CHAPTER 4

EFFECTS OF CHONDROCONDUCTIVE PORE GEOMETRY ON CHONDROGENESIS

4.1 INTRODUCTION

Cellular condensation is a pivotal stage in chondrogenesis wherein mesenchymal cells aggregate and cell-to-cell interactions increase significantly. The presence of hyaluronic acid, collagen I, and fibronectin help cells migrate as well as increase their packing density and the number of gap junctions formed.[1] Observations of limb mesenchymal cells have shown that high cellular density, spherical cell morphology, and increased cell-to-cell interactions are required for chondrogenic differentiation.[2,3]

The development of micromass, or cell pellet, culture has increased understanding of the mechanisms involved in initiating condensation and during subsequent cellular differentiation. Mesenchymal progenitor cells in micromass cultures undergo chondrogenesis in the presence of appropriate growth factors.[4] Though micromass cultures provide a good method to induce cellular differentiation, they are not ideal for articular cartilage regeneration *in situ*. A layer of fibroblastic cells encapsulates the micromasses and entrap the chondrocytic cells within. The cells also accelerate into terminal differentiation, making these conditions unfavorable for sustaining a chondrocytic phenotype.[5,6] However, the beneficial conditions of micromass culture that promote cellular differentiation, such as high cell density and low permeability, can be mimicked through engineering solid freeform fabricated (SFF) scaffolds with complex internal architectures and external geometries.[7] The pore shape, porosity,

permeability, and other characteristics of polymer scaffolds can be designed to stimulate and promote condensation directly within SFF scaffold pores. Furthermore, if the constructs are implanted at an orthotopic site for the regeneration of a clinical defect, the SFF scaffolds are capable of providing a mechanically protected environment for the developing tissue while allowing nutrient diffusion deep within the scaffold.

In our laboratory, image-based design[8] and SFF are used to design and construct poly(propylene fumarate) (PPF) scaffolds.[9,10] Pore architectures of various geometries can be patterned within three-dimensional scaffolds and parameters such as porosity, permeability, and diffusivity can be manipulated. Controlling these parameters will be critical in determining how scaffold design can be used to increase cellular density and condensation within the scaffolds, so that ultimately the amount of cartilage formation is increased. Pore geometries have been designed with increased surface area to increase cellular attachment to and retention within the polymer scaffold. The designed pore geometries also change the cell distribution within pores to help increase cell density required to attain the critical mass required for condensation.

In this study, the influence of cubical and ellipsoidal pore architectures on the induction of cellular condensation and differentiation was investigated (FIGURE 4.1). PPF scaffolds were pre-cultured *in vitro* for 2 weeks and then implanted *in vivo* in mice for 4 weeks. The ellipsoidal pores mimic the size, shape, and volume of the micromass and the interconnected pores allow signaling between cell aggregates in adjacent pores. The pore shape also alters the localized cellular density to help initiate condensation. Compared with cubic scaffolds, the ellipsoid scaffolds also have reduced permeabilities and greater surface areas for cell attachment. We hypothesize that the ellipsoidal pore shape with decreased permeability will have a greater effect on inducing chondrogenic differentiation

and extracellular matrix production by porcine bone marrow stromal cells (BMSC) and porcine chondrocytes.

4.2 MATERIALS AND METHODS

4.2.1 Cell isolation

Primary porcine chondrocytes were isolated from the metacarpophalangeal joints of domestic pigs. Cartilage slices were extracted aseptically within four hours of slaughter. Chondrocytes were isolated from the tissue using a solution of 1 mg/mL collagenase (Sigma, St. Louis, MO) and antibiotics. The isolated cells were filtered through a nylon mesh and were allowed to adhere for 24 hours. The adherent chondrocyte population was used for experimentation.

Porcine BMSC were isolated from the bone marrow aspirate of adolescent Yucatan minipigs (Seguin Animal Hospital, Seguin, TX). The aspirate was collected in a solution of heparin and antibiotics. The whole bone marrow was cultured for weeks and the entire adherent cell population was used for these studies. The cells were not passaged or passaged once prior to use.

4.2.2 *In vivo* experimentation

Scaffold design and fabrication

Image-based design (IBD) techniques[8] were used to create cylindrical scaffolds with internal pore architectures defined by periodic repetition of a base unit cell. Unit cells for cubic and ellipsoidal pore designs were [0.7, 0.7, 0.7 mm] and [0.7, 0.7, 0.9 mm], respectively (TABLE 4.1). The external dimensions of all designed scaffolds were 3 mm in height and 5 mm in diameter. Cubic scaffold struts were designed to be 350 μm thick, corresponding to pore space dimensions of 350 μm in length, width and height. The designed porosity of the scaffold was 50

percent. Ellipsoidal scaffolds pore dimensions were 700 μm in diameter and 900 μm in height. The interconnections between pores for the ellipsoidal scaffolds were 260 μm for the top and bottom and 300 μm for the sides of the ellipsoid. The designed porosity for the ellipsoid scaffold was 52 percent. Other calculations of scaffold properties shown in Table 4.1 were made using Solidview (Solidconcepts, Valencia, CA).

Wax molds were printed from design files using a three dimensional rapid prototyping system (Patternmaster™, Solidscape, Inc., Merrimack, NH). The wax molds were cast into poly(propylene fumarate) using indirect SFF fabrication techniques described previously.[10] Briefly, one gram of PPF (MW: 1,500 Da) was dissolved in one milliliter of N-vinyl-pyrrolidone (NVP). After homogenization, 0.02 grams of benzoyl peroxide was added to the PPF/NVP mixture. Then, two microliters of N, N-dimethyl-*p*-toluidene was added to induce crosslinking immediately before casting.[12] A custom polytetrafluoroethylene (PTFE) mold (diameter = 6 mm) was used to force the polymer solution through the pore spaces of the wax molds. The PTFE mold was placed in a nitrogen chamber at room temperature for 24 hours to allow the polymer to cure. The wax was then removed with acetone, and the remaining PPF scaffolds were rinsed in distilled deionized water and sterilized in 70% ethanol.

Scaffolds cast in PPF from the wax molds were scanned in air at a resolution of 16 μm using a micro-computed tomography machine (MS-130, GE Medical Systems, Toronto, Canada) to determine the accuracy of the fabrication process. The measured values of fabricated scaffolds were less than 5 percent of designed pore and strut values.

Permeability and Relative Diffusivity Calculations

A finite difference technique was used to solve creeping Stokes flow for three unit pressure gradients. Utilizing homogenization theory[19], the effective permeability tensor, K_{ij}, is calculated as:

$$K_{ij} = \frac{1}{\mu}\left\langle v_j^i \right\rangle \qquad \left(m^4 N s^{-1}\right)$$

where μ is the fluid viscosity and $\left\langle v_j^i \right\rangle$ is the average velocity field resulting from pressure gradients applied in three directions. The effective diffusivity tensor was calculated using a finite element homogenization method based on the asymptotic expansion technique to solve a multiscale version of the governing diffusion equation for three unit concentration gradients.[11] The effective diffusivity D_{ij}^{eff} tensor is then calculated from three characteristic local concentration gradients and the local diffusivity coefficients as:

$$D_{ij}^{eff} = \left\langle D_{ik}\left(\delta_{kj} + \frac{\partial \chi_j}{\partial y_k}\right)\right\rangle \qquad \left(m^2\ s^{-1}\right)$$

Where D_{ik} is the local diffusivity tensor, $\frac{\partial \chi_j}{\partial y_k}$ is the characteristic concentration gradient, δ_{kj} is the kronecker delta, and $\langle\ \rangle$ denotes an average over the unit cell.

In vitro pre-culture

In preparation for cell seeding, the PPF scaffolds were rinsed twice with sterile Hank's Balanced Salt Solution (HBSS) and once with culture medium. The PPF scaffolds were then placed in a custom made PTFE mold (d = 5.1mm). BMSC and chondrocytes were seeded into the scaffold pores using a collagen I hydrogel. For these hydrogels, 50 x 10^6 cells/mL were resuspended in a solution containing either ~3.8 mg/mL collagen I (stock concentration: 4.66 mg/mL; BD Bioscience Discovery Labs, San Jose, CA) after trypsinization. The pH of the solution was increased by the addition of 20 μL of 0.5 M sodium hydroxide

solution. The cell and hydrogel suspension was pipetted into the scaffold pores (~60 μL) immediately after sodium hydroxide addition. The gels were allowed to solidify within the scaffolds in a humidified chamber at 37°C for 30 minutes. The PPF scaffolds were then removed from the PTFE mold and placed in a 24-well tissue culture plate. Scaffold constructs were cultured in either basal medium (DMEM + 10% FBS + 1% P/S) or a chondrogenic-defined medium (basal medium supplemented with 10 ng/mL TGF-β_1, 0.1 nM dexamethasone, 50 μg/mL 2-phospho-L-ascorbic acid, 0.4 mM proline (Sigma), 5 μg/mL insulin (Gibco) and 0.1 mM non-essential amino acids (Gibco)). Chondrocyte-seeded constructs were cultured in the same chondrogenic medium, but without TGF-β_1 or dexamethasone. The scaffolds were then placed in the incubator and gently agitated on an orbital shaker. The pre-seeded scaffolds were pre-cultured for 2 weeks with the culture media changed every other day during this period.

Pre-chondrogenic condensation evaluation using PNA

Rhodamine-conjugated peanut agglutinin (PNA) (Vector Labs, Burlingame, CA) was used to identify the pre-chondrogenic aggregates at 2, 4, 5, 7, and 10 day timepoints. At each timepoint, at least 2 scaffolds seeded with BMSC were frozen embedded in OCT (Tissue-Tek®, Sakura, Torrence, CA). Serial frozen sections between 7 – 14 μm thick were cut using a cryostat (HM 500 M, Microm GmbH, Walldorf, Germany). Slides were immersed in distilled water, incubated for 30 minutes in 100 ng/mL PNA solution, rinsed with water and then DAPI was used to stain the cellular nuclei on the same sections. The sections were washed and then mounted using ProLong Gold (Molecular Probes, Carlsbad, CA). Pictures were taken using a Nikon microscope with epifluorescence attachment (TE300 Eclipse, Nikon America, Melville, NY) using SPOT image acquisition program (Diagnostic Instruments, Sterling Heights, MI) using a rhodamine and UV filter set. Pictures from at least five different areas were

taken from each slide. Two pictures were taken from each area for PNA and DAPI stains, the two pictures were then merged together using SPOT.

In vivo implantation

After pre-culture, 24 PPF scaffolds [n=3; 1) cubical or ellipsoidal pored scaffolds seeded with 2) BMSC or chondrocytes cultured in 3) basal or defined chondrogenic medium] were implanted subcutaneously in 5- to 8-week-old immunocompromised mice (N:NIH-III; Harlan, Indianapolis, IN). Six animals were anesthetized with intramuscular injections of ketamine (50 μg/g) and xylazine (5 μg/g) in saline. Four dorsal subcutaneous pockets were created by blunt dissection and one PPF scaffold from each experiment group was placed into each pouch, and in a different pouch location in each mouse. The implantation sites were closed with surgical wound clips. The animals were housed in groups for four weeks with free access to food and water.

Evaluation of implants

The mice were sacrificed after 4 weeks. The scaffolds were harvested and fixed in zinc-buffered formalin (Z-FIX, Anatech, Battle Creek, MI) overnight. The scaffolds were then dehydrated for paraffin embedding. The specimens were bisected longitudinally and embedded so that sagittal and transverse sections were cut at a thickness of 7 μm. Slides were stained with hematoxylin and eosin (H&E) or Safranin O counterstained with Fast Green FCF. Briefly, hydrated slides were immersed for 30 seconds in Harris hematoxylin then cleared in running tap water, followed by 3 minutes in 0.02% Fast Green FCF, 10 seconds in 1% acetic acid, and 6 minutes in 0.1% Safranin O. The slides were then dehydrated and mounted with permount (Sigma).

Immunohistochemistry was used to detect collagen I and collagen II as described previously (Appendix F).[10] Paraffin sections were deparaffinized and rehydrated.

Endogenous peroxidase activity was blocked and the slides were placed in sheep hyaluronidase (Sigma) solution with 1% bovine serum albumin (BSA, Sigma) to retrieve the antigen. After several TBS washes, 10% (v/v) normal goat serum was used to block non-specific background. All incubations were performed using a humidity chamber. Primary antibodies, polyclonal rabbit antisera raised against human type I collagen or type II collagen (US Biological, Swampscott, MA), was placed on the sections for 1 hour at room temperature. Excess primary antibodies were washed off and the primary antibody was detected using a biotinylated-goat anti-rabbit secondary antibody and Vectastain ABC reagent according to the manufacturer's instructions (Vectastain ABC kit for rabbit IgG, Vector Laboratories, Burlingame, CA). Peroxidase activity was visualized using diaminobenzidine (DAB substrate kit, Vector Laboratories) for 5-7 minutes. The sections were rinsed with water to stop the reaction. The sections were then counterstained using 0.02% Fast Green FCF, dehydrated and mounted using permount. Paraffin slides of a mouse joint were used as positive controls and to determine the optimal concentrations for the primary antibody. For negative controls, the primary antibodies were not added.

Histomorphometric image analysis

Histological sections taken from four planes or more (100 - 500 μm difference) of each scaffold were used for analyses. Low magnification (2X objective) images of whole cross sections of tissue samples were acquired using a CCD camera (RT Color 2.2.1, Diagnostic Instruments, Sterling Heights, MI) mounted on a Nikon Eclipse E600 microscope (Nikon America, Melville, NY). RGB images were acquired through Spot, an image acquisition program (Diagnostic Instruments, Sterling Heights, MI). The illumination of each picture was controlled so that the exposure for each image was the same; thus the brightness, contrast and saturation of the images was captured using the same parameters.

Image processing was performed on the RGB images using Matlab (The Mathworks, Natick, MA) to determine: 1) the ratio of positive Safranin O staining to the available pore space and 2) the average sGAG concentration of the section (correlated using the stain intensity). The first calculation is correlated to the total area of cartilage-like matrix present in the slide and also approximates the number of chondrocytic cells that have not dedifferentiated. The second value is correlated to the amount of sGAG present in the extracellular matrix.[12,13] The histomorphometric data was statistically analyzed using ANOVA to determine if scaffolds with elliptical pores promoted more cartilage formation and sGAG deposition than scaffolds with cubic pores. All statistical tests were performed using SPSS.

4.3 RESULTS

Cubical and ellipsoidal pore architectures were patterned within cylindrical shaped scaffolds (FIGURE 4.1). Particular consideration was given to the porosity and permeability of the designs. The porosity of the cubical scaffolds was equal to 50% and that of the ellipsoidal scaffolds was 52% (TABLE 4.1). The effective permeability of the ellipsoidal scaffold was 0.29 $x10^{-7}$ m^4/Ns in the short axes and $0.36x10^{-7}$ m^4/Ns in the long axis. The effective permeability of the cubical scaffold was six times greater, at $2.16x10^{-7}$ m^4/Ns in all three axes. The pore volume of the ellipsoid scaffold was greater than the cubic, but the difference, assuming uniform cell distribution, results in an increase of 500 cells per mm^3. Thus, theoretically there is not a difference in the cellular densities between the pore designs.

Cellular condensation during the pre-culture stage was confirmed by peanut agglutinin (PNA) staining. During the pre-culture period, some scaffolds seeded with BMSC were removed from culture at 2, 4, 5, 7, and 10 day timepoints and

cellular condensation was localized using rhodamine-conjugated PNA.[14,15] PNA recognizes galactosyl (β-1,3) N-acetylgalactosamine in pre-chondrogenic aggregates but will not bind to the aggregates after chondrogenic differentiation has occurred. In Figure 4.2, the areas of red stain corresponding to the fluorescent dye rhodamine identify the presence of pre-chondrogenic aggregates. The slides are counterstained with DAPI (4',6-diamidino-2-phenylindole), which stains the nuclei of all cells blue. Cells double-labeled with DAPI and PNA are positive aggregates considered to be pre-chondrogenic condensations. At 2 and 4 days of pre-culture, positive staining was present in both the cubic and ellipsoid scaffolds. In these areas, positively stained aggregates were in areas of increased cellular density shown by the increased number of nuclei stained with DAPI. A greater number of cellular aggregates were seen in the ellipsoid scaffolds compared to scaffolds with cubic pores. Additionally, aggregates with higher stain intensities were seen at the interconnections of pores throughout the ellipsoid scaffolds (FIGURE 4.2b), but only at the outer pores within the cubical scaffolds (FIGURE 4.2a). After 5, 7 and 10 days of pre-culture, the cells no longer stained positive for PNA and only the DAPI staining is present, indicating that the pre-cartilage condensation stage had ended and the cells have progressed further down the pathway to chondrocytic differentiation.

Chondrocytic cells proliferated and a cartilage-like extracellular matrix developed within the scaffolds during pre-culture and implantation, except for scaffolds seeded with BMSC and cultured in basal medium. Chondrogenic differentiation of the BMSC did not occur without the presence of growth factors in the chondrogenic medium (FIGURE 4.3). The BMSC seeded scaffolds cultured in chondrogenic medium exhibited pockets of chondrogenic differentiation (FIGURE 4.4a,b), while chondrocytes cultured in either medium were widespread throughout the scaffold pores and surrounded by a dense extracellular matrix (FIGURE 4.4c,d,e,f). The cells in these cartilage-like areas

were round and chondrocytic in appearance and were surrounded by well-established lacunae and pericellular matrices. The extracellular matrix in these areas stained positive with Safranin O, indicating the presence of sGAG and other cartilage proteins. Within the same sections, non-cartilaginous tissue (muscle, vasculature, fibrotic tissue) stained positive with Fast Green. Larger areas of Fast Green were found in the BMSC seeded scaffolds compared to the chondrocytes, where the areas of Fast Green were limited to the scaffold edges. Immunohistochemistry was used to detect collagen II and collagen I in the BMSC seeded scaffolds cultured in chondrogenic medium (FIGURE 4.5) and chondrocyte seeded scaffolds cultured in basal (FIGURE 4.6) and chondrogenic medium (FIGURE 4.7). The areas that were positive for collagen II and collagen I were well correlated with areas that stained positive for Safranin O and Fast Green, respectively, and further confirmed the formation of cartilage-like tissue within the scaffolds.

Histologic results showed that greater amounts of cartilage developed in the ellipsoid scaffolds compared to the cubic pored scaffolds. Histomorphometric image analysis was performed on tissue sections stained with Safranin O and Fast Green to quantify the amount of cartilage and sulfated glycosaminoglycan (sGAG) concentration present in each section. The areas of positive Safranin O staining were determined and normalized by the total available pore space. This percentage correlates to an approximate number of viable chondrocytes for the section that are capable of producing sGAG, identifying the BMSC that have differentiated into chondrocytes or the primary chondrocytes that have maintained a chondrocytic phenotype. For BMSC seeded scaffolds cultured in chondrogenic medium, the average positive Safranin O staining was 3±0.2% for the cubical scaffolds compared to 13±4% for the ellipsoidal scaffolds (FIGURE 4.4a2,b2). The quantified Safranin O stained areas for chondrocyte-seeded scaffolds cultured in basal medium (FIGURE 4.4c2,d2) were 26±7% and 28±8%

for cubic and ellipsoid pores, respectively. For chondrocyte seeded scaffolds cultured in chondrogenic medium (FIGURE 4.4e2,f2), areas of Safranin O staining were 61±15% and 79±6% for cubical and ellipsoidal scaffolds, respectively, both greater than all other groups. The ellipsoidal pore design had statistically greater areas of cartilage stained with Safranin O for both the BMSC ($p<0.001$) and chondrocytes ($p<0.05$) cultured in chondrogenic medium (FIGURE 4.8a). The areas of cartilage formation for scaffolds cultured in basal medium were not statistically different.

The intensity of the Safranin O stain was also determined using histomorphometric image analysis and correlated to the amount of sGAG present in the cartilage-like extracellular matrix. The average sGAG concentrations correlated from the Safranin O stain intensity for BMSC were 5.9±1.7 $\mu g/mm^2$ for cubic scaffolds compared to 7.0±1.3 $\mu g/mm^2$ for ellipsoid scaffolds (FIGURE 4.4a3,b3). For chondrocytes cultured in basal medium (FIGURE 4.4c3,d3), the average GAG concentration was higher than for the BMSC at 7.0±2.1 and 7.3±2.6 $\mu g/mm^2$ for cubic and ellipsoid scaffolds, respectively. The average sGAG concentrations for the chondrocytes cultured in chondrogenic medium were 7.6±1.3 $\mu g/mm^2$ for cubic scaffolds and 8.7±2.2 $\mu g/mm^2$ for ellipsoid scaffolds (FIGURE 4.4e3,f3). The stain intensities for were statistically different between the two pore designs for BMSC ($p<0.05$) and chondrocytes ($p<0.05$) cultured in chondrogenic medium (FIGURE 4.8b). The GAG concentration for the chondrocytes that were cultured in basal medium did not vary significantly between pore geometries, but the total sGAG present in the extracellular matrix was significantly greater in the ellipsoid scaffolds ($p<0.05$).

4.4 DISCUSSION

Previous research has identified conditions that work synergistically during limb morphogenesis to initiate chondrogenesis, such as high cellular density to increase cell-cell interactions, spheroid cell morphology, and signaling from growth factors.[16] In the current study, we artificially simulated these conditions to tissue engineer cartilage within computationally designed load-bearing biomaterial scaffolds. We compared cubical and ellipsoidal pore architectures to evaluate the effects of pore geometry and scaffold permeability on the formation of new cartilage tissue by porcine bone marrow stromal cells (BMSC) and primary chondrocytes. For both cell types, greater areas of cartilage and higher concentrations of cartilage proteins (sGAG) were found in the ellipsoid scaffolds cultured in chondrogenic medium.

Prior studies have shown that the cell density for monolayer culture must exceed 5000 cells/mm^2 in order for differentiation to occur.[17] Typical micromass cultures seed 250,000 cells per pellet (diameter ~ 2 mm)[18], which equates to a cellular density between 40,000 – 60,000 cells/mm^3 after centrifugation. The cellular density calculated for a single cubic pore is 49,500 cells/mm^3, which is within this range used for stimulating aggregation in micromasses. The cellular density for the ellipsoid pore is 50,000 cells/mm^3. There is a slight difference between the two designs, but the cellular densities of both should be high enough for differentiation to occur. The BMSC that were cultured in basal medium had the critical cell mass required, but other signals, such as biofactors, must be present to initiate the condensation process. Johnstone et al., also found that micromasses cultured in basal medium did not aggregate or differentiate. The presence of growth factors, particularly TGF-β, was required for initiating aggregation.[4]

The results of this study indicate that the defined chondrogenic medium may be the strongest factor determining cartilage formation with greater influence than cellular density, pore shape, or scaffold permeability. BMSC cultured in basal medium did not differentiate and the tissue formation by the chondrocytes cultured in basal medium was moderate compared to the tissue that was produced by the chondrocyte-seeded constructs that were cultured in chondrogenic medium. Significant differences between the scaffold pore designs were not found with the scaffolds cultured in basal medium, but for the scaffolds that were cultured in chondrogenic medium, greater areas of cartilage formation were quantified within the ellipsoid scaffolds compared to the cubic pored scaffolds. This emphasizes that the growth factors and proteins in the defined chondrogenic medium are essential and increase the cellular metabolic activity, stimulate chondrogenic differentiation of BMSC and promote extracellular matrix production in both cell types. The cellular activity is at a heightened level such that the differences between the scaffold pore designs are more distinguishable and the other factors such as the pore geometry and permeability are now elements that also impart some influence on the development of cartilage tissue.

The amount of cellular differentiation is determined early on during chondrogenesis. PNA staining of scaffolds during pre-culture showed that the ellipsoid pored scaffolds seeded with BMSC already had greater numbers of pre-chondrogenic cellular aggregates compared to their cubic counterparts after 2 days of pre-culture. The pre-cartilage condensations shown by the PNA staining indicate the areas where BMSC have committed to chondrogenic differentiation. During skeletogenesis *in vivo*, it is during this condensation stage that the shape, size, and numbers of skeletal elements are determined. The condensations are initiated by the aggregation of cells toward a central location, which increases the packing density of the cells.[19] Furthermore, positive PNA staining in the

ellipsoid scaffolds occurred with the highest intensities at the pore interconnections. The number of cell nuclei at and surrounding these aggregates confirm the increased cell density at these locations. These results suggest that the pore interconnections may serve as cell aggregating points for cellular condensation.

One potential explanation for the greater areas of cartilage formation in the ellipsoid scaffold is the pore shape. The pore shape was designed to induce cellular condensation within the SFF scaffolds. The external shape and size of the micromass was used as a design factor because the cell packing density is highest when the cells are in a spherical-like mass (see Figure 2.1 for examples of cellular condensation shape). The volume of a cube with length 0.35 mm is 0.043 mm^3, while the volume of the corresponding sphere is with diameter 0.35 mm is 0.022 mm^3. If the identical number of cells were put within these two geometries, then the cell density within the sphere would be almost two times greater than the density in the cube. The same principle can be applied to the ellipsoid pore shape. The curves of the ellipsoid shape change the cell distribution and increase the local cell packing density, which is beneficial for cellular differentiation. From the PNA results, we believe that the pore interconnections provide points of aggregation that help direct cell migration to these locations to further increase the cell density. The cartilage tissue that formed within the ellipsoid scaffolds were found mainly at these pore narrowings and at pore interconnections. The cubic architecture scaffolds have straight channels, without the narrowings that are found in the ellipsoid pores. The cell nuclei are more widespread throughout the pores of the cubic scaffolds. As a result, this more uniform cell density leads to fewer areas of cellular condensation. Thus, lacking a point of aggregation and areas of greater local cell density, the cells in the cubic scaffolds do not condensate in the same manner as the cells in the ellipsoid scaffolds.

Similar results were seen in the chondrocyte seeded scaffolds cultured in chondrogenic media. The cells in the ellipsoid scaffolds produced a more robust extracellular matrix with higher sGAG concentrations than the chondrocytes seeded in cubic scaffolds. Since the chondrocytes are already differentiated, cellular aggregation is not required. Other scaffold properties, such as lower permeability and the ellipsoid pore shape which promotes increased local cellular density maybe greater factors in promoting new cartilage tissue formation. There was a greater density of chondrocytic cells in the ellipsoid scaffolds, which may have contributed to the increased amount of sGAG present in the extracellular matrix. The higher cell density and lower permeability of the ellipsoid scaffolds help to preserve the chondrocytic phenotype, resulting in greater areas of Safranin O staining and reduced areas of dedifferentiation, shown by the reduced amount of Fast Green staining.

The scaffold permeability should conceivably have an effect upon cellular metabolism and tissue formation. What remains unclear is which permeability value, or values, is most suitable for either developing tissue such as differentiating BMSC or for chondrocytes that are trying to repair a defect. The permeability of native cartilage ranges between 0.1 to $2x10^{-15}$ m^4/Ns.[20] Should the scaffold permeability match that of the native tissue or is a higher scaffold permeability with greater oxygen tensions and higher diffusivity of nutrients more appropriate for cartilage tissue regeneration. Technology and increased proficiency for scaffold design have advanced so that we can begin to isolate this scaffold parameter and design scaffolds that will evaluate this uncertainty.

The calculated permeabilities of both scaffold designs are six or seven orders of magnitude larger than that of native cartilage. These values are further reduced due to the presence of the hydrogel and due to the oxygen gradient that exists

within SFF scaffolds, as shown by Malda et al.[21] Cylindrical 3D-deposited fiber (3DF) scaffolds with 4 mm diameter and 4 mm height were used for the analysis. The scaffolds had a porosity of 80% with cubic pore spacing ~ 800 x 800 x 200 μm. Oxygen tension from the scaffolds edges to the centers were measure using glass microelectrodes and the cell distribution was also analyzed over time. The measured oxygen tensions gradually decreased from 20% at the edge to about 5-8% at the center of the scaffolds. The permeability of the 3DF scaffolds were not calculated so a direct comparison can not be made. The 3DF scaffolds are similar enough to the SFF scaffolds used in this study to assume that the oxygen tension within the SFF scaffolds is around 5% oxygen.

The lower permeability of the ellipsoid scaffold results in lower oxygen tensions, which may further augment the effects of pore shape and cellular density. Other scientists have shown that cellular growth, differentiation and metabolism of BMSC and chondrocytes benefit from environments with high cellular densities and low permeabilities.[22] Mesenchymal stem cells cultured at low oxygen tensions (5% O_2) favor chondrogenic differentiation, increases in proteoglycan synthesis, inhibit collagen X expression and suppress alkaline phosphatase activity.[23] Chondrocytes cultured at lower oxygen tensions reduce the rate of senescence. The cells keep a high proliferation rate and maintain a higher ratio of chondroitin sulfate to keratan sulfate synthesis.[24] Furthermore, the reduced oxygen tensions help decrease the rate of cell ageing by reducing the amount of free radicals generated by oxidative metabolism and this further reduces the amount of extracellular matrix, proteoglycan, and DNA degeneration caused by the presence of reactive oxygen species. Thus, the lower permeability of the ellipsoid scaffolds may contribute to the increased cellular differentiation in the BMSC and the increased cell density and sGAG production of the chondrocytes.

4.5 CONCLUSION

In conclusion, scaffold design using ellipsoidal pores that have narrowed pore interconnections and lower permeability showed enhanced cartilage formation. Our results showed greater amounts of BMSC pre-cartilage condensation and subsequent chondrogenic differentiation in scaffolds with ellipsoidal pores than cubical pores. These factors also encouraged greater phenotype stability and enhanced extracellular matrix production in chondrocytes. We postulate that a combination of ellipsoid shape with narrower pore interconnection and low permeability contributed to these results.

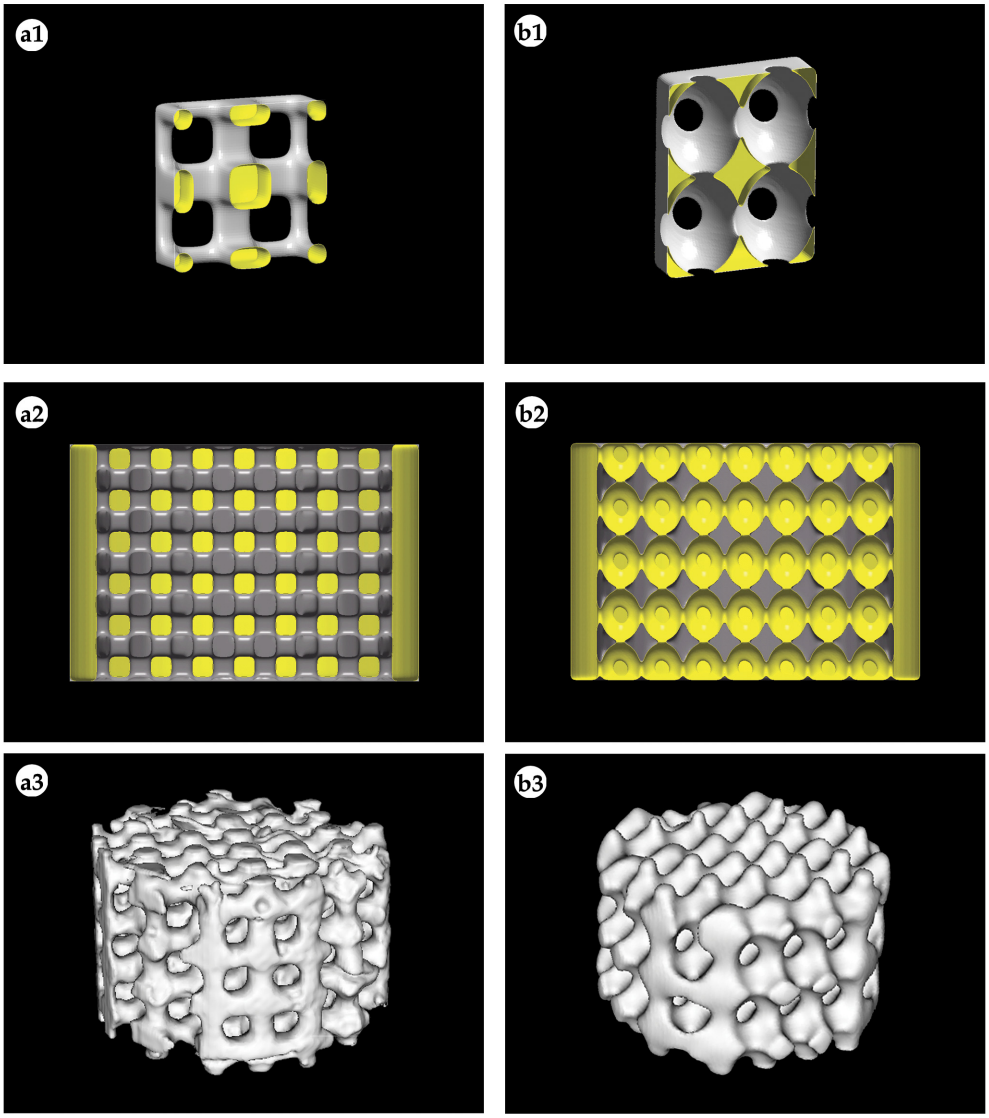

Figure 4.1: Designed scaffold architectures and fabricated SFF scaffolds. Cross-sections of four unitcell aggregates depicting the architecture and pore interconnections of cubic (a1) and ellipsoid (b1) scaffolds. Wax molds with cubic (a2) and ellipsoid (a3) architecture created using a three dimensional rapid prototyping system, the xz plane is shown and the areas shown in yellow indicate the pore space of the scaffolds. Micro-CT images of fabricated cubic (a3) and ellipsoid (b3) pored PPF scaffolds created by indirect SFF. See Table 4.1 for the parameters used for scaffold design and other calculated scaffold properties, such as surface area and scaffold diffusivity.

	Cubic Pore Scaffold	Ellipsoid Pore Scaffold
Design Unit Cell Size [x, y, z] (μm)	[700, 700, 700]	[700, 700, 900]
Designed Pore Size [x, y, z] (μm)	[350, 350, 350]	[700, 700, 900]
Pore Interconnections [x, y, z] (μm)	[350, 350, 350]	[300, 300, 260]
Porosity (%)	50	52
Permeability ([x, y, z] $x10^{-7}$ m^4/Ns)	[2.16, 2.16, 2.16]	[0.36, 0.36, 0.29]
Diffusivity ([x, y, z] m^2/s)	[0.44, 0.44, 0.44]	[0.25, 0.25, 0.27]
Surface Area, unitcell (mm^2)	2.7	4.4
Surface Area, scaffold (mm^2)	250	260
Pore Volume, unitcell (mm^3)	0.17	0.23
Pore Volume, scaffold (mm^3)	29.5	30.8
Cellular Density, unitcell (Numbers of cells/mm^3)	49,500	50,000
Cellular Density, scaffold (Total number of cells)	$3x10^6$	$3x10^6$

Table 4.1: Scaffold design parameters and properties.

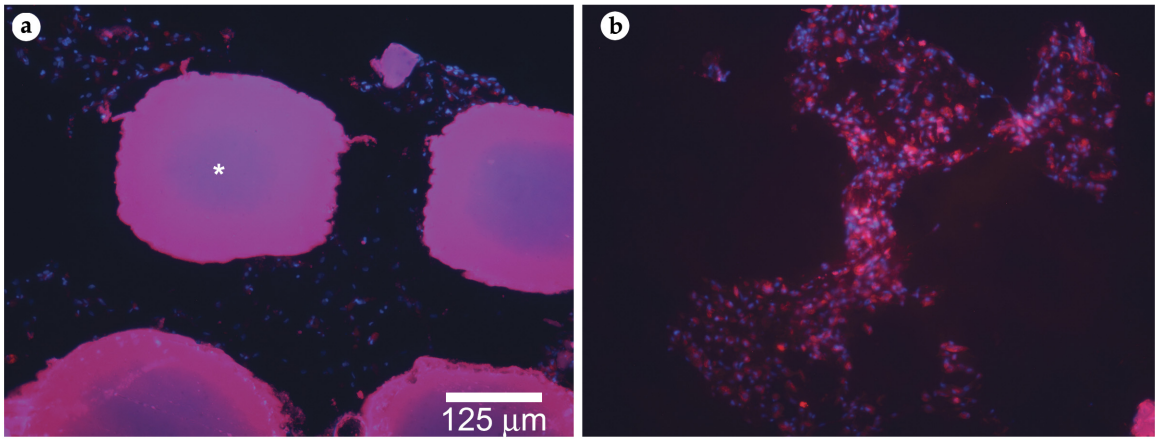

Figure 4.2: Fluorescent microscope images of pre-chondrogenic aggregates in BMSC seeded scaffolds. Pre-chondrogenic condensations stain red due to the presence of galactosyl (β-1,3) N-acetylgalactoseamine. Nuclei of all cells, whether positive or negative for PNA, stain blue with DAPI nuclear stain, while the residual PPF scaffold is autofluorescent as indicated with the white asterisk. After 2 days of pre-culture, cubic scaffolds (a) showed small isolated areas of PNA staining mostly at the outer scaffold edges, while in ellipsoid (b) scaffolds positive staining was seen throughout the scaffolds with higher intensities at the pore interconnections. DAPI staining in these positively stained areas confirmed the increased cell density of these aggregates. After day 5, little to no PNA staining was seen in either cubic or ellipsoid scaffolds.

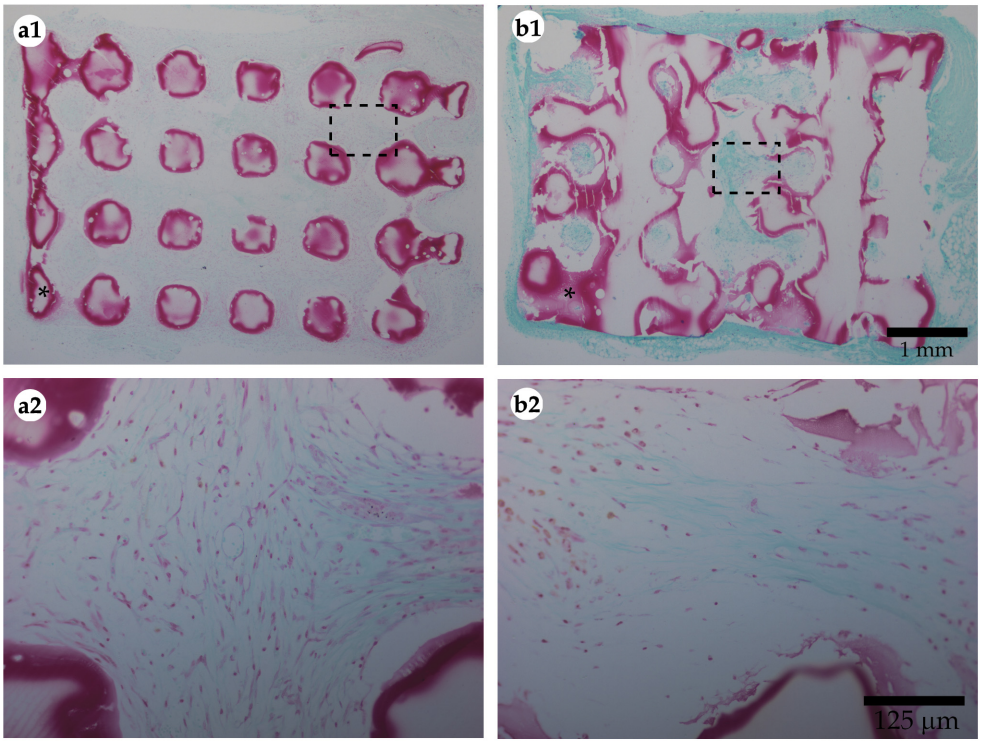

Figure 4.3: BMSC seeded into PPF scaffolds using collagen I hydrogels and cultured in basal medium showed no areas of chondrogenesis. Constructs were pre-cultured *in vitro* for 2 weeks, then implanted for 4 weeks *in vivo*. Neither scaffolds with cubical (a) or ellipsoidal (b) architecture showed any areas of positive Safranin O staining. a2,b2) The pores of the scaffolds were filled mainly with fibroblastic cells that stained positive with Fast Green. The residual PPF scaffolds are visible in the sections (indicated with *).

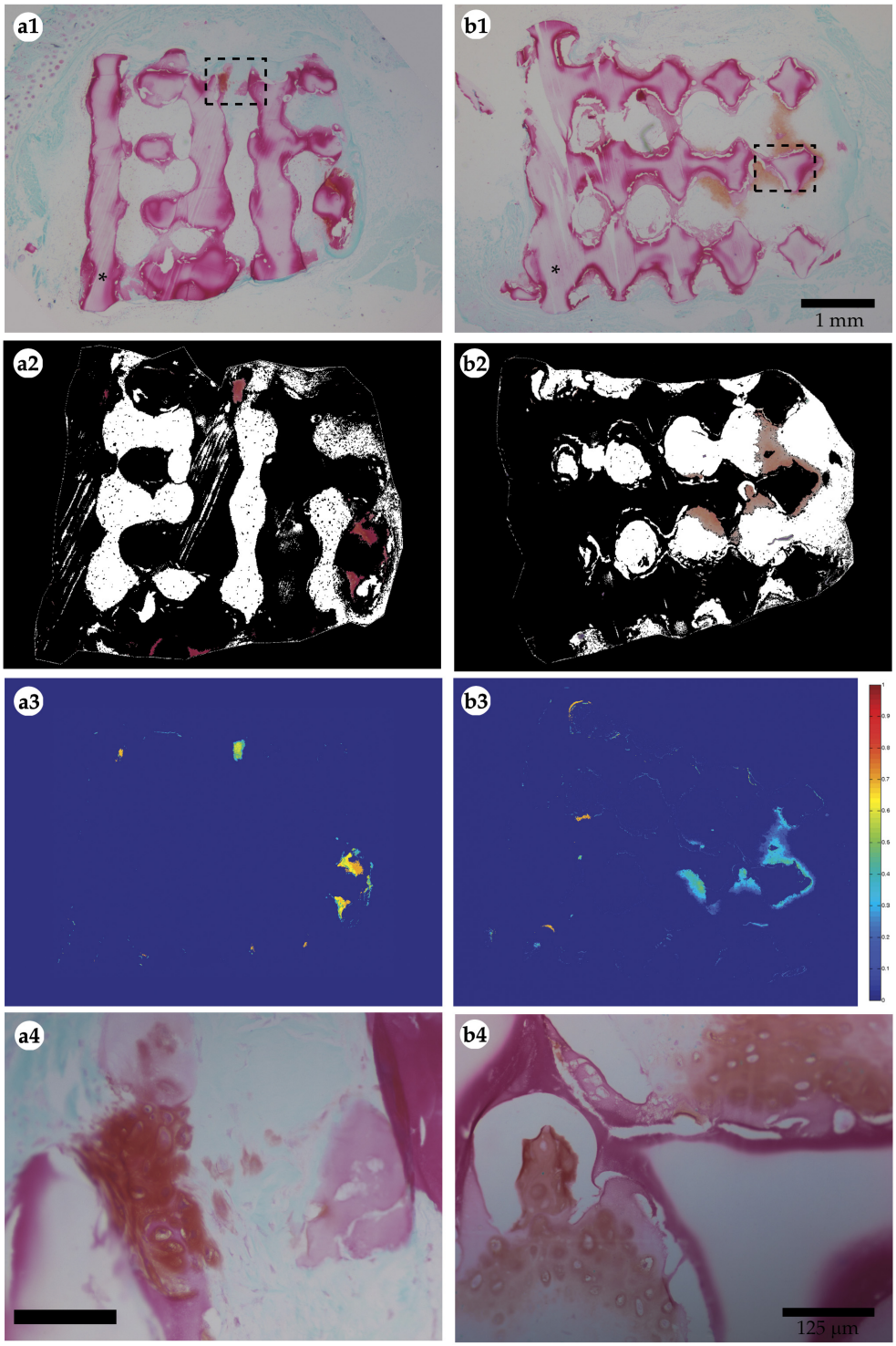

Figure 4.4: Cartilage formation in PPF scaffolds. BMSC seeded in cubic (a) and ellipsoid (b) scaffolds and cultured in chondrogenic medium. Sections are stained with Safranin O/Fast Green. Residual PPF is visible in the sections (labeled with *). a2,b2) The positive areas of Safranin O shown were extracted using image processing. These areas were quantified to determine the amount of cartilage matrix formation and the stain intensity was correlated to sGAG concentration. The amount of cartilage for these representative specimens is 3% for a2, 13% for b2. a3,b3) The average sGAG concentration calculated for the sections are 5.86 µg/mm^2 for a3, 7.01 µg/mm^2 for b3. a4,b4) Higher magnifications of the areas within the dashed boxes show round chondrocytic cells surrounded by positively stained extracellular matrix.

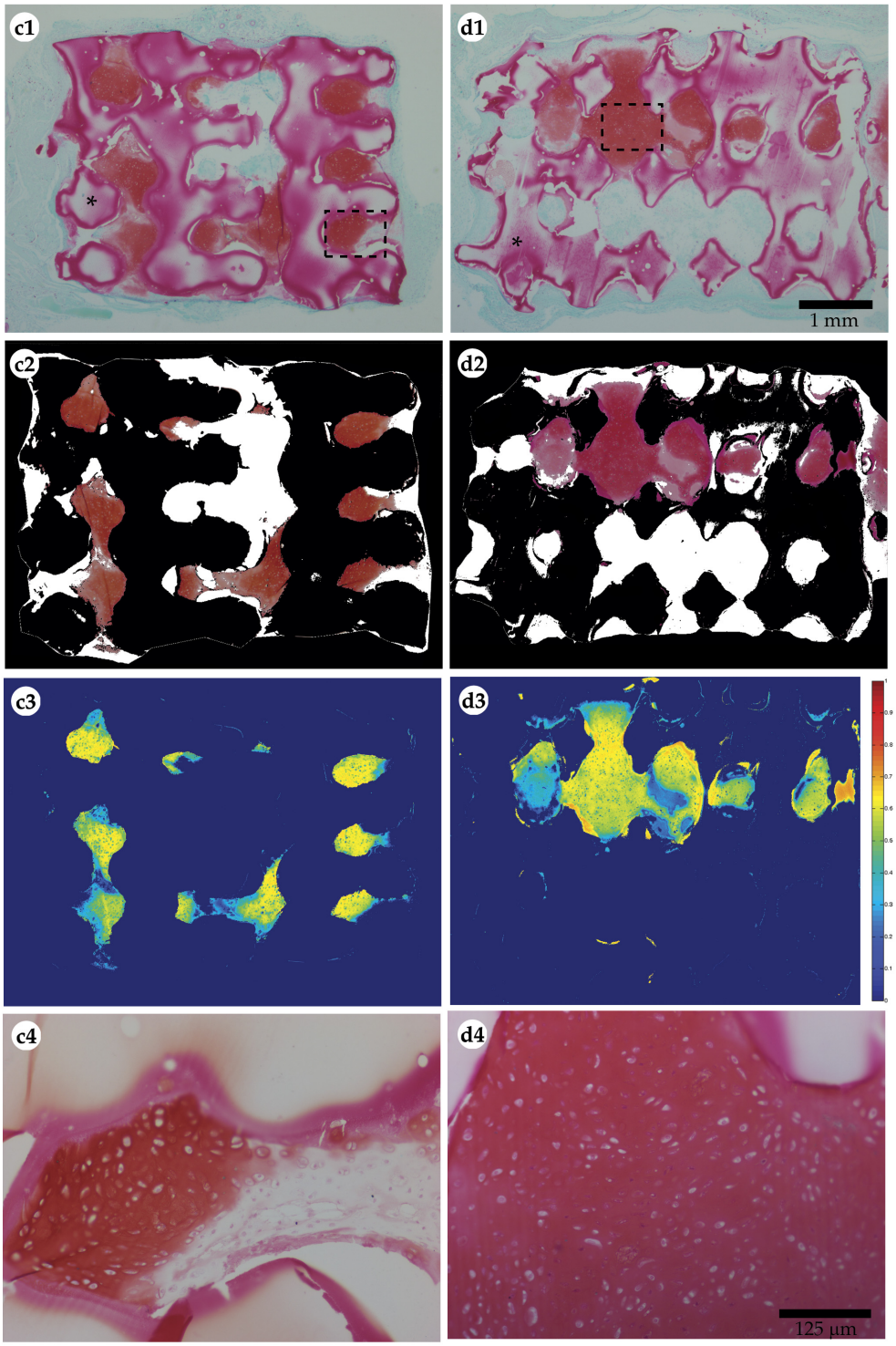

Figure 4.4: (continued) Cartilage formation in PPF scaffolds. Chondrocytes seeded in cubic (c) and ellipsoid (d) scaffolds cultured in basal medium. Residual PPF is visible in the sections (labeled with *). c2,d2) The calculated percentages of positive Safranin O staining for these representative specimens is 37% for c2 and 40% for d2. c3,d3) The average sGAG concentration calculated for the sections is 9.5 µg/mm^2 for c3 and 9.8 µg/mm^{2}for d3. c4,d4) Higher magnifications of the areas within the dashed boxes show round chondrocytic cells surrounded by positively stained extracellular matrix.

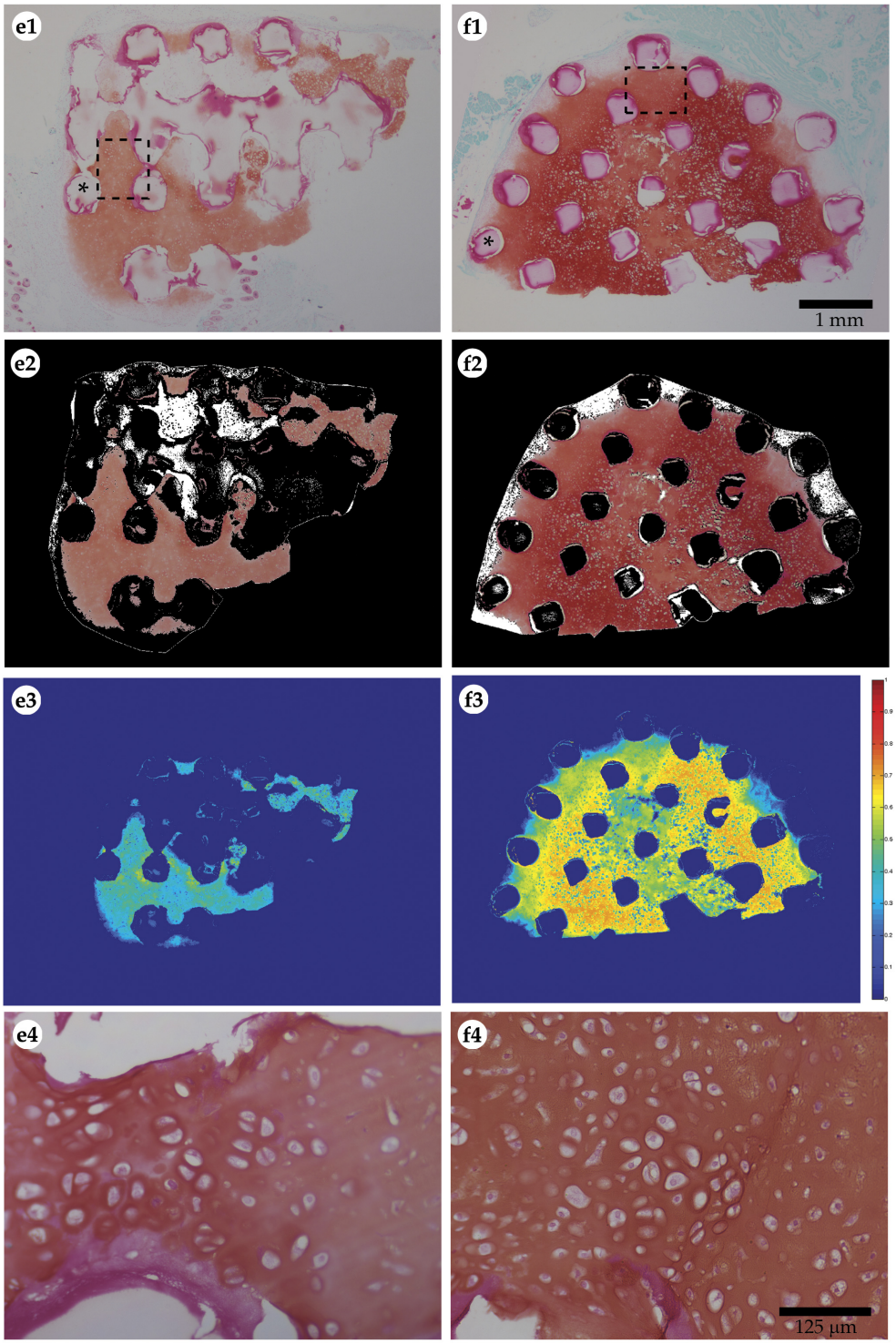

Figure 4.4: (continued) Cartilage formation in PPF scaffolds. Chondrocytes seeded in cubic (e) and ellipsoid (f) scaffolds and cultured in chondrogenic medium. Residual PPF is visible in the sections (labeled with *). e2,f2) The areas of cartilage that stained positive for Safranin O for these representative specimens is 60% for e2 and 84% for f2. e3,f3) The average sGAG concentration calculated for the sections is 7.9 µg/mm^2 for e3 and 10.8 µg/mm^2 for f3. e4,f4) Higher magnifications of the areas within the dashed boxes show round chondrocytic cells surrounded by positively stained extracellular matrix.

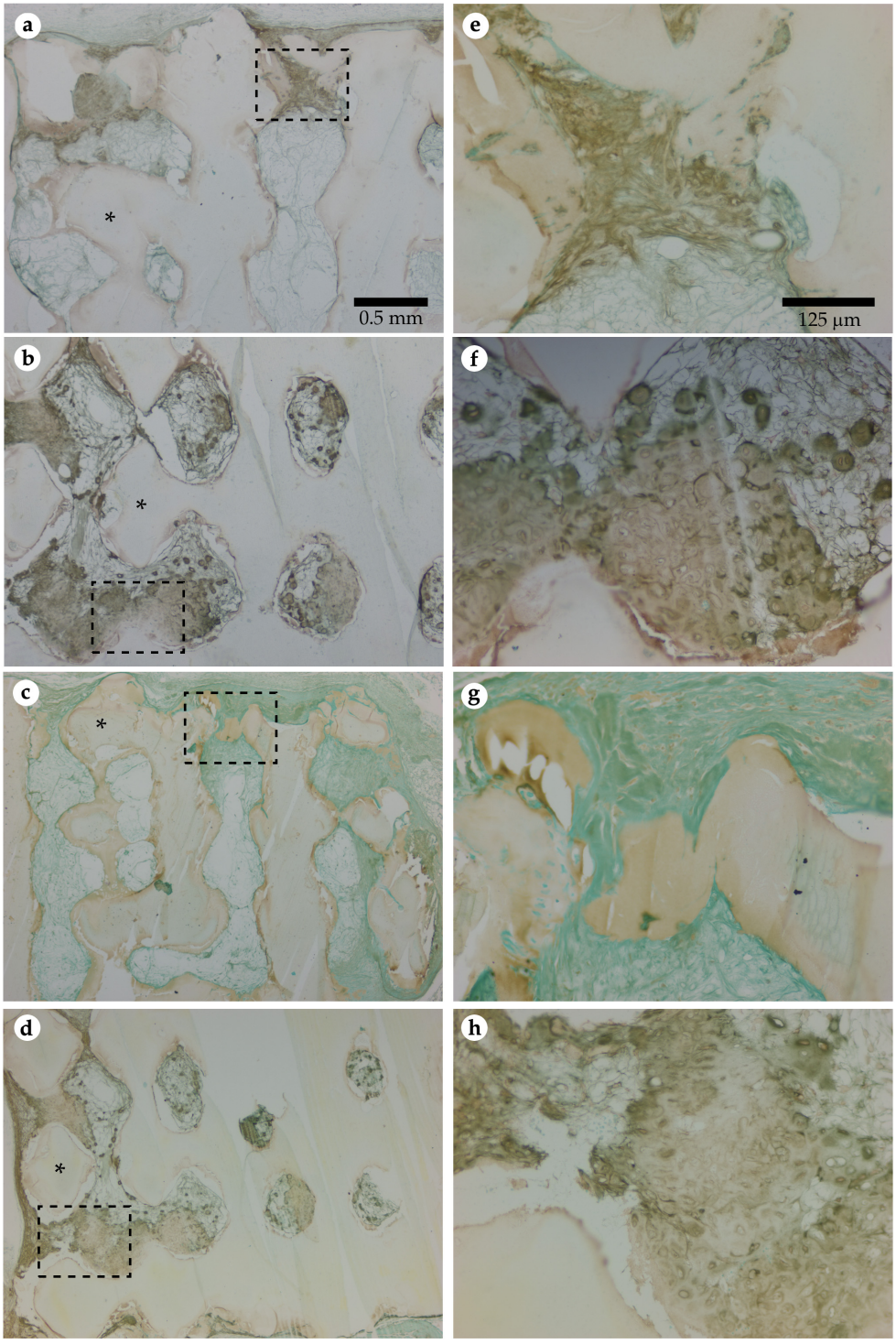

Figure 4.5: Immunohistochemistry detecting collagen II and collagen I in SFF scaffolds seeded with BMSC and cultured in chondrogenic medium. Residual cubic (a,c) and ellipsoid (b,d) PPF scaffolds are indicated with *. Positive staining (brown) for collagen II (a,b) is seen in the extracellular matrix surrounding the differentiated cells. Collagen I (c,d) is localized at the scaffold edges and in some outer pores. Higher magnification of areas in dashed boxes showing chondrocytic cells (e - h).

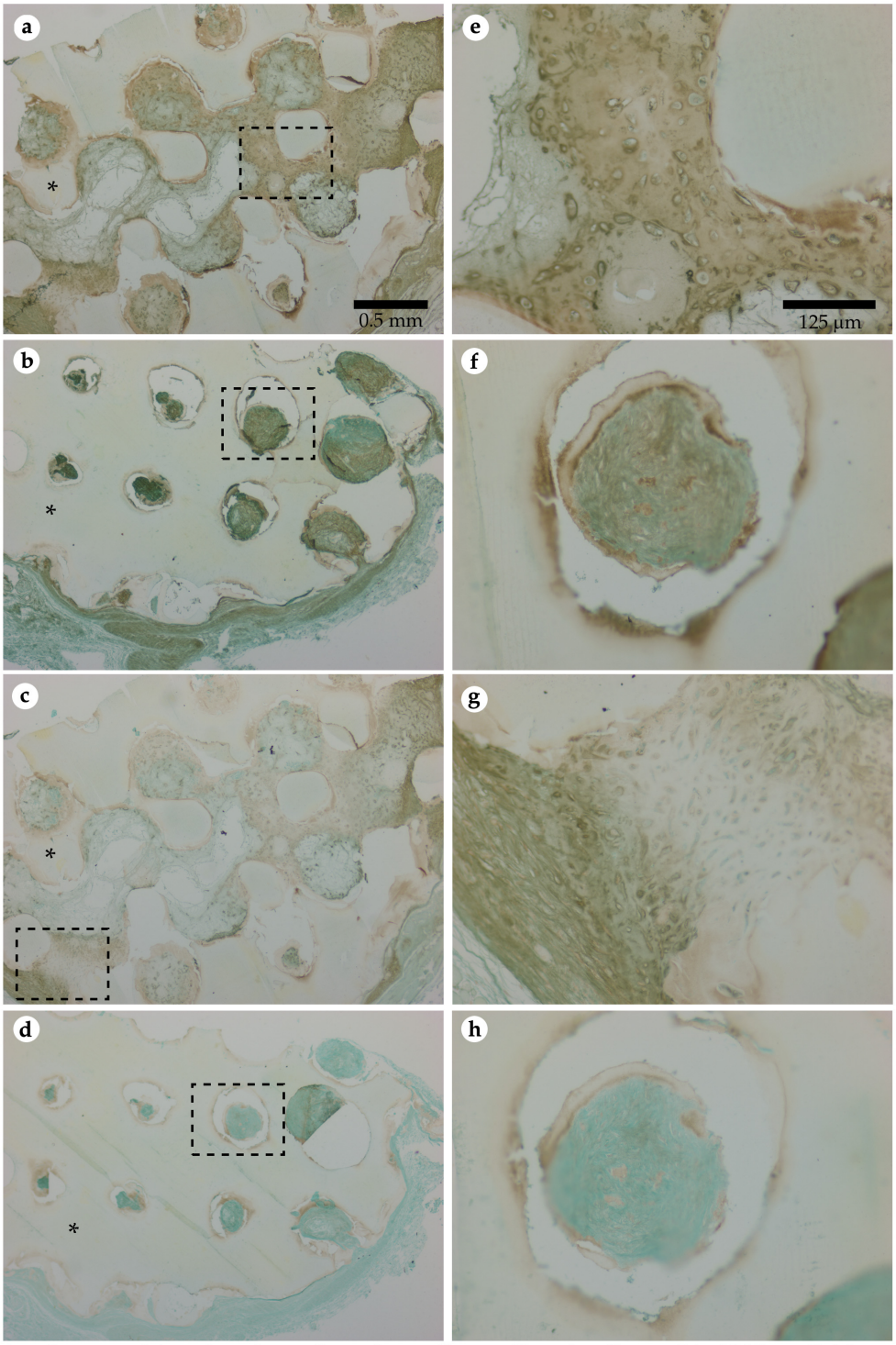

Figure 4.6: Immunohistochemistry detecting collagen II and collagen I in PPF scaffolds seeded with chondrocytes and cultured in basal medium. Residual cubic (a,c) and ellipsoid (b,d) PPF scaffolds are indicated with *. Positive staining (brown) for collagen II (a,b) is seen in the extracellular matrix surrounding the cells. Collagen II is widespread throughout the pores of the scaffolds, whereas collagen I (c,d) is mostly at the scaffold edges. Higher magnification of areas in dashed boxes showing chondrocytic cells (e - h).

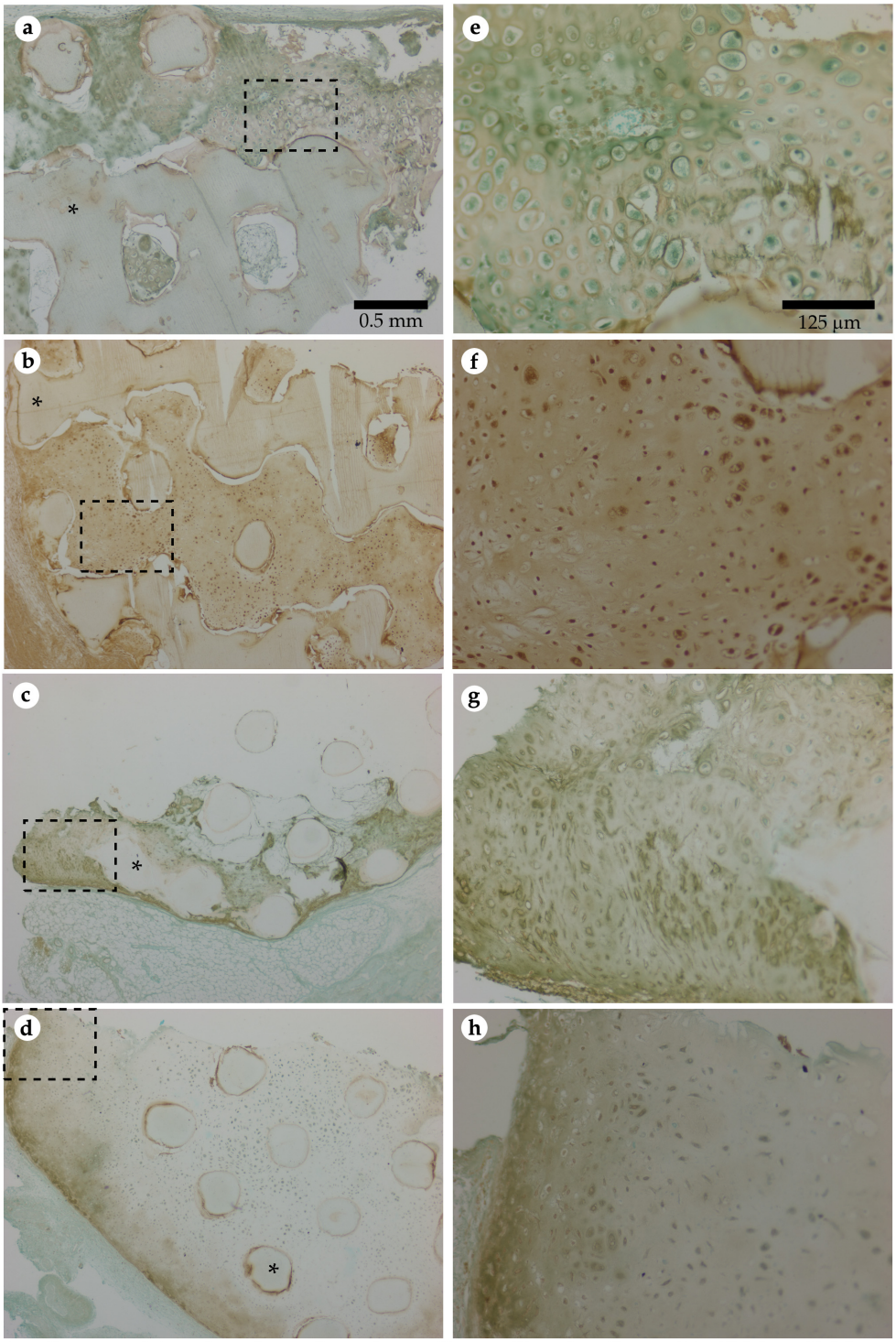

Figure 4.7: Immunohistochemistry detecting collagen II and collagen I in PPF scaffolds seeded with chondrocytes and cultured in chondrogenic medium. Residual cubic (a,c) and ellipsoid (b,d) PPF scaffolds are indicated with *. Positive staining (brown) for collagen II (a,b) is seen in the extracellular matrix surrounding the cells. Collagen II is more widespread throughout the pores of the ellipsoid scaffolds. Collagen I (c,d) was localized to the scaffold edges. Higher magnification of areas in dashed boxes showing cell morphology (e -h).

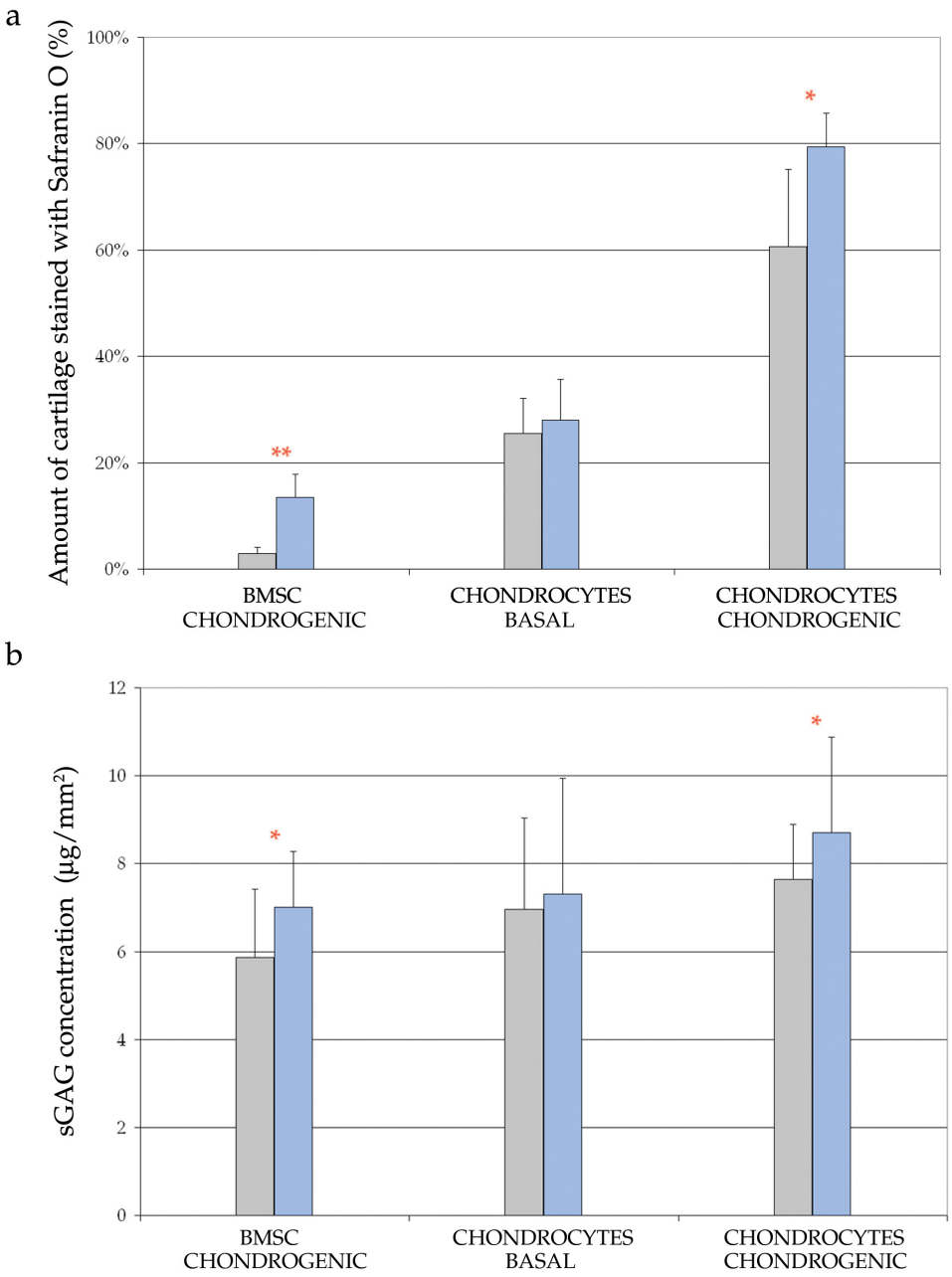

Figure 4.8: Impact of scaffold architecture on chondrocytic activity using histomorphometric data. Analysis of Safranin O stained sections of cubic (gray bars) and ellipsoid (blue bars) pored scaffolds seeded with BMSC or chondrocytes and cultured in basal or chondrogenic medium. a) The area of positive Safranin O is equated to the amount of cartilage matrix present in the sections. The area is normalized by the total pore space available and is expressed as a percentage. Significantly greater areas of cartilage was quantified for BMSC and chondrocytes seeded into scaffolds with ellipsoid pores that were cultured in chondrogenic medium. b) The Safranin O stain intensity is correlated to the GAG concentration in the extracellular matrix. Significant differences between the cubic and ellipsoid scaffolds indicate that the ellipsoid scaffolds have a higher quality matrix that is more robust and cells may be more metabolically active. Overall, comparison between the two scaffold designs suggests that the characteristics of the ellipsoid scaffolds have a greater influence on cartilage tissue formation.[* indicates $p<0.05$ and ** indicates $p<0.001$ for comparisons between cubic and ellipsoid pore geometry]

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CHAPTER 5

COMBINED EFFECTS OF HYALURONIC ACID AND PORE GEOMETRY ON CHONDROGENESIS

5.1 INTRODUCTION

In the previous two chapters, studies were conducted to test the response of BMSC and chondrocytes to hyaluronic acid (HyA) and also to examine the effects of scaffold pore geometry on cellular condensation and cartilage tissue formation. The previous experiments indicated that both of these factors have positive effects on the cellular activity and the tissue regeneration capabilities of the cells. In this chapter, we will examine the effects of combining 5% HyA/collagen hydrogels with designed cubic and ellipsoid pore geometries on cartilage morphogenesis by BMSC and chondrocytes.

Hyaluronic acid is a ubiquitous glycosaminoglycan that is present in the cartilage matrix and has many chondroinductive and chondroprotective properties. Our previous investigations of HyA showed that stimulation of cellular aggregation and GAG production was highest within the composite 5% HyA/collagen I hydrogels, which have a concentration of 0.23 mg/mL HyA. Both *in vitro* and *in vivo* data analysis showed that the hydrogels and scaffolds with 5% HyA hydrogels had greater sGAG concentrations and larger areas of cartilage formation for both BMSC and chondrocytes.

Designed ellipsoid pore geometries were able to stimulate more chondrogenic differentiation than cubic pore geometries. The pore shape helped increase the local cellular density in the ellipsoid pores, which help the cells attain the critical

mass required for chondrogenic differentiation to occur. The low permeability of the ellipsoid scaffolds may have also contributed to the increased differentiation and sGAG concentration.

In this study, the composite 5% HyA/collagen hydrogels were combined with the cubical and ellipsoidal pore architectures to determine their influence on the induction of cellular condensation and differentiation. The same scaffold designs used in Chapter 4 were used to fabricate poly(ε-caprolactone fumarate) (PCLF) scaffolds with cubical and ellipsoidal pore geometries. BMSC and chondrocytes were seeded into PCLF scaffolds using either collagen only or composite 5% HyA/collagen hydrogels. The PCLF constructs were cultured *in vitro* in defined chondrogenic medium for 5 weeks. Both cell types have increased their cartilage tissue forming capabilities in the presence of these two factors separately. The addition of HyA to the hydrogels may help to further increase cellular condensation and differentiation within the ellipsoid pores. The potential benefit of combining the 5% HyA hydrogels increases the probability of regeneration of cartilage tissue within the scaffolds. Statistical analysis between the groups will also reveal if there is synergistic effect between the 5%HyA and the ellipsoid pore geometry.

5.2 MATERIALS AND METHODS

5.2.1 Cell isolation

Primary porcine chondrocytes were isolated from the metacarpophalangeal joints of domestic pigs. Cartilage slices were extracted aseptically within four hours of slaughter. Chondrocytes were isolated from the tissue using a solution of 1 mg/mL collagenase (Sigma, St. Louis, MO) and antibiotics. The isolated

cells were filtered through a nylon mesh and were allowed to adhere for 24 hours. The adherent chondrocyte population was used for experimentation.

Porcine BMSC were isolated from the bone marrow aspirate of adolescent Yucatan minipigs (Seguin Animal Hospital, Seguin, TX). The aspirate was collected in a solution of heparin and antibiotics. The whole bone marrow was cultured for weeks and the entire adherent cell population was used for these studies. The cells were not passaged or passaged once prior to use.

5.2.2 *In vitro* experimentation

Scaffold design and fabrication

Image-based design (IBD) techniques[8] were used to create cylindrical scaffolds with internal pore architectures defined by periodic repetition of a base unit cell. Unit cells for cubic and ellipsoidal pore designs were [0.7, 0.7, 0.7 mm] and [0.7, 0.7, 0.9 mm], respectively (FIGURE 5.1). The external dimensions of all designed scaffolds were 3 mm in height and 5 mm in diameter. Cubic scaffold struts were designed to be 350 μm thick, corresponding to pore space dimensions of 350 μm in length, width and height (TABLE 4.1). The designed porosity of the scaffold was 50 percent. Ellipsoidal scaffolds pore dimensions were 700 μm in diameter and 900 μm in height. The interconnections between pores for the ellipsoidal scaffolds were 260 μm for the top and bottom and 300 μm for the sides of the ellipsoid. The designed porosity for the ellipsoid scaffold was 52 percent. Other calculations of scaffold properties were made using Solidview (Solidconcepts, Valencia, CA).

Wax molds were printed from design files using a three dimensional rapid prototyping system (Patternmaster™, Solidscape, Inc., Merrimack, NH). The wax molds were scaled to 1.5 times greater in all axes to account of polymer

shrinkage after casting. The poly(ε-caprolactone fumarate) scaffolds were created using indirect SFF fabrication techniques described previously (Appendix B).[10] Briefly, 0.3 grams of N-vinyl-pyrrolidone (NVP) was added to 0.65 grams of PCLF. Then 1.5 milliliters of $CHCl_3$ were added to the PCLF/NVP mixture. After homogenization, 0.03 grams of benzoyl peroxide was added to the PCLF/NVP/$CHCl_3$ mixture. Then, four microliters of N, N-dimethyl-*p*-toluidene was added to induce crosslinking immediately before casting.[12] A custom polytetrafluoroethylene (PTFE) mold (diameter = 9 mm) was used to force the polymer solution through the pore spaces of the wax molds. The molds were placed under the hood for two hours to allow the polymer to cure. The wax was then removed with 70% ethanol under gentle agitation. The remaining PCLF scaffolds were rinsed in many changes of 70% ethanol and then air dried. The scaffolds were then rehydrated in distilled water and sterilized in sterile 70% ethanol.

Scaffolds cast in PCLF from the wax molds were scanned in air at a resolution of 16 μm using a micro-computed tomography machine (MS-130, GE Medical Systems, Toronto, Canada) to determine the accuracy of the fabrication process. The measured values of fabricated scaffolds were less than 5 percent of designed pore and strut values.

In vitro pre-culture

In preparation for cell seeding, the PCLF scaffolds were rinsed twice with sterile Hank's Balanced Salt Solution (HBSS) and once with culture medium. The PCLF scaffolds were then placed in a custom made PTFE mold (d = 5.1mm). BMSC and chondrocytes were seeded into the scaffold pores using either a 5% HyA/collagen or a collagen I only hydrogel. For these hydrogels, 50 x 10^6 cells/mL were resuspended in a solution containing either ~3.8 mg/mL collagen I (stock concentration: 4.66 mg/mL; BD Bioscience Discovery Labs, San Jose, CA)

after trypsinization (Appendix D). The pH of the solution was increased by the addition of 20 μL of 0.5 M sodium hydroxide solution. For 5% HyA hydrogels, HyA was added to the cell/collagen suspension prior to the addition of the sodium hydroxide. The cell and hydrogel suspension was pipetted into the scaffold pores (~60 μL) immediately after sodium hydroxide addition. The gels were allowed to solidify within the scaffolds in a humidified chamber at 37°C for 30 minutes. The PCLF scaffolds were then removed from the PTFE mold and placed in a 12-well tissue culture plate. Scaffold constructs seeded with BMSC were cultured in a chondrogenic-defined medium (DMEM, 10% FBS, 1% P/S supplemented with 10 ng/mL TGFβ_1, 0.1 nM dexamethasone, 50 μg/mL 2-phospho-L-ascorbic acid, 0.4 mM proline (Sigma), 5 μg/mL insulin (Gibco) and 0.1 mM non-essential amino acids (Gibco)). Chondrocyte-seeded constructs were cultured in the same chondrogenic medium, but without TGFβ_1 or dexamethasone. The scaffolds were then placed in the incubator and gently agitated on an orbital shaker. The scaffold constructs were cultured *in vitro* for 5 weeks, during which the culture media was changed every other day.

In vitro evaluation

Twenty-four PCLF scaffolds [n=3; 1) cubic or ellipsoid pores were seeded with 2) BMSC or chondrocytes using 3) 5% HyA/collagen or collagen I hydrogel] were used to assess the ability of BMSC and chondrocytes to form new cartilage matrix within the scaffolds with only *in vitro* culture. Cell-seeded constructs were cultured for 5 weeks in defined chondrogenic medium. The culture medium for chondrocyte-seeded scaffolds did not contain TGF-β_1 or dexamethasone.

Pre-chondrogenic condensation evaluation using PNA

Rhodamine-conjugated peanut agglutinin (PNA) (Vector Labs, Burlingame, CA) was used to identify the pre-chondrogenic aggregates at a 2 day timepoint. Two

additional scaffolds from each group [n=2; 1) scaffolds with cubic or ellipsoid pores were seeded with BMSC using 2) 5% HyA/collagen or collagen I hydrogel] were frozen embedded in OCT (Tissue-Tek®, Sakura, Torrence, CA). Serial frozen sections between 7 – 14 μm thick were cut using a cryostat (HM 500 M, Microm GmbH, Walldorf, Germany). Slides were immersed in distilled water, incubated for 30 minutes in 100 ng/mL PNA solution, rinsed with water and then DAPI was used to stain the cellular nuclei on the same sections. The sections were washed and then mounted using ProLong Gold (Molecular Probes, Carlsbad, CA). Pictures were taken using an Olympus confocal microscope (FV-500, Olympus America, Inc., Center Valley, PA) using the FluoView image acquisition program (Olympus). Concurrent PNA and DAPI images were taken using an Argon (488 nm) and Green Helium Neon (543 nm) lasers. Pictures from at least five different areas were taken from each slide and merged together using FluoView.

Evaluation of constructs

The scaffolds were fixed in zinc-buffered formalin (Z-FIX, Anatech, Battle Creek, MI) overnight. The scaffolds were rinsed and prepared for snap freezing. The specimens were bisected longitudinally and embedded so that sagittal and transverse sections were cut at a thickness of 7 μm. The constructs were frozen embedded in OCT (Tissue-Tek®, Sakura, Torrence, CA). Serial frozen sections between 7 – 14 μm thick were cut using a cryostat (HM 500 M, Microm GmbH, Walldorf, Germany). Slides were stained with hematoxylin and eosin (H&E) or Safranin O counterstained with Fast Green FCF. Briefly, hydrated slides were immersed for distilled water for 30 seconds, followed by 3 minutes in 0.02% Fast Green FCF, 10 seconds in 1% acetic acid, and 6 minutes in 0.1% Safranin O. The slides were then dehydrated and mounted with permount (Sigma).

Histomorphometric image analysis

Serial cryosections were cut through the entire scaffolds. At least four planes or more (100 - 500 μm difference) of each scaffold were used for analyses. Low magnification (2X objective) images of whole cross sections of tissue samples were acquired using a CCD camera (RT Color 2.2.1, Diagnostic Instruments, Sterling Heights, MI) mounted on a Nikon Eclipse E600 microscope (Nikon America, Melville, NY). RGB images were acquired through Spot, an image acquisition program (Diagnostic Instruments, Sterling Heights, MI). The illumination of each picture was controlled so that the exposure for each image was the same; thus the brightness, contrast and saturation of the images was captured using the same parameters.

Image processing was performed on the RGB images using Matlab (The Mathworks, Natick, MA) to determine: 1) the ratio of positive Safranin O staining to the available pore space and 2) the average sGAG concentration of the section (correlated using the stain intensity). The first calculation is correlated to the total area of cartilage-like matrix present in the slide and also approximates the number of chondrocytic cells that have not dedifferentiated. The second value is correlated to the amount of sGAG present in the extracellular matrix.[1,2] Small adjustments were made to the previous histomorphometric methods due to the formation of the layer of hyaline cartilage that surrounded the scaffolds. These layers were included in the area calculations, but were excluded for determination of the sGAG concentration so that the true concentration within the scaffold pores was determined and not skewed by the sGAG rich cartilage surrounding the scaffolds. The histomorphometric data was statistically analyzed using ANOVA to determine which combination of pore geometry (cubic or ellipsoid) and hydrogel type (5% HyA or collagen) promoted more cartilage formation and sGAG deposition by BMSC and chondrocytes. All statistical tests were performed using SPSS.

5.3 RESULTS

BMSC and primary chondrocytes were seeded into cubic or ellipsoid pored PCLF scaffolds with 5% HyA/collagen or collagen only hydrogels. After 5 weeks of *in vitro* culture in chondrogenic medium, all cells produced a cartilage-like matrix. Large areas of hyaline-like cartilage were found within the scaffolds pores and a dense extracellular matrix rich with sGAG also enveloped the constructs.

The formation of cellular aggregates during *in vitro* culture was confirmed by peanut agglutinin (PNA) staining. PNA binds to galactosyl (β-1,3) N-acetylgalactosamine, which is expressed in pre-chondrogenic aggregates. BMSC were seeded into PCLF scaffolds with cubic and ellipsoid architecture with either 5% HyA/collagen or collagen only hydrogels. After 2 days of culture in chondrogenic medium, cellular condensations were seen within all scaffolds groups analyzed. Confocal images show positive areas of red staining in Figure 5.2, which identify the pre-chondrogenic aggregates. Strong staining was seen throughout the cubic and ellipsoid pores of scaffolds seeded using 5% HyA hydrogels. Fewer and more isolated areas of PNA were seen in the scaffolds with collagen hydrogels. Of all the groups, the ellipsoid scaffolds with 5% HyA had the greatest areas of PNA staining. The blue DAPI (4',6-diamidino-2-phenylindole) stain further shows that the cellular nuclei are at highest density in these areas of positive PNA. The cellular densities in both cubic and ellipsoid scaffolds also seemed greater in 5% HyA hydrogel group.

Chondrocytic cells proliferated and a cartilage-like extracellular matrix developed within the scaffolds during the *in vitro* culture period. The BMSC seeded scaffolds exhibited pockets of chondrogenic differentiation (FIGURE 5.3 a,b,c,d), while chondrocytes were widespread throughout the scaffold pores and

surrounded by a dense extracellular matrix (FIGURE 5.4 a,b,c,d). The cells in these cartilage-like areas were round and chondrocytic in appearance and were surrounded by well-established lacunae and pericellular matrices. The extracellular matrix in these areas stained positive with Safranin O, indicating the presence of sGAG and other cartilage proteins. Within some of these sections, non-cartilaginous tissue (fibrous tissue) was identified with Fast Green staining. Larger areas of Fast Green were found in the BMSC seeded scaffolds compared to the chondrocytes, where the areas of Fast Green were limited to the scaffold edges.

Histomorphometric image analysis was performed on tissue sections stained with Safranin O and Fast Green to quantify the amount of cartilage and sulfated glycosaminoglycan (sGAG) concentration present in each section. The areas of positive Safranin O staining were determined and normalized by the total available pore space. This percentage correlates to an approximate number of viable chondrocytes for the section that are capable of producing sGAG, identifying the BMSC that have differentiated into chondrocytes or the primary chondrocytes that have maintained a chondrocytic phenotype.

BMSC showed extensive chondrogenic differentiation whether cultured in cubic or ellipsoid pore geometry and greater differences in cartilage formation were seen between the collagen and 5% HyA groups. The histomorphometric analysis showed an increasing trend between the experimental groups, from lowest to highest amount of cartilage formed (FIGURE 5.5): 1) cubic pore with collagen hydrogel (51±12%), 2) ellipsoid pore with collagen hydrogel (67±10%), 3) cubic pore with 5% HyA hydrogel (77±10%), and 4) ellipsoid pore with 5% HyA hydrogel (92±9%). Significant differences were found in comparisons between hydrogel types (collagen and 5% HyA, $p<0.02$) and between pore geometry (cubic and ellipsoid) with the collagen hydrogel ($p<0.03$) and with the 5% HyA

hydrogel ($p<0.01$). The sGAG concentration did not show a similar trend. The average sGAG concentrations of the scaffolds with collagen hydrogels were comparable, at 6.9±0.7 and 7.1±1.5 μg/mm^2 for cubic and ellipsoid pored scaffolds, respectively. The average sGAG concentrations of scaffolds with 5% HyA hydrogels were 8.5±0.8 μg/mm^2 for cubic scaffolds and 10±1.1 μg/mm^2 for ellipsoid scaffolds. A significant difference was found between the pore geometries of scaffolds with 5% HyA hydrogels ($p<0.001$). ANOVA analysis showed that there is not interaction between the pore geometry and hydrogel type for either the cartilage area or the sGAG concentration.

Chondrocytes filled the scaffold pores with hyaline-like cartilage after 5 weeks of *in vitro* culture, regardless of hydrogel type or pore geometry. A sGAG rich extracellular matrix filled the entire scaffold with very little areas of Fast Green. The pore geometry did not have an effect of the synthesis of new cartilage, but differences between the collagen and 5% HyA hydrogels were significant (FIGURE 5.6). The average areas of cartilage formation for all scaffold groups were similar: 1) cubic pore scaffold with collagen hydrogel (95±2%), 2) ellipsoid pore scaffold with collagen hydrogel (97±2%), 3) cubic pore scaffold with 5% HyA hydrogel (98±2%), and 4) ellipsoid pore scaffold with 5% HyA hydrogel (99±2%). Statistical analyses revealed no significant differences between any of these groups. However, differences in the quality of the tissue, as shown by the sGAG concentration, were different between the hydrogel types. For scaffolds with collagen hydrogels, the average sGAG concentrations were 10±0.7 μg/mm^2 for cubic scaffolds and 10±0.8 μg/mm^2 for ellipsoid scaffolds. The 5% HyA scaffolds had significantly greater sGAG concentrations at 13±0.8 μg/mm^2 for both cubic and ellipsoid geometries. ANOVA analysis showed no significant interactions between the hydrogel and pore geometries.

5.4 DISCUSSION

Experiments conducted in the previous chapters have shown that the addition of hyaluronic acid to a collagen hydrogel increased chondrogenic differentiation of BMSC and promoted the development of a hyaline-like extracellular matrix by chondrocytes. Our studies of scaffold pore geometry also showed that an ellipsoid pore geometry was able to increase chondrogenic differentiation of BMSC and increase sGAG concentration and cartilage formation by chondrocytes. The previous histomorphometric analyses of BMSC differentiation promoted by these two factors separately showed that the amount of cartilage formed by the cells filled at most 13% of the available pore space. Therefore, it is believed that a combination of the 5% HyA hydrogel with the ellipsoid pore geometry may help stimulate more extensive cartilage formation throughout the scaffold pores.

The amount of cellular differentiation is determined early on during chondrogenesis. The pre-chondrogenic condensations shown by the PNA staining indicate the areas where BMSC have committed to chondrogenic differentiation. After 2 days of culture in chondrogenic medium, the BMSC seeded into the PCLF scaffolds were expressing galactosyl (β-1,3) N-acetylgalactosamine and were able to bind PNA. In general, scaffolds with collagen I hydrogels showed smaller and more isolated areas of staining compared to the scaffolds with 5% HyA hydrogels, which had more overt and widespread areas of PNA indicating greater areas of condensation. The addition of the HyA to the hydrogels seem to stimulate not only aggregation, but cellular proliferation as well.[3,4] The increased cellular density within the scaffolds with HyA hydrogels can be seen with the DAPI nuclear stain (FIGURE 5.2). The cell nuclei are more numerous in the pores of the HyA scaffolds with either pore geometry compared to the scaffolds with collagen only hydrogels. The areas

have stained positive for PNA also have numerous cells surrounding the location. The 5% HyA also stimulated greater areas of aggregation, in some cases the entire pore space stained red, especially in the ellipsoid scaffold with HyA (FIGURE 5.2d).

The data show that the 5% HyA hydrogels have a much larger effect on the formation of cartilage than the scaffold pore geometry for both cell types. The BMSC cultured in 5% HyA had significantly greater areas of cartilage than the scaffolds with collagen alone. The Safranin O staining agrees with the results shown by the PNA staining, where larger areas of cellular condensation went on to form greater areas of cartilage in the 5% HyA hydrogels. The chondrocytes cultured in 5% HyA hydrogels also had significantly greater concentrations of sGAG and slightly greater areas of cartilage, though this difference was not significant.

The long term *in vitro* culture period further elucidated the similarity between the pore geometries. Although the areas of cartilage formation were significantly different for the two pore designs for the BMSC, we believe that longer culture periods beyond 5 weeks may reduce this significance. With the constant presence of TGF-β in the culture medium, new areas of cellular condensation will continue to form over time. The TGF-β increases cellular proliferation and aids in aggregation, so that the areas that did not have the critical mass required for differentiation will continue to grow and eventually differentiate as well. Continued examination of PNA staining at longer timepoints will further support this hypothesis. However, the significant result of this experiment shows that after 5 weeks, the amount and quality of cartilage within the ellipsoid scaffolds is greater than the cubic. Due to increases in cellular density and lower permeability, the ellipsoid pore shape may be better at initiating cartilage formation with shorter culture periods. This is a compelling discovery because

clinically, shorter culture periods and faster turnaround time between implant preparation and implantation into patient are preferred.

The *in vitro* only culture promoted mature hyaline cartilage matrix to develop within the scaffold pores. This tissue was more robust and larger in quantity than the tissue that was in scaffolds that were subjected to the combination of *in vitro* pre-culture and *in vivo* implantation (results of Chapters 3 and 4). The absence of vasculature, fibroblast invasion, and inflammatory cells that the tissue constructs are subjected to *in vivo* helped increase the development of a robust tissue *in vitro*. The continued presence of TGF-β and other proteins that sustain extracellular matrix production also helped the development of hyaline tissue. The formation of the thick hyaline cartilage that enveloped the scaffolds may be attributed to the TGF-β and insulin in the culture medium. The continued presence of these factors stimulated cellular proliferation beyond the confines of the scaffold pores.[5,6] This matrix that developed is higher rich in sGAG because it is surrounded by a nutrient-rich medium. This layer of cartilage may have simulated *in vivo* conditions were nutrients diffuse through the ECM in order to get to the deepest layers of cartilage. The diffusion of nutrients through this dense matrix may have been lower, but the tissue within the pores of scaffolds still showed healthy chondrocytic cells with high GAG concentrations in the extracellular matrix for both BMSC and chondrocytes. Furthermore, due to the formation of this thick cartilage layer, comparisons between the scaffold permeabilities and amount of cartilage formation can not be made.

The tissue formation within the SFF scaffolds during the *in vitro* only culture period was comparable between the BMSC and chondrocytes. Hyaline-like matrix filled the scaffold pores, especially in the HyA groups. The BMSC cultured in 5% HyA and ellipsoid pores had sGAG concentrations that were similar to the chondrocytes cultured in collagen only hydrogels in either scaffold

design. The chondrocytes cultured in 5% HyA had sGAG concentrations that were close to the middle/deep zone of native cartilage, which has a sGAG concentration between 13 -14 μg/mm^2. Although the sGAG concentrations are approaching hyaline-like quantities, the structural organization of the extracellular matrix is still missing within these scaffolds for all groups. The tissue within the scaffolds is still a random mixture of cells surrounded by sGAG rich extracellular matrix without the zonal arrangement that is seen in articular cartilage. The addition of a mechanical stimulation during the culture period[7,8,9] or designing SFF scaffolds with zonal architecture[10] have been shown to help to achieve more structural organization within the extracellular matrix.

5.5 CONCLUSION

In conclusion, the 5% HyA hydrogels have a greater overall effect on cartilage formation than the scaffold pore geometry. For BMSC, the combination of 5% HyA and ellipsoid pore geometry did significantly increase the areas of cartilage formation and sGAG concentration over all other groups. The primary effector is 5% HyA and the pore geometry is a weaker secondary effect. We believe that over time in the presence of TGF-β, the BMSC tissue formation in the two pore designs would become equal. Longer term tissue formation would be similar to the chondrocyte-seeded scaffolds, where significant differences were not found between the amount of cartilage that formed or the sGAG concentration.

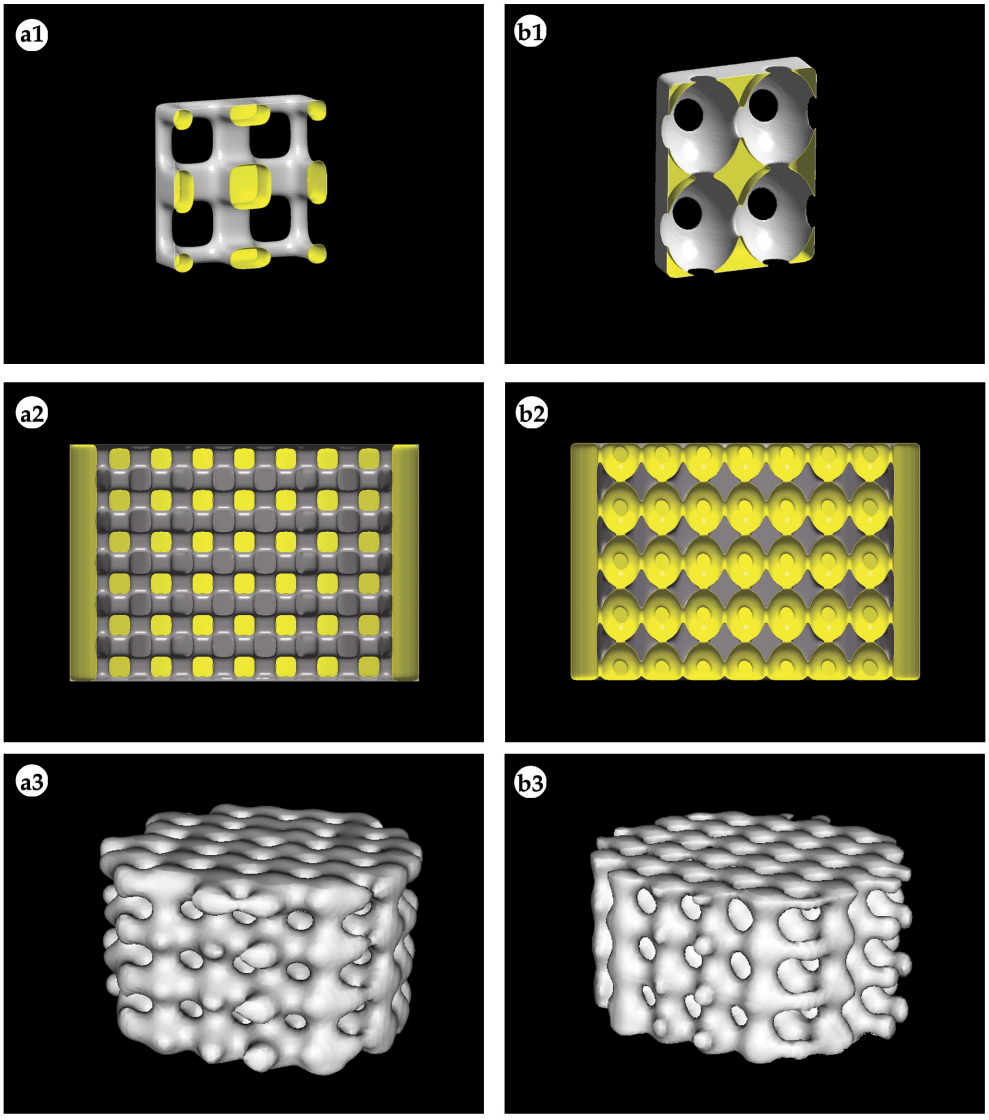

Figure 5.1: Designed scaffold architectures and fabricated PCLF scaffolds. Cross-sections of four unitcell aggregates depicting the architecture and pore interconnections of cubic (a1) and ellipsoid (b1) scaffolds. Wax molds with cubical (a2) and ellipsoidal (a3) architecture created using a three dimensional rapid prototyping system, the xz plane is shown and the areas shown in yellow indicate the pore space of the scaffolds. Micro-CT images of fabricated cubic (a3) and ellipsoid (b3) pored PCLF scaffolds created by indirect SFF. See Table 4.1 for the parameters used for scaffold design and other calculated scaffold properties, such as surface area and scaffold diffusivity.

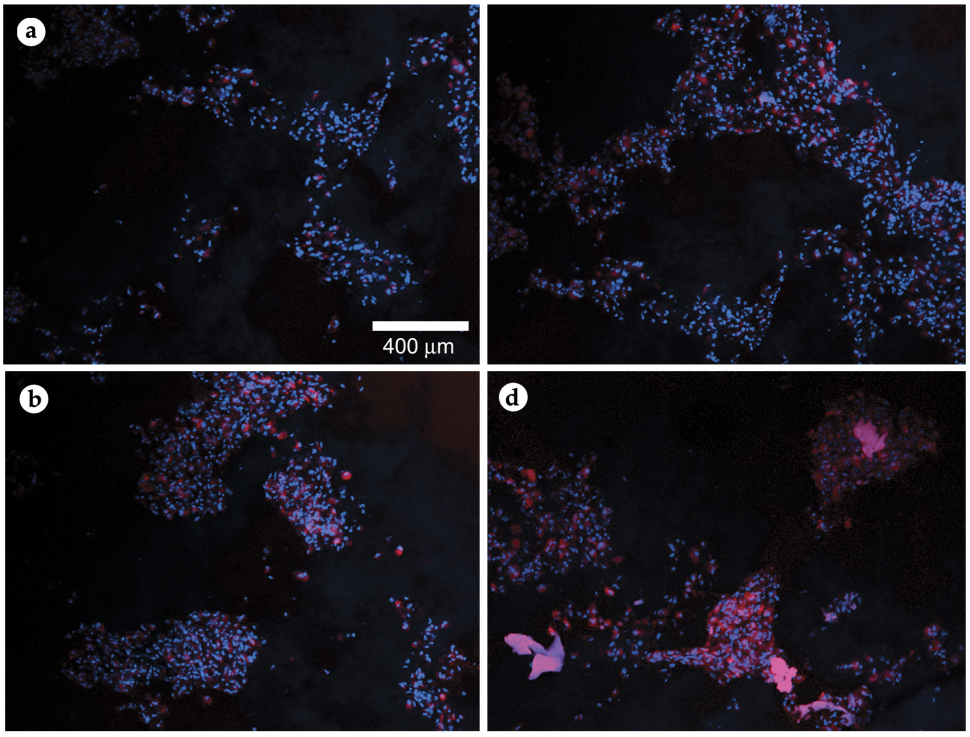

Figure 5.2: Visualization of pre-chondrogenic cellular aggregates using rhodamine-conjugated peanut agglutinin (PNA). PCLF scaffolds with cubical (a,b) or ellipsoidal (c,d) architecture were seeded wtth BMSC using collagen I (a,c) or 5% HyA/collagen (b,d) hydrogels. After two days of *in vitro* culture in chondrogenic medium, positive PNA staining was found in all scaffolds. More extensive staining was seen in the scaffolds with HyA and the most widespread staining was observed in the ellipsoid pored PCLF scaffolds with 5% HyA/collagen.

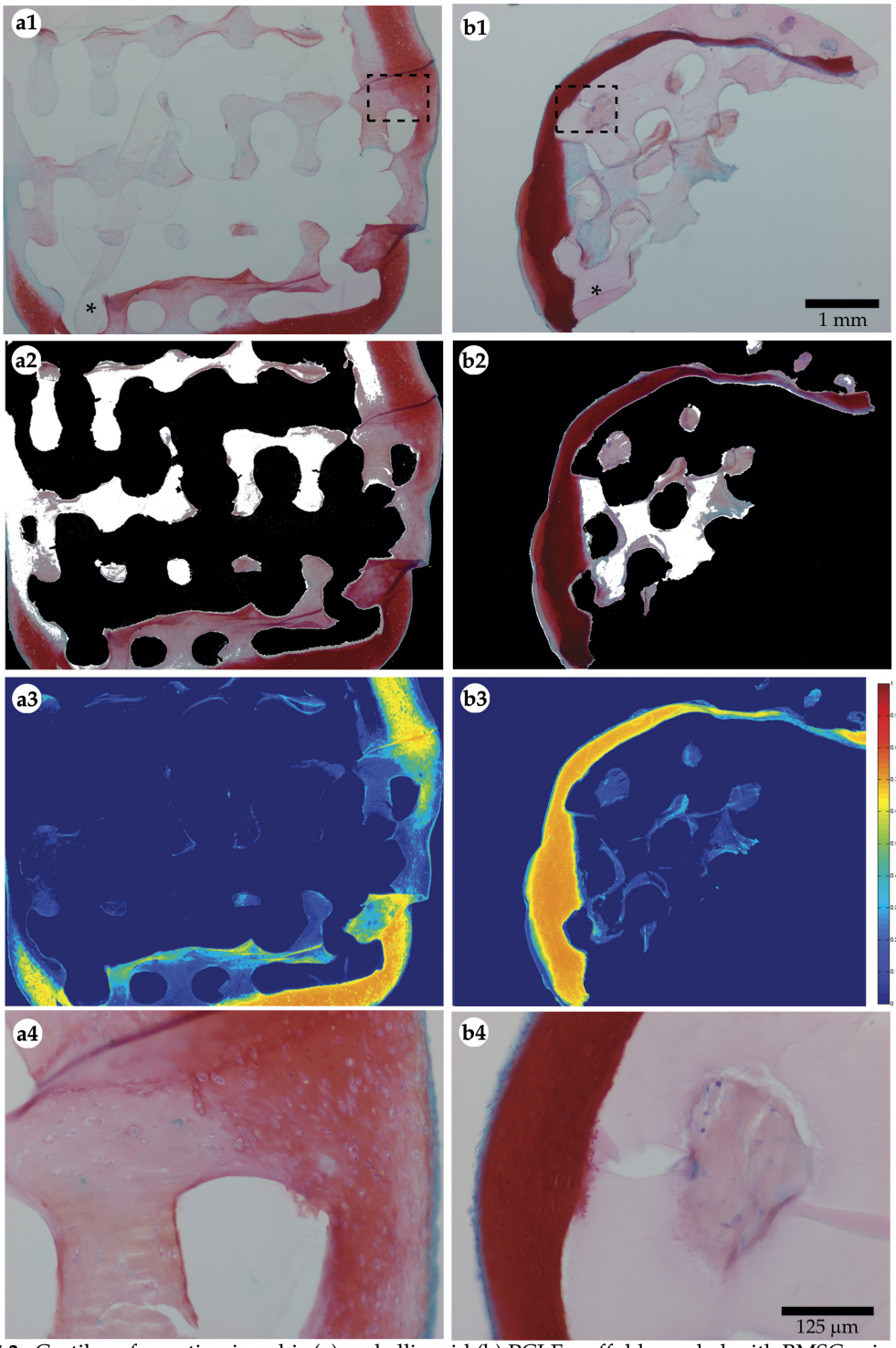

Figure 5.3: Cartilage formation in cubic (a) and ellipsoid (b) PCLF scaffolds seeded with BMSC using collagen hydrogels. Sections are stained with Safranin O/Fast Green FCF. Residual PCLF is visible in the sections (labeled with *). a2,b2) The positive areas of Safranin O shown were extracted using image processing. These areas were quantified to determine the amount of cartilage matrix formation and the stain intensity was correlated to sGAG concentration. The calculated percentages of positive Safranin O staining for these representative specimens is 57% for a2, 72% for b2. a3,b3) The average sGAG concentration in the scaffold pores calculated for the sections are 6.7 µg/mm^2 for a2, 7.4 µg/mm^2 for b2. a4,b4) Higher magnifications of the areas within the dashed boxes show round chondrocytic cells surrounded by positively stained extracellular matrix.

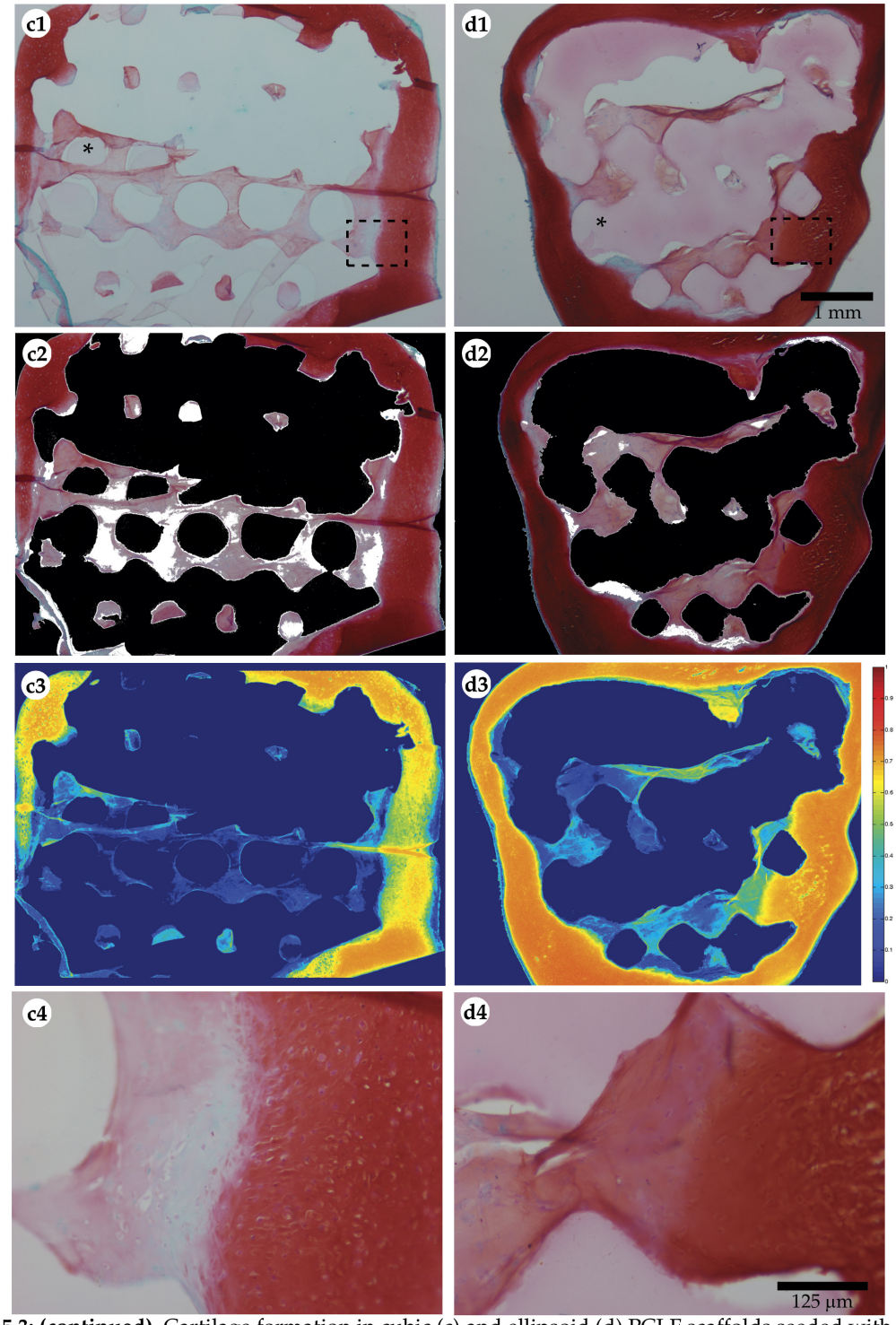

Figure 5.3: (continued) Cartilage formation in cubic (c) and ellipsoid (d) PCLF scaffolds seeded with BMSC using 5% HyA/collagen hydrogels. Sections are stained with Safranin O/Fast Green FCF. Residual PCLF is visible in the sections (labeled with *). The amount of cartilage calculated for these representative specimens is 82% for c2 and 90% for d2. The average sGAG concentration in the scaffold pores calculated for these sections are 7.2 µg/mm^2 for c2 and 9.4 µg/mm^2 for d2. c4,d4) Higher magnifications of the areas within the dashed boxes show round chondrocytic cells surrounded by positively stained extracellular matrix.

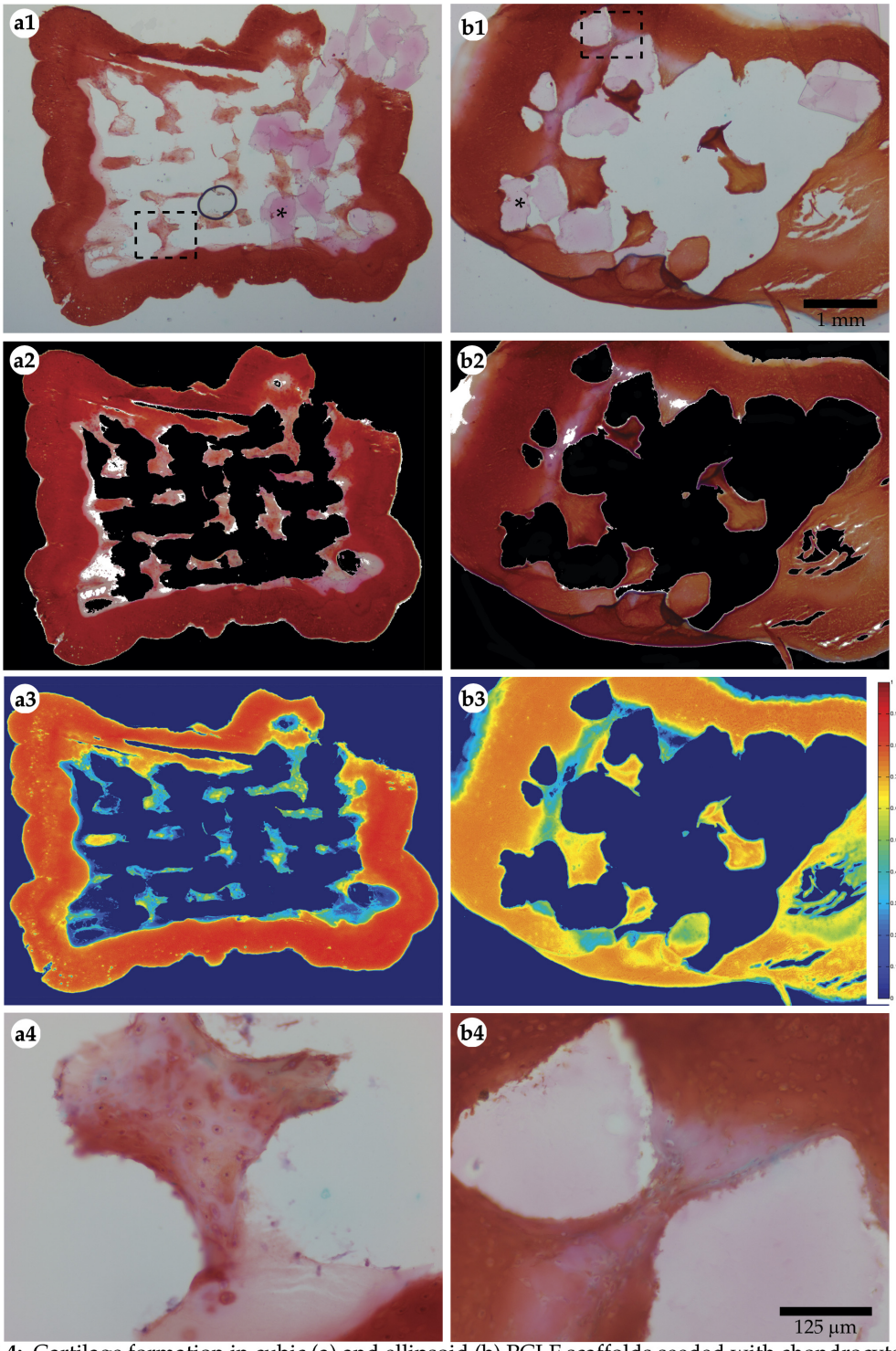

Figure 5.4: Cartilage formation in cubic (a) and ellipsoid (b) PCLF scaffolds seeded with chondrocytes using collagen hydrogels. Sections are stained with Safranin O/Fast Green. Residual PCLF is visible in the sections (labeled with *). a2,b2) The positive areas of Safranin O shown were extracted using image processing. These areas were quantified to determine the amount of cartilage matrix formation and the stain intensity was correlated to sGAG concentration. The calculated percentages of positive Safranin O staining for these representative specimens is 96% for a2 and 97% for b2. The average sGAG concentration within the scaffold pores calculated for these sections are 9.6 µg/mm^2 for a3, 10.3 µg/mm^2 for b3. a4,b4) Higher magnifications of the areas within the dashed boxes show round chondrocytic cells surrounded by positively stained extracellular matrix.

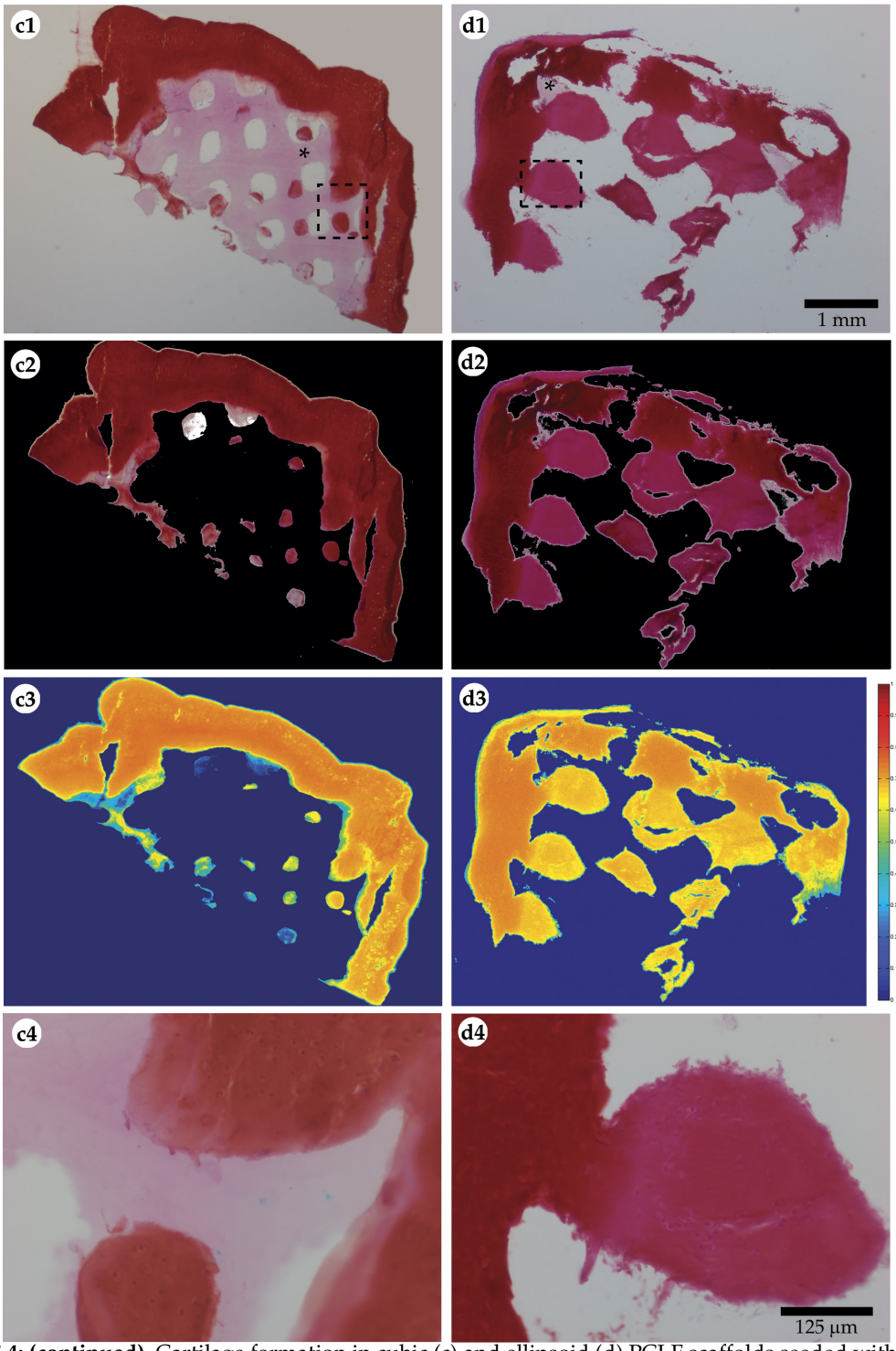

Figure 5.4: (continued) Cartilage formation in cubic (c) and ellipsoid (d) PCLF scaffolds seeded with chondrocytes using 5% HyA/collagen hydrogels. Sections are stained with Safranin O/Fast Green. Residual PCLF is visible in the sections (labeled with *). c2,d2) The calculated areas positive Safranin O staining for these representative specimens is 98% for c2 and 99% for d2. The average sGAG concentration within the scaffold pores calculated for these sections are 12.1 µg/mm^2 for c3 and 12.9 µg/mm^2 for d3. c4,d4) Higher magnifications of the areas within the dashed boxes show round chondrocytic cells surrounded by positively stained extracellular matrix.

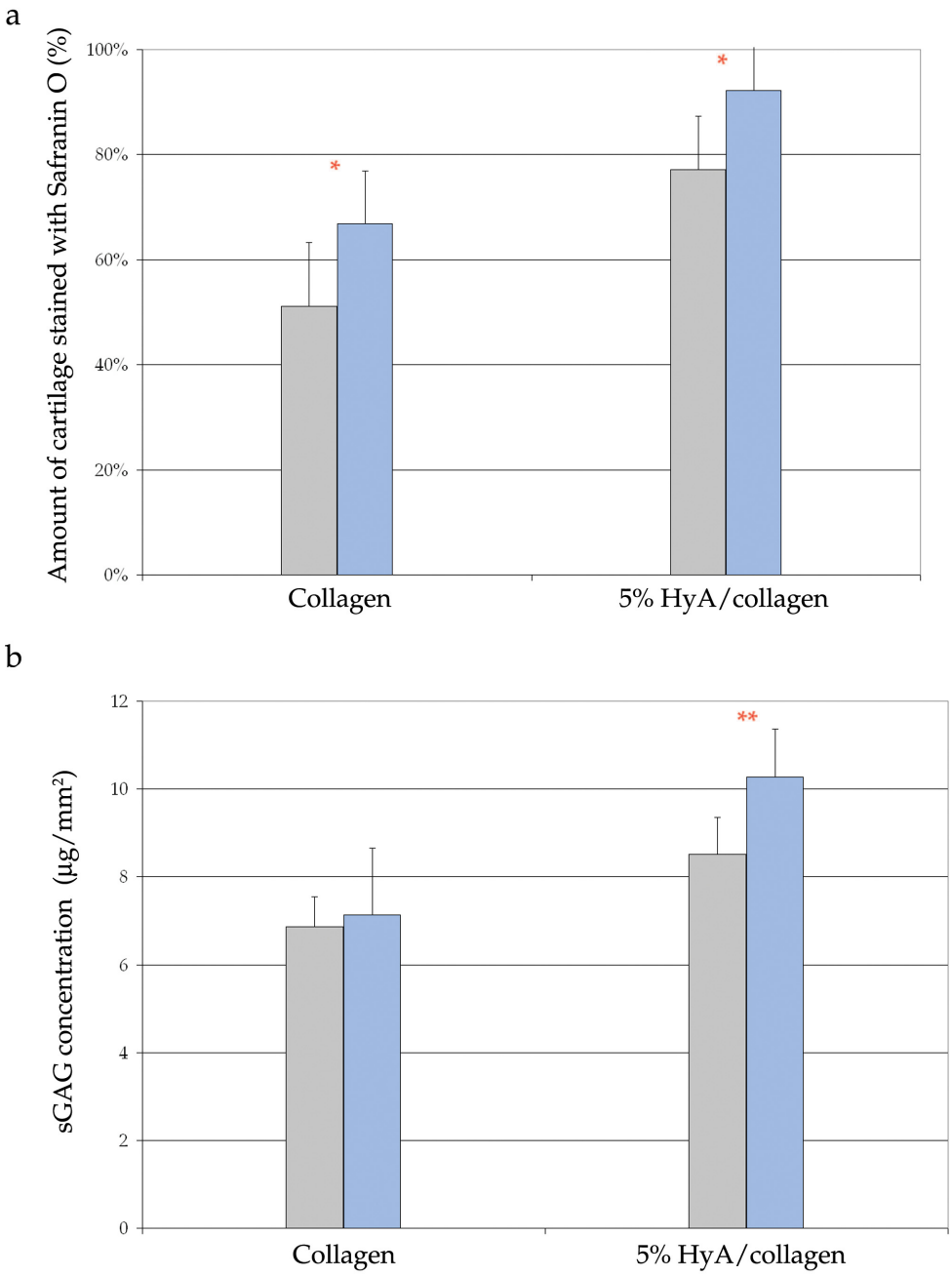

Figure 5.5: Evaluation of the impact of hydrogel type and pore geometry on BMSC activity using histomorphometric data. Analysis of Safranin O stained sections of cubic (gray bars) and ellipsoid (blue bars) pored scaffolds seeded with BMSC using collagen or 5% HyA/collagen hydrogels. a) The area of positive Safranin O is equated to the amount of cartilage matrix present in the sections.The area is normalized by the total pore space available and is expressed as a percentage. The largest areas of cartilage were in the ellipsoid scaffolds with 5% HyA hydrogels. b) The Safranin O stain intensity is correlated to the GAG concentration in the extracellular matrix. Significant differences in GAG concentration indicate the presence of a higher quality matrix that is more robust with increased celllular activity. [* indicates $p<0.03$ and ** indicates $p<0.001$ for the comparisons indicated]

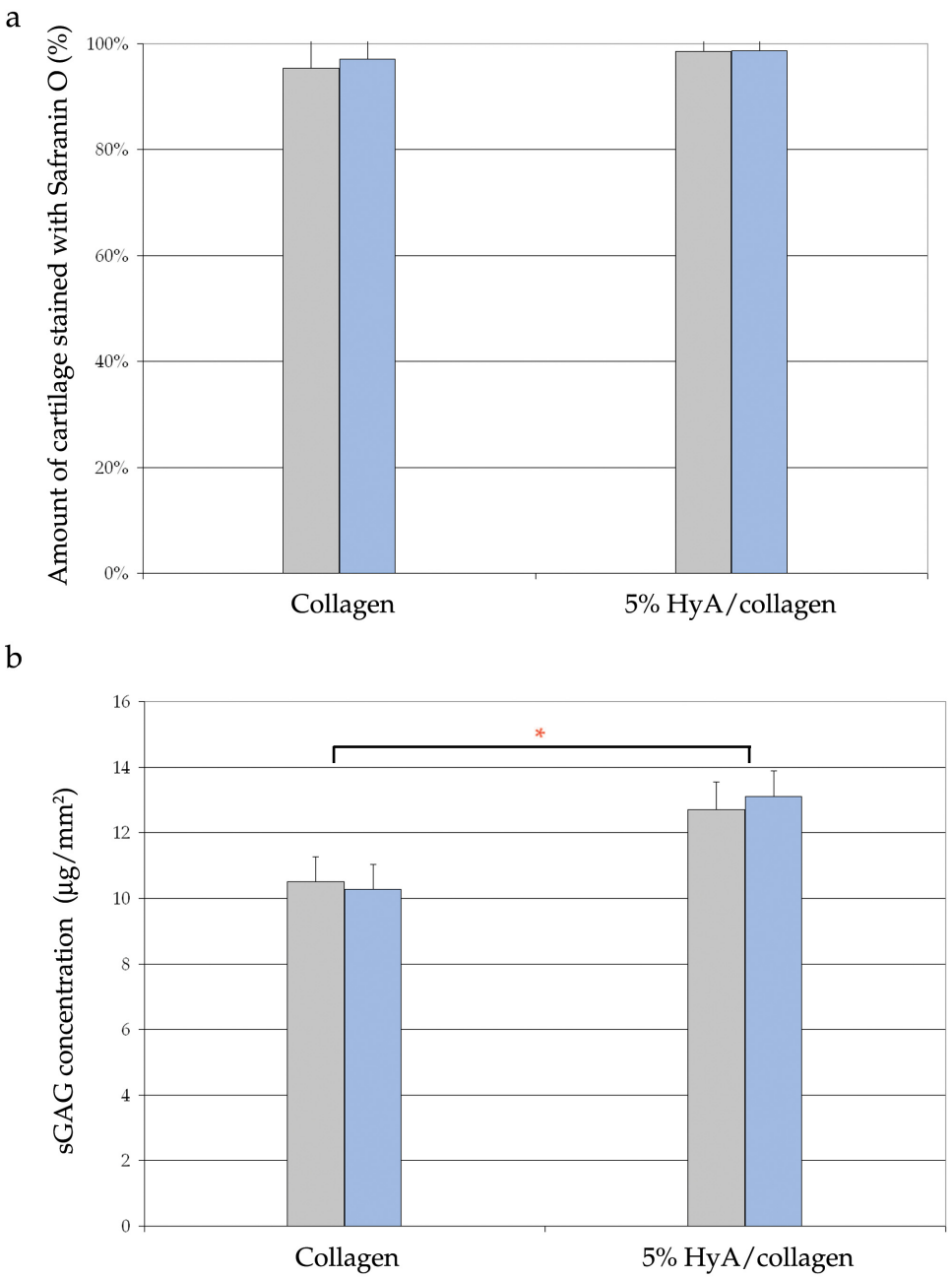

Figure 5.6: Evaluation of the impact of hydrogel type and pore geometry on chondrocytic activity using histomorphometric data. Analysis of Safranin O stained sections of cubic (gray bars) and ellipsoid (blue bars) pored scaffolds seeded with chondrocytes using collagen or 5% HyA hydrogels. a) The area of positive Safranin O is equated to the amount of cartilage matrix present in the sections. The area is normalized by the total pore space available and is expressed as a percentage. The amount of cartilage were not significantly different between the groups. b) The Safranin O stain intensity is correlated to the GAG concentration in the extracellular matrix. Significant differences between the GAG concentration of collagen and 5% HyA hydrogels indicates the presence of a more hyaline-like extracellular matrix and more metabolically active cells. [* indicates $p<0.01$ for comparisons between hydrogel types]

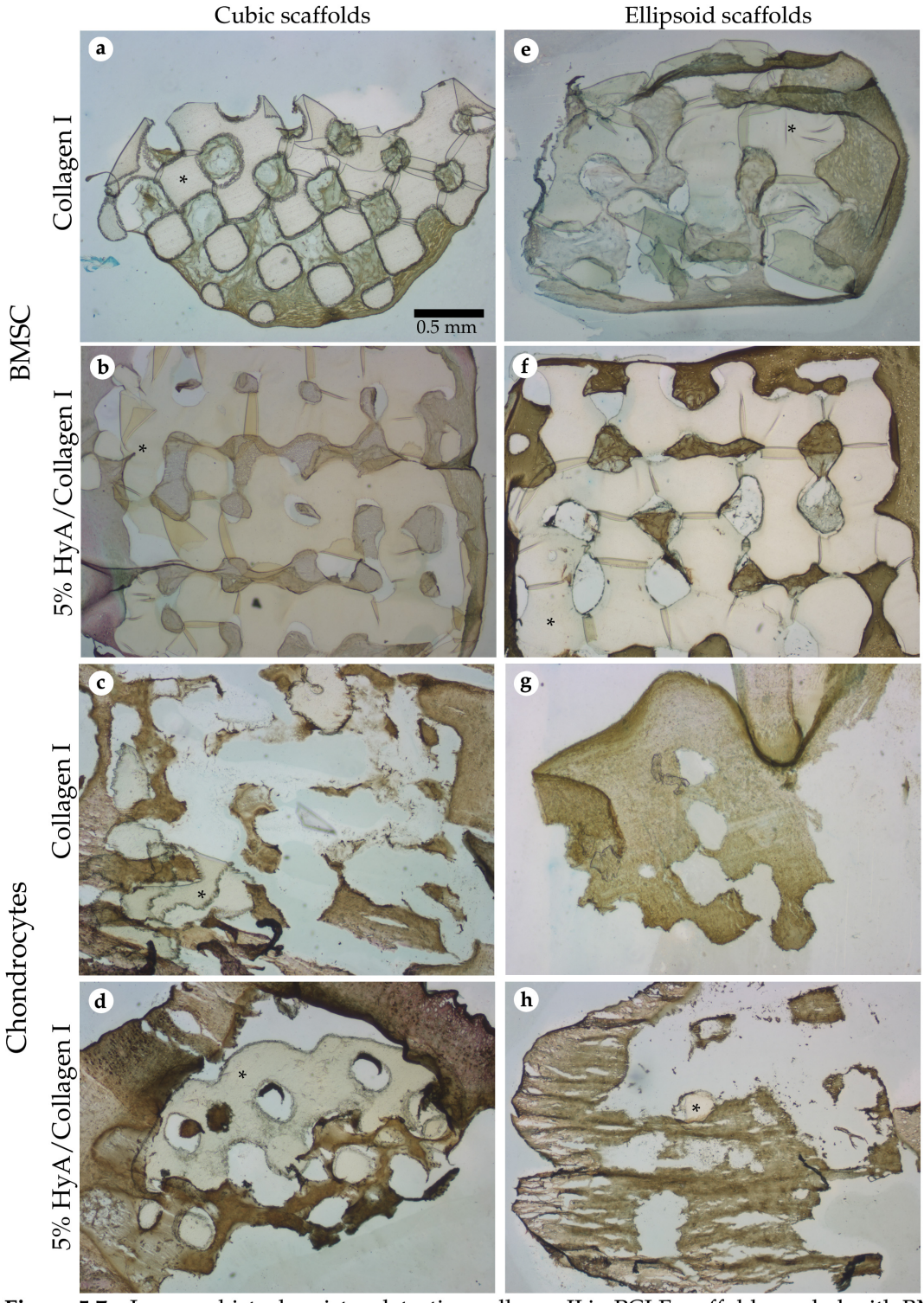

Figure 5.7: Immunohistochemistry detecting collagen II in PCLF scaffolds seeded with BMSC and chondrocytes. Residual cubic (a - d) and ellipsoid (e - h) scaffolds are indicated with *. Positive staining (brown) is seen in the extracellular matrix surrounding the cells. Collagen II is widespread throughout the pores of the scaffold with chondrocytes and in more isolated areas for the scaffolds with BMSC. More areas of positive staining are seen with the scaffolds seeded using 5% HyA/collagen hydrogels compared with scaffolds with collagen I only.

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CHAPTER 6
CONCLUSIONS AND FUTURE WORK

6.1 INTRODUCTION

Articular cartilage is a complex organ with poor regenerative capacity. With the prevalence of osteoarthritis and rheumatoid arthritis in a growing population of elderly patients, it will be important to find better methods that enhance chondrogenesis in cartilage defects and improve the quality of repair tissue.

The use of solid freeform (SFF) scaffolds not only allows the design of specific scaffold properties for evaluation, but also provides mechanical stability to the developing tissue, which is lacking in many conventional scaffolds being researched for cartilage repair. We use cellular condensation as a model, not as a recapitulation step, but as a starting point to examine how to combine cells, growth factors and organizational matrices to increase chondrogenesis within SFF scaffolds. Another advantage is that the SFF scaffolds can be made from a wide range of biomaterials to meet desired mechanical properties and degradation profiles. Furthermore, the design of these scaffolds will advance the understanding of how physical scaffold properties can influence cartilage tissue formation.

6.2 EXPERIMENTAL CONCLUSIONS

6.2.1 Hyaluronic Acid Stimulated Chondrogenesis

Hyaluronic acid (HyA) is an essential glycosaminoglycan that serves a large role in cellular condensation and in the maintenance of the extracellular matrix in cartilage. It also has many beneficial chondroinductive properties which stimulate cellular proliferation, migration and differentiation.[1] We found that certain concentrations of HyA between 0.05 and 0.23 mg/mL were able to stimulate cellular condensation and initiate chondrogenesis in BMSC. A 5% HyA/collagen hydrogel, 0.23 mg/mL HyA concentration, stimulated the most sGAG produced by BMSC and chondrocytes. Above the 5% HyA concentration, steric inhibition, HyA chain entanglement, and saturation of HyA receptors limit cellular metabolism and aggregation. The 5% HyA hydrogels were used to seed BMSC and chondrocytes into SFF scaffolds. After a combination of 2 week *in vitro* pre-culture and 4 week *in vivo* implantation, increased amounts of cartilage tissue formed within scaffold pores seeded using the 5% HyA hydrogels. The addition of HyA to collagen hydrogels increased the amount of cartilage formation from 3% to 10% for BMSC and from 29% to 63% for chondrocytes. The tissue that formed within the SFF scaffolds with chondrocytes in 5% HyA were not only larger, but more hyaline-like with greater concentrations of sGAG. The chondroinductive properties of HyA make it a good substrate for use as a cell carrier for SFF scaffolds, that also increases chondrogenesis by BMSC and chondrocytes.

6.2.2 Ellipsoid Pore Geometry Induced Chondrogenesis

SFF scaffolds with chondroconductive pores were evaluated for their ability to promote chondrogenesis in BMSC and chondrocytes. Two pore geometries were

used to assess cartilage tissue formation of BMSC and chondrocytes: 1) cubic pore architecture with square channels and 2) ellipsoid pore architecture with lower permeabilities. The ellipsoid pore geometry imitates the size, volume, and shape of micromass cultures, which induce chondrogenic differentiation in the presence of chondroinductive growth factors.[2] The ellipsoid geometry combines high cellular density, low permeability, and spheroid shape to induce cellular condensation within SFF scaffold pores. The cellular densities within the two pore designs were comparable to each other and to the initial cellular densities within micromass cultures. It is believed that the lower permeability and the ellipsoid shape increased cellular packing density within the pores to promote cellular condensation. Peanut agglutinin staining for pre-cartilage condensations showed more areas of staining and stronger stain intensities in the ellipsoid pores, particularly at the pore interconnections. The chondroconductive ellipsoid pore design was able to stimulate greater areas of pre-cartilage condensations, which differentiated into larger areas of cartilage. BMSC seeded into ellipsoid pores produced greater areas of cartilage at 13% than BMSC in cubic pores, which was around 3%. Chondrocytes seeded in ellipsoid scaffolds seeded also had increased the amounts of cartilage (79%) above the amounts of cartilage that formed in cubic scaffolds (61%). Chondrocytes cultured in the ellipsoid scaffolds also exhibited greater phenotype stability and enhanced matrix production compared to chondrocytes in cubic scaffolds.

6.2.3 Combined Effects of Hyaluronic Acid and Pore Geometry

The results from the previous studies showed that a 5% HyA hydrogel and ellipsoid pore geometry were able to enhance chondrogenesis of BMSC and chondrocytes, separately. A combination of these two factors together may further increase the amount of cartilage formation and increase the quality of repair tissue that develops within SFF scaffolds. Permutations of hydrogel type,

collagen I or 5% HyA/collagen, and pore geometries, cubic or ellipsoid, were tested for their effects on the cartilage tissue forming capabilities of BMSC and chondrocytes during 5 weeks of *in vitro* culture. Hyaline-like extracellular matrix was found in all scaffolds, regardless of cell type, hydrogel type, or scaffold pore design. A trend was observed in the amount of cartilage formed by BMSC between the groups: with the lowest amounts of cartilage in the cubic pored scaffold with collagen hydrogel, then the ellipsoid pored scaffold with collagen, the cubic scaffold with 5% HyA, and with the highest amount of cartilage in the ellipsoid scaffold with 5% HyA. The combined effect of the chondroinductive HyA and the chondroconductive ellipsoid pore geometry was able to stimulate greater areas of cartilage formation by BMSC and greater sGAG concentrations than all other groups. The tissue within the ellipsoid scaffolds with 5% HyA was comparable to the chondrocytes seeded in collagen hydrogels, similar in area of cartilage formed, cell morphology, and sGAG concentrations. Significant differences between the chondrocyte-seeded constructs were only found in comparisons of sGAG concentrations between the hydrogel types, collagen and 5% HyA.

6.2.4 Comprehensive Summary

A summary of all the experiments conducted within this thesis is presented in Table 6.1. It is organized by the scaffold biomaterial, pore architecture, hydrogel type, culture medium, and culture conditions. The main conclusions of this thesis are that 5% hydrogels and ellipsoid pore geometry are able to enhance chondrogenesis in SFF scaffolds by increasing cellular condensation. The HyA has a greater and wider range of effects over both BMSC and chondrocytes than the pore geometry. Although a combination of the chondroinductive HyA and chondroconductive ellipsoid pore was able to increase differentiation and cartilage formation with shorter culture periods.

6.3 SIGNIFICANCE AND IMPLICATIONS

The experiments conducted in this thesis are an initial step in understanding how scaffold design can influence cartilage tissue generation. The ability to design scaffolds with chondroconductive properties that help stimulate chondrogenesis will be an advancement in the field of cartilage tissue engineering. Although, the effect of scaffold pore geometry was a secondary effector, other scaffold properties such as porosity or pore size may be more influential. The advantage with SFF over conventional matrices is that these scaffold properties can be studied easily, due to the design capabilities and also due to the ease of scaffold fabrication, which has a high degree of accuracy and reproducibility. Conducting further studies on how scaffold design can enhance the cartilage formation within SFF scaffolds may ultimately lead to the development of a tissue construct that can bear mechanical load, while encouraging the formation of hyaline-like cartilage.

The chondroinductive properties of HyA have a greater effect on cartilage formation than the scaffold pore geometry. The effect of the chondroconductive scaffolds is more effective during the initial pre-cartilage stages, while the effects of HyA are much longer lasting. The effects of the HyA stimulation can be seen well after the HyA has degraded. After long term culture, 5 weeks of *in vitro* culture or the combination of 2 week *in vitro* pre-culture and 4 week *in vivo* implantation, differences in the amount and biochemical composition of tissue where HyA was present are still observed. Significantly greater areas of cartilage were found for all groups tested over time and repair tissue that was more hyaline-like with higher sGAG concentrations developed within most groups tested. The addition of HyA helps both chondrocytes and BMSC to become better established within the SFF scaffolds. After the trauma of cell isolation from their native niches, the addition of HyA helps the chondrocytes rebuild pericellular

matrices [3] and may help shield them from binding to collagen I, which destabilizes their chondrocytic phenotype. [4] Chondrocytes that had intact chondrons and pericellular matrices synthesize more proteoglycans and collagen II than chondrocytes with digested chondrons. [5] Although the scaffold pore geometry has a weaker secondary effect compared to the HyA, we still found greater areas of cartilage formation within the ellipsoid pores. Compared with cubic pores, the ellipsoid pore shape may decrease the culture time required for the formation of healthy repair tissue within SFF scaffolds.

The culture methods used to evaluate the tissue formation had large effects on the amount of cartilage that was present within the scaffolds. A cartilage-like matrix filled the pores of the scaffolds that were cultured *in vitro* only, while the constructs that were also subjected to *in vivo* implantation showed significantly less areas of cartilage. The presence of the chondrogenic biofactors during the continuation of the *in vitro* culture period helped stimulate increased extracellular matrix production. The scaffolds were implanted at an ectopic location and do not have a nutrient-rich environment stimulating further extracellular matrix production. In fact, the ectopic implant location exposes the scaffold construct to a variety of agents that decrease the amount of cartilage the formed over time. Factors such as fibroblast and vascular invasion and inflammatory cells contribute to the lower areas of cartilage present within the implanted scaffolds. Two elements that helped to reduce the 'dedifferentiation' of the cartilage tissue within the scaffolds were evaluated. The addition of HyA to the collagen hydrogel helped to improve the amount and quality of matrix that is maintained *in vivo*. HyA has many chondroprotective properties, especially in the formation of the pericellular matrix. A well-established chondrocytes will produce chondromodulin-I and secrete other factors that limit or decrease the invasion of non-cartilaginous cells.[6] The second factor that may have decreased the amount of dedifferentiation was the pore geometry.

Differences were found between the pore geometries for both the BMSC and chondrocytes that were cultured *in vitro* for 2 weeks and then implanted *in vivo* for 4 weeks. We believe that the permeability of scaffold and ellipsoid pore shape may have decreased the areas where non-cartilaginous cells can invade.

A comparison between the cartilage tissue formation by BMSC and chondrocytes reveal differences in both the areas and quality of extracellular matrix. The BMSC have to progress through all the steps of cartilage morphogenesis from condensation to differentiation, so comparatively they require longer time periods to develop a mature cartilage matrix. Our results show that with some additional supplements, such as HyA and ellipsoid pore, the tissue generated by BMSC can be almost analogous to the tissue formed by chondrocytes in collagen hydrogels. The BMSC filled 92% of the scaffold pore space, while the chondrocytes in collagen filled an average of 96% of the pore space. The BMSC cultured in 5% HyA and ellipsoid pores also had sGAG concentrations that were equivalent to the chondrocytes cultured in collagen only hydrogels, both at 10 $\mu g/mm^2$. Thus, as assessed by areas and the biochemical composition, the BMSC in ellipsoid scaffolds and 5% HyA hydrogels are similar. Further tests to assess the mechanical properties of the tissue within these constructs will reveal any differences.

The ability of hyaluronic acid to protect damaged tissue is being studied both clinically and scientifically. The chondroprotective glycosaminoglycan has been shown to help facilitate repair after an injury and also increase collagen II and aggrecan expression in chondrocytes. Williams *et al*. found that after the administration of a fibronectin fragment mediated injury or chymopapain mediated injury, the pre-administration of HyA helped decrease the amount of damage incurred by the fibronectin fragments and chymopapain digestion. The

more significant finding was that weekly administration of HyA post-injury substantially restored proteoglycan content for both injury types.[7,8]

Intra-articular injections of HyA are widely used, but the lifetime of the injected HyA *in vivo* is limited to a couple days.[9] Thus, the incorporation of the HyA into a hydrogel will help increase the residence time and extend its chondroprotective properties *in vivo*. Cross-linking and modification of the HyA molecule have also been conducted to increase its resistance against the degradative mechanisms of the body and to facilitate the formation of more durable matrices and hydrogels.[10,11] The effect of a 50% cross-linked HyA hydrogel was studied in a chondral defect in rabbits. The cross-linked hydrogel increased chondrocyte density at the defect and was longer lasting than the linear non-crosslinked HyA hydrogel.[12] Thus, if we wanted to further increase the timeframe of the chondroprotective effects of HyA and improve the *in vivo* residence time, we could cross-link the composite HyA/collagen hydrogels with a variety of agents, such as carbodiimide[13], photocross-linker, disulfide cross-linking[14], metal cation-mediated cross-linking.[15]

Non-woven matrices made from modified HyA have also been created for use in cartilage tissue engineering applications. Hyaff is a non-woven mesh that is fabricated through the esterfication of hyaluronan derived from rooster combs. Hyaff-7, modified HyA ethyl ester, and Hyaff-11, modified HyA benzylic ester, were evaluated for their ability to sustain avian chondrocytes during 21 days of *in vitro* culture. Comparisons between the two modified HyA derivatives revealed that avian chondrocytes prefer the HyA benzylic ester. About three hours after seeding cells within these scaffolds, the formation of chondrocyte aggregates were seen between the fibers of the HyA-derived scaffolds. These aggregates were similar to the aggregates that were observed within the chondrocyte-seeded HyA hydrogels. The chondrocytes within these aggregates

stained metachromatically with Toluidine blue, indicating the presence of a high concentration of sGAG.[16]

The implantation of the Hyaff-11 meshes at clinical defect sites has also been documented. The procedure is very similar to a matrix-associated autologous chondrocyte implantation, where the donor chondrocytes are expanded in monolayer for 2 weeks and then seeded into the Hyaff matrix and cultured for an additional 2 weeks prior to implantation. The Hyaff construct is then fixed to the defect site with fibrin glue. Significant improvements after 1 year and continued improvement was found at the 2 and 3 year follow-up evaluations. The data indicate that the use of the Hyaff matrix is comparable to autologous chondrocyte implantation and is particularly favorable for younger patients with single defects.[17] Although, the implantation of these HyA derived matrices seems favorable, the mechanical properties of the repair tissue were not evaluated and the use of the Hyaff for multiple defects did not significantly improve the patient scores. From this data, we can see that the use of HyA matrices is favorable to cartilage formation, but perhaps if they are used in conjunction with a load-bearing scaffold, such as a designed SFF scaffold, the outcomes maybe even more favorable for cartilage tissue formation. Using the same study design, a comparison between a Hyaff matrix and the SFF designed scaffold with HyA hydrogel would further elucidate the requirements for enhancing tissue repair within chondral defects and potential benefits of using load-bearing scaffolds.

Culturing BMSC and chondrocytes in a three-dimensional environment seems to stimulate differentiation or redifferentiation of the cells into a chondrocytic phenotype. Three common three-dimensional systems used to stimulate differentiation have been established: 1) high-density cell pellets[2,18], 2) micromass cultures[19], 3) alginate beads[20,21,22]. More recently, hollow spherical alginate

beads were used to stimulate BMSC condensation.[23] In the study, the hollow alginate beads were used to assess cellular condensation and expression of cartilage specific markers, collagen II, Sox-9 and Sox-5, were analyzed. The gene expression showed that the cells were differentiating chondrogenically, but did not determine if the hollow beads were better for inducing cellular condensation.

A few other studies have created sponges and other non-woven matrices that have spherical macropores. The spherical macropore geometry is created by using spherical porogens, such as rounded salt crystals[24], paraffin spheres[25], or spherical PEMA beads[26]. In comparison with the SFF scaffolds tested in this thesis, the greatest distinction is in the biomaterial and fabrication methods used to produce these matrices, which still result in a scaffold with weaker mechanical properties. For example, in one study, nanofibrous poly(lactic acid) scaffolds with spherical macropores were created using a spherical paraffin network.[14] The compressive moduli of these scaffolds ranged from 125 – 200 kPa, which is still weaker than that of articular cartilage. Due to the fabrication methods, the interconnections between the pores can not be controlled and therefore the interconnected pore network is still very tortuous and unpredictable compared with designed SFF pores. Furthermore, the size of the macropores within these scaffolds were not uniform and fall within the range of 150 – 500 μm. These studies with spherical macroporous scaffolds only present the ability to make spherical interconnected pore architectures, and do not evaluate cellular viability or other biological assays to determine how the pores may influence cell metabolism or behavior.

6.4 FUTURE DIRECTIONS

6.4.1 Evaluation of constructs at an orthotopic site

To further evaluate the cartilage-like tissue development within SFF scaffolds with designed pore architectures and the HyA composite hydrogels used to seeded cells within the scaffolds, we will implant these pre-cultured constructs at an orthotopic site. The study should be conducted within a partial thickness defect, in rabbits or another large animal model. Since the constructs will be cultured *in vitro* prior to implantation, we will inject a 5% HyA/collagen hydrogel with cells into the defect to increase integration between the repair construct and the native tissue and to also fill in the defect void completely. The microenvironment that is present in the articulating joint will decrease the fibroblastic and vascular invasion that decreases the amount of cartilage tissue formed after *in vivo* implantation at an ectopic site. A benefit of using SFF is that we can adjust the scaffold design parameters to easily customize the scaffolds to fit the defect for new animal model. The assessment at the orthotopic site will also allow us to gain a better understanding of repair tissue formation within the SFF scaffolds when external loads are applied and this may allow us to distinguish between the three biomaterials that were used in these studies: PCL, PCLF, and PPF.

6.4.2 Effects of pore size and cellular density

The cellular density used in these studies ($3x10^6$ cells/scaffold) is within range of the densities used to stimulate chondrogenesis for cartilage tissue engineering.[27,28,29] Some studies have used higher cellular densities as high as $10x10^6$ cells/scaffold[30,31,32] which would equate to a pore cellular density of 170,000 cells/mm^3 for both cubic and ellipsoid designs. Seeding the scaffolds at

higher cellular densities will increase the cell packing densities within the pores and will lead to an increased amount of condensation within the scaffolds. There is of course a ceiling limiting the number of cells that are able to survive within the confines of the scaffold pores due to limitations of available nutrients and metabolites. Varying the cellular densities within the cubic and ellipsoid pore geometries would further elucidate the differences between the pore designs.

An alternative method to increasing the cellular density is to increase the pore sizes of the scaffolds. A larger pore will have greater volume, and therefore higher cellular densities. The same cubic and ellipsoid geometries were designed with larger sizes while the remaining scaffold properties, porosity, permeability, and diffusivities are kept constant between the small and large pore designs (Table 6.2). Preliminary studies of pore size effect on BMSC chondrogenesis were evaluated using these scaffold designs in PCLF scaffolds (cultured *in vitro* in chondrogenic medium for 4 weeks). Chondrogenesis was seen within the all scaffolds cultured, but stained very weakly for Safranin O. An example of chondrogenesis within cubic and ellipsoid large pore designs is shown in Figure 6.1. Since the culture conditions were the same as the previous PCLF scaffolds cultured for 5 weeks *in vitro*, we believe that the results are due to the quality of the BMSC used and not an effect of the scaffolds or culture conditions, so more experiments should be conducted to examine the real effect of the larger pores.

6.4.3 Non-invasive Methods of Cartilage Evaluation

The ability to quantitatively measure the collagen content in cartilage tissues using non-invasive methods of detection will be extremely beneficial for clinical applications. We describe an optical spectroscopic approach to detect the natural fluorophores that are present in articular cartilage, particularly the pyridinoline cross-links between collagen fibers.[33] The reflectance and lifetime of NADH in

chondrocytes and the pyridinoline cross-links in collagen was collected in samples of articular cartilage slivers. By generating a correlation between the fluorescence of the tissues and quantifying the collagen content with biochemical assays, we can begin to generate a correlation between the amount of collagen present in the samples and the fluorescence absorption. The development of this technology would be a very useful minimally invasive tool to measure the quality of tissue within scaffolds or even repair tissue within a clinical defect.

Using similar minimally invasive optic technologies, the scaffold permeabilities can be detected by using an oxygen-sensitive dye, RTDP (ruthenium tris(-dipyridyl) dichloride). The small dye particles enter into the cells through phagocytosis. Then by looking at the fluorescence lifetime of the dye, we can determine the intercellular oxygen tensions through an established correlation.[34] Examining the oxygen tensions within the cells will help to develop an idea of the oxygen consumptions required for the cells. Through this method we can also be able to relate the scaffold permeability with the metabolic activities of the cells and determine ultimately scaffold permeabilities that are most suitable for cartilage formation.

6.5 CLOSING REMARKS

Solid freeform fabrication is a versatile technology that can be used for a wide range of tissue engineering applications. The ability to design specific scaffold properties tailored to those applications is a very valuable tool. There are many scaffold design parameters that still need to be investigated, but ultimately, the determination of the physical scaffold properties that are best able to stimulate chondrogenesis or the morphogenesis of any organ, will be a significant advancement in tissue engineering.

Scaffold Biomaterial and Pore geometry	BMSC Chondrogenic		CHONDROCYTES Basal		CHONDROCYTES Chondrogenic		Culture Period
	collagen I	5% HyA	collagen I	5% HyA	collagen I	5% HyA	
PPF cubic (n=3)	3%/5.9	-	29%/7.0	63%/9.5	61%/7.6	-	2 wk *in vitro* /4wk *in vivo*
PCL cubic (n=4)	4%/3.2	10%/3.6	-	-	63%/5.0	74%/6.4	2 wk *in vitro* /4wk *in vivo*
PCLF cubic (n=3)	51%/6.9	77%/8.5	-	-	95%/10	98%/12	5 wk *in vitro*
PPF ellipsoid (n=3)	13%/7.0	-	28%/7.3	59%/9.5	79%/8.7	-	2 wk *in vitro* /4wk *in vivo*
PCLF ellipsoid (n=3)	67%/7.1	92%/10	-	-	97%/10	99%/13	5 wk *in vitro*

Table 6.1: Summary of the histomorphometric analyses of all conducted experiments. The results are organized by scaffold biomaterial and pore geometry, cell type, hydrogel type, culture medium and culture period conditions. The first number, shown as a percentage, is the area of cartilage that stained with positive with Safranin O. The second number shown is the average sGAG concentration (μg/mm²) for the group. Experiments were not conducted for some of the groups and are marked with a dash.

	Cubic Small Pore	Ellipsoid Small Pore	Cubic Large Pore	Ellipsoid Large Pore
Design Unit Cell Size [x, y, z] (mm)	[0.7, 0.7, 0.7]	[0.7, 0.7, 0.7]	[1.4, 1.4, 1.4]	[1.26, 1.26, 1.63]
Designed Pore Size [x, y, z] (mm)	[0.35, 0.35, 0.35]	[0.7, 0.7, 0.7]	[0.7, 0.7, 0.7]	[1.26, 1.26, 1.63]
Pore Interconnections [x, y, z] (mm)	[0.35, 0.35, 0.35]	[0.3, 0.3, 0.26]	[0.7, 0.7, 0.7]	[0.3, 0.3, 0.26]
Porosity (%)	50	52	50	55
Permeability ([x, y, z] $x10^{-7}$ m^4/Ns)	[2.16, 2.16, 2.16]	[0.36, 0.36, 0.29]	[2.12, 2.12, 2.12]	[0.31, 0.31, 0.20]
Diffusivity ([x, y, z] m^2/s)	[0.44, 0.44, 0.44]	[0.25, 0.25, 0.27]	[0.39, 0.39, 0.39]	[0.22, 0.22, 0.19]
Surface Area, unitcell (mm^2)	2.7	4.4	12.5	15.4
Surface Area, scaffold (mm^2)	250	260	157	178
Pore Volume, unitcell (mm^3)	0.17	0.23	1.37	1.36
Pore Volume, scaffold (mm^3)	29.5	30.8	29.5	32.4
Cellular density, unitcell (Number of Cells/pore)	8500	11,500	68,600	68,000
Cellular density, scaffold (Total number of Cells)	$3x10^6$	$3x10^6$	$3x10^6$	$3x10^6$

Table 6.2: Designed scaffold parameters and physical properties. Comparison between small and large pore designs of the same cubic and ellipsoid pore geometries. The large pore designs have comparable scaffold permeabilities, porosities and diffusivities, but the cellular densities are almost seven times greater than the small pore designs.

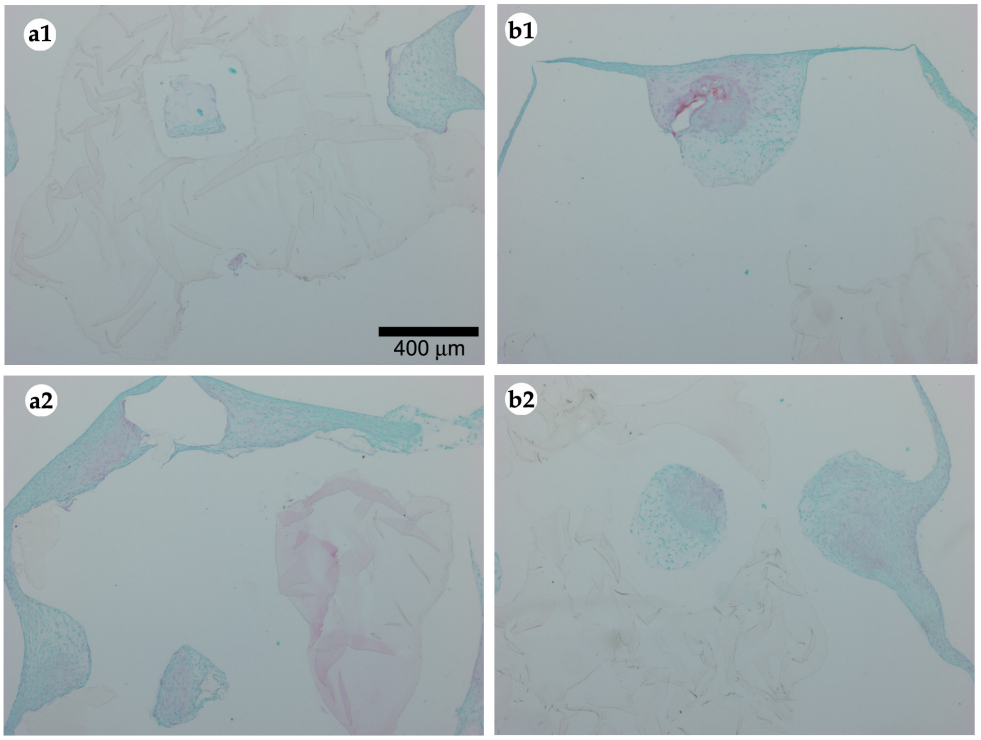

Figure 6.1: Cartilage formation in PCLF scaffolds with designed large pore cubic and ellipsoid geometries. Weak Safranin O staining is seen at the periphery and at the central pores of the cubic (a) and ellipsoid (b) scaffolds. More studies need to be conducted, but initial results suggest that a larger pore with higher cellular density may help increase chondrogenesis of BMSC within SFF scaffolds.

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APPENDICES

APPENDIX A
PRIMARY CHONDROCYTE AND BMSC ISOLATION

A.1 INTRODUCTION

The isolation of primary cells is an important determinant because the quality of the cells will partially determine the experimental outcome. After one or two days of monolayer culture, chondrocytes begin to dedifferentiate and acquire a fibroblastic phenotype. The addition of ascorbic acid to the culture medium helps to maintain the chondrocytic phenotype, but the chondrocytes should be used one or two days after the digestion step. Passaging the chondrocytes also decreases their ability to redifferentiate and with each passage, the cells lose their chondrogenic capabilities. For the BMSC, it is well know that the number of multipotent cells present in the bone marrow aspirates decreases with age. The bone marrow aspirates used for these experiments were all taken from male adolescent Yucatan minipigs. Furthermore, passaging the BMSC decreases the multipotent capacity of the cells and also segregates the adherent cell population in favor of the faster growing cells. Ideally, passage 1 or 2 BMSC are favored for use, the differentiation capacity of BMSC above passage 3 will be limited.

A.2 PROTOCOL FOR PRIMARY CHONDROCYTE ISOLATION

Solutions:

1. Solution for storing excised cartilage:
   Hank's Balanced Salt Solution (HBSS, Gibco, cat#14175-095)
   2:100 penicillin/streptomycin (Gibco, cat#15140-122)
   1:100 MEM non-essential amino acids (Gibco, cat#11140-050)

1:500 fungizone (Gibco, cat#15290-018)

(I generally make about 50 mL and wrap the tube with aluminum foil to protect the fungizone and MEM from light)

2. Digestion solution:

   DMEM high glucose (**serum-free**) (Gibco, cat#11195-065)
   1 mg/ml collagenase II-S (Sigma, cat#C1764)
   2:100 penicillin/streptomycin
   2:100 kanamycin monosulfate (Sigma, cat#K1377)
   1:500 fungizone

Autoclaved materials required:

1. 250 mL Erlenmeyer flask with small stirbar inside
2. Small sheet of Milliwrap (to cover top of Erlenmeyer flask)
3. Scalpel handles (no. 3), forceps with teeth, large Tuffier forceps for flipping knee, smaller forceps.
4. Millipore filter units with 22 μm nylon mesh

Other materials:

1. Aluminum foil, heavy duty is preferred
2. no. 15 scalpel blades
3. 35 mL of 100% ETOH in 50 mL vial for sterilizing instruments
4. 70% ETOH and 100% ETOH
5. 60mL or 30mL syringes

Sterilization:

Wash knees/ankles (muscle still attached) with 70% ETOH.
Remove the outer skin.
Place tissue on foil inside culture hood with hood on and lights OFF (so that you can see the flame)
Douse tissue with 100% ETOH.
Light on fire.
Flip over a few times to make sure all sides cook. Check to make sure fire is out before continuing.

Dissection:

Use sterile scalpels and forceps. Re-sterilize frequently by rinsing in 100% ETOH and flashing in alcohol candle.
Remove excess muscle, fascia, and ligaments without puncturing synovial

capsule.
Sterilize instrument and change scalpel blade if desired.
Open synovial capsule at articulating surface.
Separate articulating surfaces by cutting ligaments and tissue surrounding the joint. Sterilize instruments before trying to cut the ACL.
Cut away soft tissue to expose articular surface.
Cut away cartilage tissue sliver by sliver and place in HBSS on ice.
Try not to get too close to the subchondral bone. You can tell you did if it starts to bleed.
Try to keep the cartilage slivers the same size so that they digest evenly over time.

Digestion:

Aspirate off the storage solution and rinse with serum-free DMEM or HBSS.
Place pieces in a 250 mL flask with a spinner (autoclave flask with spinner inside)
Add 100 mL of digestion solution per 2 knees worth of tissue
Digest for 6 hours or less (or until cartilage slices are mostly gone) with agitation;

*ONLY required if using an old stir plate that exudes a lot of heat during stirring: attach timer to stir plate so digest is agitated for 30 minute intervals, with 30 minute rest intervals in between.

DO NOT leave in digest solution for more than 6 hours, otherwise most of your cells will be dead. If there are still a lot of chunks remaining after 6 hours, filter the solution and put the remaining cartilage chunks in fresh digest solution.
After digest is complete, few pieces of cartilage should be visible in the medium.

Filter and Culture:

Filter digest solution through the Millipore filter units. Centrifuge chondrocytes at 2000 rpm for 8 minutes (be sure to check supernatant for any remaining cells in suspension). Plate out overnight on tissue culture polystyrene in DMEM (+10% FBS + 1%P/S and 50 μg/mL 2-phospho-L-ascorbic acid). Chondrocytes de-differentiate in monolayer culture, so trypsinize the cells the next day (24 hours after plating) for use or freeze.

Tips: It's hard to avoid at least some contamination of the samples, so try to keep them in antibiotics as much as possible. If you're very concerned, you may also add some kanamycin or fungizone when you plate them out, but

the fungizone will also alter the cells behavior in unquantified, though probably small ways.

Check for infections:

Digestion media should not turn orange. When you spin down after filtering, the media above the cell pellet should look clear.

Alternative timing to spread the harvest/digest over 2 days:

Follow all instructions prior to Digestion.
After the all cartilage slices have been excised, aspirate off the HBSS storing solution. Try to get as much of the solution out as possible.
Wash once with sterile HBSS.
Pour some sterile DMEM+10% FBS+1%P/S on the cartilage slices. Swirl around to suspend the slices in media and then pour into an autoclaved 250 mL Erlenmeyer flask with stirbar. Repeat until all the cartilage slices are in the flask.
Add more DMEM+10%FBS+1%P/S to the flask so that total is about 75 mL per knee. Add 50 μg/mL 2-phospho-L-ascorbic acid to the media in the flask.
Cover with Milliwrap and place in incubator with gentle agitation overnight.

Next morning:

Aspirate off the DMEM solution. Wash with either HBSS or serum-free DMEM to get rid of the remaining FBS, which interferes with digest enzymes.
Continue with the Digestion step.

A.3 PROTOCOL FOR BONE MARROW STROMAL CELL ISOLATION

Heparin solution for bone marrow aspirate collection:

DMEM, hi-glucose
10% fetal bovine serum
1:50 penicillin/streptomycin
1:250 fungizone
100 units/mL heparin sulfate
1:1000 gentamicin (optional; Sigma, cat#15710-064)

Fill 50 mL conical vials with 20 mL of heparin solution.
Ship the vials with ice packs or dry ice (NOTES: FedEx drop off locations will not accept packages with dry ice. Do not use crushed ice.)

In Texas, at Seguin Animal Hospital:

Add 10 – 20 mL of bone marrow aspirate per vial
Tighten vials and swirl solution briefly.
Return ship overnight with ice packs.

Upon the arrival of the aspirate, the process of plating the cells and removing the blood cells that come with them takes approximately two weeks. During this time, you must supervise the cells to make sure that they are not confluent. The day they become confluent is the day in which they must be passaged. Otherwise, their proliferative capacity is diminished.

Bone marrow aspirate culture medium:

DMEM, hi-glucose
10% FBS
1:50 penicillin/streptomycin
1:500 fungizone

Day 1: Arrival day

1. Label (cell type, passage, specimen #, date) and fill T75 Falcon flasks with 10-15 mL of media or T225 flasks with 30 mL of media each.
2. Resuspend the cells in the vial by gently pipetting up and down to redistribute settled components.
3. Place 10-15 mL of bone marrow aspirate into each flask.
4. Gently swish media/cell mix on bottom of flask.

Day 7:

1. Change half of the media.
2. You may lightly swish the media around to start removing some of the blood cells. LIGHTLY.

Day 11:

1. Change half of the media.
2. You may lightly swish the media around to start removing some of the blood cells. LIGHTLY.

Day 14:

Passage the cells. You may need to passage anywhere between days 11-18.

APPENDIX B
SCAFFOLD BIOMATERIAL AND SFF FABRICATION

B.1 INTRODUCTION

Three biomaterial polymers will be used for the experiments in this study: poly(propylene fumarate) (PPF), poly(ε-caprolactone) (PCL) and their copolymer, poly(ε-caprolactone fumarate) (PCLF). All three materials can be used in combination with indirect SFF fabrication techniques to create SFF scaffolds with the designed architectures.[1] These three-dimensional matrices with defined pores and pore interconnections are capable of supporting load. These scaffolds will provide a mechanically protective environment for the hydrogels and allow nutrient diffusion to support the developing tissue.

Poly(propylene fumarate) is a biodegradable, biocompatible polymer that has been used previously for orthopaedic applications.[2,3] PPF is an unsaturated linear polyester that can be cross-linked through its fumarate double bonds using N-vinyl pyrrolidone. The cross-linking reaction is propagated by free radical polymerization using benzoyl peroxide and N, N dimethyl-*p*-toluidine. The result is an injectable polymer that is moldable to fill irregularly shaped defects.[4]

Poly(ε-caprolactone) is biodegradable polymer that is FDA-approved for suture material. Its biocompatibility and flexibility make it a good potential biomaterial for tissue engineering applications. PCL is better for tissue regeneration because the bulk degradation of the biomaterials has decreased acidic degradation products and inflammatory response like some biomaterials that are harmful to

the developing tissue.[5] (Hutmacher) There are a few casting issues with using PCL. A lower molecular weight or lower viscosity PCL is needed in order to cast directly with the wax molds from the Solidscape machines. The alternative is to make hydroxyapatite scaffolds and then cast into the PCL.

Poly(ε-caprolactone fumarate) (PCLF) is a new polymer that is made with by the combination of PPF and PCL. The combined polymer has the benefits of both original polymers. The flexibility and self-crosslinking characteristics of PCL is combined with the injectable biocompatible properties of the PPF.[6] The replacement of propylene glycol with PCL also reduces steric hindrance and makes the fumarate double bonds more accessible for crosslinking. The self-crosslinking properties of PCLF also decrease the amount of NVP, initiator, and accelerator that are required.

Using various indirect casting techniques, we can create scaffolds from these three polymers. The indirect casting methods used to fabricate PPF and PCLF will be described here. The mechanical properties and degradation profile of both polymers can be varied depending on the amount of NVP used. A 1:1 ratio of polymer:NVP gives 100% cross-linked polymer. A lower percentage of crosslinks results in a more malleable the scaffold that also degrades faster.

Differences in tissue formation due to the biomaterial are believed to be minimal. The PCL has been approved by the FDA and biocompatibility studies of PPF[7] and PCLF shown little inflammatory response. Although with the casting method used for the PCLF scaffolds, some residual chloroform may still be present within the scaffolds. An appropriate time may be required to allow the chloroform to leach out of the scaffolds.

B.2 PROTOCOL FOR CASTING PPF SCAFFOLDS WITHOUT β-TCP

Materials:

Poly(propylene fumarate) (PPF) – from Michael Yaszemski's Lab
N-vinyl-2-pyrrolidinone (NVP) – Fisher Scientific (cat# AC14092-5000)
Benzoyl peroxide (BPO) – Sigma Catalog #
N,N dimethyl-p-toluidene (DMT) – Sigma Catalog #
Hot plate
Glass scintillation vials
Glass pipette and rubber dropper
Weigh boats
Spatula
Pipetteman (1 – 10 μL) and tips
Wax molds (blue or green) and PTFE mold for casting

WEAR ALL PERSONAL PROTECTIVE EQUIPMENT (lab coat, gloves, googles) AT ALL TIMES.

Procedure:

1. Turn on the hot plate. Break up the PPF into smaller chunks with spatula, so that it is measureable.

2. Measure out 0.7 grams of PPF into a glass scintillation vial.

3. Tare the glass vial with PPF, and add 0.7 grams of NVP to the glass. Slowly add the NVP because the amount on the scale increases rapidly after every drop.

4. In a small weight boat, measure out 0.02 grams of BPO.

5. Put the glass vial with PPF and NVP on the hot plate and stir the mixture until the chunks of PPF have dissolved. Use the spatula to smash the chunks of PPF against the side of the glass to help it dissolve. Remove the vial from the heat when the two monomers have a smooth liquid consistency without chunks. Cool the solution back to room temperature before proceeding.

<u>Conduct the rest of the steps under a well-ventilated hood</u>.

6. Carefully crush the BPO against the sides of the weigh boat until it is a fine powder.

7. Prepare the Pipetteman for drawing up 2 μL of DMT. Get your wax molds and PTFE mold or external mold ready for casting.

8. Add the crushed BPO into the glass vial with the PPF and NVP. Stir for 10 seconds and then add 2 μL of DMT to the side of the glass. DO NOT ADD directly to the solution because it will cure too quickly.

9. Stir the entire mixture for 5 seconds and then pour into external molds. Immediately push the wax molds into the PPF solution.

10. Allow the PPF to solidify overnight in the sealed chamber and pass some nitrogen gas over the scaffolds, if desired.

11. Label the glass scintillation vials and allow them to vent under the hood overnight. Place them in the appropriate WASTE buckets in the morning. Place any paper towels, plastic droppers, weigh boats, and other solid waste in the appropriate containers.

12. Remove the PPF from the external molds and trim (either use the dremel, grinding machine, or razor blade) to expose the wax so that it may be removed.

B.3 PROTOCOL FOR CASTING PCLF

Materials:

Poly(ε-caprolactone fumarate) in -20°C freezer – allow to warm up to room temperature for 20 – 30 minutes prior to removing the parafilm to reduce the amount of moisture within the bottle
N-vinyl-2-pyrrolidinone (NVP) – Fisher Scientific (cat# AC14092-5000)
Benzoyl peroxide (BPO) – Sigma Catalog #
N,N dimethyl-p-toluidene (DMT) – Sigma Catalog #
Chloroform ($CHCl_3$)
Glass scintillation vials
Glass pipette and rubber dropper
5 mL glass pipette
Weigh boats
Spatula
Pipetteman (1 – 10 μL) and tips
Timer
Green wax molds scaled to 1.5x greater than the design and casting cups

Formulation:

1. 0.65 gm PCLF in 1.5 mL of $CHCl_3$ with 1:1 ratio of PCLF to NVP
2. 0.65 gm PCLF in 1.5 mL of $CHCl_3$ with 1:4 ratio of PCLF to NVP

WEAR ALL PERSONAL PROTECTIVE EQUIPMENT (lab coat, gloves, googles) AND WORK UNDER HOOD AT ALL TIMES.

Procedure:

1. Measure out the PCLF into glass scintillation vials. The polymer becomes statically-charged, so be aware that it may stick to everything.

2. Tare and measure out desired amount of NVP (according to the formulations above for desired amount of cross-linking) using the glass pipette and dropper and put into the glass vial with the PCLF.

3. Measure out 30 mg of BPO into a small weight boat.

4. Get the wax molds and cups ready for casting. I cast about 3 – 4 molds that are about 9 mm in diameter and 10 mm in height.

5. Add 1.5 mL of $CHCl_3$ to the PCLF and NVP. Stir the mixture until the PCLF is completely dissolved.

6. Crush the BPO to a fine powder and add it to the PCLF/NVP/$CHCl_3$ mixture.

7. Add 4 μL of DMT to the PCLF mixture. Stir well and pour into the cups. Wait 1 minute before pushing the wax molds into the polymer solution. The polymer should start to cure after 3 or 4 minutes.

8. Allow the polymer to cross-link completely under the hood for about 1.5 hours.

9. After removing the cups, rinse off the polymer with some water. Trim off the excess polymer to prepare for wax removal.

10. Use 70% ethanol to remove the wax. Change the ethanol at least two or three times to get rid of the chloroform.

11. Air dry the polymer scaffolds overnight. The scaffolds will shrink, but they will swell back after they are rehydrated.

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APPENDIX C

COMPOSITE HYALURONIC ACID/COLLAGEN FABRICATION AND SFF SCAFFOLD SEEDING

C.1 INTRODUCTION

The cells in the experiment were encapsulated in either collagen I or composite hyaluronic acid/collagen I hydrogels. The addition of hyaluronic acid hydrogels serves to increase the chondrogenic potential and stabilize the phenotype of both BMSC and chondrocytes. This appendix describes the procedure for fabricating hydrogels alone and also for using hydrogels to seed cells into scaffolds.

C.2 FABRICATION OF COMPOSITE HYA/COLLAGEN HYDROGELS

The cells were encapsulated in either collagen I or composite HyA/collagen I gels. After trypsinization, cells were resuspended at 3.5×10^6 cells/mL in 2 mL of collagen I (BD Bioscience Discovery Labs, San Jose, CA) with 200 μL of culture medium. The pH of the collagen/cell suspension was increased with the addition of 20 μL of 0.5 M sodium hydroxide with 220 mg/mL sodium bicarbonate. The mixture was pipetted in 145 μL aliquots into 48-well tissue culture plates. The resulting collagen I hydrogels were 1.5 mm in height and 11 mm in diameter. To create the composite HyA/collagen I gels, hyaluronic acid (stock concentration: 3 mg/mL in 1.5 M NaCl, MW: 3×10^6 Da; Hyalogic LLC, Edwardsville, KS) was added to the collagen I cell suspension to produce 1, 2, 5, and 10% (w/w) HyA/collagen I gels. The HyA concentrations for the corresponding weight percent gels were 0.05, 0.09, 0.23, and 0.47 mg/mL,

respectively. The pH was raised to physiologic levels with NaOH. (The amount of collagen I used to resuspend the cells in composite hydrogels was adjusted so that the final volumes of the HyA/collagen cell suspension was similar to collagen I only hydrogels.) All hydrogels were placed in an incubator at 37°C and allowed to solidify for 30 minutes. Either basal medium or defined chondrogenic medium was then added to the tissue culture wells. Hydrogels were cultured under gentle agitation on an orbital shaker and with the media replaced every other day.

C.3 CELL SEEDING OF SFF SCAFFOLD USING HYDROGELS

In preparation for cell seeding, scaffolds were sterilized in sterile 70% ethanol, rinsed twice with sterile HBSS. The scaffolds were then soaked in culture medium to allow proteins to adhere to the surface. The cells were seeded into the scaffold pores using the either collagen only or HyA composite hydrogels. After trypsinization, the cells were resuspended at 40 x 10^6 cells/mL in a solution containing either ~3.8 mg/mL collagen I or 5% (w/w) HyA/collagen I. The pH of the solution was increased with the addition of sodium hydroxide, and ~60 μL aliquots of the cell/hydrogel suspension was immediately pipetted into the wells of a PTFE mold (diameter = 5.1 mm). The scaffolds were then pushed into the wells to force the cell suspension through the pores of the scaffold. The gels were allowed to solidify within the scaffolds in a humidified chamber at 37°C for 30 minutes. The hydrogel/scaffold constructs were then removed from the PTFE mold and placed in a 12-well tissue culture plate. Basal medium or defined chondrogenic medium was added to the wells. The plate was placed in the incubator and gently agitated on an orbital shaker. The culture media was changed every other day during the *in vitro* pre-culture period.

C.4 PROTOCOL FOR HYA/COLLAGEN HYDROGEL FABRICATION

Solutions:

1. Collagen I, solubilized from rat tail; stock concentration should be at least 3.5 mg/mL (BD Bioscience Discovery Labs)

2. Hyaluronic acid, stock: 3 mg/mL in 1.5 M NaCl solution (Molecular Weight: 3 x 10^6 Da; Hyalogic LLC)

3. 0.5 M Sodium hydroxide (NaOH) with 220 mg/mL sodium bicarbonate ($NaHCO_3$)

Keep all solutions sterile and on ice. Work under the sterile culture hood.

Procedure:

1. Trypsinize cells, resuspend and count number of cells. Keep on ice.

2. Use the count number to determine the cell density desired for each hydrogel

3. Determine the volume of the hydrogel desired and calculate the volume of collagen I required, then add 10-20 μL to account for collagen viscosity
   Example: Volume = 60 μL per hydrogel x 10 hydrogels = 0.6 mL => 0.62 mL

4. Calculate the amount of hyaluronic acid need by computing:

$$\frac{VolumeofCollagen \times CollagenConcentration \times PercentHyAHydrogelDesired}{HyaluronicAcidStockConcentration} =$$

$$\text{Example: } \frac{0.62mLofCollagen \times 3.5mg/mLCollagenStock \times 5\%HyA}{3mg/mLHyAStock} = 36\mu LofHyA$$

5. Spin down/pellet cells

6. Aspirate off all media, be careful to not suck up cells.

7. Add 200 μL of media per mL of collagen I (amount from step#3)

8. Add amount of collagen I from step #3 and resuspend the cells with gentle pipetting. Try not to make bubbles when resuspending, keep pipetting until the cells are mostly homogenous throughout the collagen

9. Gently invert the hyaluronic acid to agitate the solution. Then add the amount of hyaluronic acid from step #4. Mix the HyA with the cell/collagen suspension with the pipette immediately, sometimes the HyA and collagen will form an ionic precipitate – if this happens, you will need to add more NaCl to your HyA stock solution.

10. Prepare your culture well plate, scaffolds, PTFE mold, etc. for the hydrogel. If you take longer than 1 minute, put the cell/collagen/HyA suspension in ice.

11. Shake the NaOH/$NaHCO_3$ solution so that the bicarbonate is dispersed throughout. Pipette up and down to get the amount desired, make sure there is some bicarbonate particles in the solution in your pipette. Add the NaOH/$NaHCO_3$ solution to the cell/collagen/HyA suspension and mix immediately. You can test a small amount of the suspension on litmus paper to get the pH of the hydrogel – it should be around 7.2 – 7.6.

12. Aliquot the mix into your prepared molds, plates, scaffolds, etc.

13. Place the hydrogels in the incubator at 37°C to allow the collagen to set. Wait 30 minutes or longer for the hydrogels to completely solidify before putting adding culture media.

NOTES: For collagen I hydrogels only, skip steps #4 and #9.

APPENDIX D
DMMB ASSAY FOR GAG QUANTIFICATION

D.1 INTRODUCTION

Dimethyl-methylene blue (DMMB) is an extremely useful assay that is used to quantify the amount of sulfated glycosaminoglycans (sGAG). The DMMB is binds specifically to sGAG, even in the presence of other polyanions and can be used to determine the type of sGAG present in the sample. Often times the sGAG is normalized to the number of cells in the sample (either cell count or quantify the amount of DNA using Hoechst dye).

D.2 PROTOCOL FOR DMMB ASSAY

Solutions:

1. Hank's Balanced Salt Solution (HBSS, Gibco, cat#14175-095)

2. Proteinase K digest solution[1]:
   78 μg/mL proteinase K (Sigma, cat#82456)
   in 100 mM sodium acetate (Sigma, cat#S1429)

3. DMMB reagent[2]:
   16 mg 1,9 dimthyl-methylene blue dye (Sigma, cat#341088)
   3.04 g Glycine
   2.37 g NaCl
   95 mL 0.1 M HCl
   in 1 liter of dIH_2O, pH 3.0
   (The reagent solution is stable for at least 3 months when stored in a brown bottle at room temperature)

4. Chondroitin-6-sulfate for calibration (shark cartilage; Sigma, cat#C4384)

Materials:

1.5 mL centrifuge tubes with lock
0.5 mL centrifuge tubes

Procedure:

1. Rinse your samples with HBSS.

2. Place your samples in 1.5 mL centrifuge tubes with locks. Also make a proteinase K blank for the negative control and a CS standard (10 – 20 μg) for positive control/reference.

3. Add about 200 μL of proteinase K digest solution to each tube.

4. Incubate for 2 hours (or until sample is digested) at 65°C in water bath. Vortex gently to aid digestion.

5. Increase the temperature to 100°C for 10 – 15 minutes to inactivate the proteinase K enzyme. Keep watch on the caps so that they don't pop open.

6. Centrifuge the samples for 10 minutes at 14,000 rpm at 4°C.

7. Label and record the weights of a new set of 0.5 mL centrifuge tubes.

8. Draw off the supernatant, be careful not to disturb the pellet. Put the supernatant in the new 0.5 mL tubes.

9. Reweigh and record the weights of the tubes.

10. Get a flat bottomed 96 well plate for the DMMB assay.

11. Save two rows for the chondroitin sulphate standard calibration. Make at least 5 concentrations between 0 and 2.5 μg/μL with 2 replicates each.

12. Use the rest of the plate for your samples. Add 5 μL of supernatant to each well and have 3 replicates of the same sample.

13. Add 200 μL of DMMB reagent to each well. Tap on the side of the plate gently to mix.

14. Immediately read the absorbance in the plate reader at 525 nm.

15. Use the calibration curve to determine the amount of sGAG in 5 μL of each sample.

16. Use then subtract the (tube+supernant weight) from the (empty tube weight) to determine the total amount of supernatant and calculate the total sGAG concentration for the sample.

NOTE: If the GAG concentration in your sample is too high, the DMMB will change to a deep magenta or violet color and a precipitate will form. Dilute your samples accordingly and repeat the measurement.

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APPENDIX E

SAFRANIN O QUANTIFICATION BY HISTOMORPHOMETRICAL IMAGE ANALYSIS

E.1 INTRODUCTION

Quantification of the amount of sulfated glycosaminoglycans (sGAG) from histology sections is a very useful tool. Since histology is primarily a qualitative analysis of the tissue, this histomorphometric image analysis significantly enhances the data gathered from the Safranin O stain. Safranin O is a cationic dye that binds specifically and stoichiometrically (1:1) to polyanions, such as chondroitin sulphate and keratan sulphate. Thus, Safranin O stain intensity can be directly correlated to the sGAG concentration present in the tissue.[1]

The first step in the analysis was to determine the correlation between the Safranin O stain intensity and the GAG concentration. We used a variety of cartilage samples to get a wide range of Safranin O stain intensities and used the DMMB assay to determine the GAG concentrations of the samples. The second step was to write a Matlab program that could isolate the Safranin O stained areas, determine the intensity of the stain and correlate that to the concentrations determined with the DMMB assay. In order to use the linear correlation that was derived, the Safranin O staining protocol and the camera settings should be identical.

E.2 CORRLEATION OF SAFRANIN O STAIN INTENSITY TO sGAG CONCENTRATION

A correlation between the Safranin O stain intensity and the sGAG concentration was determined using a dimethyl-methylene blue (DMMB) assay. Hydrogels seeded with BMSC (FIGURE E.1a) or chondrocytes and full-thickness cartilage plugs removed from porcine metacarpophalangeal joints using a trephine (d = 5 mm) were used for the analysis. Specimens were snap-frozen in OCT and serial transverse sections were cut at 12 μm thickness. Sections were taken from four layers: superficial (0 – 120 μm), transitional (120 – 360 μm, FIGURE E.1b), middle (360 – 720 μm), and deep (720 – calcified tissue, FIGURE E.1c). A few sections from each layer were fixed to slides and stained with Safranin O. The remaining sections were digested using a solution of proteinase K, as described previously (Appendix D).[2] The supernatants from the digest were used to determine the sGAG content using a DMMB assay. Known amounts of chondroitin sulphate were used as the calibration standard. Histomorphometric image analysis was used to determine the Safranin O stain intensities of the articular cartilage slices and hydrogels. The averaged stain intensities were then correlated to the averaged sGAG contents determined with the DMMB assay. In Figure E.1d, the linear correlation derived from these data (r = 0.98) is shown: $y = 18.75x + 1.27$, where y is the sGAG concentration ($\mu g/mm^2$) and x is the Safranin O stain intensity, x = [0 1].

E.3 HISTOMORPHOMETRIC IMAGE ANALYSIS USING MATLAB

Histological sections taken from many planes of each scaffold were used for analyses. Low magnification (2X objective) images of whole cross sections of tissue samples were acquired using a CCD camera (RT Color 2.2.1, Diagnostic Instruments, Sterling Heights, MI) mounted on a Nikon Eclipse E600 microscope

(Nikon America, Melville, NY). RGB images were acquired through Spot, an image acquisition program (Diagnostic Instruments, Sterling Heights, MI). The illumination of each picture was controlled so that the exposure for each image was the same; thus the brightness, contrast and saturation of the images was captured using the same parameters.

Image processing was performed on the RGB images in Matlab (The Mathworks, Natick, MA) to determine: 1) the ratio of positive Safranin O staining to the available pore space and 2) the average stain intensity of the section. The prior calculation is correlated to the number of chondrocytic cells that have not dedifferentiated while the latter is correlated to the amount of sGAG present in the extracellular matrix.[3,4] Matlab code was written to convert the RGB image (FIGURE E.2a) to hue saturation value (HSV) and threshold out the scaffold and background, so that only the areas of positive Safranin O staining remained. (FIGURE E.2b) The pixel area was calculated for these extracted areas to determine the area of positive Safranin O staining for the section. The background was subtracted from the picture and was thresholded again to remove the scaffold, but this time pore space that did not stain for Safranin O was selected. (FIGURE E.2c) The two extracted areas were combined to calculate the total pore space that was available for that particular section. (FIGURE E.2d) With these two areas, the percentage of positive Safranin O staining for the section is calculated. The second set of histomorphometric data assessed was the overall intensity of the stain. The extracted areas of positive Safranin O staining were used for this calculation. The intensity (I) was calculated using the equation, $I = (R+G+B)/3$.[5] A value between 0 and 1 corresponding to the overall density of stain was assigned for each pixel. The image was divided into 50 x 50 pixel blocks (186 μm = 50 pixels) and the average intensity of each block was calculated. (FIGURE E.2e) The values from each 50 x 50 pixel block were used to determine the average intensity of the entire section. The linear correlation,

described previously, was then used to determine the average sGAG concentration within the positively stained Safranin O areas.

E.4 PROTOCOL FOR SAFRANIN O/FAST GREEN FCF STAINING

Materials:

Harris Hematoxylin (Sigma cat#HHS-16)
0.02% Fast Green FCF, CI#42053 in distilled water (0.1 gm in 500 mL dIH_2O)
1% Acetic Acid (make fresh day of use)
0.1% Safranin O, CI#50240 in distilled water (0.5 gm in 500 mL dIH_2O)
Xylene
Xylol (50:50 xylene:ETOH)
Ethanol (ETOH)

WORK UNDER HOOD AT ALL TIMES. WEAR ALL PPE, LAB COAT, NITRILE GLOVES.

Procedure:

1.	Deparaffinize in xylene	2 x 5 min
2.	Xylol	1 x 5 min
3.	100% ETOH	2 x 2 min
4.	95% ETOH	2 x 2 min
5.	70% ETOH	1 x 2 min
6.	Distilled Water	1 x 2 min
7.	Harris Hematoxylin (optional)	30 sec
8.	Wash in running distilled water in sink until water runs clear (only if staining with Harris)	1 min
9.	Fast Green	1 x 3 min
10.	1% acetic acid	several washes
11.	Safranin O	1 x 6 min
12.	95% ETOH	1 x 1 min
13.	100% ETOH	several washes
14.	100% ETOH	several washes
15.	Xylol	several washes
16.	Xylene	several washes

17. Coverslip with Permount. Do not leave in xylene, but wash in between coverslipping to prevent sections from drying out.
18. Leave slides under hood for 24 hours to allow Permount to set.

Results:
Nuclei = black
Cytoplasm = gray green
Mucus, cartilage, most cell granules = orange red

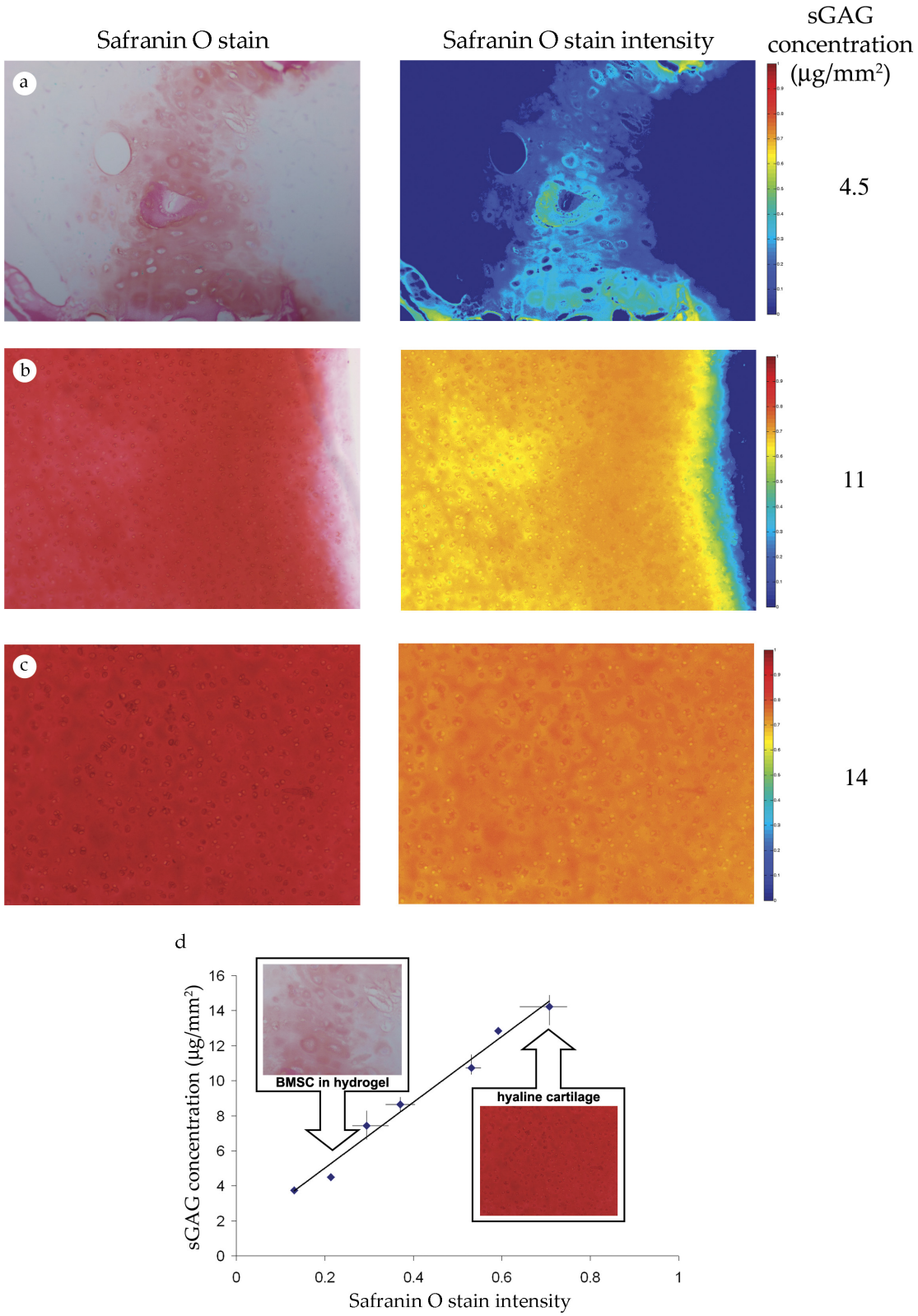

Figure E.1: Examples of Safranin O stain intensity correlation to sGAG concentration. a) BMSC in hydrogel, b) superficial/middle layer of cartilage, c) deep layer of cartilage, and d) linear correlation between stain intensity and GAG concentration.

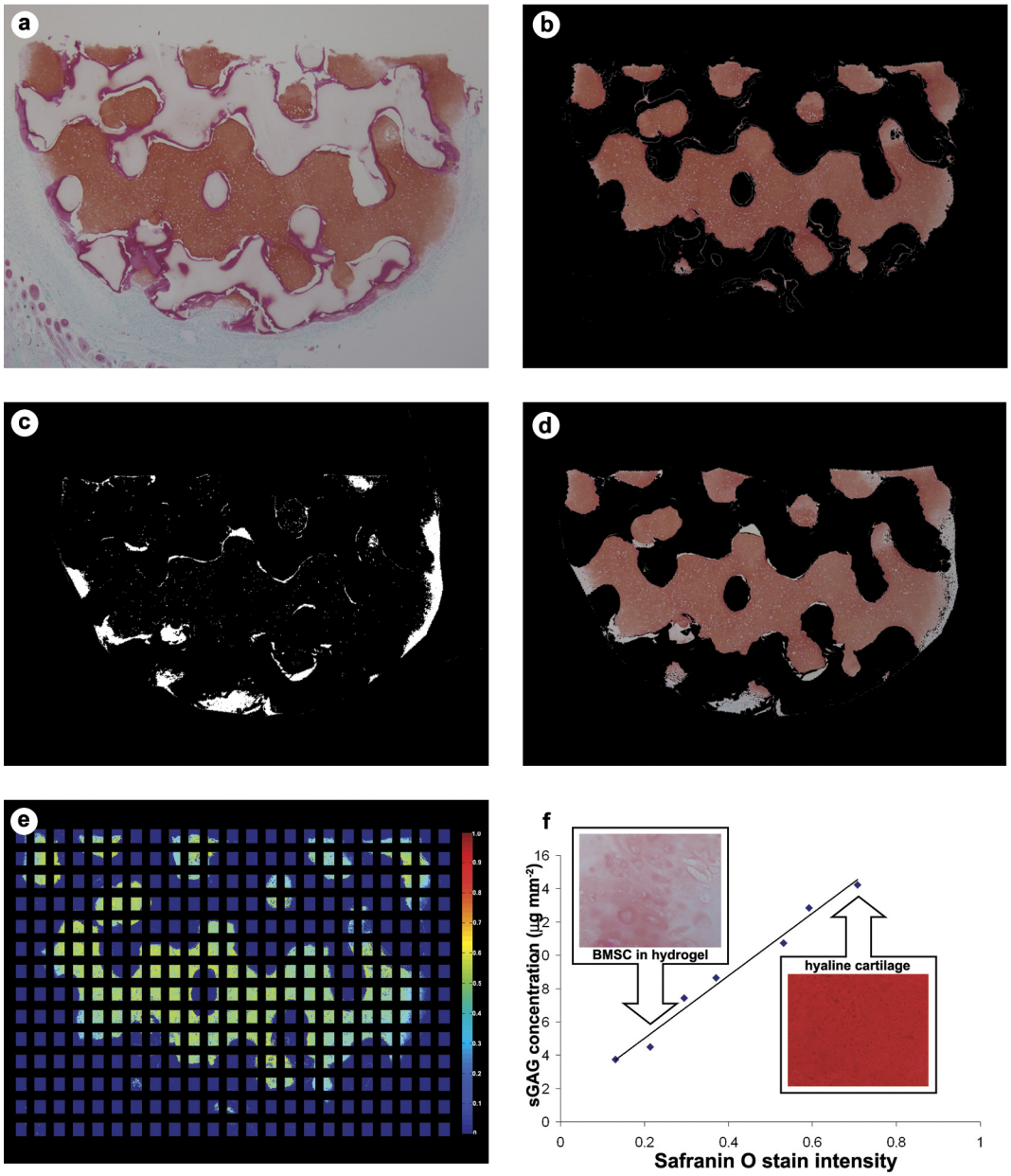

Figure E.2: Safranin O quantification by histomorphometrical analysis. a) Section of a chondrocyte-seeded scaffold after 2 week pre-culture and 4 week implantation. Residual scaffold is indicated by the asterisk. b) Tissue stained positive by Safranin O and c) remaining, non-stained non-scaffold, areas identified through image processing. d) Total scaffold pore space created from composite of (b) & (c). The ratio of positive Safranin O staining to total pore space, 78% in this specimen, is used as a metric for chondrocytic viability and metabolic activity, whether the cells are BMSC that have differentiated into chondrocytes or chondrocytes that have not dedifferentiated. e) Safranin O stain intensity of each 186 μm by 186 μm region determined from the correlation, I = (R+G+B)/3. I is a scaled value between 0 and 1. f) Calibration curve derived from a dimethyl-methylene blue (DMMB) assay relating the amount of sGAG (μg/mm^2) to the Safranin O stain intensity. The linear correlation is used to quantify the amount of sGAG present in the section using the Safranin O stain intensity calculated in e. The amount of sGAG calculated for this specimen is 9.68 μg/mm^2.

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APPENDIX F
IMMUNOHISTOCHEMISTRY

F.1 INTRODUCTION

Immunohistochemistry uses antibodies to detect proteins that are present in histologic sections. Antibodies targeting collagen I and collagen II were used on paraffin and frozen sections. Optimal titers of the primary antibodies were evaluated on paraffin sections of a decalcified mouse joint. If staining cryosections, start with step#2 (H_2O_2) and after washing (step#3), skip to step #7 (blocking with normal goat serum). The targeted collagen sequences of the antibodies are homologous between species, so that although the target immunogens are for human collagens, there is cross-reactivity with porcine collagens. Representative images of negative controls, where the primary antibody was not added, and positive controls, where sections of porcine articular cartilage were stained, are shown in Figure F.1.

For more information on immunohistochemistry, antibody titers, background, etc., the Immunohistochemical Staining Methods Education Guide, 4th edition from the Dako website is a good resource (www.dakousa.com).

F.2 PROTOCOL FOR IMMUNOHISTOCHEMISTRY FOR DETECTING COLLAGEN I AND COLLAGEN II

Materials:

Rabbit anti-human collagen type I (polyclonal IgG) USBiological #C7510-11C
Affinity Purified in 0.125 sodium borate, 0.075 M NaCl, pH 8.0
Recommended dilution: 1:100

Rabbit anti-human collagen type II (polyclonal IgG) USBiological #C7510-20F
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Recommended dilution: 1:100

Xylol (50% Xylene: 50% Ethanol)
Bovine Serum Albumin (BSA)
50 mU/mL Sheep hyaluronidase + 1% BSA (Sigma)
0.3% v/v H_2O_2 in methanol
Tris-Buffered Saline (TBS): 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.6
Vectastain ABC for Rabbit IgG Kit (Vector Labs)
3′,3′ diaminobenzidine (DAB) Substrate Kit for Peroxidase (Vector Labs)
PAP Immunobarrier pen (optional)

Procedure:

1. Deparaffinize and rehydrate slides:

Xylene	2 x 5 min
Xylol	1 x 5 min
100% ETOH	2 x 2 min
95% ETOH	1 x 2 min
80% ETOH	1 x 2 min
70% ETOH	1 x 2 min
30% ETOH	1 x 2 min
dI H_2O	1 x 2 min

2. 0.3% v/v H_2O_2 in methanol — 1 x 15 min

3. Wash in distilled H_2O — 2 x 5 min

4. Antigen retrieval:

   a. Warm slides to 37°C in dIH_2O — 1 x 5 min

4. Antigen retrieval: (cont.)

b. Hyaluronidase @ 37°C 1 x 10 – 30 min

(The time you leave in hyaluronidase is dependent on your sections, adjust as necessary to keep background low and stain intensity high, I usually use 30 min)

5. Wash in distilled H2O 1 x 5 min

During this step, I circle the sections with PAP pen so that I have to use less antibodies per section. (optional)

6. Wash in TBS 2 x 5 min

7. Diluted normal serum (NS) from Vector ABC Kit 1 x 30 min

3 drops of stock (yellow label) to 10 mL of TBS

8. BLOT excess serum from sections

9. 1° antibody diluted in TBS + 1% BSA 1 x 60 min @ RT

I've used dilutions of 1:100, 1:500 or you can leave it overnight @ 4°C with lower concentration of antibody. (Also have a negative control slide, or section where 1° antibody is not added)

10. Wash in TBS 3 x 5 min

11. 2° antibody from Vector ABC kit 1 x 30 min

1 drop of stock (blue label) to 10 mL of TBS

12. Make up Vectastain ABC reagent:

2 drops of Reagent A (orange bottle) to 10 mL of TBS

2 drops of Reagent B (brown bottle) mixing bottle with Reagent A/TBS

Mix immediately and let stand for 30 min

13. Wash in TBS 2 x 5 min

14. Vectastain ABC reagent from ABC kit 1 x 30 min

15. Wash in TBS 1 x 5 min

16. Prepare DAB substrate per manufacturer's instructions: (make just before use – the DAB enzyme degenerates after 6 hours at 4°C) Keep it protected from LIGHT!

To 5 mL of distilled H_2O
Add 2 drops of Buffer Stock Solution and mix well
Add 4 drops of DAB Stock Solution and mix well
Add 2 drops of H_2O_2 Solution and mix well

DAB is a suspected **carcinogen**. Protect yourself appropriately. Neutralize any unused DAB with an equal volume solution of 3% potassium permanganate ($KMnO_4$) and 2% sodium carbonate (Na_2CO_3) in deionized or distilled water. Dispose of all waste in appropriately labeled DAB waste containers.

17. Incubate sections in DAB substrate for 2 – 10 min until good stain intensity develops. I usually leave it on between 5 – 7 minutes.

18. Rinse in distilled water to get rid of DAB.

19. Wash in distilled water for 5 min.

20. Counterstain with Fast Green:
    a. Stain in 0.02% Fast Green for 1 min.
    b. Rinse in 1% acetic acid for several washes

21. Dehydrate, clear and mount in Permount

22. Leave slides under hood to allow Permount to set overnight and let the xylene evaporate off.

NOTES: **NEVER** let sections dry out. Use a humidification chamber when necessary.

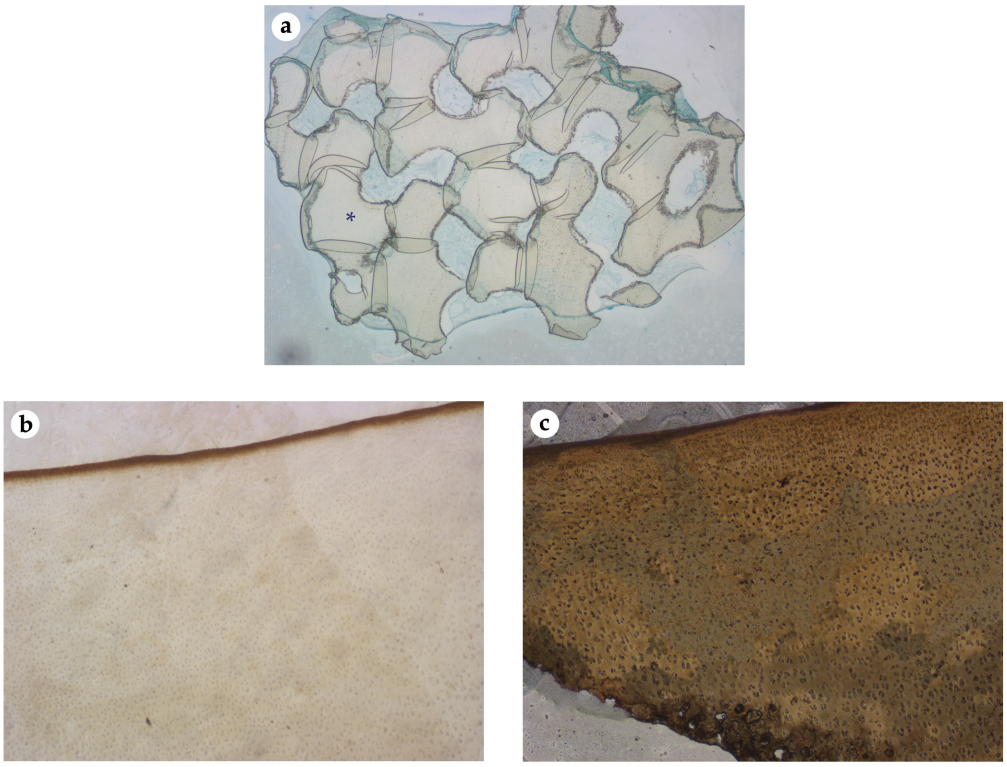

Figure F.1: Images of negative and positive controls for immunohistochemistry staining for collagen I and collagen II. a) Negative control where primary antibody was not added to the section. Residual scaffold is stained weakly with light orange-brown color (indicated by *). b) Positive control showing staining for collagen I in a slice of porcine articular cartilage. Collagen I is detected at the edge of the section, shown by the brown color. c) Positive staining of collagen II in a slice of porcine articular cartilage. The dark brown staining is seen throughout the section indicating the widespread presence of collagen II.