

Optimal Seeding Densities for *In Vitro* Chondrogenesis of Two- and Three-Dimensional-Isolated and -Expanded Bone Marrow-Derived Mesenchymal Stromal Stem Cells Within a Porous Collagen Scaffold

Troy D. Bornes, MD, Nadr M. Jomha, MD, PhD, Aillette Mulet-Sierra, MSc, and Adetola B. Adesida, PhD

Divisions of Orthopaedic Surgery and Surgical Research, Department of Surgery, University of Alberta, Edmonton, Canada

Abstract

Bone marrow-derived mesenchymal stromal stem cells (BMSCs) are a promising cell source for treating articular cartilage defects. The objective of this study was to assess the impact of cell seeding density within a collagen I scaffold on *in vitro* BMSC chondrogenesis following isolation and expansion in two-dimensional (2D) and three-dimensional (3D) environments. It was hypothesized that both expansion protocols would produce BMSCs capable of hyaline-like chondrogenesis with an optimal seeding density of 10×10^6 cells/cm³. Ovine BMSCs were isolated in a 2D environment by plastic adherence, expanded to passage two in flasks containing an expansion medium, and seeded within collagen I scaffolds at densities of 50, 10, 5, 1, and 0.5×10^6 BMSCs/cm³. For 3D isolation and expansion, aspirates containing known quantities of mononucleated cells (bone marrow-derived mononucleated cells [BMNCs]) were seeded on scaffolds at 50, 10, 5, 1, and 0.5×10^6 BMNCs/cm³ and cultured in the expansion medium for an equivalent duration to 2D expansion. Constructs were differentiated *in vitro* in the chondrogenic medium for 21 days and assessed with reverse-transcription quantitative polymerase chain reaction, safranin O staining, histological scoring using the Bern Score, collagen immunofluorescence, and glycosaminoglycan (GAG) quantification. Two-dimensional-expanded BMSCs seeded at all densities were capable of proteoglycan production and displayed increased expressions of aggrecan and collagen II messenger RNA (mRNA) relative to pre-differentiation controls. Collagen II deposition was apparent in scaffolds seeded at 0.5 – 10×10^6 BMSCs/cm³. Chondrogenesis of 2D-expanded BMSCs was most pronounced in scaffolds seeded at 5 – 10×10^6 BMSCs/cm³ based on aggrecan and collagen II mRNA, safranin O staining, Bern Score, total GAG, and GAG/deoxyribonucleic acid (DNA). For 3D-expanded BMSC-seeded scaffolds, increased aggrecan and collagen II mRNA expressions relative to controls were noted with all densities. Proteoglycan deposition was present in scaffolds seeded at 0.5 – 50×10^6 BMNCs/cm³, while collagen II deposition occurred in scaffolds seeded at 10 – 50×10^6 BMNCs/cm³. The

Address correspondence to: Adetola B. Adesida, PhD, Divisions of Orthopaedic Surgery and Surgical Research, Department of Surgery, University of Alberta, 3-002E Li Ka Shing Centre for Health Research Innovation, 112th Street and 87th Avenue, Edmonton T6G 2E1, Alberta Canada, adesida@ualberta.ca.

Disclosure Statement

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highest levels of aggrecan and collagen II mRNA, Bern Score, total GAG, and GAG/DNA occurred with seeding at 50×10^6 BMNCs/cm³. Within a collagen I scaffold, 2D- and 3D-expanded BMSCs are capable of hyaline-like chondrogenesis with optimal cell seeding densities of $5\text{--}10 \times 10^6$ BMSCs/cm³ and 50×10^6 BMNCs/cm³, respectively.

Introduction

Bone marrow-derived mesenchymal stromal stem cells (BMSCs) are a promising cell source for treating articular cartilage defects.¹ BMSCs seeded within biomaterial scaffolds and implanted into focal chondral defects are capable of resurfacing cartilage in animal and human joints, although inconsistent outcomes have been reported based on macroscopic assessment, histological analysis, magnetic resonance imaging, and clinical scoring.^{2–7} Repair tissue quality has been shown to correlate with functional outcome.^{6–8} Therefore, tissue-engineering variables, such as cell expansion environment and seeding density of scaffolds, are currently under investigation with the goal of improving neocartilage quality.

BMSCs have conventionally been isolated by plastic adherence and expanded in a two-dimensional (2D) environment within tissue culture flasks.^{9,10} Although this method has been shown to produce cells capable of chondrogenic differentiation,^{11–14} major drawbacks include loss of multi-potent differentiation, inability to produce cartilaginous extracellular matrix (ECM) proteins and cellular senescence during prolonged expansion periods.^{15–18} Three-dimensional (3D) isolation and expansion of BMSCs have been proposed as a method of mimicking the natural bone marrow microenvironment and maintaining multipotency and chondrogenic capacity.^{19,20} Cell collections containing bone marrow-derived mononucleated cells (BMNCs)—a small fraction of which are BMSCs—are seeded within biomaterials for 3D isolation, expansion, and subsequent differentiation.^{20,21}

Cell seeding density is a transplantation variable that has not been evaluated in detail to date for either 2D- or 3D-expanded BMSCs. Healthy articular cartilage naturally contains 9.6×10^6 chondrocytes/cm³.²² The optimal BMSC seeding density required for cell organization, chondrogenic differentiation, and ECM production to create engineered tissue that resembles native cartilage is currently unknown. For implantation purposes *in vivo*, BMSC seeding densities of $1\text{--}50 \times 10^6$ cells/cm³ of biomaterial matrix have been used in preclinical animal studies.^{1,14,23–32} In clinical studies, a density of 5×10^6 cells/cm³ has been reported although the rationale for adopting this seeding density was not described.^{3,4}

The objective of this study was to assess the impact of cell seeding density within a clinically relevant, collagen I scaffold on *in vitro* BMSC chondrogenesis following 2D and 3D isolation and expansion. Two-dimensional isolation was performed by seeding whole bone marrow aspirates (BMAs) into tissue culture flasks, while 3D isolation involved seeding whole BMAs within collagen scaffolds. Collagen was used as a biomaterial given that it is used routinely in preclinical animal and human studies.^{3–5,14} Ovine cells were studied as sheep are emerging as a useful animal model for the study of cell transplantation techniques for cartilage repair.¹⁴ It was hypothesized that hyaline-like cartilage would be produced within collagen scaffolds by 2D- and 3D-expanded BMSCs with an optimal seeding density of 10×10^6 cells/cm³.

Materials and Methods

Bone marrow aspiration and BMNC counting

BMAs were obtained from the iliac crest of six female Suffolk sheep (mean age \pm standard error of the mean [SEM] of 3.3 ± 0.8 years) as previously described following ethical approval from the University of Alberta's Animal Care and Use Committee (Table 1).¹³ Staining with crystal violet (Sigma-Aldrich, Oakville, Canada) and hemocytometer counting were used to determine the number of BMNCs in each BMA.

Culture of 2D-expanded BMSCs

BMSCs were isolated in a 2D environment by plastic adherence from BMAs and expanded in tissue culture flasks to passage two (P2) within the expansion medium (Fig. 1). Expanded BMSCs were then seeded onto porous collagen scaffolds at 50, 10, 5, 1, or 0.5×10^6 BMSCs/cm³. Thereafter, BMSC-scaffold constructs were differentiated within the chondrogenic medium for 21 days.

Two-dimensional isolation and expansion involved seeding whole BMA collections containing 8×10^7 BMNCs within each 150 cm² tissue culture flask and submersing each BMA collection in the expansion medium containing the alpha-minimal essential medium (α -MEM; Corning-Mediatech, Manassas, VA) supplemented with 8.8% vol/vol heat-inactivated fetal bovine serum (FBS), 88.5 units/mL penicillin, 88.5 μ g/mL streptomycin, 258.4 μ g/mL L-glutamine, 8.8 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 885.0 μ M sodium pyruvate (all from Life Technologies, Burlington, Canada), and 5 ng/mL fibroblast growth factor-2 (FGF-2; Neuromics, Edina, MN).

Cells were allowed to adhere and grow statically for 7 days in a humidified incubator. The humidified incubator used throughout the entirety of the study for cell and tissue culture contained air with 3% O₂ and 5% CO₂ heated to 37°C. After 7 days, the media were changed twice per week. Once 80% cell confluence was attained, adherent BMSCs were washed with phosphate-buffered saline, detached using 0.05% wt/vol trypsin-ethylenediaminetetraacetic acid (EDTA; Corning-Mediatech), and expanded to P2.

For chondrogenic differentiation, 2D-expanded BMSCs were suspended in the chondrogenic medium consisting of Dulbecco's modified Eagle's medium containing 4.5 mg/mL D-glucose, 110 μ g/mL sodium pyruvate and L-glutamine (Sigma-Aldrich) supplemented with 9.6 mM HEPES, 95.6 units/mL penicillin, 95.6 μ g/mL streptomycin, 279.2 μ g/mL L-glutamine (all from Life Technologies), 1 \times insulin-transferrin-selenium (ITS)+ premix (BD Biosciences, Mississauga, Canada), 365 μ g/mL ascorbic acid 2-phosphate, 40 μ g/mL L-proline, 100 nM dexamethasone, 125 μ g/mL human serum albumin (all from Sigma-Aldrich), and 10 ng/mL transforming growth factor-beta three (TGF- β 3; ProSpec, East Brunswick, NJ).

Cylindrical collagen scaffolds (3.5 mm thickness; 6 mm diameter) were created using a biopsy punch on sheets of type I collagen sponge ($125 \times 100 \times 3.5$ mm³ dimension; 115 ± 20 μ m pore size; Integra LifeSciences, Plainsboro, NJ). Cell counts were calculated using trypan blue staining and hemocytometer counting, and BMSCs were micro-pipetted onto

each scaffold within a 20 μL chondrogenic medium suspension. Scaffold seeding was performed at five BMSC densities (Table 2). Seeded scaffolds were incubated for 15 min followed by addition of 100 μL of chondrogenic medium to the base of each scaffold. Thereafter, constructs were incubated for an additional 30 min to promote cell adhesion and then submersed in 1 mL of chondrogenic medium. Constructs were cultured statically for 21 days within a humidified incubator. Media were changed twice per week.

Culture of 3D-expanded BMSCs

BMAs containing known numbers of BMNCs were seeded onto collagen scaffolds at densities of 50, 10, 5, 1, or 0.5×10^6 BMNCs/cm³ and cultured in the expansion medium to foster 3D isolation and expansion of BMSCs. Following the expansion period, all constructs were differentiated within the chondrogenic medium for 21 days (Fig. 1).

For 3D isolation and expansion, scaffolds were prepared in an identical manner to the protocol used for seeding of 2D-expanded BMSCs. During seeding, BMAs containing known numbers of BMNCs were centrifuged and suspended within the expansion medium to create 20 μL seeding collections that were micropipetted onto scaffolds. Scaffold seeding was performed at five BMNC densities (Table 2).

Seeded scaffolds were incubated for 15 min followed by addition of 100 μL of expansion medium to the base of each scaffold. Constructs were incubated for an additional 30 min to promote cell adhesion and were subsequently immersed in 1 mL of expansion medium and cultured within a humidified incubator. In an identical manner to the 2D expansion group, BMA-seeded scaffolds were cultured statically for 7 days undisturbed. Media were changed twice weekly thereafter. For each donor, 3D expansion was carried out for the equivalent amount of time as 2D expansion.

To induce chondrogenic differentiation, the medium used for culture of 3D-expanded BMSC-seeded scaffolds was switched from 1 mL of expansion medium to 1 mL of chondrogenic medium at the same time point as chondrogenic culture commenced for 2D-expanded BMSC-seeded scaffolds. Constructs were cultured statically for 21 days within a humidified incubator. Media were changed twice per week.

Cell count and population doubling during isolation and expansion

A colony-forming unit fibroblastic (CFU-F) assay of P0 BMSCs was performed as previously described.¹³ Each colony was considered to have derived from a single isolated BMSC, which enabled calculation of the number of BMSCs isolated from 1×10^5 BMNCs that were seeded. The ratio of isolated BMSCs to seeded BMNCs was used to calculate the number of BMSCs arising from 8×10^7 BMNCs plated in each tissue culture flask at P0. At the end of P0, P1, and P2, cell counts of BMSCs were determined using trypan blue staining and hemocytometer counting.

In the 3D expansion group, crystal violet staining and hemocytometer counting were used to calculate the quantity of BMNCs per volume of BMA. Known numbers of BMNCs were seeded within BMA collections onto scaffolds before expansion. The number of seeded BMSCs was estimated using the BMSC-to-BMNC ratio calculated from the CFU-F assay.

Postexpansion cell counts were calculated from a standard curve (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tec) based on deoxyribonucleic acid (DNA) content derived from a Cy-QUANT Cell Proliferation Assay (Life Technologies). Population doublings were determined using the method described by Solchaga *et al.*³³

Trilineage differentiation potential of BMSCs

To confirm the presence of cells with properties of mesenchymal stem cells within the BMAs, plastic adherent P2 BMSCs were cultured in osteogenic, adipogenic, and chondrogenic media and trilineage differentiation potential was demonstrated as previously described.¹³

Assessment of chondrogenesis

Tissue-engineered constructs were assessed with reverse-transcription quantitative polymerase chain reaction for gene expression (primers are shown in Table 3), safranin O staining of ECM proteoglycans, and biochemical quantification of glycosaminoglycan (GAG) and DNA as described previously.¹³ Histological scoring was also performed, and depositions of collagens I and II were assessed with immunofluorescence.

Histological scoring using the Bern Score

Constructs containing BMSCs from five donors were fixed in 10% wt/vol buffered formalin, processed into paraffin wax, and sectioned at a thickness of 5 μm . Sections were stained with safranin O and fast green, as previously described,¹³ and graded using the Bern Score based on ECM staining, intercellular distance and ECM accumulation, and cell morphology.³⁴ Each category was scored out of three with a combined maximal Bern Score of nine. Nonhomogeneous constructs were evaluated through assessing distinct regions and combining scores based on the percentage area represented as described by Grogan *et al.*³⁴

Immunofluorescence

Sections with a thickness of 5 μm were treated with Protease XXV (Fisher Scientific, Ottawa, Canada) and hyaluronidase (Sigma-Aldrich) and incubated with rabbit anti-collagen I (NBP1-30054, 1:250 dilution; Novus Biologicals, Oakville, Canada) and mouse anti-collagen II (II-II6B3, 1:200 dilution; Developmental Studies Hybridoma Bank, Iowa City, IA). Immune localized antigens were visualized with Texas Red anti-rabbit IgG (Vector Laboratories, Burlington, Canada) and fluorescein isothiocyanate (FITC) goat anti-mouse Ig (BD Biosciences). Sections were mounted using the Biotium EverBrite Mounting Medium with DAPI (4',6-diamidino-2-phenylindole; Cedarlane Laboratories, Burlington, Canada).

Images were captured using an Eclipse Ti-S microscope (Nikon Canada, Mississauga, Canada) fitted with NIS Elements (version 4.20; Nikon Canada) and assembled in Photoshop (Adobe Systems, San Jose, CA).

Statistical analysis

Analyses were performed using SPSS Statistics 22 (IBM, Armonk, NY) and significance was concluded when $p < 0.05$.

Repeated measures analysis of variance (ANOVA) was used for assessment of cell count and doubling over time during expansion and differentiation. For assessment of cell count over time within specific seeding density groups, a paired *t*-test was used. When different constructs were used at different time points, as in the analysis of peri-differentiation cell count of 3D-expanded BMSC-seeded scaffolds, a *t*-test was used given that pairing was not possible. Comparison of gene expressions, biochemical quantities, cell counts, and histological scores following chondrogenic differentiation between seeding groups required a Kruskal–Wallis one-way ANOVA with pairwise comparisons. A Mann–Whitney *U* test was used to compare predifferentiation to post-differentiation gene expressions.

Results

Isolation and expansion within 2D and 3D environments

The duration of time from plating of BMNCs to reach 80% confluence at P2 during 2D expansion was 23.8 ± 0.8 days. The mean number (\pm SEM) of BMSCs present per flask at the completion of P0 was $7.9 \pm 1.4 \times 10^6$ and this number increased to $16.1 \pm 1.6 \times 10^6$ and $27.6 \pm 4.4 \times 10^6$ at the completion of P1 and P2, respectively ($p < 0.001$; Supplementary Fig. S2). BMSC count following 2D expansion was $34.2\% \pm 4.5\%$ of the BMNC count initially seeded. There were 12.3 ± 1.0 cumulative BMSC population doublings during expansion. Based on the CFU-F assay, 18.0 ± 8.5 BMSCs were isolated from 1×10^5 seeded BMNCs. Therefore, $0.018\% \pm 0.009\%$ of all seeded BMNCs was BMSCs.

Following 3D expansion, the number of BMSCs present was significantly lower than the initial number of BMNCs seeded in scaffolds at densities of 50, 10, and 5×10^6 BMNCs/cm³ ($p < 0.001$), while there was not a significant difference noted for densities of 1 and 0.5×10^6 BMNCs/cm³ ($p = 0.87$ and 1.0 , respectively; Supplementary Fig. S2). For seeding densities of 50, 10, 5, 1, and 0.5 BMNCs/cm³, BMSC counts following 3D expansion were 0.29 ± 0.05 , 0.15 ± 0.06 , 0.12 ± 0.05 , 0.09 ± 0.06 , and $0.05 \pm 0.05 \times 10^6$ BMSCs. These BMSC counts equated to $5.8\% \pm 0.9\%$, $14.9\% \pm 6.2\%$, $24.2\% \pm 9.9\%$, $89.5\% \pm 59.2\%$, and $100.3\% \pm 92.2\%$ of initial BMNC counts, respectively.

Following expansion, cell densities were calculated to be 2.9 ± 0.5 , 1.5 ± 0.6 , 1.2 ± 0.5 , 0.9 ± 0.6 , and 0.5 ± 0.5 BMSCs/cm³, respectively. Cumulative BMSC population doublings were 9.3 ± 1.1 , 9.7 ± 1.4 , 10.2 ± 1.2 , 11.1 ± 1.2 , and 10.7 ± 1.2 , respectively.

Trilineage differentiation of BMSCs

Spindle-shaped BMSCs demonstrated plastic adherence and were differentiated into cells capable of producing cartilaginous proteoglycans, adipose droplets, and calcified bone matrix (Supplementary Fig. S2).

Seeding density of 2D- and 3D-expanded BMSCs affected chondrogenic gene expression

Constructs containing 2D- and 3D-expanded BMSCs had varying levels of cartilage-related genes that were dependent on seeding density following chondrogenic culture. In 2D-expanded BMSC-seeded scaffolds, messenger RNA (mRNA) expressions of hyaline

cartilage-related collagen II and aggrecan were highest in scaffolds seeded at $5\text{--}10 \times 10^6$ BMSCs/cm³ (Fig. 2A, E).

For aggrecan mRNA expression, constructs containing 10 and 5×10^6 BMSCs/cm³ had significantly enhanced aggrecan mRNA expression in comparison to scaffolds seeded with more cells (50×10^6 BMSCs/cm³; $p < 0.001$) and less cells (0.5×10^6 BMSCs/cm³; $p < 0.01$; Fig. 2A). Seeding at 5×10^6 BMSCs/cm³ also resulted in improved aggrecan mRNA expression relative to 1×10^6 BMSCs/cm³ ($p < 0.01$).

Densities of 10 and 5×10^6 BMSCs/cm³ corresponded to a greater collagen II mRNA expression than densities of 50, 1, and 0.5×10^6 BMSCs/cm³ ($p < 0.01$; Fig. 2E). Ratios of collagen II/I and collagen II/X also followed this pattern ($p < 0.05$). Expression of transcription factor sex determining region Y (SRY)-box 9 (SOX9) was not different between scaffolds seeded at 50, 10, and 5×10^6 BMSCs/cm³ ($p = 0.29$), but these densities led to increased expression relative to 0.5×10^6 BMSCs/cm³ ($p < 0.05$; Fig. 2I).

With respect to fibrocartilage-related collagen I mRNA expression, constructs seeded at 5, 1, and 0.5×10^6 BMSCs/cm³ had a greater collagen I mRNA expression than 50×10^6 BMSCs/cm³ ($p < 0.05$; Fig. 2C). Expression of hypertrophic cartilage-associated collagen X was greater in constructs containing 10 and 5×10^6 BMSCs/cm³ than those containing 1 and 0.5×10^6 BMSCs/cm³ ($p < 0.05$; Fig. 2G).

In 3D-expanded BMSC-seeded scaffolds, aggrecan and collagen II mRNA expressions were highest in scaffolds seeded with BMA collections containing 50×10^6 BMNCs/cm³ (Fig. 2B, F). A significantly higher aggrecan mRNA expression was found in scaffolds seeded at 50, 10, and 5×10^6 BMNCs/cm³ than 0.5×10^6 BMNCs/cm³ ($p < 0.05$), although the difference was most significant between 50 and 0.5×10^6 BMNCs/cm³ ($p < 0.01$; Fig. 2B). Hyaline cartilage-related collagen II mRNA expression was significantly higher in scaffolds seeded at 50×10^6 BMNCs/cm³ than 5, 1, or 0.5×10^6 BMNCs/cm³ ($p < 0.05$) and approached a significantly higher level in scaffolds seeded at 50×10^6 BMNCs/cm³ relative to 10×10^6 BMNCs/cm³ ($p = 0.07$; Fig. 2F). Postdifferentiation collagen II mRNA was significantly greater than predifferentiation collagen II mRNA in all seeding density groups ($p < 0.05$).

For fibrocartilage-related collagen I, mRNA expression was more pronounced in constructs containing 50×10^6 BMNCs/cm³ than 1 or 0.5×10^6 BMNCs/cm³ ($p < 0.05$; Fig. 2D). Significant differences were not noted between seeding density groups for collagen X and SOX9 mRNA expressions ($p = 0.13$ and 0.25 ; respectively; Fig. 2H, J). The collagen II/X ratio was higher in scaffolds seeded at 50×10^6 BMNCs/cm³ than 10, 1, and 0.5×10^6 BMNCs/cm³ ($p < 0.05$). The collagen II/I ratio was higher in scaffolds seeded at 0.5×10^6 BMNCs/cm³ than 50 and 5×10^6 BMNCs/cm³ ($p < 0.05$).

To determine whether BMNC seeding density during 3D isolation and expansion primed chondrogenic gene expression before onset of differentiation, 3D-expanded BMSC-seeded scaffolds were also assessed for gene expression directly following expansion. Seeding density did not significantly impact mRNA expressions of aggrecan, collagen I, collagen II, collagen X, or SOX9 ($p = 0.82, 0.09, 0.64, 0.40, 0.40$, respectively; Fig. 2 right column).

Seeding density of 2D- and 3D-expanded BMSCs affected chondrogenic ECM deposition

In 2D-expanded BMSC-seeded scaffolds, safranin O staining of proteoglycans appeared to be most pronounced in scaffolds seeded at 10 and 5×10^6 BMSCs/cm³, although abundant staining was also apparent in scaffolds seeded at other densities (Fig. 3A and Supplementary Fig. S3). Accordingly, Bern Scores were highest in scaffolds seeded at 10 and 5×10^6 BMSCs/cm³ (7.8 ± 0.4 and 7.7 ± 0.3 , respectively; Fig. 3C and Supplementary Fig. S4). Scores for these densities were significantly larger than those for 50 and 0.5×10^6 BMSCs/cm³ ($p < 0.05$).

Analysis of proteoglycan quantity demonstrated that total GAG, GAG/DNA, and GAG/cell were highest in scaffolds seeded at 10 and 5×10^6 BMSCs/cm³ (Fig. 4A, E, G). There was not a significant difference between 10 and 5×10^6 BMSCs/cm³ for these parameters ($p = 0.72$, 0.18 , and 0.21 , respectively). Constructs containing 5×10^6 BMSCs/cm³ had significantly higher GAG/DNA than constructs containing 50, 1, and 0.5×10^6 BMSCs/cm³ ($p < 0.01$; Fig. 4E). Constructs containing 10×10^6 BMSCs/cm³ had significantly higher GAG/DNA than scaffolds seeded with 50 and 0.5×10^6 BMSCs/cm³ ($p < 0.01$), but were marginally different than 1×10^6 BMSCs/cm³ ($p = 0.50$; Fig. 4E).

Collagen II deposition was pronounced in scaffolds seeded at 10, 5, 1, and 0.5×10^6 BMSCs/cm³, while scaffolds seeded at all densities appeared to lack abundant collagen I deposition (Fig. 5A; see Supplementary Fig. S5 for controls).

During chondrogenic differentiation of 2D-expanded BMSCs, cell counts decreased in scaffolds that were seeded at densities of 50 and 10×10^6 BMSCs/cm³ ($p < 0.05$), while cell counts increased in scaffolds that were seeded at densities of 5, 1, and 0.5×10^6 BMSCs/cm³ ($p < 0.05$; Fig. 6A). At the end of the differentiation period, cell counts were not significantly different between scaffolds seeded at 10 and 5×10^6 BMSCs/cm³ ($p = 0.19$). There was also not a significant difference between 1 and 0.5×10^6 BMSCs/cm³ ($p = 0.27$). Cell counts between other seeding groups were notably different ($p < 0.05$).

Scaffolds seeded with 3D-expanded BMSCs contained cartilaginous proteoglycans following differentiation. Proteoglycan staining appeared to be most pronounced in scaffolds seeded at 50, 10, and 5×10^6 BMNCs/cm³ (Fig. 3B), although variability was noted between donors, and cells from one donor (Z33) produced large depositions of proteoglycans at 1×10^6 BMNCs/cm³ (Supplementary Fig. S3). Bern Scores were highest in scaffolds seeded at 50×10^6 BMNCs/cm³ (7.2 ± 0.5 ; Fig. 3). However, a significant difference in scores between seeding densities was not present based on Kruskal–Wallis ANOVA ($p = 0.16$).

Quantitative analysis of proteoglycan deposition demonstrated that seeding at 50×10^6 BMNCs/cm³ led to higher total GAG, GAG/DNA, and GAG/cell than scaffolds seeding at 1×10^6 BMNCs/cm³ ($p < 0.01$) and 0.5×10^6 BMNCs/cm³ ($p < 0.001$; Fig. 4B, F, H). For these parameters, less significant differences were noted between 10×10^6 BMNCs/cm³ and lower densities of 1×10^6 BMNCs/cm³ ($p < 0.05$) and 0.5×10^6 BMNCs/cm³ ($p < 0.01$). Scaffolds seeded at 5×10^6 BMNCs/cm³ also had higher total GAG, GAG/DNA, and GAG/cell than scaffolds seeded at 0.5×10^6 BMNCs/cm³ ($p < 0.05$). Immunofluorescence

indicated deposition of both collagens I and II, particularly in scaffolds seeded at 50 and 10×10^6 BMNCs/cm³ (Fig. 5B).

Following chondrogenic culture of 3D-expanded BMSC-seeded scaffolds, cell counts were not significantly different between scaffolds seeded at 50 and 10×10^6 BMNCs/cm³, or between scaffolds seeded at 1 and 0.5×10^6 BMNCs/cm³ ($p = 0.22$; Fig. 6B). Seeding at 1 and 0.5×10^6 BMNCs/cm³ resulted in postdifferentiation BMSC counts of 0.21 ± 0.11 and $0.06 \pm 0.03 \times 10^6$. Considerable variation in BMSC counts between donors was noted at these lowest BMNC seeding densities as some scaffolds appeared to have very low cell numbers present following differentiation, while others contained sufficient cell numbers to produce significant levels of cartilaginous ECM (Supplementary Fig. S3 and Fig. 5B).

Discussion

The major finding of this study is that isolation and expansion of ovine BMSCs under 2D and 3D conditions yielded cell populations capable of producing hyaline-like neocartilage *in vitro*, and chondrogenic differentiation of these cells was dependent on seeding density within collagen I scaffolds. Optimal seeding densities were identified for 2D- and 3D-expanded BMSCs.

Two-dimensional isolation and expansion were performed using a conventional protocol within tissue culture flasks.^{9,10} Whole BMA was seeded into flasks containing the expansion medium in an attempt to maintain the natural bone marrow microenvironment on the 2D surface of the tissue culture flask during isolation and the first week of expansion,^{11,13,35} while avoiding the loss of cells and mediators that could be discarded through the use of cell separation techniques.^{9,10} An extended expansion period beyond P2 was not used in this study as it was previously shown that prolonged expansion leads to dedifferentiation, senescence, and loss of multipotential capacity of BMSCs.^{17,18}

Three-dimensional isolation and expansion were performed by seeding BMA collections containing defined numbers of BMNCs within scaffolds followed by culture in the expansion medium. The goal of this technique was to maintain BMSCs within a 3D environment and to avoid subjecting cells to the artificial environment within tissue culture flasks. Presumably, BMSCs adhered to the scaffold through integrin-mediated binding to type I collagen,³⁶ while other cellular and chemical components of bone marrow³⁷⁻⁴⁰ were entrapped within scaffold pores but washed away over the course of the expansion period with media changes. BMA collections were seeded within scaffolds to create seeding densities of 50, 10, 5, 1, and 0.5×10^6 BMNCs/cm³.

The total number of BMSCs present following isolation and expansion was 5.8% of the initial BMNC count in the scaffolds seeded at 50×10^6 BMNCs/cm³. The percentage of postexpansion BMSCs increased as BMNC seeding density decreased and was measured to be 100.3% in scaffolds seeded at the lowest density of 0.5×10^6 BMNCs/cm³. Increased proliferation may have played a role in this effect, as population doublings were higher in constructs seeded at lower densities. Augmented proliferation at lower density was reported previously during both 2D and 3D expansion,⁴¹⁻⁴⁴ and could involve reduced contact

inhibition and increased nutrient availability per cell.⁴⁵ Higher probability of attachment at the time of seeding due to a relatively increased availability of scaffold binding sites could also increase BMSC count at lower BMNC seeding densities.

The impact of seeding density on chondrogenesis was evaluated after 21 days of *in vitro* culture. To date, multiple parameters have been used in the literature to assess hyaline-like chondrogenic capacity, including proteoglycan content, histological scoring, GAG/DNA, collagen II quantity, and collagen II/I ratio.^{9,10,12,13,33,34} These parameters involve either gene expression or measurement of macromolecule deposition in the ECM.

A number of assays were used in the study at hand to provide a rigorous and multimodal evaluation of engineered tissue. Neocartilage was considered hyaline like if (1) collagen II was present based on collagen II mRNA expression, collagen II/I mRNA ratio and collagen II immunofluorescence; and (2) proteoglycan was abundant as measured through aggrecan mRNA expression, safranin O staining, Bern Score, total GAG, and GAG/DNA. Neocartilage was considered to be superior if these parameters were more pronounced in one construct relative to another construct.

Following 2D expansion, BMSCs seeded at all densities were shown to be capable of hyaline-like cartilaginous tissue formation based on increased collagen II and aggrecan mRNA expressions relative to controls, and proteoglycan deposition. Collagen scaffolds seeded at 10 and 5×10^6 BMSCs/cm³ had the highest mRNA expressions of hyaline cartilage-related collagen II, aggrecan, and SOX9, although expressions of collagens I and X that relate to fibrocartilage and hypertrophic cartilage, respectively, were also highest in these constructs.

Scaffolds seeded at 10 and 5×10^6 BMSCs/cm³ produced the highest levels of ECM proteoglycan deposition and were found to be superior based on histological scoring. Collagen II deposition was apparent in scaffolds seeded at $0.5\text{--}10 \times 10^6$ BMSCs/cm³, while collagen I was lacking. Constructs containing 50×10^6 BMSCs/cm³ at the time of seeding displayed stunted chondrogenesis in comparison to 10 and 5×10^6 BMSCs/cm³, which could relate to cell crowding, nutrient limitation, and relatively less volume available for ECM deposition.^{45,46} These findings suggest that the optimal seeding density for chondrogenesis of 2D-expanded BMSCs within collagen scaffolds is $5\text{--}10 \times 10^6$ BMSCs/cm³.

In 3D-expanded BMSC-seeded scaffolds, constructs seeded at all densities produced increased collagen II and aggrecan mRNA expressions relative to controls, and proteoglycan deposition. Constructs seeded at 50×10^6 BMNCs/cm³ had the highest levels of aggrecan mRNA expression and ECM proteoglycan deposition, although scaffolds seeded at 10 and 5×10^6 BMNCs/cm³ also had higher levels of proteoglycans in comparison to lower seeding densities. The Bern Score was highest in scaffolds seeded at 50×10^6 BMNCs/cm³, but a significant difference was not demonstrated between seeding groups. Collagen II mRNA expression was highest in scaffolds seeded at 50×10^6 BMNCs/cm³.

Based on immunofluorescence, deposition of collagen II was particularly notable within scaffolds seeded at both 50 and 10×10^6 BMNCs/cm³. Collagen I mRNA expressions was

highest in scaffolds seeded at 50 , 10 , and 5×10^6 BMNCs/cm³, while immunofluorescence showed significant deposition in scaffolds seeded at 50 and 10×10^6 BMNCs/cm³. The results of this study suggest that a seeding density of 50×10^6 BMNCs/cm³ leads to the highest level of hyaline-like chondrogenesis, although it is possible that higher densities that were not assessed in this study could further augment chondrogenesis.

At present, there is a paucity of literature examining the impact of BMSC seeding density on chondrogenesis. Several *in vitro* studies have assessed the impact of mature chondrocyte density on chondrogenesis. Hansen *et al.* demonstrated that seeding at 1.2×10^6 chondrocytes/cm³ augmented chondrogenesis within a porous methoxypolyethylene glycol-poly(lactic-co-glycolic acid) scaffold relative to higher densities of 4 – 20×10^6 chondrocytes/cm³.³²

Mahmoudifar and Doran and Vunjak-Novakovic *et al.* showed superior chondrogenesis within polyglycolic acid scaffolds seeded with 25 – 27×10^6 chondrocytes/cm³ in comparison to lower densities of 5 – 15×10^6 chondrocytes/cm³.^{47,48} Francioli *et al.* reported that chondrogenesis improved by increasing cell density within porous collagen II scaffolds from 25 to 66×10^6 chondrocytes/cm³.⁴⁹ In agarose hydrogels, Huang *et al.* demonstrated that a density of 9×10^6 chondrocytes/cm³ was superior to 3 – 6×10^6 chondrocytes/cm³, while Mauck *et al.* found that 60×10^6 chondrocytes/cm³ augmented chondrogenesis relative to 20×10^6 chondrocytes/cm³.^{50,51}

Cell density analysis in this study was based on the number of cells and volume of scaffold present during seeding, as this is the time point at which these parameters would be known in a clinical setting.⁴ It was demonstrated that cell count following differentiation varied in comparison to predifferentiation cell count, and this could have involved a balance between multiple factors, including cell loss during scaffold seeding and culture, and ongoing proliferation during the differentiation period.⁵² With the assumption that construct volume was maintained during chondrogenesis, final cell densities at the end of *in vitro* chondrogenesis were 22.3 , 8.4 , 6.5 , 3.4 , and 2.4×10^6 BMSCs/cm³ for 2D-expanded BMSCs seeded at 50 , 10 , 5 , 1 , and 0.5×10^6 BMSCs/cm³, respectively, and 7.1 , 5.6 , 4.8 , 2.1 , and 0.6×10^6 BMSCs/cm³ for 3D-expanded BMSCs seeded at 50 , 10 , 5 , 1 , and 0.5×10^6 BMNCs/cm³, respectively.

Cell-seeded collagen scaffolds have been shown previously to decrease in volume during chondrogenesis as a result of cell-mediated contraction and degradation of scaffold biomaterials.⁵³ Therefore, cell densities at the end of chondrogenic culture were potentially higher than calculated.

Although both 2D- and 3D-expanded BMSC-seeded collagen scaffolds were assessed and shown to produce bioengineered tissue with properties of hyaline cartilage, a direct comparison of these methods was not performed given that the properties of the cells populating scaffolds at the time of isolation and following culture may have differed. Two-dimensional-expanded BMSCs were shown to have qualities of plastic adherence and trilineage differentiation. Previously, our group characterized cell surface markers of BMSCs isolated and expanded using this isolation method.¹¹

Although 3D-expanded BMSCs were derived from the same BMA collections as 2D-expanded BMSCs, the cells that adhered to the collagen scaffold during 3D isolation were not characterized and may have derived from a different subpopulation of BMSCs than those that adhered to plastic during 2D isolation. It was shown previously that different subpopulations of BMSCs are present based on adherence and cell surface markers.^{20,54} Future study is required to characterize cells derived from 3D isolation and to compare these cells to those derived from 2D methods utilizing whole BMA and cell fractions (BMNCs).

This study has some limitations. Static culture conditions were used, which may have led to less uniform densities throughout scaffolds relative to dynamic conditions.

Second, ovine rather than human BMSCs were used. Sheep are routinely involved as animal models in cartilage engineering studies.^{14,55,56} However, differences between species could impact the clinical applicability of our findings. Aspirates were derived from sheep at an age that is consistent with skeletal maturity and cartilage maturity based on zonal architecture, presence of a continuous calcified cartilage layer, and lack of spontaneous cartilage repair.⁵⁷ Given that sheep have a life expectancy of 10–12 years, the sheep in this study (mean age of 3.3 years) could correspond to an adult human population that would be amenable to cell-based cartilage restoration techniques.

Only female sheep were included as donors. A difference in *in vitro* chondrogenesis between cells from male and female animals and humans has been demonstrated previously with a higher chondrogenic capacity demonstrated in males.^{58,59} Although the use of female sheep potentially reduced variability that may have been caused through the use of a mixed sample of animals, the impact of seeding density on chondrogenesis could be different in male animals.

Third, an *in vitro* model was used, and the applicability of the conclusions to clinical use is unclear. It is possible that remodeling that occurs *in vivo* over time could offset differences that are apparent within this *in vitro* model.^{32,60}

Fourth, seeding of cells within biomaterial scaffolds was considered to be an effective process in this study and the number of cells that did not adhere was not determined.

Finally, during the calculation of population doublings during 3D expansion, the initial number of BMSCs present following isolation required the use of a ratio derived from the CFU-F assay. Given that the CFU-F involved plastic adherence, it is possible that the fraction of BMSCs present from the BMNC pool following 3D isolation was different than that calculated from the CFU-F assay.

In conclusion, BMSCs isolated and expanded in 2D and 3D environments are capable of producing hyaline-like cartilaginous tissue within a porous collagen I scaffold. Chondrogenesis appeared to be most pronounced with seeding densities of $5\text{--}10 \times 10^6$ BMSCs/cm³ and 50×10^6 BMNCs/cm³ for 2D and 3D expansion protocols, respectively. Accordingly, these densities could be considered when seeding collagen I scaffolds in BMSC transplantation protocols.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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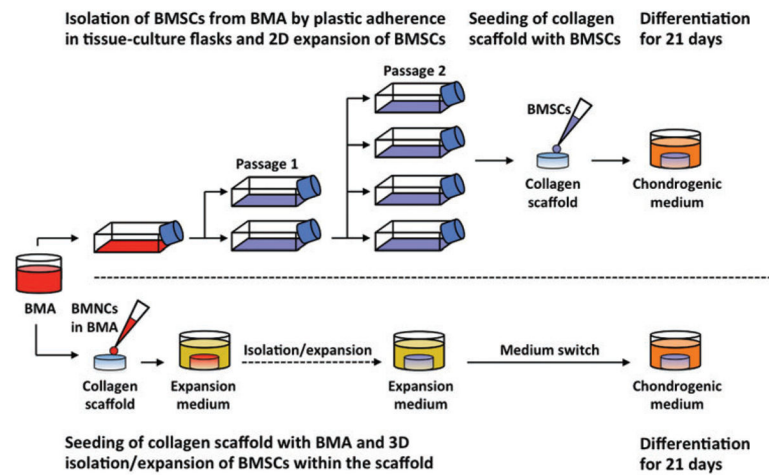
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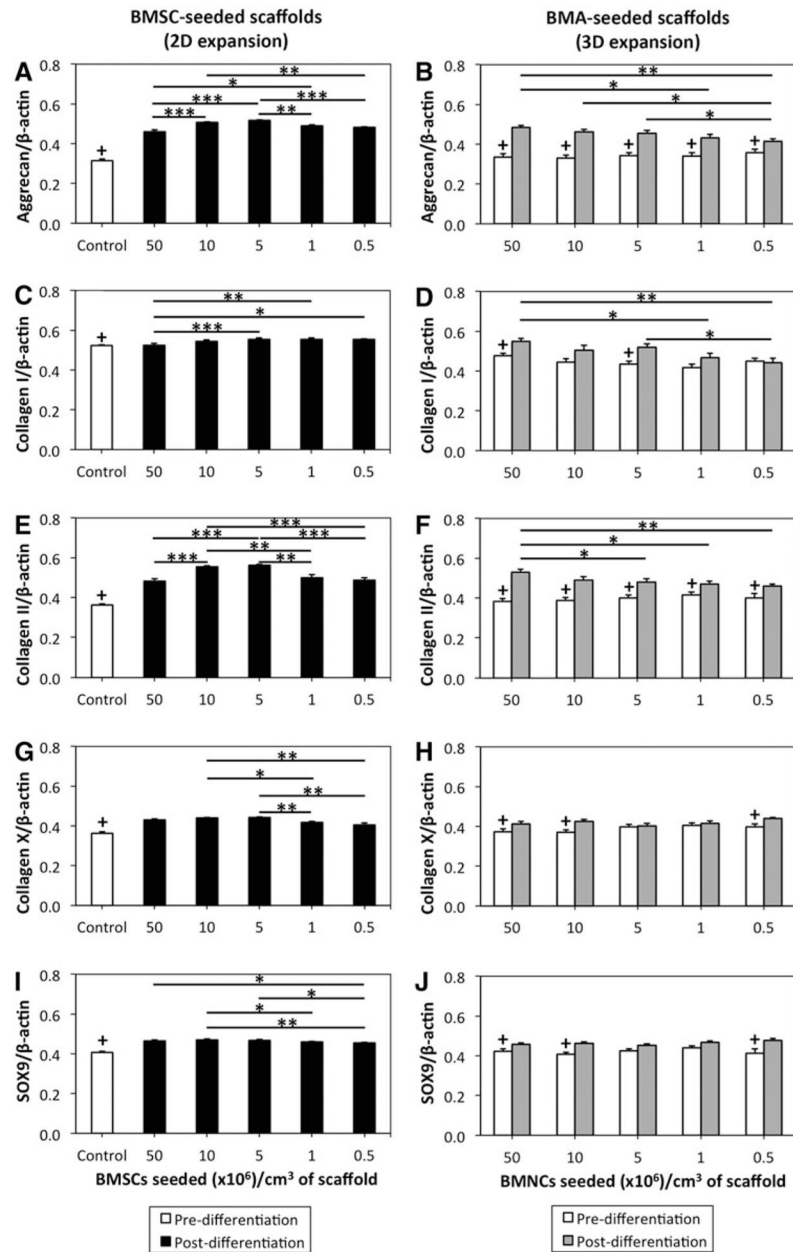
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**FIG. 1.**

In vitro cartilage engineering from two-dimensional (2D)-expanded and three-dimensional (3D)-expanded bone marrow-derived mesenchymal stromal stem cells (BMSCs). BMSCs were isolated in a 2D environment by plastic adherence from bone marrow aspirates (BMAs) and expanded in tissue culture flasks to passage two (P2) within expansion medium. BMSCs were then seeded onto porous collagen scaffolds at $50, 10, 5, 1, \text{ or } 0.5 \times 10^6$ BMSCs/cm³. For 3D isolation and expansion, BMAs containing known numbers of bone marrow-derived mononucleated cells (BMNCs) were seeded onto porous collagen scaffolds at densities of $50, 10, 5, 1, \text{ or } 0.5 \times 10^6$ BMNCs/cm³ and cultured in expansion medium. Thereafter, all constructs were differentiated within chondrogenic medium for 21 days. Color images available online at www.liebertpub.com/tec

**FIG. 2.**

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) for gene expression analysis of 2D- and 3D-expanded BMSCs seeded within collagen scaffolds. BMSCs were isolated and expanded within 2D and 3D environments, differentiated within collagen scaffolds for 21 days and assessed with RT-qPCR using SYBR Green detection. (A, C, E, G, I) Data represent the mean \pm standard error of the mean (SEM) of 2D-expanded BMSCs in doublets based on seeding density group. (B, D, F, H, J) Data represent the mean \pm SEM of 3D-expanded BMSCs in doublets based on seeding density group. Statistical analysis is represented by unlabeled, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$;

[†]predifferentiation control value that is significantly different than differentiated BMSC-seeded scaffold ($p < 0.05$).

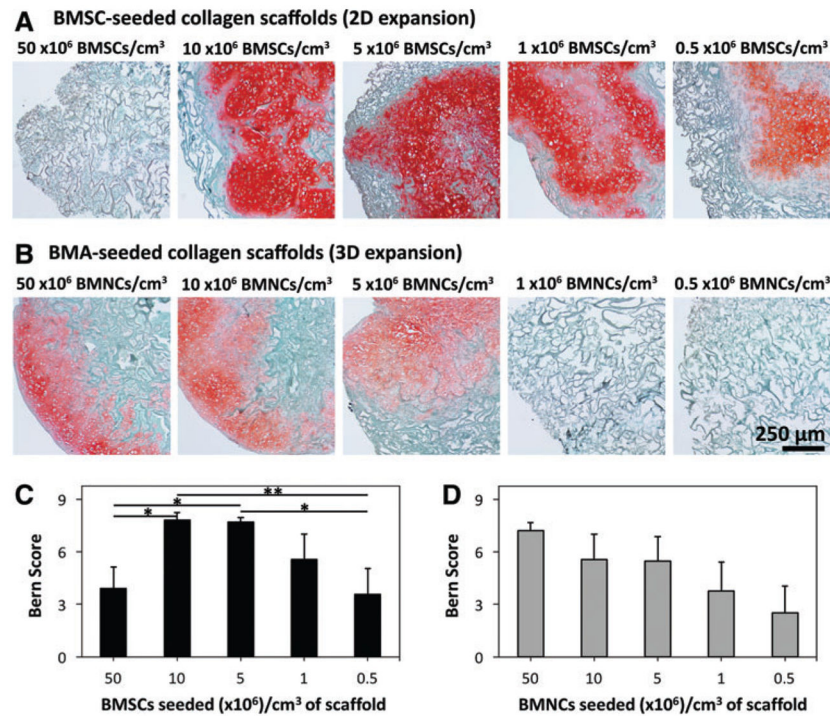


FIG. 3. Histological analysis and scoring of 2D- and 3D-expanded BMSC-seeded collagen scaffolds. BMSCs were isolated and expanded within 2D and 3D environments, and differentiated within collagen scaffolds for 21 days. Thereafter, constructs were fixed, sectioned at 5 μ m thickness, and stained with safranin O and fast green. Presented photomicrographs represent cell–scaffold constructs derived from (A) 2D-expanded BMSCs seeded at 50, 10, 5, 1, or 0.5 $\times 10^6$ BMSCs/cm³, and (B) 3D-expanded BMSCs seeded at 50, 10, 5, 1, or 0.5 $\times 10^6$ BMNCs/cm³ (cells from donor Z28; 10 \times magnification). (C) Mean (\pm SEM) Bern Score of 2D-expanded BMSCs from five donors based on seeding density group. (D) Mean (\pm SEM) Bern Score of 3D-expanded BMSCs from five donors based on seeding density group. Statistical analysis is represented by unlabeled, not significant; * $p < 0.05$; ** $p < 0.01$. Color images available online at www.liebertpub.com/tec

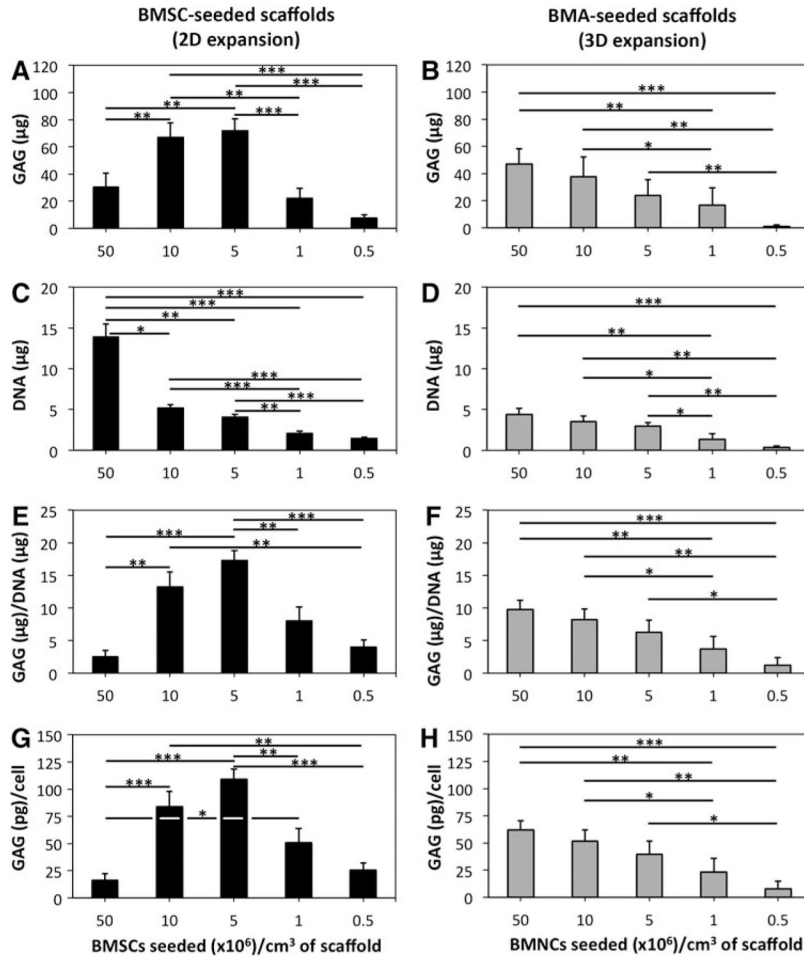
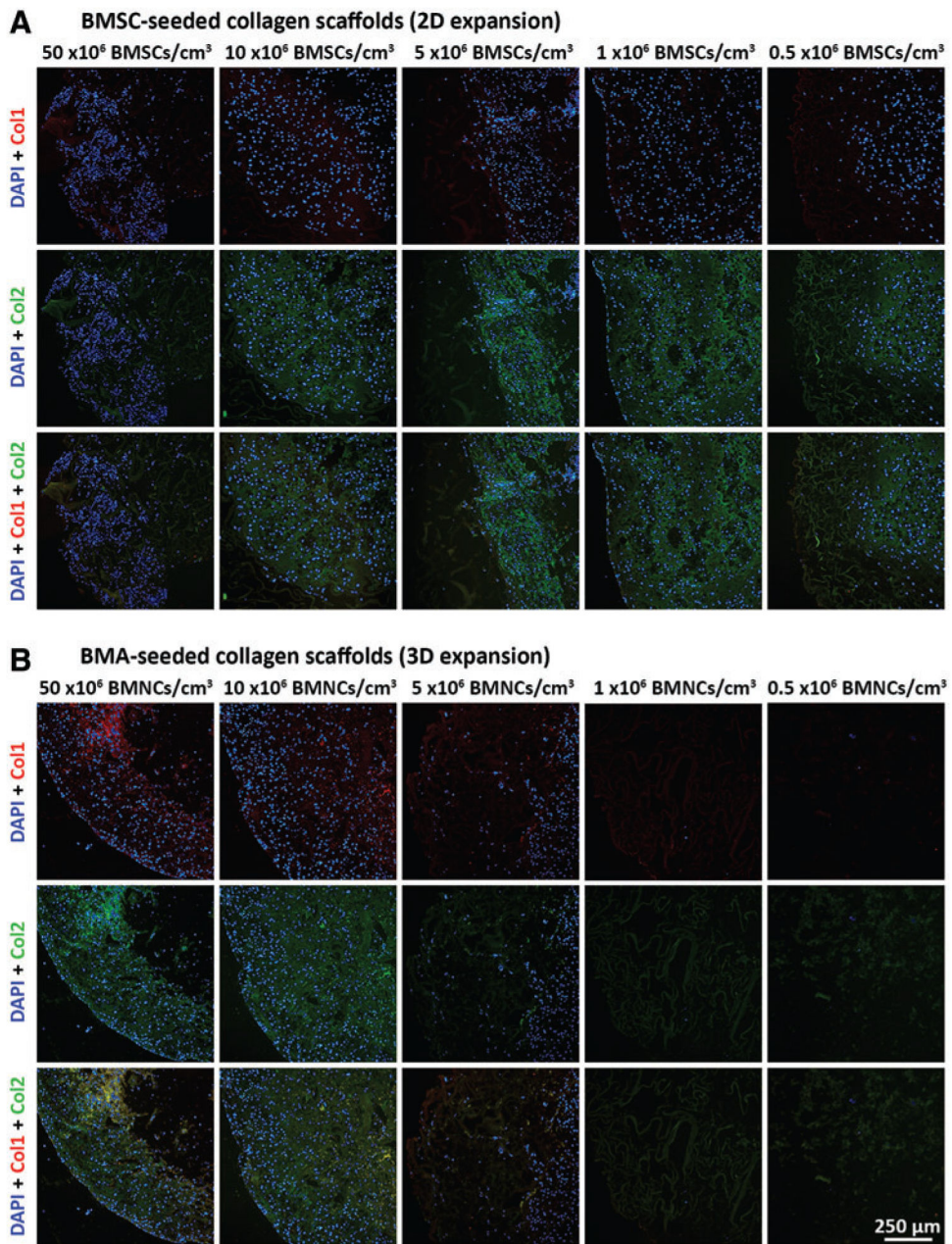


FIG. 4. Glycosaminoglycan (GAG) and deoxyribonucleic acid (DNA) quantification of 2D- and 3D-expanded BMSC-seeded collagen scaffolds. BMSCs were isolated and expanded within 2D and 3D environments and differentiated within collagen scaffolds for 21 days. Thereafter, constructs were assessed for GAG and DNA quantities. **(A, C, E, G)** Data represent the mean \pm SEM of GAG quantity, DNA quantity, GAG/DNA, and GAG/ cell within constructs from six donors in doublets based on BMSC seeding density in 2D-expanded BMSC-seeded scaffolds. **(B, D, F, H)** Data represent the mean \pm SEM of GAG quantity, DNA quantity, GAG/DNA, and GAG/ cell within constructs from six donors in doublets based on BMNC seeding density in 3D-expanded BMSC-seeded scaffolds. Statistical analysis is represented by unlabeled, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

**FIG. 5.**

Immuno-fluorescence analysis of collagen I and collagen II content within 2D- and 3D-expanded BMSC-seeded collagen scaffolds. BMSCs were isolated and expanded within 2D and 3D environments and differentiated within collagen scaffolds for 21 days in chondrogenic medium. Thereafter, constructs were fixed, sectioned at 5 μm thickness, and processed for visualization of cells (*DAPI*), collagen I (Col I; *Texas Red*) and collagen II (Col II; *FITC*). Presented photomicrographs represent cell–scaffold constructs derived from (A) 2D-expanded BMSCs seeded at 50, 10, 5, 1, or 0.5 × 10⁶ BMSCs/cm³, and (B) 3D-expanded BMSCs seeded at 50, 10, 5, 1, or 0.5 × 10⁶ BMNCs/cm³ (cells from donor Z28;

10× magnification). DAPI, 4',6-diamidino-2-phenylindole. FITC, fluorescein isothiocyanate. Color images available online at www.liebertpub.com/tec

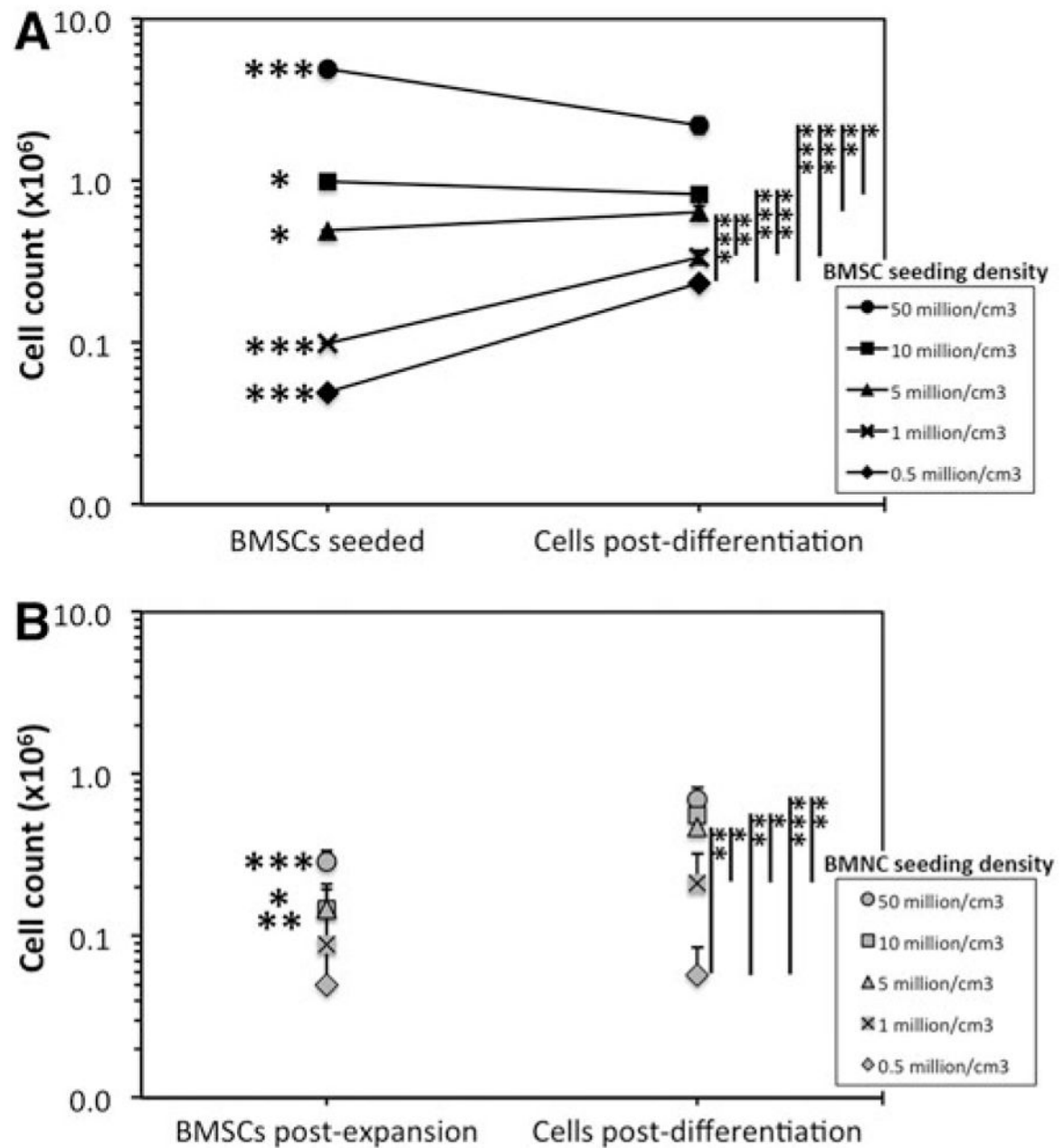


FIG. 6. Peri-differentiation cell count of 2D- and 3D-expanded BMSC-seeded collagen scaffolds. (A) BMSCs were isolated and expanded within tissue culture flasks containing expansion medium (2D environment), seeded at specific cell counts within collagen scaffolds to create five seeding densities and differentiated for 21 days in chondrogenic medium. DNA levels were then quantified and used to calculate cell count following differentiation. (B) BMAs were seeded onto collagen scaffolds (3D environment) to create five BMNC densities, cultured in expansion medium, and subsequently differentiated within chondrogenic medium for 21 days. DNA levels were quantified in one group of scaffolds after expansion and another group of scaffolds after differentiation. Data represent the mean \pm SEM of cell

counts from six donors. Statistical analysis is represented by unlabeled, not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 1

Bone Marrow Donor Information

Donor	Gender	Age (years)	Mass (kg)
Z28	Female	2.0	66
Z01	Female	2.2	71
Z33	Female	2.3	81
Y19	Female	3.2	63
Y08	Female	3.3	94
T10	Female	7.0	74

Table 2

Cell Seeding Density

Density ($\times 10^6$ cells/cm ³)	Number of cells seeded
50	4,948,000
10	989,600
5	494,800
1	98,960
0.5	49,480

Table 3

Ovine Primer Sequences Used in RT-qPCR Analysis

<i>Gene</i>	<i>Primer sequences</i>		<i>NCBI reference</i>
β-actin (<i>ACTB</i>)	5'-CGGCGGGACCACCAT-3'	Forward	NM_001009784.1
	5'-GCAGTGATCTCTTTCTGCATCCT-3'	Reverse	
Aggrecan (<i>ACAN</i>)	5'-TGGAATGATGTCCCATGCAA-3'	Forward	XM_004018048.1
	5'-GCCACTGTGCCCTTTTACAG-3'	Reverse	
Collagen I (<i>COL1A1</i>)	5'-CGCCCCAGACCAGGAATT-3'	Forward	XM_004012773.1
	5'-GTGGAAGGAGTTTACAGGAAGCA-3'	Reverse	
Collagen II (<i>COL2A1</i>)	5'-ACCTCACGTCTCCCCATCA-3'	Forward	XM_004006408.1
	5'-CTGCTCGGGCCCTCCTAT-3'	Reverse	
Collagen X (<i>COL10A1</i>)	5'-CAGGCTCGAATGGGCTGTAC-3'	Forward	XM_004011185.1
	5'-CCACCAAGAATCCTGAGAAAGAG-3'	Reverse	
SRY-Box 9 (<i>SOX9</i>)	5'-GCTGCTGGCCGTGATGA-3'	Forward	XM_004013527.1
	5'-GGGTCGCGGTTTGT-3'	Reverse	

β-actin, beta-actin; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; SOX9, sex determining region Y (SRY)-box 9.