic pore group within the same timepoint. This initial difference is diminished at later timepoints, with the results of histological analysis indicating that the cubic pore group appears to catch up and pass the cylindrical pore group in terms of calcification, as indicated through the quantification of alizarin red histological staining for calcified regions. Image analysis results show that at day 21, the cubic pore group shows a significantly greater number of calcified regions when compared to the cylindrical pore group (Figure 5).

For adipogenesis, the cubic pore group at day 21 shows significantly increased expression of adiponectin, when compared to the corresponding cylindrical pore group (see Figure 4C). For both pore geometries, the expression of adiponectin is significantly upregulated on day 21. Based on histological evidence, there appears to be no significant difference between pore geometries when quantifying the number of lipid deposits per cell for both groups.

As with the adipogenic group, the cubic pore samples showed significantly upregulated type II collagen expression compared to the cylindrical pore samples for the group undergoing chondrogenesis (see Figure 4D). At day 21, both cubic and cylindrical pore scaffolds show an

upregulation of type II collagen expression, with cubic pore scaffolds resulting in the highest expression. Based on histological evidence, there appears to be no significant difference between pore geometries when quantifying the number of proteoglycan-rich regions per cell for both groups.

4. Discussion

The ability to both enrich MSCs from isolated tissue and subsequently control MSC differentiation is of great importance to the field of cell therapy and regenerative medicine. In addition to chemical and biomolecular cues, MSCs have been shown to respond to scaffold parameters including substrate stiffness, surface roughness, pore size, and 2D geometry, such as curvature. In the work presented here, we used 3D printing technology to evaluate the impact of 3D pore geometry on human MSC enrichment and differentiation. Scaffolds were printed out of a resin of PPF, a biodegradable polymer that has previously been shown to result in accurately printed scaffolds, with parameters such as pore size, porosity, and wall thickness exhibiting accuracies of up to 91% [18]. One of the most important criteria for the culture and differentiation of MSCs on 3D scaffolds is an interconnected porous network to promote the exchange of nutrients, soluble factors, and cell signals. Here, we showed the ability to achieve an interconnected network of evenly sized pores that could be printed with high reproducibility. Scaffolds were designed to be composed of repeatable units such that feature size and shape could be easily altered. Design parameters were altered based on the outcome of each print. PPF is a viscous polymer, a characteristic that can be troublesome for printing systems based on the use of liquid resins. Polymer resin viscosity has been noted in previous studies as a potential source of printing inaccuracies, particularly for small feature sizes [30]. Lowering of the viscosity through the use of a solvent is a simple way to combat this issue, as we have done here using DEF, the monomer of PPF.

PPF forms a crosslinked network through the crosslinking of a carbon-carbon double bond within the backbone of the PPF chain. DEF also contains a crosslinkable carbon-carbon double bond and therefore serves as an excellent candidate for the dilution of PPF to achieve a desirable viscosity for printing. Previous studies have shown that the inclusion of a percentage of DEF within the PPF resin improves the mechanical properties of the scaffold, as it increases the

number of crosslinks formed between polymer chains [23]. PPF is a hydrolytically degradable polymer, with fumaric acid and propylene glycol as the byproducts of degradation. While the degradation of the scaffolds used in the current study was not investigated, our lab has previously studied how 3D printed PPF scaffolds degrade *in vitro*. During a 224 day degradation period, the structural, mechanical, and cytocompatibility properties of three different 3D printed PPF scaffold designs were evaluated [17]. Results indicate that the scaffolds undergo surface degradation, which was confirmed through an increase in pH and the formation of micropores and surface channels. Additionally, extracts of the degradation products were found to not induce significant cell death.

PPF scaffolds with cubic pores were first investigated for their utility in MSC enrichment from unprocessed human bone marrow. Here, we showed that cubic pore scaffolds, both unmodified and modified with biomolecules, supported the attachment of cells with similar phenotypic signatures to those cultured on TCPS, the current gold standard for MSC enrichment and expansion. Through the direct seeding of whole bone marrow onto 3D scaffolds, naïve and early progenitor cells are preserved, which are hypothesized to be lost during density gradient centrifugation [5, 31]. In addition to preserving these cell types, direct seeding into 3D scaffolds results in cells maintaining their native morphology, which has been shown to influence MSC proliferation and differentiation capacity [32]. Through the use of surface modification techniques, we were able to attach proteins from the MSC ECM as well as growth factors to the material surface. For MSC isolation and enrichment, there were not large differences between modified and unmodified scaffolds, though there were modest increases in the expression of surface markers for those modified with type I collagen and TGF- β 3 on day 7 compared to unmodified scaffolds and TCPS. While these increases may indicate that surface modification improves the scaffold for MSC enrichment, further studies need to be completed to elucidate the role of biochemical cues in addition to physical cues for the 3D enrichment of MSCs. In addition to playing a role in enrichment, surface modifications may be of greater importance for the priming of cultures for differentiation towards a specific mesenchymal lineage.

In order to elucidate how MSCs would respond to cubic and cylindrical pore geometries presented, cell viability had to be first tested to ensure that the post-processing methods did

negatively affect cell viability. Culture over a 7 day period showed that cells had a metabolic activity level that was not significantly different from the control, indicating that the post-processing methods used resulted in scaffolds that supported MSC cell viability. Differentiation of MSCs down the three main mesenchymal lineages was assessed over a period of 21 days and pore geometries were compared for each group at each timepoint. Results indicated that there were significant differences between the gene expressions of phenotypic markers depending on the 3D pore geometry of the scaffold.

The pore sizes of our scaffolds are around 830 μm for the cubic pore design and 730 μm for the cylindrical pore design. There have been numerous studies that have investigated the influence of pore size for mesenchymal target tissues, especially bone and cartilage. Here we aimed to minimize the impact of pore size by designing the pores to be equivalent in size, and focus on the impact of pore geometry on cell behavior, a concept that has been much less studied. Trabecular bone has a macroporous structure, with open marrow space pores on the order of 1 mm in size [33]. Previous studies have shown that larger pore sizes (on the order of 1-3 mm) allow for greater interaction between cells and the underlying biomaterial, improving the osteogenic properties of the tissue and resulting in a thicker tissue formation along the material surface [34, 35]. For chondrogenesis applications, there are a large range of pore sizes that have been investigated, with disagreement over which size is appropriate for the promotion of the chondrogenic phenotype. Studies have indicated that smaller pore sizes (on the range of 150 μm) are successful for the promotion of type II collagen and aggrecan production [14], while larger pores (250-500 μm) increased cell proliferation and ECM deposition [15]. Additional studies investigating scaffold permeability showed that pore sizes on the order of 900 μm supported cartilage matrix production both *in vitro* and *in vivo* [36, 37]. While the feature sizes in this study are greater than the subcellular curvatures that are commonly investigated in 2D studies of cell behavior [10, 13], we showed that scaffolds fabricated with larger pore sizes were capable of supporting MSC attachment, proliferation, and differentiation. Due to the tunable nature of our scaffold designs, the future investigation of a range of pore sizes would be both easily achievable as well as beneficial to the field.

Based on the 2D studies that have investigated the impact of geometry on MSC behavior [10, 12, 13], we hypothesized that the cubic pore geometry would best support osteogenesis, while the cylindrical pore geometry would promote the adipogenic and chondrogenic groups. Chondrogenic differentiation is typically completed using a 3D high density pellet culture system [38] which promotes increased cell-cell connections to facilitate cell signaling necessary for differentiation. Likewise, adipogenesis is induced using high density culture and has been shown to be promoted in environments with less cytoskeletal stress, such as a rounded geometry [10]. Environments with increased tension, such as a square or cubic geometry have been shown to support osteogenesis, and cytoskeletal tension has been found to be an important factor in the regulation of cell fate [13]. Here in 3D scaffolds, we observe a nearly opposite trend. While the results of histological analysis show no differences between groups, qRT-PCR analysis shows that for both the adipogenic and chondrogenic groups, the cubic pore geometry results in a significant upregulation of the expression of key markers of cell phenotype for each respective lineage. For the osteogenic group, there is an initial upregulation of osteogenic expression in the cylindrical pore group at day 7, with ALP expression, a marker known to peak at day 7 during osteogenesis, significantly upregulated in the cylindrical pore geometry. However, by day 21, the cubic pore scaffold shows a significantly higher number of calcified regions, quantified using alizarin red staining. Overall, the cubic pore geometry appears to have a greater influence over the promotion of differentiation regardless of lineage. We hypothesize that the success of the cubic pore scaffolds may be attributed to the increased elastic modulus as well as the high level of pore size and porosity uniformity that was achieved with this design.

5. Conclusion

In the work presented here, we were able to show the ability to successfully and reproducibly print 3D scaffolds with an interconnected porous network. Optimization of post processing methods provides a clean scaffold with little to no uncrosslinked resin remaining that encourages cell attachment and proliferation. The mechanical properties of the scaffolds can be tuned by varying the length of post curing as well as the molecular weight of the polymer. This would allow for the material properties to be altered based on the application, providing a means to match the mechanical properties of the scaffold with the target tissue. Cubic pore scaffolds were supportive of MSC enrichment from unprocessed human bone marrow, indicating the potential

to utilize 3D printed PPF scaffolds for direct MSC isolation and differentiation. In addition, the 3D environments studied here were supportive of human MSC differentiation down all three of the main mesenchymal lineages. Cubic pore geometry was shown to significantly support adipogenic and chondrogenic lineage commitment on the gene expression level compared to its cylindrical pore counterpart, although these differences were not detected at the protein level. For osteogenic differentiation, gene expression data suggested an early role for cylindrical pore geometry, while protein level expression appears to be influenced by cubic pore geometry. The development of a tunable 3D culture system for optimized culture of MSCs would have great potential in the field of tissue engineering and regenerative medicine.

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Tables

Table 1: Flow Cytometry Analysis for the Panel of Positive Surface Markers For Human MSCs Following Bone Marrow Seeding on Unmodified and Biomolecule Modified 3D Printed PPF Scaffolds. Data is shown as the percent of P1, which was the cell gate set using human MSC positive controls. On day 1, both modified and unmodified scaffolds demonstrate expression levels equivalent to the TCPS control. Surface modified scaffolds have a marginally increased expression of positive surface markers. Scaffolds modified with a mixture of collagen and HA, as well as scaffolds modified to contain the growth factor TGF- β 3 showed the highest level of expression at day 1, with levels above that of the control. On day 7, all experimental groups show expression levels similar to the control. Both the unmodified scaffold group and the collagen modified scaffold group exhibit expression levels that are increased over the control, with the collagen modified scaffold group having the highest overall expression compared to all groups.

Percent of P1 (cell gate)	CD90	CD73	CD105
On day 1:			
Unmodified scaffold	8.6	61.4	58.2
Collagen modified scaffold	19.8	49.9	54.5
Collagen + HA modified scaffold	32.3	63.9	68.1
TGF- β 3 modified scaffold	24.5	66.0	66.1
TCPS	15.7	47.8	52.3
On day 7:			
Unmodified scaffold	17.5	61.2	71.7
Collagen modified scaffold	35.0	67.7	75.3
Collagen + HA modified scaffold	18.7	57.3	54.6
TGF- β 3 modified scaffold	21.2	59.5	72.5
TCPS	25.2	58.6	60.2

Figure Captions

Figure 1: 3D Printed Scaffolds. (A) Analysis of the final designs of each pore geometry including 3D images resulting from μ CT imaging. The results from μ CT show structures with highly defined and interconnecting pores. (B) The tabulated pore size and porosity are close to the theoretical for both pore geometries.

Figure 2: Post Printing Processing and Characterization of Scaffolds. (A) Modulus resulting from compressive testing of scaffolds exposed to an increasing total energy delivered through post processing UV crosslinking. There were no statistical differences between 25 and 100% acetone washes, however, as the total energy increased there was a significant increase in the modulus of the scaffold up to 1100 J ($p < 0.05$). Between 1100 and 2200 J, there were no significant differences (*, $p > 0.05$). The PPF used in this experiment had a MW = 1133 Da. (B) Modulus of cubic and cylindrical pore scaffolds subjected to a wash of 30 min IPA, 30 min 100% acetone, 110 J, and 24h 70% ethanol. The MW of PPF for this experiment was 1632 Da. Cubic pore scaffolds exhibited a statistically greater elastic modulus when compared to the cylindrical pore scaffolds (*, $p < 0.05$). The sol fraction of all scaffolds was less than 5%.

Figure 3: Cell Viability on 3D Printed PPF Scaffolds. (A) XTT data following 1, 4, and 7 days of culture. * indicates samples on day 7 of both groups were statistically greater than day 1 ($p < 0.05$). There were no statistical differences between cells grown on 3D printed scaffolds and the control at any timepoint. (B) Live-dead viability staining of samples at days 1, 4, and 7 of culture. The cells exhibit high viability (green) and localization within the pores of the scaffolds. Scale bar = 100 μ m.

Figure 4: qRT-PCR Assessment of MSC Differentiation on 3D Printed Scaffolds. (A and B) Fold change in expression of osteogenic markers alkaline phosphatase (ALP) and osteopontin (OPN). For both markers, the cylindrical pore group at day 7 exhibited a statistically greater expression level compared to all other groups. (C) Fold change in the expression of the adipogenic marker, adiponectin. At day 21, the cubic pore group showed the highest expression. (D) Fold change in the expression of type II collagen, a marker for chondrogenesis. At day 21, the cubic pore group had statistically greater expression compared to the cylindrical pore group. * indicates $p < 0.05$.

Figure 5: Quantification of Histological Assessment of MSC Differentiation on 3D Printed Scaffolds. (A) Comparison of the number of calcified regions, an indicator of osteogenesis. Data is shown as the number of calcified regions per mm². At day 21, the cubic pore geometry had a statistically greater (* indicates $p < 0.05$) number of calcified regions compared to the cylindrical pore group. (B) For adipogenic samples, the percentage of oil red o positive cells are reported. There was no statistical difference between pore geometries at day 21 ($p > 0.05$). (C) Quantification of chondrogenesis was computed by dividing the number of proteoglycan positive regions by the number of cells. At day 21 there was no statistical difference between pore geometries ($p > 0.05$).

Figure 1

A

B

Figure 2

A

B

Figure 3

Figure 4

Figure 5

Graphical Abstract