**SPECIAL FOCUS: STRATEGIC DIRECTIONS
IN MUSCULOSKELETAL TISSUE ENGINEERING***

Harnessing External Cues: Development and Evaluation of an *In Vitro* Culture System for Osteochondral Tissue Engineering

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Over the last decade, engineered structures have been developed for osteochondral (OC) tissue regeneration. While the optimal structure design is yet to be determined, these scaffolds require *in vitro* evaluation before clinical use. However, the means by which complex scaffolds, such as OC scaffolds, can be tested are limited. Taking advantage of a mesenchymal stem cell's (MSC's) ability to respond to its surrounding we harness external cues, such as the cell's mechanical environment and delivered factors, to create an *in vitro* culture system for OC tissue engineering with a single cell source on a gradient yet integrated scaffold system. To do this, the effect of hydrogel stiffness on the expression of human MSCs (hMSCs) chondrogenic differentiation was studied using histological analysis. Additionally, hMSCs were also cultured in different combinations of chondrogenic and osteogenic media to develop a co-differentiation media suitable for OC lineage differentiation. A uniquely graded (density-gradient matrix) OC scaffold with a distal cartilage hydrogel phase specifically tailored to support chondrogenic differentiation was cultured using a newly developed "simulated *in vivo* culture method." The scaffold's culture in co-differentiation media models hMSC infiltration into the scaffold and subsequent differentiation into the distal cartilage and proximal bone layers. Cartilage and bone marker staining along with specific matrix depositions reveal the effect of external cues on the hMSC differentiation. As a result of these studies a model system was developed to study and culture OC scaffolds *in vitro*.

Keywords: chondrogenesis, co-differentiation media, human mesenchymal stem cell, matrix stiffness, osteogenesis, polymer-gel matrix

Introduction

OSTEochondral (OC) DEFECT REPAIR is a significant challenge in orthopedic surgery. Although allografts have been used for OC defect repair, they present shortcomings in terms of disease transmission and the degree of host tissue integration. Therefore, there is a growing need to develop engineered grafts for extended cartilage/OC defect repair.^{1,2}

OC tissue, by design, is a complex tissue as it consists of both articular cartilage and subchondral bone connected by a bone–cartilage interface.¹ Additionally, articular cartilage is further divided into four zones (the superficial, middle, deep, and calcified cartilage zones) that each differ in the amounts

and orientation of the extracellular matrix (ECM) component collagen they contain, among other differences. To add to this complexity, the last zone of this cartilage tissue gives way to a transitional zone, which leads to the bone end.^{1,3} To mimic this layered structure, bi-phasic and tri-phasic scaffolds, along with, recently, gradient scaffolds, have been developed for OC tissue engineering.^{4–7}

The advantage of the gradient as opposed to other designs is believed to be that the smooth materials transitions will support continuous strength along the scaffold length without potential abrupt breakpoints, resulting in better-integrated OC tissue layers.^{1,8} Furthermore, the gradient design might be useful in mimicking the layered but continuous arrangement

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of articular cartilage zones that are present in *in vivo* OC tissue, in which mineral content and matrix density is greatest at the deep end of the articular cartilage as it nears the subchondral bone.⁹

Cartilage tissue is at a regenerative disadvantage since it contains no vasculature and is comprised of cells that are bound by lacunae. As such, upon damage this complex tissue requires aid to heal. To aid in its repair a cell source, of some type, is oftentimes utilized. Tissue-specific cells, such as chondrocytes and osteoblasts, have been used to fabricate a stratified scaffold with biomimetic multi-tissue regions.⁶ However, chondrocytes have been known to drift in phenotype during extended culture *in vitro* or postimplantation *in vivo*.^{10,11} As an alternative to the use of tissue-specific cells, progenitor cells can also be used. These cells, which include mesenchymal stem cells (MSCs), are highly utilized due to their ability to differentiate into a variety of cell types including both chondrocytes and osteoblasts.¹² As such, MSCs have since developed a history of successful use in OC regeneration strategies.¹³

For a single cell source, such as MSCs, to be applied to OC tissue engineering strategies these cells must either be allowed to differentiate separately before use or methods of selectively differentiating the cells must be determined. While many earlier studies simply differentiate the cells separately before implantation onto the OC grafts or provided proof of concept results derived from separate chondrogenic and osteogenic cultures, these methods do not provide a realistic example of how these OC grafts will perform clinically.^{14,15} To overcome this problem, a bioreactor capable of delivering both chondrogenic and osteogenic differentiation mediums separately to a biphasic scaffold loaded with MSCs was developed and proved to aid in the upregulation of genes consistent with selective differentiation.¹⁶ Yet, since most bioreactors described for OC tissue engineering are used to aid with cellular distribution, bioreactor use for OC tissue engineering is still in its infancy.¹⁷

In general, it is known that how cartilage cells respond to certain stimuli depends on many factors including their location within the OC tissue.¹⁸ Furthermore, when it comes to stem cells overall, it has more recently been stated that the mechanical environment that the cells are in can also affect gene expression and cell fate.^{19,20} More specifically, it has been determined that human MSCs (hMSCs) retain memory of their past physical environments, which acts as mechanical dosing and influences cell fate decisions.²¹ Taking these facts into consideration, studies often select biomaterials with mechanical properties closer to that of the tissue targeted for regeneration to take advantage of these mechanical cues in complex tissue regeneration.²²

Along with these mechanical cues, chemical cues have always been utilized in the differentiation of MSCs. However, due to its complex nature, OC scaffolds that contain MSCs require a variety of different chemical cues to support the differentiation of these cells into chondrocytes, osteoblasts, and cells of the interface. As previously mentioned, these chemical cues have been delivered separately as proof of concept, and technologies are being developed to aid in this use.^{16,23} However, in addition to this, some researchers have also explored the development of a common media that could support and deliver the chemical cues necessary to achieve both chondrogenesis and osteogenesis simulta-

neously, yet little is understood about the media composition and its effect on MSC OC lineage development.²⁴

To successfully develop an OC graft many factors such as cell source and material selection, among others, must be taken into account. Furthermore, the integration of these different factors must be conducted *in vitro* before use *in vivo*. This said, by harnessing a MSCs ability to respond to external cues, we develop a co-differentiation media and determine its true effectiveness in supporting the chondrogenic and osteogenic differentiation of an OC scaffold.

Materials and Methods

Human mesenchymal stem cell isolation and characterization

Freshly isolated human bone marrow (BM) was purchased from Lonza (Walkersville, MD) and processed using the fully automated Magellan device (Arteriocyte[®]; Arteriocyte Medical Systems, Hopkinton MA) to obtain MSCs. In brief, the BM was transferred into a 60 mL syringe and injected into the inner chamber of the device, which uses a series of sequential centrifugations to separate the red blood cell population, platelet-rich population, and mononuclear layer.²⁵ The platelet-rich plasma, containing the mononuclear fraction, was collected into a 10 mL syringe and subjected to MACS (magnetic-activated cell sorting) using CD271 columns (Miltenyi Biotec, Inc., San Diego, CA) to isolate cells expressing the MSC marker, CD271.

Briefly, an anti-Fc receptor antibody was added to the mononuclear fraction to reduce nonspecific binding and CD271-conjugated microbeads were added to the fraction. Following this the fraction was passed through a column containing metallic beads set in a magnetic field. Upon removal of the column from the magnetic field, the CD271-positive cells were released, and immediately subjected to *in vitro* culture. For *in vitro* culture, the CD271-positive cells obtained from 5 mL of platelet-rich plasma were plated on 150×20 mm polystyrene tissue culture dishes (USA Scientific, Inc., Ocala, FL) and cultured in basal media (Dulbecco's modified Eagle's medium [DMEM]/F-12+GlutaMAX, with 10% fetal bovine serum [FBS] and 1% penicillin/streptomycin [P/S]) at 5% CO₂ and 87% humidity.

At passage 3 the hMSCs were subjected to fluorescence-activated cell sorting (FACS) to quantify expected expression of the cell surface stem cells markers CD105 (1:200), CD90 (1:400), and CD73 (1:50) and to confirm expected absence of hematopoietic, CD34 (1:50), and leukocytic, CD45 (1:50), markers (Becton, Dickinson and Company, Franklin Lakes, NJ). Briefly, the cultured hMSCs were released from the plate through the addition of Trypsin-EDTA (0.25%), centrifuged at 1200 rpm for 7 min, and ~1 million hMSCs were resuspended in 1 mL of staining buffer containing 1 µL of live/dead dye and incubated for 30 min on ice. Cells were washed and resuspended in a staining buffer solution containing the fluorescently tagged antibody of interest. After 30 min on ice, the samples were subjected to FACS in an LSR-II (Becton, Dickinson and Company) and output data were assessed using FlowJo software v.10 (TreeStar, Inc.).

Tri-lineage differentiation into the osteoblast, chondrocyte, or adipose lineage by the CD271-positive hMSCs was confirmed using a standard protocol similar to those described previously.¹² Briefly, the hMSCs were either plated in a 24-

well plate at a density of 25,000 cells/well (for osteogenesis or adipogenesis) or seeded in a microcentrifuge tube for a pellet culture containing 500k cells/pellet. For this pellet culture the 500k cells were pipetted onto the bottom of a microcentrifuge tube at a high density and media was placed on top of the suspension. Due to the fact that the cells are incapable of adhering onto the walls of the tube they form a pellet.

For osteogenesis the cells were allowed 1 day to attach using basal media (before treatment in osteogenic media [basal media; Gibco] supplemented with 100 ng/mL BMP-2, 10 mM β -glycerophosphate, and 50 μ g/mL ascorbate-2-phosphate) for 21 days. For the adipogenic differentiation the StemPro adipogenesis differentiation kit was purchased from Life Technologies (Grand Island, NY) and used as instructed. Finally, for chondrogenic differentiation the pellets were treated with chondrogenic media (serum-free, high glucose, basal media [Gibco] supplemented with ITS+, 100 μ L/mL sodium pyruvate, 40 μ g/mL L-proline, 50 μ g/mL ascorbate-2-phosphate, 10^{-7} M dexamethasone, and 10 ng/mL TGF- β 1) for 21 days. For all cultures media was changed every 2–3 days, and the hMSCs in this study were used between passages 3–5.

Hydrogel selection

Hyaluronan gels of different stiffness were made by mixing Glycosil[®], a thiol-modified hyaluronan gel with the cross-linking agent polyethylene glycol (PEG) at ratios ranging from 1:1 to 7:1 (ESI BIO, Alameda, CA) ($n=3$ each).²⁶ Stiffness (Storage Modulus) was determined using a Discovery HR-3 Hybrid Rheometer (TA Instruments, New Castle, DE). Five hundred thousand hMSCs were mixed with each of three hyaluronan:PEG gels, and the gel-cell mixtures were added into 1 mL microcentrifuge tubes during the gels cross-linking. These samples were then compared to hMSCs mixed with PuraMatrix, a widely used peptide-based hydrogel matrix for stem cell chondrogenesis as well as cartilage tissue engineering studies^{27–29} (Becton, Dickinson and Company), and pellets of hMSCs created by adding 500,000 hMSCs directly into microcentrifuge tubes.

The gels/pellets were cultured for 21 days in standard chondrogenic media, previously described, with media changes every 2–3 days. Gels/pellets were fixed in 10% formalin, embedded in paraffin, and cut into 5 μ m sections. Sections were stained with hematoxylin and eosin (H&E) to visualize cell and matrix morphology ($n=2$). Matrix glycosaminoglycan (GAG) content was quantified using dimethylmethylene blue (DMMB) assay, absorbance read at 520 nm, and DNA content was quantified by PicoGreen staining with fluorescence measurement at 485/535 nm ($n=4$). Material controls, containing gel alone, were cultured for 21 days alongside the aforementioned groups and their quantitative results from DMMB and PicoGreen were subtracted out to remove any background effects.

Immunostaining was performed using primary antibodies to the cartilage marker proteins Collagen Type II (1:100), Aggrecan (1:50), and Sox9 (1:50) (Abcam, Cambridge, MA); an antibody against tubulin (EMD Millipore, Billerica, MA) was used as a housekeeping protein. Secondary antibodies were Alexa Fluor 488 and Alexa Fluor 546 while nuclei were counter-stained with NucRed[®] Dead 647 (Thermo Fisher Scientific, Waltham, MA) ($n=2$). Imaging

was carried out using a confocal microscope, and quantification of images in a single image stack (minimum $n=6$) was determined through the use of ImageJ software.

Selection of chondrogenic-osteogenic co-differentiation media

hMSCs were prepared for osteogenic or chondrogenic lineage differentiation as follows: for osteogenic differentiation, hMSCs were seeded onto a 24-well tissue culture polystyrene (TCPS) plate at a density of 25,000 cells/well, and maintained for 2 days in basal media before treatment with the various co-differentiation media types (see Table 1). For chondrogenic differentiation, pellets of hMSCs were formed by the addition of the cells into a 1 mL screw cap microcentrifuge tube, at a density of 500,000 cells/tube. The hMSCs adhered onto the TCPS and the hMSCs within the screw cap tube were then subjected to culture in each of five different co-differentiation media types to test the ability of the various co-differentiation mediums to support further osteogenic or chondrogenic differentiation, respectively.

The five different media types were as follows: 100% chondrogenic media (100C); 75% chondrogenic: 25% osteogenic media (75C); 50% chondrogenic: 50% osteogenic media (50C); 25% chondrogenic: 75% osteogenic media (25C); and 100% osteogenic media (0C). The composition of each media type is shown in Table 1. The hMSCs on the hard surface of the TCPS and those surrounding each other in a soft cell pellet were treated with each distinct co-differentiation media type for 21 days with media changes every 2–3 days and chondrogenic or osteogenic lineage differentiation was evaluated as follows:

To assess osteogenic differentiation, plates of hMSCs maintained on TCPS in each of the distinct co-differentiation media were subjected to whole mount Alizarin Red staining as previously described.^{30–32} Briefly, samples were rinsed with water and fixed in 70% ethanol (EtOH) for 1 h at 4°C. The samples were then air dried for 5–10 min and rinsed with water before being incubated in 40 mM Alizarin Red solution (pH 4.2) for 10 min at room temperature. Each well was then washed several times with water and once with phosphate-buffered saline (PBS) before imaging. The Alizarin Red stain was quantified through extraction using a 10% cetylpyridinium chloride (CPC) solution; absorbance was measured at a 562 nm wavelength using a Biotek Synergy HT plate reader (Winooski, VT).

To assess chondrogenic differentiation, hMSC pellets maintained in each of the distinct co-differentiation media were fixed for 1 h in 10% buffered formalin, embedded in paraffin, and cut into 5 μ m sections. Sections were stained with Alcian Blue, pH 2.5, overnight, rinsed, and counterstained in Nuclear Fast Red. Quantification of GAG content was performed using DMMB assay as previously described.^{33,34} Samples were digested in Proteinase K solution, at a concentration of 1 mg per million cells for a minimum of 16 h at 56°C. Once digested, 50 μ L of the digested sample was combined with 200 μ L of DMMB solution, in a 96-well plate, which was then read for absorbance at 520 nm.

Production of gradient matrix PLGA microsphere scaffold

A single, continuous cylindrical scaffold consisting of fused polymer microspheres with an end-to-end gradient in

TABLE 1. COMPOSITION OF THE VARIOUS CO-DIFFERENTIATION MEDIA TYPES TESTED

<i>Media compositions</i>									
<i>100C</i>			<i>75C</i>			<i>50C</i>			<i>0C</i>
<i>Item</i>	<i>Final conc.</i>	<i>Item</i>	<i>Final conc.</i>	<i>Item</i>	<i>Final conc.</i>	<i>Item</i>	<i>Final conc.</i>	<i>Item</i>	<i>Final conc.</i>
DMEM high glucose w/ Glutamax	50 mL	DMEM high glucose w/ Glutamax	37.5 mL	DMEM high glucose w/ Glutamax	25 mL	DMEM high glucose w/ Glutamax	12.5 mL	DMEM low glucose w/ Glutamax	50 mL
P/S	100 U/mL	DMEM Low glucose w/ Glutamax	12.5 mL	DMEM low glucose w/ Glutamax	25 mL	DMEM low glucose w/ Glutamax	37.5 mL	P/S	1%
Sodium pyruvate	100 µg/mL	P/S	100 U/mL	P/S	100 U/mL	P/S	100 U/mL	FBS	10%
Human recombinant insulin	6.25 µg/mL	FBS	2.5%	FBS	5.0%	FBS	7.5%	Ascorbic acid	50 µg/mL
Selenous acid	6.25 ng/mL	Human recombinant insulin	4.68 µg/mL	Human recombinant insulin	3.13 µg/mL	Human recombinant insulin	1.56 µg/mL	β-glycerophosphate	10 mM
Ascorbic acid	50 µg/mL	Human transferrin	4.68 µg/mL	Human transferrin	3.13 µg/mL	Human transferrin	1.56 µg/mL	BMP-2	100 ng/mL
Dexamethasone	0.1 µM	Selenous acid	4.68 ng/mL	Selenous acid	3.13 ng/mL	Selenous acid	1.56 ng/mL		
L-Proline	40 µg/mL	Ascorbic acid	50 µg/mL	Ascorbic acid	50 µg/mL	Ascorbic acid	50 µg/mL		
TGF-β	10 ng/mL	Dexamethasone	0.075 µM	Dexamethasone	0.05 µM	Dexamethasone	0.025 µM		
		L-Proline	30 µg/mL	L-Proline	20 µg/mL	L-Proline	10 µg/mL		
		TGF-β	7.5 ng/mL	TGF-β	5 ng/mL	TGF-β	2.5 ng/mL		
		β-glycerophosphate	2.5 mM	β-glycerophosphate	5 mM	β-glycerophosphate	7.5 mM		
		BMP-2	25 ng/mL	BMP-2	50 ng/mL	BMP-2	75 ng/mL		

100% Chondrogenic media (100C); 75% chondrogenic:25% osteogenic media (75C); 50% chondrogenic media:50% osteogenic media (50C); 25% chondrogenic media:75% osteogenic media (25C); 100% osteogenic media (0C).

DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; P/S, penicillin/streptomycin.

pore volume was formed using a method we have previously reported.^{1,7,35} In brief, poly(85 lactide-co-15 glycolide) (PLGA) polymer, purchased from Evonik Industries (Birmingham, AL), was formed into microspheres via an oil-in-water emulsion method. Batches of microspheres in the size range of 355–425 μm were then combined with porogen (NaCl) at five different ratios ranging from 0 to 40 weight percent, and the microsphere-porogen mixtures were layered into a cylindrical scaffold mold beginning with the highest concentration of porogen and ending with the lowest. The scaffolds were heated for 1 h at 100°C to sinter the microsphere layers and washed in water to leach the remaining porogen.

This process produces a single scaffold with an end-to-end porosity gradient.³⁵ The gradient scaffold is uniquely constructed to provide strength throughout the OC implant, while at the same time creating microenvironments along the scaffold length that differ in stiffness, which can be further modified by infusion of a gel. The intent of these microenvironments is to support differential MSC differentiation and hence formation of cartilage tissue at the top end, and mineralized bony tissue at the bottom end in a structurally integrated OC matrix. Before use, the gradient matrix scaffolds were sterilized by soaking in 70% EtOH for 15 min, followed by two cycles of ultraviolet (UV) treatment for 15 min.

Scaffold cartilage layer formation

Fifty microliters of the selected hydrogel-cell mixture (see Hydrogel selection) was pipetted onto the distal (more porous) end of the gradient matrix PLGA scaffold and allowed to solidify at room temperature for 5 min. This created a hydrogel layer along the length of the scaffold with the majority of the gel in the distal end of the scaffold with less gel in proximal portion. The scaffolds were then cultured for 7 days in chondrogenic media (see Table 1) at 37°C.^{36,37}

Simulated in vivo scaffold culture

PLGA microsphere gradient matrix scaffolds containing a distal cartilage layer generated as above were transferred to an ultra-low attachment 24-well tissue culture plate (Corning, Corning, NY) containing 100,000 hMSCs in 1 mL of the selected co-differentiation media type. Care was taken to place the scaffold in the plate such that the hydrogel-cartilage-containing layer faced up, and the PLGA-polymer-rich, end of the scaffold was down. The plate was alternated between a rocker at room temperature under sterile conditions and static culture at 37°C for ~5 h to allow the nonadherent hMSCs to infiltrate the bottom end of the scaffold. Using this method gravity alone would not dictate the seeding of the additional cells, although it might play a minor role, especially when the samples were left static.

The plate was then cultured for an additional 21 days and media was replaced every 2–3 days. After 21 days, scaffolds were removed from the simulated *in vivo* culture system and cut into top and bottom halves. Chondrogenic and osteogenic differentiation was assessed as follows: ECM GAG content was quantified using DMMB assay as described above ($n=4$). DNA content was quantified by the PicoGreen double stranded (dsDNA) assay and GAG content was normalized to DNA content in each sample. Mineralization

was quantified using Alizarin Red staining as described above, and normalized to DNA content as well ($n=4$).

Whole-mount immunofluorescence to detect cartilage- and bone-characteristic matrix proteins was carried out on uncut scaffolds. Briefly, scaffolds were fixed for 1 h in 10% formalin, washed with PBS, permeabilized with 0.25% triton for 10 min, and bovine serum albumin (BSA) was used to block nonspecific binding. The primary antibody, prepared in 1% BSA, was added and incubated for 1 h in a humidified chamber. Samples were washed three times with PBS and the secondary antibody, also prepared in 1% BSA, was added and incubated at room temperature for 1 h in the dark.

Immunostaining was performed using primary antibodies to both cartilage and bone marker proteins Collagen Type II (1:100), Sox9 (1:50), Collagen I (1:100), and RUNX2 (1:50) (Abcam). The secondary antibody used was Alexa Fluor 546 while nuclei were counter-stained with NucRed Dead 647 (Thermo Fisher Scientific). Imaging was carried out using a confocal microscope, and quantification of images in a single image stack (minimum $n=9$) was determined through the use of ImageJ software.

Statistical analysis

All statistical analyses in the studies were performed using a one-way analysis of variance (ANOVA) with a Tukey post test. Quantitative data were reported as mean and a significance level of $p<0.05$ was used in all statistical tests performed.

Results

Processed and isolated hMSCs display characteristic stem cell markers

The steps involved in the cell isolation process are shown in Figure 1A. Human BM aspirate processed via the Magellan[®] system produced platelet-rich plasma, platelet-poor plasma, and red blood cell fractions. Immuno-selection of the platelet-rich plasma using MACS[®] resulted in CD271-positive cells. *In vitro* culture of the CD271-positive hMSCs showed that they displayed typical MSC morphology (not shown); and FACS confirmed appropriate high level expression of the typical stem cell markers CD105, CD90, and CD73. hMSCs minimally expressed CD45 and CD34, indicating no/minimal hematopoietic cell contamination. Successful tri-lineage differentiation of the CD271-positive hMSCs into osteogenic, chondrogenic, and adipogenic lineages was confirmed *in vitro* (Fig. 1B). The CD271-positive hMSCs were subsequently referred to in this study as “hMSCs.”

Hydrogel stiffness and composition influence hMSC chondrogenic differentiation

To form a distal cartilage layer in our gradient matrix scaffold that would emulate the morphological and mechanical properties of native articular cartilage, it was necessary to first identify a substrate that would recapitulate the external cues that help drive chondrogenic differentiation by mesenchymal progenitors during normal development and growth. Hyaluronan is a naturally occurring GAG that is abundant in articular cartilage, and has a history of success in cartilage tissue engineering.^{38–40} We developed hyaluronan-containing matrices

FIG. 1. Depiction of the method used to isolate hMSCs. (A) Bone marrow aspirate was processed using the fully automated Magellan[®] device, yielding platelet-rich plasma containing hMSCs, which was further processed using magnetic-activated cell sorting (MACS[®]) to isolate CD271-positive cells. The resultant hMSCs were plated on tissue culture plastic and passage 3 cells were subjected to FACS. (B) Trilineage differentiation of the CD271-positive cells depicting osteogenesis (Alizarin Red), chondrogenesis (Alcian Blue), and Adipogenesis (Oil Red O). FACS, fluorescence-activated cell sorting; hMSCs, human mesenchymal stem cells; MACS, magnetic-activated cell sorting. Color images available online at www.liebertpub.com/tea

FIG. 2. Cartilage hydrogel stiffness. Rheological analysis of the different hyaluronan:PEG ratios including average storage moduli. Note that as the hyaluronan:PEG ratio decreases, stiffness increases. [#]Significant when compared to all other groups; ⁺significant when compared to all other groups excluding [#] ($p < 0.05$). PEG, polyethylene glycol.

with different stiffnesses, and compared their ability to direct hMSC chondrogenic differentiation *in vitro*.

As shown in Figure 2, rheological assessment of the different gels confirmed that modulation of the ratio of hyaluronan hydrogel to PEG cross-linker, produced gels with storage moduli ranging from 10 to 45 Pa. The 2:1 hyaluronan:

PEG combination lead to the formation of a gel that was significantly stiffer than all of the other gel:cross-linker combinations, with the 3:1 ratio following as the second stiffest gel. The overall storage moduli of the remaining gel combinations decrease with the 7:1 being the softest.

Three gels with varying hyaluronan:PEG ratios (and hence different stiffnesses) were selected for hMSC seeding and subsequent *in vitro* culture. As shown, the gels with the highest and lowest storage moduli were selected for further studies (2:1 and 7:1, respectively) along with the 4:1 ratio.

The selected gels were combined with hMSCs and cultured in microcentrifuge tubes for 21 days in chondrogenic media (see Materials and Methods section for details). As controls, we used PuraMatrix-hMSC (used previously) and gel-free hMSC cell pellets.³⁵ As seen in Figure 3A, H&E staining of sections of the samples after 21 days showed uniform ECM deposition and morphology, including relatively uniform distribution of chondrocytes in lacunae, in the gel-free cell pellet, and in the cell-matrix samples composed of the 2:1 and 4:1 ratio hyaluronan:PEG matrix. In contrast, ECM morphology was poor in cell-matrix samples composed of 7:1 ratio hyaluronan:PEG matrix. ECM morphology in the PuraMatrix sample was highly disorganized, and few/no lacunae were observed.

The effect of gel composition on cell number as determined by DNA content is shown in Figure 3B. All cell-gel samples containing hyaluronan had significantly more cells than the PuraMatrix-containing sample. Interestingly,

FIG. 3. Effect of ECM stiffness on hMSC chondrogenic differentiation. (A) Sections of control, gel-free hMSC pellet (left) and hMSC-seeded hyaluronan:PEG gel constructs at different ratios (2:1, 4:1, 7:1), and PuraMatrix (right) after 21 days of culture, stained with hematoxylin and eosin. Note the relatively uniform ECM deposition and morphology, including relatively uniform distribution of chondrocytes in lacunae, in the control cell pellet and in constructs with 2:1 and 4:1 hyaluronan:PEG ratios, compared to constructs of 7:1 hyaluronan:PEG or PuraMatrix, which are disorganized and have few/no cells with lacunae. (B) DNA quantification and (C) normalized GAG quantification of the constructs shown in (A). [#]Significant when compared to all other groups ($p < 0.05$). ECM, extracellular matrix; GAG, glycosaminoglycan. Color images available online at www.liebertpub.com/tea

inclusion of hyaluronan in the samples even significantly increased cell number after 21 days over the positive control, cell-only pellet. As a measure of cartilage matrix production, the amount of GAGs formed in the matrices was quantified using the DMMB assay, and normalized to DNA content. As shown in Figure 3C, the amount of GAG produced per cell was significantly higher for chondrocytes in

the matrix composed of 2:1 ratio hyaluronan:PEG when compared to all other groups.

We also used immunofluorescence to examine production of the cartilage marker proteins, Aggrecan, Collagen Type II, and Sox 9, by the differentiated hMSCs in the various samples. As shown in Figure 4A, only the cells maintained for 21 days in the hyaluronan:PEG construct at a 2:1 ratio

FIG. 4. Effect of hydrogel stiffness on expression of hMSC chondrogenic marker proteins. (A) Visual sections of control, gel-free hMSC pellet (left) and hMSC-seeded hyaluronan:PEG gel constructs at different ratios (2:1, 4:1, 7:1), and PuraMatrix (right) after 21 days of culture, subjected to immunofluorescence to reveal localization of the chondrogenic protein markers Aggrecan, Collagen type II, and Sox9, and Tubulin as a housekeeping protein. Only the 2:1 ratio hyaluronan:PEG construct co-expressed all three proteins. Scale=100 μ m. (B) Quantification of the respective images. Asterisks depict different levels of significance ($p < 0.05$). Color images available online at www.liebertpub.com/tea

produced all three cartilage marker proteins. Aggrecan and Collagen Type II were also detected in the 4:1 ratio construct, but Sox9 was not co-expressed. Interestingly, quantification of fluorescence staining intensity further confirmed that the 2:1 ratio construct (the stiffest one) contained significantly more cartilage marker protein than the 4:1 or 7:1 ratio constructs (the softer ones) (Fig. 4B). Moreover, the 2:1 gel contained more cartilage marker protein overall than

the PuraMatrix construct, and intriguingly, even more than the control, gel-free cell pellet.

Our results showed that the 2:1 hyaluronan:PEG hydrogel (the stiffest matrix) provided better external matrix cues for supporting hMSCs chondrogenic differentiation than the hyaluronan:PEG gels we tested at other stiffnesses, and was far superior to PuraMatrix for supporting chondrogenic differentiation. Furthermore, the 2:1 hyaluronan:PEG gel

FIG. 5. Effect of different co-differentiation media types on hMSC osteogenic and chondrogenic lineage differentiation after 21 days *in vitro*. (A) hMSCs were maintained on tissue culture plastic and stained whole-mount with Alizarin Red to detect mineralization. Quantification of Alizarin Red staining by the cultures is shown in (A). *Significant when compared to 100C. (B) hMSCs were maintained as cell pellets and sections of formalin-fixed paraffin-embedded pellets were stained with Alcian Blue to detect GAG accumulation. Quantification of GAG accumulation via DMMB stain is shown in (B). #Significant when compared to all other groups; +significant when compared to all other groups excluding #, **significant when compared to all other groups excluding # and + ($p < 0.05$). DMMB, dimethylmethylene blue. Color images available online at www.liebertpub.com/tea

even supported better chondrogenic differentiation overall than that observed by hMSCs in the gel-free cell pellet.

Effect of media conditions on hMSC chondrogenic and osteogenic differentiation *in vitro*

To evaluate the performance of our novel gradient matrix scaffold for creating useful OC grafts, it was necessary to first develop culture conditions that would support simultaneous differentiation of hMSCs into both cartilage and bone lineages. We developed five different media types consisting of varying ratios of chondrogenic:osteogenic components, and tested their ability to influence chondrogenic and osteogenic differentiation by hMSCs *in vitro*. The five different media compositions tested were as follows: 100% chondrogenic (100C); 75% chondrogenic:25% osteogenic (75C); 50% chondrogenic:50% osteogenic (50C); 25% chondrogenic:75% osteogenic (25C); and 100% osteogenic (0C). The composition of each media type is shown in Table 1.

The effects of each media type on osteogenic lineage differentiation by hMSCs in monolayer culture on TCPS is

shown in Figure 5A, and the effects of each media type on chondrogenic lineage differentiation by hMSCs in pellet culture is shown in Figure 5B. As expected, pure chondrogenic media (100C) produced greatest GAG accumulation by the cells, while conversely; pure osteogenic media (0C) led to the highest amount of mineral formation. However, a ratio of 50% chondrogenic:50% osteogenic (50C) media supported significantly better chondrogenic differentiation than that observed in 0C or 25C media, and also supported significantly better osteogenic differentiation than that observed in 100C or 75C media.

Development of a “simulated *in vivo*” culture system

A “simulated *in vivo*” culture system was developed to assess and predict the ability of our novel gradient matrix scaffold to support formation of OC tissue (depicted in Fig. 6A). First, cylindrical scaffolds made of PLGA microspheres arranged in a continuous porosity gradient (“density-gradient matrix”) were made in which the distal (top) end of the scaffold was less dense, and the proximal (bottom) end of

FIG. 6. Engineered osteochondral graft design. (A) Gradient matrix PLGA scaffolds with a *top-to-bottom* density gradient (*top*=less dense; *bottom*=more dense) were created by heat-sintering layers of PLGA microspheres with decreasing porogen content. (B) hMSC-seeded hyaluronan:PEG hydrogel was loaded at the *top* of the scaffold and the scaffold was cultured for 7 days in chondrogenic media to prime the loaded hMSCs toward the chondrogenic lineage. (C) “Simulated *in vivo*” culture in co-differentiation media was used to model hMSC infiltration into the scaffold and subsequent differentiation into distal cartilage (*top*—hyaluronan stained pink) and proximal bone layers (*bottom*—scaffold stained blue). (D) DNA content in the *top* and *bottom* halves of the scaffold after 7 and 28 days. Asterisks depict different levels of significance ($p < 0.05$). Color images available online at www.liebertpub.com/tea

the scaffold was more dense (see Methods for details). A distal cartilage layer was initiated by infiltrating the top of the scaffold with hMSC-seeded 2:1 hyaluronan:PEG hydrogel, and preculturing the scaffold for 7 days in chondrogenic media to prime the hMSCs toward the chondrogenic lineage (Fig. 6B).

To model the infiltration into an OC defect that occurs by BM hMSCs *in vivo*, the scaffolds were placed into ultra-low attachment surface-coated dishes containing 50C co-differentiation media (the media type that we found was optimal for simultaneous chondrogenic and osteogenic lineage differentiation), in the presence of 100,000 hMSCs in suspension. Care was taken to place the proximal PLGA-rich end of the scaffold down, in closest proximity with the non-adherent hMSCs and the scaffold was maintained for another 21 days (Fig. 6B).

Cell number, as assayed by DNA content, was compared in the two ends of the scaffold, as depicted in Figure 6C, before and after coculture with the nonadherent hMSCs. While initially the two ends of the scaffold had similar amounts of DNA, most likely due to the infiltration of the cell containing hydrogel into the lower portion of the scaffold, after further incubation with the additional 100,000 hMSCs there was a significant increase in DNA content in the bottom half of the scaffold. The amount of DNA present in the bottom half of the scaffold after day 28 of culture was also significantly greater than that present in the bottom half of the scaffold at day 7, and was also significantly greater than the amount of DNA present in the distal end of the

scaffold (Fig. 6D). These results suggest that the non-adherent hMSCs preferentially colonized the proximal, PLGA-rich end of the scaffold, and that they proliferated there.

OC differentiation in “simulated *in vivo*” culture

To assess continued chondrogenic differentiation by the chondrogenic-primed hMSCs in the distal cartilage layer of the scaffold, and *de novo* osteogenic differentiation by newly infiltrated hMSCs in the proximal end of the scaffold, we examined GAG deposition, mineralization, and cartilage and bone marker protein expression in the distal and proximal scaffold ends after 7 and 28 days of “simulated *in vivo*” culture.

As shown in Figure 7A and B, GAG and mineral deposition in the scaffold increased between days 7 and 28 of culture. Moreover, when these data were normalized to DNA content, we observed that the top half of the scaffold had significantly more GAG than the bottom half of the scaffold (Fig. 7C), while mineral content in top and bottom halves was not significantly different (Fig. 7D). This suggested that the top half of the scaffold was preferentially undergoing chondrogenic differentiation during simulated *in vivo* culture compared to the bottom half.

Immunofluorescence staining for cartilage and bone marker proteins is shown in Figures 8 and 9. After 7 days of chondrogenic culture (Fig. 8), there was very little visibly detectable RUNX2 but some detectable Sox9 and Collagen Type II in either half of the scaffold; however, there was strong staining for Collagen Type I in both the top and

FIG. 7. Quantification of GAG deposition and Alizarin Red staining (as a measure of mineralization) by the simulated *in vivo* cultured scaffold. (A) GAG and (B) mineral deposition in the top and bottom halves of the scaffold after 7 and 28 days of simulated *in vivo* culture. (C) GAG and (D) mineral deposition normalized to DNA content. Asterisks depict different levels of significance ($p < 0.05$).

FIG. 8. Whole-mount immunofluorescence staining to visualize production of the cartilage and bone markers by simulated *in vivo* cultured scaffolds after 7 days. Sox9, RUNX2, Collagen type II, and Collagen type I are shown by *red* staining; nuclei were counterstained with NucRed Dead 647 (*pseudocolored blue*). Scale = 200 μ m. Color images available online at www.liebertpub.com/tea

bottom halves. At day 28, after simulated *in vivo* culture, Sox9 immunoreactivity decreases, but intense fluorescence was observed for RUNX2, especially in the bottom half, and Collagen Type I throughout. Collagen Type II also increased when compared to day 7.

Figure 10 shows quantification of the immunofluorescence images shown in Figures 8 and 9. Image analysis revealed amounts of RUNX2 and Collagen Type II were relatively low and not significantly different between the scaffold's top and bottom halves after 7 days of chondrogenic culture. By day 28, the amounts of RUNX2 and Collagen Type II were significantly greater than they were at day 7 (Fig. 10B, C). On the other hand, the amount of Sox9 in the top of the scaffold at day 7 is significantly greater than the amount seen in the bottom half at the same time point.

By day 28 the amount of Sox9 in the top half decreases while the amount in the bottom half increases (Figure 10A). In contrast, while the amount of Collagen Type I also increased in both the top and bottom halves of the culture between days 7 and 28, there was only a minimal difference in the amount of Collagen Type I between the top and bottom halves at each time point (Fig. 10D).

Discussion

Regeneration of OC tissue is a particularly challenging problem, as it requires formation of two separate tissues (articular cartilage and subchondral bone) joined together by an integrated cartilage–bone interface. To develop tissue-engineered constructs for clinical use in OC defect repair, it

FIG. 9. Whole-mount immunofluorescence staining to visualize production of cartilage and bone markers by simulated *in vivo* cultured scaffolds after 28 days. Sox9, RUNX2, Collagen type II, and Collagen type I are shown by *red* staining; nuclei were counterstained with NucRed Dead 647 (*pseudocolored blue*). Scale = 200 μ m. Color images available online at www.liebertpub.com/tea

will be necessary to (1) identify a readily available cell source(s) capable of generating appropriate cartilage and bone tissue; (2) develop a structure (i.e., scaffold) that will support formation of distinct but integrated cartilage and bone layers; (3) utilize external cues (i.e., growth factors and mechanical signals) to promote and sustain formation of OC tissue by the cell source; and (4) develop an *in vitro* system that mimics *in vivo* OC defect repair, to assess, optimize and predict performance of tissue-engineered constructs before *in vivo* testing.

In this study, we describe a novel OC tissue-engineering approach and companion “simulated *in vivo*” testing system that incorporates each of the four strategies above. We developed a gradient-matrix, clinically compliant scaffold material for OC tissue engineering, and we identified a cell source capable of OC lineage differentiation in this scaffold

material. We also showed that certain external cues can be harnessed to direct chondrogenic and osteogenic lineage differentiation within the scaffold, and we developed a culture system for testing the performance of OC tissue-engineered constructs *in vitro*.

Bedside-capability to isolate cell source for OC engineering

BM aspirate is clinically used as a source of “stem” cells for musculoskeletal regenerative medicine^{41–43}; however, BM contains a heterogeneous mix of differentiated cell types and hematopoietic progenitors, and it is estimated that BM-MSCs comprise <0.001% of the total cell population.^{12,44} Accordingly, BM aspirate is typically processed to enrich

FIG. 10. Quantification of the immunofluorescence staining of the simulated *in vivo* cultured scaffolds after 7 and 28 days of simulated *in vivo* culture. (A–D) Sox9, RUNX2, Collagen type II, and Collagen type I. #Significant when compared to all other groups; +significant when compared to all other groups excluding #. Asterisks depict different levels of significance ($p < 0.05$).

for BM stromal cells that will participate in the regeneration process.^{45,46}

In this study, we utilize two clinically relevant and commercially available BM concentration devices (Magellan and MACS) to isolate BM-MSCs. To facilitate clinical use, a method of isolation of cells that is rapid, efficient, and perhaps even automated, as the Magellan system is, is likely to be beneficial. Since the source of cells used for OC engineering is a critical component to the future success of the clinical application of these procedures, the ease and feasibility with which the cells are obtained is an important aspect to take note of. Our study supports use of CD271-positive cells isolated from human BM aspirate via Magellan and MACS as a feasible and readily obtainable source of cells for the development of clinically relevant OC tissue engineering strategies.⁴⁴

Presence of the CD271 cell-surface antigen is a widely accepted indicator of early stemness in MSCs; in addition, CD271-positive cells have good osteogenic and chondrogenic differentiation capabilities.^{47,48} The CD271-positive cells isolated using the protocol we developed here expressed typical stem cell markers upon culture (CD105, CD90, and CD73) and demonstrated appropriate *in vitro* tri-lineage differentiation into bone, cartilage, and fat. These results identify human BM aspirate-derived MSCs as a feasible and readily obtainable source of cells for bedside tissue engineering of OC defects.

Generation and *in vitro* evaluation of a clinically compliant gradient-matrix scaffold

Mechanical cues, provided by the extracellular environment, influence the attachment, proliferation, and differentiation of MSCs.¹⁹ We leveraged this characteristic in the

overall development of our OC scaffold. The scaffold substrate, PLGA, was chosen because it is an FDA approved and clinically compliant material in wide use in the medical field,⁴⁹ and because it can support both chondrogenic and osteogenic differentiation of MSCs.^{35,50–53} To optimize differentiation into two different tissues by a single cell source, within a single structure, the initial scaffold framework was constructed of PLGA microspheres layered in a continuous porosity gradient such that the distal (top) end of the scaffold is less dense, and the proximal (bottom) end of the scaffold is more dense. This physical arrangement allowed us to introduce a hydrogel matrix at the less-dense end, which would direct and support optimal differentiation of hMSCs into the chondrogenic lineage, creating a distal (top) cartilage layer.

A gel matrix with mechanics matching those of articular cartilage would be ideal to drive MSCs into the chondrogenic lineage; however, for tissue engineering, the ideal gel matrix would also need to be biodegradable to allow for gradual replacement of the gel substrate by a natural cartilage matrix. Since a biodegradable gel with mechanics matching native articular cartilage is not available, we used hyaluronic acid as the gel substrate. Hyaluronan is a long-chain sugar polymer abundant in native cartilage and is well established as a chondrogenic substrate in tissue engineering.⁵⁴ By varying the amount of cross-linking agent, we generated hyaluronan gels with the identical component ingredients but with variable stiffness, as measured by their storage moduli.

At 45 Pa, the stiffest gel we generated was substantially weaker than naturally occurring cartilage, which reported storage modulus is around 35–50 MPa or higher depending on the frequency used for testing.⁵⁵ Nonetheless, it outperformed a hMSC cell pellet alone in terms of cartilage-

characteristic matrix formation and also out-performed PuraMatrix, a commercially available gel with a very low storage modulus (5 Pa) used to support various types of cells including cartilage cells.⁵⁶ These studies identify a hyaluronan gel matrix for hMSC chondrogenesis *in vitro* and furthermore, demonstrate the feasibility of a temporary artificial ECM for chondrogenic differentiation of hMSCs during *in vivo* OC defect repair.⁵⁷

MSC migration from the subchondral bone marrow into an OC defect is thought to be a natural repair response to full-thickness articular cartilage damage,⁵⁸ and is also the rationale and mechanism underlying microfracture, a clinical procedure used for post-traumatic knee injury.¹ In the microfracture procedure, holes surgically punched through the articular cartilage allow BM cells to enter the defect region, where the BM-MSCs are thought to differentiate *in situ* into cartilage repair tissue. However, because the repair tissue formed in microfracture is fibrocartilage, and not hyaline cartilage, the repair lacks the structural integrity and durability to sustain long-term use.^{59,60}

Here, we have chosen a hyaluronan matrix to direct and support chondrogenic differentiation of hMSCs. By providing a scaffold that contains a distal layer of this pro-chondrogenic matrix, preseeded with hMSCs and primed by *in vitro* culture toward the chondrogenic lineage, we predict that MSCs migrating into the scaffold *in vivo* will attach to the proximal portion of the scaffold primarily and be directed into the osteogenic lineage, while the preseeded cells form hyaline-like cartilage that better approximates native articular cartilage tissue.

In a clinical setting, we envision that our novel gradient-matrix scaffold will be inserted into an OC defect such that the distal cartilage layer, formed by MSCs seeded into the chosen hyaluronan gel, will be contiguous with the articular cartilage, and the proximal, polymer-rich end will be contiguous with the subchondral bone. This placement will facilitate colonization of the proximal, polymer-rich end of the scaffold, by endogenous MSC present in the subchondral BM.

To test the feasibility of this design, we developed a “simulated *in vivo*” culture system to mimic *in vivo* scaffold infiltration by MSCs and assess formation of the OC tissue scaffold. OC scaffold culture *in vitro* necessitates the development of a co-differentiation media that supports MSC chondrogenesis and osteogenesis. Nevertheless, current means to simply and efficiently analyze OC repair constructs *in vitro* are greatly lacking.^{14,15} While there have been reports on the use of a coculture or co-differentiation media for *in vitro* preparation of OC engineered scaffolds, no systematic study has quantified the effects of co-differentiation media on chondrogenic and osteogenic differentiation.²⁴

The co-differentiation media we have developed contains growth factors and chemical agents known to influence the differentiation of MSCs into the chondrogenic or osteogenic lineage. Typically, treatment of MSCs with TGF- β 1 promotes chondrogenic differentiation, while treatment with BMP-2 can promote either chondrogenic or osteogenic differentiation.^{1,61,62} Other media factors that can influence cell fate include ascorbate, which is required for maintenance of chondrocyte and osteoblast phenotype by articular chondrocytes and these bone forming cells *in vitro* and β -glycerol phosphate, which is required for MSC osteogenic differentiation and matrix mineralization.^{63–66}

Although hMSC differentiation into the chondrogenic lineage was optimal in 100% chondrogenic media, and differentiation into the osteogenic lineage was similarly best in 100% osteogenic media, by combining media at different ratios, we identified co-differentiation conditions that supported chondrogenic and osteogenic differentiation *in vitro*. The optimal co-differentiation media contains both TGF- β 1 and BMP-2, in addition to ascorbate and inorganic phosphate; however, with the exception of ascorbate that remains the same, the concentrations of each agent are substantially less than in the original pure osteogenic or chondrogenic media. Identifying a co-differentiation media that supports simultaneous osteogenic and chondrogenic lineage differentiation overcomes a major barrier to preclinical testing of scaffolds for OC engineering.

Our observation that cell number was significantly increased in the scaffold 21 days after incubating it in co-differentiation media in the presence of free-floating hMSCs, demonstrates that the scaffold is successful in supporting hMSC attachment and proliferation. Importantly, significantly more cells were present in the proximal (bottom) end of the scaffold at the end of the culture period. This is likely due the presence of the hyaluronan-containing hydrogel in the distal end of the scaffold, which contains abundant ECM, likely limiting penetration by free MSCs. Also, while some amount of gel penetrates into the proximal portion of the scaffold there is most likely a greater amount of available pore space at this end that allows the cells to attach to this region first, leading us to predict that *in vivo*, when the scaffold is positioned in the OC defect region, it will also encourage migration into this proximal portion and proliferation by endogenous BM-derived MSCs.

Chondrogenic and osteogenic lineage differentiation by mesenchymal progenitor cells *in vitro* and *in vivo* can be monitored by assessing GAG deposition, matrix mineralization, and expression of characteristic markers including Collagen Type I, Sox9, Collagen Type II, and RUNX2. Our observation that the distal (top) half of the scaffold contains significantly more GAG than the proximal (bottom) half is consistent with enrichment of chondrocytes synthesizing a GAG-rich ECM in the distal part of the scaffold.

Although we find differences between the designated top (cartilage-forming) and bottom (bone-forming) regions of the scaffold suggesting the potential of our system for differential differentiation of MSC into chondrogenic and osteogenic lineages, the co-differentiation media and the scaffold system conditions did not produce exclusively site-specific chondrogenesis and osteogenesis.

Surprisingly, the distal (top) layer of the scaffold also contained Collagen Type I. In fact, there was just as much Collagen Type I protein present in the distal scaffold half as there was in the proximal half. There are several possible explanations for the presence of Collagen Type I in the top half of the scaffold. One is that since Collagen Type I is a marker for bone, it is possible that bone formation is occurring in the top half of the scaffold; however, for this to occur we would expect concurrent high levels of RUNX2, the master regulator of bone formation and in fact, RUNX2 levels are lower in the top than the bottom half of the scaffold.⁶⁷

Another possible explanation for presence of Collagen Type I in the top of the scaffold is that since Collagen Type I expression precedes formation of precartilage mesenchymal

condensations during chondrogenic differentiation *in vitro* and *in vivo*, the Collagen Type I in the distal end of the scaffold may represent prechondrogenic MSCs that are committed to undergo overt chondrogenic differentiation, but which have not yet done so.⁶⁸ Our observation that Sox9 was transiently present in the top half of the scaffold at day 7, but not at day 28, is consistent with progressive induction of MSCs into the chondrogenic phenotype in the top half of the scaffold.⁶⁹

Additionally, we also observed GAG deposition and Collagen Type II in the bottom half of the scaffold. This may be due to the fact that hydrogel diffusion into the gradient PLGA matrix is not a well-controlled process, and some of the hMSC-seeded gel may have infiltrated into the intended bone layer, positioning chondrogenic cells that are depositing GAG inside the bottom half of the matrix.

Furthermore, the hMSCs that were added in the media may have attached to both the top and bottom halves of the scaffold during the *in vivo* mimicking protocol. When cultured in media containing both BMP-2 and TGF- β 1 growth factors or when there are a variety of opposing cues, these un-primed MSCs have tendency to differentiate to either lineage.^{20,70} This is probably the reason why we see some mineralization in the cartilage layer of the graft. Nevertheless, we do not predict this to be a problem when our scaffolds are implanted *in vivo* as cells in the BM should not be able to access the cartilage portion of the graft.

Furthermore, to achieve durable osseochondral repair, it is important to direct the formation of hyaline cartilage, not fibrocartilage. In tissues primarily composed of fibrocartilage, such as the meniscus or the annulus fibrosis of the intervertebral disc, Collagen Type I makes up ~90% and 80% of the total collagen protein, respectively, while Collagen Type II makes up only 1% or 2%.⁷¹ When we use ImageJ to compare the semi-quantitative amounts of Collagen Types I and II present at either end of the scaffold, we observed that neither the distal nor the proximal contain these proteins in a ratio similar to what has been reported in fibrocartilaginous tissue, reducing the possibility of fibrocartilage formation in our scaffold.^{72,73}

As OC tissue regeneration is a growing issue worldwide, researchers must focus a great deal of time and effort to the creation of viable technologies. However, while many solutions have been proposed no one technique has set itself apart as superior. To more efficiently arrive at a solution an *in vitro* culture system, such as the one established in this article, can be beneficial. Yet, the benefits of this system do not end with this use. In future studies the media optimized here can be combined with bioreactor technologies that utilize mechanical stimuli to further improve OC scaffold *in vitro* analysis.^{74,75} Also, systems such as the one described can be used to establish more mature OC grafts that can be cultured before implantation to reduce or completely eliminate inclusion of growth factors *in vivo*.

Conclusions

Through this study, we proposed and designed an *in vitro* culture system for OC tissue engineering. Using chondrogenic and osteogenic media, along with matrix mechanics, as external cues, the newly designed scaffold system was studied for its ability to promote and sustain the selective

chondrogenesis and osteogenesis required for OC regeneration. As a result, the study establishes a co-differentiation media suitable for supporting both chondrogenic and osteogenic differentiation, and validates the appropriate chondrogenic and osteogenic differentiation of hMSCs in the developed medium. The results demonstrate differences between the designated top (cartilage-forming) and bottom (bone-forming) regions of the scaffold, suggesting the potential of our system for differential differentiation of MSCs into chondrogenic and osteogenic lineages. Overall, this study develops an *in vitro* culture system for evaluation of OC tissue scaffolds and establishes a method to culture and study complex tissue regeneration *in vitro*.

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Disclosure Statement

No competing financial interests exist.

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