ACS Biomaterials SCIENCE & ENGINEERING

Article

pubs.acs.org/journal/abseba

Guiding Chondrogenesis and Osteogenesis with Mineral-Coated Hydroxyapatite and BMP-2 Incorporated within High-Density hMSC Aggregates for Bone Regeneration

Phuong N. Dang,[†] Neha Dwivedi,[†] Xiaohua Yu,[‡] Lauren Phillips,[†] Caitlin Bowerman,[†] William L. Murphy,[§] and Eben Alsberg*[,∥]

[†]Department of Biomedical Engineering, Case Western Reserve University, Wickenden 218, 10900 Euclid Avenue, Cleveland, Ohio 44106, United States

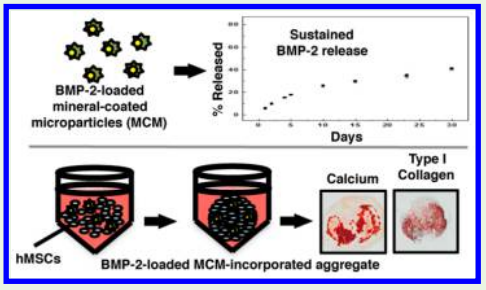
[‡]Department of Biomedical Engineering, Wisconsin Institute for Medical Research, University of Wisconsin, Room 5405, Madison, Wisconsin 53706, United States

[§]Departments of Biomedical Engineering and Orthopedics and Rehabilitation, Wisconsin Institute for Medical Research, University of Wisconsin, Room 5405, Madison, Wisconsin 53706, United States

[∥]Departments of Biomedical Engineering and Orthopaedic Surgery, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106, United States

S *Supporting Information*

ABSTRACT: Since hydroxyapatite and bone morphogenetic protein-2 (BMP-2) can regulate chondrogenesis and osteogenesis, their individual and combined effects on endochondral ossification within human bone marrow-derived stem cell (hMSC) aggregates were investigated. Hydroxyapatite was presented in the form of mineral-coated hydroxyapatite microparticles (MCM) capable of controlled BMP-2 delivery. Aggregates were treated with varied BMP-2 concentrations supplemented in the media and loaded onto MCM to examine the influence of BMP-2 amount and spatial presentation on regulating chondrogenesis and osteogenesis. MCM alone induced GAG and type II collagen production by week 5 for two of three donors, and BMP-2 may have accelerated MCM-induced chondrogenesis. ALP activity and calcium content of cells-only aggregates suggest that the BMP-2-induced osteogenic response may be concentration-dependent. Treatment with MCM and BMP-2 resulted in chondrogenesis as early as week 2, which may have promoted additional mineralization by week 5, suggesting the induction of endochondral ossification. Released BMP-2 had similar if not higher levels of bioactivity compared to that of exogenous BMP-2 with regard to chondrogenesis and osteogenesis. In addition to providing localized and sustained BMP-2 delivery, MCM incorporation within aggregates yields a self-sustaining system that may be injected or implanted more rapidly to heal bone defects through endochondral ossification without extended *in vitro* culture.



KEYWORDS: *bone tissue engineering, endochondral ossification, mineral-coated microparticles, growth factor delivery*

INTRODUCTION

Critical-sized bone defects resulting from trauma, tumor resection, infection, and congenital malformations are too large for the body to heal itself, and this motivates the need for strategies to treat this clinical problem. Bone graft procedures are used in over 2 million orthopedic procedures worldwide annually, at a cost of $2.5 billion a year.[1] However, even the gold standard, autografts, are associated with a series of limitations such as pain and donor site morbidity, size and shape restrictions, and limited graft availability.[2] While allografts and xenografts are more readily available, they present risk of infection and immunogenicity. Distraction osteogenesis, a surgical technique that gradually increases a reduced gap between two bone segments to promote bone renewal, is another widely used treatment, but it is a very long, painful process that is prone to pin-tract infection.[3] Treating defects with bone morphogenetic proteins (BMPs) released from a biomaterial scaffold, on the other hand, can be very costly, as it requires supraphysiologic dosage and/or results in uncontrolled bone growth if release is not properly controlled.[4,5] Alternative therapies for critical-sized bone defects are therefore necessary, and cell-based tissue engineering is a promising candidate to address or bypass the issues faced by current approaches.

Human bone marrow-derived mesenchymal stem cells (hMSCs) are an attractive therapeutic cell source for tissue regeneration. They are easily accessible in adult bone marrow. Importantly, they are capable of differentiating into multiple

Received: June 26, 2015
Accepted: November 20, 2015
Published: November 20, 2015

DOI: 10.1021/acsbiomaterials.5b00277
ACS Biomater. Sci. Eng. 2016, 2, 30−42

cell types such as adipocytes, chondrocytes, and osteoblasts, and can maintain their differentiation potential after expansion through several passages.[6,7] As a result, hMSCs have been widely used in a variety of tissue engineering strategies attempting to regenerate bone, cartilage, and other connective tissues.[8] One method is to culture hMSCs in high-cell density cultures such as aggregates, micromasses, and self-assembled sheets in media supplemented with growth factors. These high-cell density systems are easy to produce with high-throughput,[9] provide a 3-dimensional (3D) environment that mimics the local environment of cells in some developing and healing tissues,[10] and can enhance cell survival and function compared to those of a conventional monolayer culture.[11,12] Unlike scaffold-based tissue engineering approaches, this scaffold-free approach does not require synchronization of scaffold degradation with new bone formation, which is very challenging and can greatly compromise the integrity of the engineered tissue if not achieved. High-density hMSC cultures have been extensively studied for cartilage tissue engineering,[9,13] and more recently, they have been explored for bone formation via both the intramembranous[14−17] and endochondral ossification[18−20] pathways. For intramembranous ossification, high-density cultures have been cultured in osteogenic media containing factors capable of inducing direct differentiation of hMSCs into osteoblasts. For endochondral ossification, aggregated hMSCs have been first cultured in chondrogenic media to induce cartilage formation followed by either osteogenic media,[18,19] chondrogenic media with the addition of β-glycerophosphate,[19] or hypertrophic media containing β-glycerophosphate and l-thyroxin[20] to induce chondrocyte hypertrophy and tissue mineralization. While these approaches were successful in modulating bone formation, they necessitate expensive and lengthy *in vitro* culture that requires repeated supplementation of inductive factors in the media. Additionally, spherically shaped cultures may be restricted in size due to diffusional limitations of the factors from the media to the construct interior. Therefore, nonuniform distribution of biological signals and subsequently tissue formation may result.

To address these issues, we aimed to develop a system of hMSC aggregates incorporated with BMP-2-releasing mineral-coated hydroxyapatite microparticles (MCM) for engineering bone without the need for exogenous growth factor supplementation. Microparticle incorporation allows for controlled local delivery, which may circumvent issues with diffusion limitations and lengthy culture with minimal interference with cell−cell interactions. BMP-2 and hydroxyapatite (HAp) are used extensively in strategies for bone regeneration. BMP-2, a well-known osteogenic factor, has been extensively used to treat delayed union of fractures and spinal fusion[21] and has been shown to induce ectopic cartilage and bone formation *in vivo*.[22,23] In addition to inducing hMSC chondrogenesis,[24] BMP-2 can also stimulate chondrocyte proliferation and hypertrophy, a key aspect of endochondral ossification.[25] As for HAp, its osteoconductive and potentially osteoinductive nature enables it to be used as surface coating on orthopedic implants to enhance bone growth and implant fixation.[26−28] Interestingly, the presence of HAp has also been shown to promote cartilage formation *in vivo*.[29−31] Because of its high affinity for proteins, HAp has been used for growth factor delivery,[32−36] and we recently demonstrated controlled dual delivery of vascular endothelial growth factor (VEGF) and BMP-2 from multilayered MCM.[35] By varying the composition of the modified simulated body fluid (mSBF) in which the HAp microparticles were coated, the degradability of the mineral coated layers, comprised mainly of low crystalline carbonated HAp, was controlled to regulate growth factor release.

Since HAp and BMP-2 have both been implicated in important processes involved in endochondral ossification (e.g., chondrogenesis, chondrocyte maturation, and osteogenesis), their effects on bone formation via this pathway were investigated in hMSC aggregates. Unlike the intramembranous ossification pathway, which requires the rapid establishment of a functional vascular network to supply cells in large defects with nutrients and oxygen *in vivo*,[37,38] the initial need for vascularization is delayed in the endochondral ossification approach, rendering it more attractive for bone tissue engineering applications. Endochondral ossification begins with the formation of cartilage whose resident cells can survive in an environment with limited nutrients and oxygen due to their low metabolic need.[39,40] The endochondral ossification pathway also has an internal mechanism for stimulating angiogenesis by yielding hypertrophic chondrocytes that secrete angiogenic factors such as VEGF to recruit blood vessels.[41] MCM capable of tunable growth factor release were synthesized using the biomimetic coating process, loaded with BMP-2, and then incorporated within hMSC aggregates. Concentrations of exogenous BMP-2 and BMP-2 loaded in the MCM were varied to study the effects of growth factor amount on regulating chondrogenesis and osteogenesis. In addition to providing localized and sustained growth factor delivery, this microparticle-incorporated system also presents a mineral source that may be both osteo- and chondro- inductive. Microparticle incorporation for BMP-2 delivery within hMSC aggregates may address the inefficiencies of exogenous supplementation with regard to time, cost, and diffusion limitations. This work lays the foundation for an injectable high-cell density system that can promote bone repair without prior extended *in vitro* culture.

MATERIALS AND METHODS

Mineral-Coated Microparticle Synthesis and Characterization. HAp microparticles (3−5 μm in diameter) from Plasma Biotal LTD (Derbyshire, UK) were coated with a mineral layer using a biomimetic process as previously described.[35] Briefly, the microparticles were incubated at 37 °C and pH 6.8 in modified simulated body fluid (mSBF) containing 141 mM NaCl, 4.0 mM KCl, 0.5 mM $MgSO_4$, 1.0 mM $MgCl_2$, 20.0 mM HEPES, 5.0 mM $CaCl_2$, 2.0 mM KH_2PO_4, and 4.2 mM $NaHCO_3$. This particular mSBF formulation was chosen for MCM fabrication because the resulting MCM released BMP-2 in the most sustained manner (41 ± 1% released after 30 days) compared to formulations with higher $NaHCO_3$ concentrations.[35] HAp microparticles were incubated in mSBF at a ratio of 100 mg of HAp microparticles to 50 mL of mSBF at 37 °C on a rotation device. After 7 days, the resulting MCM were rinsed with deionized water and lyophilized.

Size and surface morphology of the MCM were examined by LEO 1530 field emission scanning electron microscopy (FESEM, Zeiss, Germany) after sputter-coating with gold. Radiolabeled BMP-2 (^{125}I labeled BMP-2, PerkinElmer, Waltham, MA) was used to analyze BMP-2 binding efficiency and release from MCM as previously described.[35] Briefly, 1 mL of PBS solution containing 8 or 32 mg of BMP-2 ("1X BMP-2" or "4X BMP-2"; Dr. Walter Sebald, Department of Developmental Biology, University of Würzburg, Germany; 0.5% ^{125}I-BMP-2) was incubated with 5 mg of MCM at 37 °C. After 4 h, BMP-2-loaded MCM were centrifuged at 8000*g* for 2 min, the supernatant was aspirated, and MCM was washed with PBS. To determine the amount of BMP-2 in the supernatant and PBS used for washing, the radioactivity in each solution was measured with a Packard Cobra II gamma counter (PerkinElmer). BMP-2 binding

DOI: 10.1021/acsbiomaterials.5b00277
ACS Biomater. Sci. Eng. 2016, 2, 30−42

Table 1. Experimental Conditions for MCM-Incorporated Human Mesenchymal Stem Cell Aggregate Study

group	description	short name	MCM per aggregate (mg)	exogenous TGF-β1 (ng/mL)	exogenous BMP-2 (ng/mL)	loaded BMP-2 (ng/mg)
1	no MCM, exo. TGF-β1	no MCM, TGF-β1	0	10	0	0
2	no MCM, no GF	no MCM, no GF	0	0	0	0
3	MCM, no GF	MCM, no GF	0.05	0	0	0
4	no MCM, exo. BMP-2 (1×)	no MCM. exo. 1×	0	0	25 (1×)	0
5	MCM, exo. BMP-2 (1×)	MCM, exo. 1×	0.05	0	25 (1×)	0
6	MCM, loaded BMP-2 (1×)	MCM, loaded 1×	0.05	0	0	1600 (1×)
7	no MCM, exo. BMP-2 (4×)	no MCM, exo. 4×	0	0	100 (4×)	0
8	MCM, exo. BMP-2 (4×)	MCM, exo. 4×	0.05	0	100 (4×)	0
9	MCM, loaded BMP-2 (4×)	MCM, loaded 4×	0.05	0	0	6400 (4×)

efficiency was determined by calculating the difference in protein concentration in the supernatant before and after binding. The amount of BMP-2 released was determined by incubating BMP-2-loaded MCM in PBS at 37 °C and measuring the radioactivity of the supernatant at specific time points.

hMSC Isolation and Expansion. hMSCs were isolated from bone marrow aspirates from the posterior iliac crest of 3 healthy donors (43 ± 5 years) using a protocol approved by the University Hospitals of Cleveland Institutional Review Board and cultured as previously described.[42] Briefly, the aspirates were rinsed with expansion medium comprising low-glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) with 10% prescreened fetal bovine serum (Gibco, Grand Island, NY). A Percoll (Sigma-Aldrich) density gradient was used to isolate mononucleated cells, which were then seeded on tissue culture plastic at a density of 1.8×10^5 cells per cm^2 in expansion medium and cultured at 37 °C with 5% CO_2. The medium was changed to remove nonadherent cells after 4 days and every 3 days thereafter. After 10−14 days, primary hMSC cultures were trypsinized and plated at 5×10^3 cells/cm^2. Cells were used at passage 3 (P3).

Aggregate Production. To form microparticle-incorporated aggregates, UV-sterilized MCM (0.05 mg/aggregate), with or without BMP-2 (1600 or 6400 ng/mg MCM), were suspended with P3 hMSCs (1.25×10^6 cells/mL) in a serum-free chemically defined medium containing high-glucose DMEM, 100 nM dexamethasone, 37.5 μg/mL L-ascorbic acid-2-phosphate, and 5 mM β-glycerophosphate. Two hundred microliter aliquots were added to the wells of sterile V-bottom polypropylene plates and then centrifuged to form aggregates as previously reported.[43]

MCM incorporation efficiency into hMSC aggregates (N = 21) of 99.99 ± 0.01% was measured as follows. MCM-incorporated aggregates sans media were removed from their respective wells. Media from the wells were then transferred to preweighed microcentrifuge tubes (3 wells per tube; 7 tubes total). For a total of 3 washes, the tubes were centrifuged at 17,000g for 5 min using an accuSpin Micro 17R Microcentrifuge (Fisher Scientific, Waltham, MA), the supernatant removed, and rinsed with diH_2O. After 2 days of lyophilization, the tubes were weighed, and the final mass was subtracted from the initial tube mass to determine unincorporated MCM mass. MCM incorporation efficiency was determined from the following equation:

$$\%\text{incorporated} = 100 \times \frac{\text{initial MCM mass} - \text{unincorporated MCM mass}}{\text{initial MCM mass}}$$

Furthermore, the microparticles, which stained positively for calcium with Alizarin Red S, appear to be evenly distributed throughout the aggregates (Supporting Information, Figure S1).

The conditions for the aggregates studied are shown in Table 1. Aggregates containing MCM without growth factor loaded ("empty MCM") were cultured without exogenously supplemented (exo.) BMP-2, with 25 ng/mL exo. BMP-2, or with 100 ng/mL exo. BMP-2, the conventional concentration for *in vitro* osteogenic differentiation of hMSCs reported in the literature.[44] Growth factor loading onto MCM was carried out by incubating MCM in PBS containing BMP-2 for 4 h at 37 °C. A binding efficiency of 60% was taken into account during loading.[35] The BMP-2 loading concentrations chosen, 1600 and 6400 ng/mg, are equivalent to the total amount of exo. BMP-2 supplemented at 25 and 100 ng/mL, respectively, during the 5-week culture. Cells-only aggregates were cultured with exo. BMP-2 concentrations of 0, 25, or 100 ng/mL. To compare the extent of chondrogenesis induced by MCM and/or BMP-2, cells-only aggregates cultured in exo. TGF-β1 (10 ng/mL) were used as a positive control for chondrogenesis.[7] Media were changed every other day.

Biochemical Analysis. At 2 and 5 weeks, aggregates (N = 4 for each donor) were digested and assayed for chondrogenic and osteogenic markers. Briefly, aggregates were homogenized in papain buffer for 1 min. Half of the sample was transferred to a new tube and treated with alkaline phosphate (ALP) lysis buffer containing 1 mM $MgCl_2$, 20 μM $ZnCl_2$, and 0.1% octyl-beta-glucopyranoside in 10 mM tris buffer (pH 7.4). Using an ALP Assay kit (Sigma-Aldrich) according to the manufacturer's instructions, ALP activity was determined by measuring its conversion of *p*-nitrophenol phosphate to 4-nitrophenol using standards of 4-nitrophenol. The other half of the sample was placed in a 65 °C water bath for further digestion with papain. The next day, 10% EDTA in 0.05 M Tris-HCl buffer (pH 7.4) was added to a fraction of the sample to disassociate DNA and/or glycosaminoglycan (GAG) that may have adhered to the MCM. DNA was then measured using PicoGreen dye (Invitrogen, Carlsbad, CA), and GAG content was quantified with a dimethyl methylene blue assay.[45] The rest of the papain-digested sample was treated with 1 M HCl to completely dissolve the MCM. As an indicator of mineralization, calcium content was measured with an *o*-cresophthalein complexone assay (Sigma-Aldrich) according to the manufacturer's instructions.

Histological Analysis. After 2 and 5 weeks of culture, four aggregates per group were fixed in 10% neutral-buffered formalin, dehydrated in 70% ethanol, and embedded in paraffin. Five micrometer-thick sections were stained for GAG and calcium with Safranin O (Acros Organics, Geel, Belgium) with a Fast Green counterstain (Fisher Chemical, Fairlawn, NJ) and Alizarin Red S (Fisher Chemical), respectively. Aggregate sections were also stained immunohistochemically for types I and II collagen, osteopontin, and osteocalcin. Briefly, sections were deparaffinized with xylene then rehydrated with decreasing concentrations of ethanol. Endogenous peroxidase activity was quenched via incubation in a 1:9 mixture of 30% v/v hydrogen peroxide and methanol for 10 min. For antigen retrieval, sections for collagen staining were treated with Protease (Sigma-Aldrich) at RT for 15 min, and the rest were incubated in citrate buffer at 60 °C for 20 min. Anticollagen type I (ab34710; Abcam, Cambridge, MA), anticollagen type II (ab34712; Abcam), antiosteopontin (ab8448; Abcam), and antiosteocalcin (ab93876; Abcam) were used as primary antibodies, and rabbit IgG (Vector Laboratories, Burlingame, CA) was used as a negative control (Supporting Information, Figure S3). The Histostain-Plus Bulk kit (Invitrogen) containing a biotinylated secondary antibody and enhanced horseradish peroxidase (HRP) conjugated streptavidin was used in accordance with the manufacturer's instructions.[46] Aminoethyl carbazole (Invitrogen) was used as the substrate for the HRP. Slides were mounted with glycerol vinyl alcohol (Invitrogen) and imaged

DOI: 10.1021/acsbiomaterials.5b00277
ACS Biomater. Sci. Eng. 2016, 2, 30−42

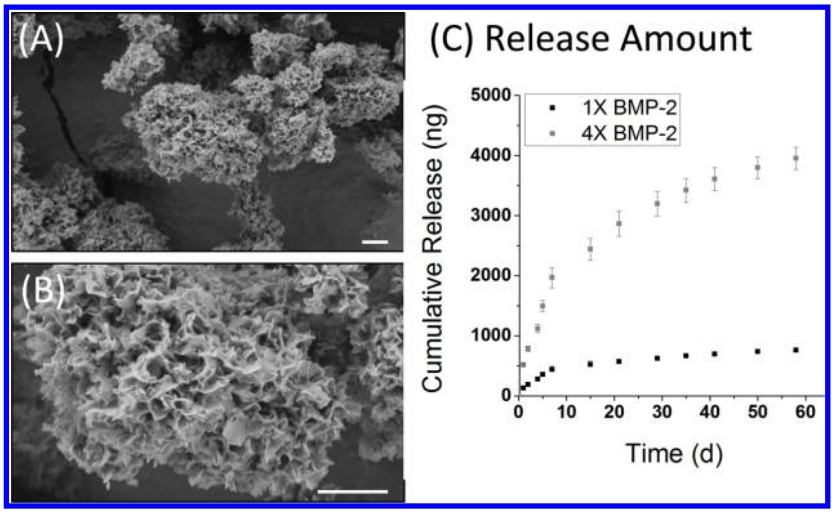


Figure 1. (A) Low and (B) high magnification scanning electron photomicrographs of MCM and (C) release profiles of BMP-2 from MCM. Scale bars = 1 μm.

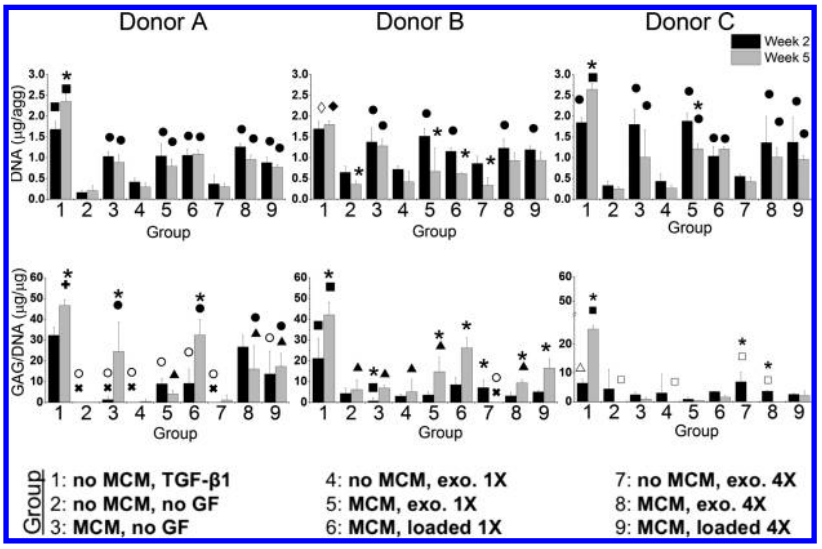


Figure 2. DNA (top) and GAG normalized to DNA (bottom) content in week 2 (black) and 5 (gray) aggregates from three different hMSC donors. *, significantly different between time points. Significantly different from ■, all other groups; △, all other groups except 7; ●, groups 2, 4, and 7; ◊, all other groups except 3 and 5; ◆, all other groups except 3; +, all other groups except 6; ×, groups 5, 6, and 9; ○, groups 1 and 8; ▲, group 6; and □, group 9 at specific time point.

using an Olympus BX61VS microscope (Olympus, Center Valley, PA) with a Pike F-505 camera (Allied Vision Technologies, Stadtroda, Germany).

The ARS staining intensity of calcium-stained sections (N = 4 per group) was quantified using a modification of a previously published protocol.[47] Briefly, Photoshop CS6 software (Adobe Systems, San Jose, CA) was used to create black-and-white image masks of the ARS-stained color images. Specifically, the wand tool (tolerance: 60) was used to highlight areas of calcification throughout each section, which were then designated black with the background designated white. The wand tool in ImageJ software (NIH, Washington, DC) was then used to calculate the black-colored areas in each section.

Statistical Analysis. One-way ANOVA with Tukey's *posthoc* tests was performed using InStat 3.06 software (GraphPad Software Inc., La Jolla, CA) to compare between the 9 conditions at each time point as well as between time points for each condition. $p < 0.05$ was considered statistically significant.

RESULTS

MCM Characterization and BMP-2 Release from MCM. After a 7-day incubation in mSBF, MCM were uniformly coated with a plate-like structure (Figure 1A,B). This typical morphology presents a high surface area for abundant protein interaction. X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FTIR) analyses indicate that the mineral coating is comprised mainly of low crystalline carbonated HAp.[35] Sustained release of BMP-2 from MCM was achieved for over 60 days in mSBF with minimal initial burst release (Figure 1C). Cumulative release, expressed in total ng of BMP-2, was significantly different between the two conditions with 4× BMP-2 releasing ~4 times more than 1× BMP-2 after 58 days.

Biochemical Analysis of hMSC Aggregates. Nine experimental conditions were examined using hMSCs from three donors (Table 1). The effects of HAp and mode of delivery of BMP-2 at various concentrations on cell number

DOI: 10.1021/acsbiomaterials.5b00277
ACS Biomater. Sci. Eng. 2016, 2, 30−42

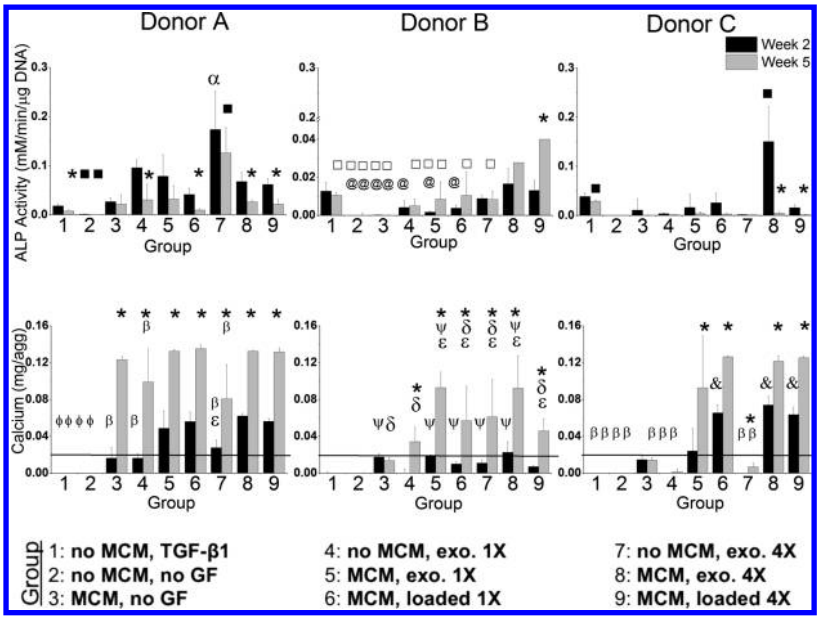


Figure 3. Alkaline phosphatase activity (top) and calcium content (bottom) in week 2 (black) and 5 (gray) aggregates from three different hMSC donors. The line denotes the theoretical amount of calcium initially incorporated within each MCM-containing aggregate. *, significantly different between time points. Significantly different from ■, all other groups; α, all other groups except 4; □, group 9; @, group 8; β, groups 5, 6, 8, and 9; δ, groups 1 and 2; Ψ, groups 1, 2, and 4; ε, group 3; Φ, groups 3−9 and &, groups 1−5 at specific time point.

and MSC chondrogenesis were determined by measuring DNA and GAG content, respectively (Figure 2). Cells-only aggregates treated with exo. TGF-β1 (group 1) were used as a positive chondrogenic control to determine to what extent MCM and/or BMP-2 induce cartilage formation in this system. To assess the degree of bone formation, ALP activity and calcium content were also quantified (Figure 3). Comparisons for DNA, GAG normalized to DNA, ALP activity normalized to DNA, and calcium content were made among the 9 groups for each individual donor at each time point.

Cell Content of hMSC Aggregates. For all three donors, cell content, measured indirectly via DNA assay, was maintained from weeks 2 to 5 of culture for most groups with the exception of group 1 for donors A and C, groups 2, 5, 6, and 7 for donor B, and group 5 for donor C (Figure 2). While it was maintained for donor B, DNA content in group 1 significantly increased after week 2 for donors A and C. Except for groups 3 and 5 in donors B and C, group 1 had significantly higher DNA content than that of all other groups at both time points. Although significant decreases in DNA content were observed in MCM- and 1× BMP-2-treated groups 5 and 6 for donor B and group 5 for donor C, the resulting DNA levels were still either similar to (donor B) or higher than (donor C) those of the cells-only groups treated with no growth factor (group 2) or with exo. BMP-2 (groups 4 and 7). In fact, all MCM-treated aggregates (groups 3, 5, 6, 8, and 9) had significantly higher DNA content than the cells-only aggregates in groups 2, 4, and 7 at both time points for donors A and C. This was also true for donor B at week 2. However, by week 5, DNA content decreased in groups 2, 5, 6, and 7. While the MCM-treated groups 3, 5, 6, 8, and 9 exhibited higher average DNA content than the cells-only groups not treated with TGF-β1 (groups 2, 4, and 7), only group 3, which did not receive growth factor treatment, remained significantly higher. Nevertheless, groups receiving MCM or TGF-β1 treatment generally resulted in higher cell content.

GAG/DNA Content within hMSC Aggregates. Increased GAG production after 2 weeks was observed in TGF-β1-treated group 1 for all donors with a lower GAG/DNA content at both time points for donor C (Figure 2). While little to no GAG/DNA content was observed in the cells-only groups not treated with TGF-β1 (groups 2, 4, and 7), GAG production was observed by week 5 in MCM-treated groups 3, 5, 6, 8, and 9 for donors A and B. In donor B, GAG/DNA content was detected in small amounts in all cells-only groups 2, 4, and 7. However, this was significantly less than that in group 6, in which incorporated MCM were loaded with 1× BMP-2, at week 5. Although not statistically significant, other aggregates treated with both MCM and BMP-2 (donor B: groups 5, 8 and 9) also exhibited higher GAG/DNA compared to that of the cells-only groups treated with either no growth factor (group 2) or BMP-2 (groups 4 and 7) at week 5. For both donors A and B, GAG production was delayed until after 2 weeks in aggregates treated with MCM alone (group 3). However, aggregates treated with both MCM and BMP-2 (groups 5, 6, 8, and 9) for these donors exhibited accelerated GAG production with significantly higher GAG/DNA than group 3 at week 2.

While chondrogenesis was induced in all MCM-treated aggregates for donors A and B, it was not to the same extent as in group 1 by week 5. Notably, while the TGF-β1-treated group 1 had significantly higher GAG/DNA content than all other groups, statistical significance was not found with group 6, which was treated with loaded 1× BMP-2, at week 5 for donor A. Comparing among the MCM-treated groups for donor A, group 6 showed significantly higher GAG/DNA content by week 5 than group 5, which was treated with an equivalent concentration of BMP-2 exogenously. Aggregates presented with the higher exo. BMP-2 concentration (donor A: group 8) exhibited significantly higher GAG production than those treated with the lower concentration (donor A: groups 5 and 6) at week 2, but group 6, which had 1× BMP-2 loaded, had the highest GAG/DNA content by week 5. Similarly for donor B, group 6 exhibited significantly higher GAG/DNA content than

DOI: 10.1021/acsbiomaterials.5b00277
ACS Biomater. Sci. Eng. 2016, 2, 30−42

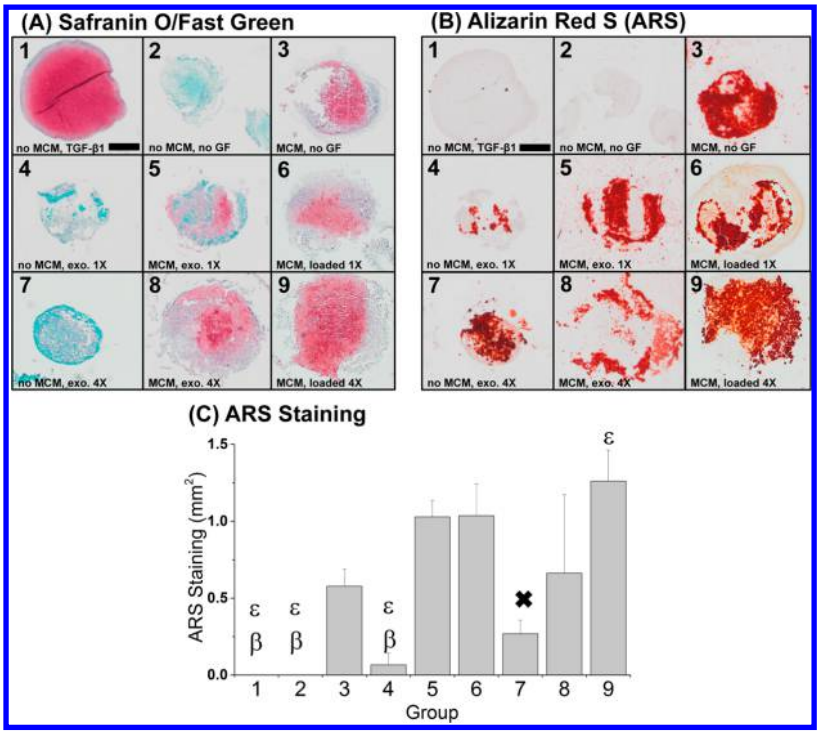


Figure 4. (A, B) Photomicrographs of Safranin O/Fast Green and Alizarin Red S (ARS) histology of week 5 aggregates from donor A. Scale bar = 500 μm. All images are at the same scale. (C) Quantification of ARS staining of week 5 aggregates from donor A. Significantly different from ε, group 3; β, groups 5, 6, 8, and 9; and ×, groups 5, 6, and 9.

most other groups (donor B: groups 2, 3, 4, 7, and 8) and higher but not statistically significant than groups 5 and 9 at week 5. Other aggregates treated with both MCM and BMP-2 (donor B: groups 5, 8, and 9) also had higher but not quite significant GAG/DNA compared to that of the cells-only groups not treated with TGF-β1 (groups 2, 4, and 7) at this time point.

Compared to the other donors, GAG production in aggregates for donor C was much weaker, which may be due to the lower chondrogenic potential of hMSCs from this donor (Figure 2, TGF-β1-treated group 1). At week 2, GAG/DNA content in TGF-β1-treated group 1 was ~6 and ~4 times lower than that in the same group for donors A and B, respectively. By week 5, it was about half of that for the other donors. Little to no GAG/DNA was detected in the other groups at both time points. Similar GAG/DNA content was observed for all groups at week 2, but no GAG content was observed in groups 2, 4, 5, 7, and 8 by week 5. Some GAG/DNA content was present in MCM-only aggregates (donor C: group 3) and aggregates with loaded BMP-2 (donor C: groups 6 and 9) at week 5.

ALP Activity within hMSC Aggregates. ALP activity, an early marker of osteogenesis, was then quantified in the aggregates (Figure 3). Interestingly, TGF-β1-treated group 1 for all three donors had detectable ALP activity, which may indicate the presence of mature cartilage, where ALP expression has been shown to localize.[48] For donor A, cells-only aggregates not treated with a growth factor (group 2) exhibited no ALP activity, while those receiving BMP-2 treatment (groups 4 and 7) showed increasing ALP activity with increasing exo. BMP-2 concentration. When aggregates were incorporated with MCM (group 3), ALP activity was higher than that of the cells-only aggregates (group 2). When aggregates were treated with both MCM and BMP-2 (groups 5, 6, 8, and 9), ALP activity was either higher than (week 2) or similar to (week 5) that of group 3. At both time points, MCM-incorporated groups 5, 6, 8, and 9 had similar levels of ALP activity compared to that of group 4 but significantly lower than that of group 7 in which exo. BMP-2 concentration was increased to 4×.

For donor B, ALP activity was much lower than that for donor A, which may be due to the difference in osteogenic potential between the donors (Figure 3). Among cells-only groups treated with either no growth factor or BMP-2 (groups 2, 4, and 7), ALP activity increased with increasing BMP-2 concentration. At week 2, ALP activity in group 8, in which MCM-treated aggregates were presented with exo. 4× BMP-2, were significantly higher than that in groups receiving no growth factor treatment or 1× BMP-2 treatment (groups 2–6). Aggregates loaded with MCM loaded with 4× BMP-2 (group 9) exhibited higher ALP expression than groups 1, 2, 3, and 5. By week 5, group 8 had significantly greater ALP activity than groups 2 and 3, while group 9 showed significantly higher ALP activity than all other groups except group 8.

For donor C, cells-only groups 2, 4, and 7 had no observable ALP activity at both time points (Figure 3). While ALP activity was detectable in MCM-treated aggregates (groups 3, 5, 6, 8, and 9), it significantly decreased by week 5. At week 2, ALP activity in MCM-incorporated group 8 treated with exo. 4× BMP-2 was significantly higher than that of all other groups, suggesting that exogenous treatment of BMP-2 at this concentration is necessary to promote enhanced ALP activity in this donor.

Calcium Content within hMSC Aggregates. The degree of mineralization was analyzed by measuring calcium content within each aggregate (Figure 3). For all three donors, no calcium was detected in groups 1 and 2, which were treated with TGF-β1 and no growth factor, respectively. When cells-only aggregates were treated with exo. BMP-2 (groups 4 and

DOI: 10.1021/acsbiomaterials.5b00277
ACS Biomater. Sci. Eng. 2016, 2, 30–42

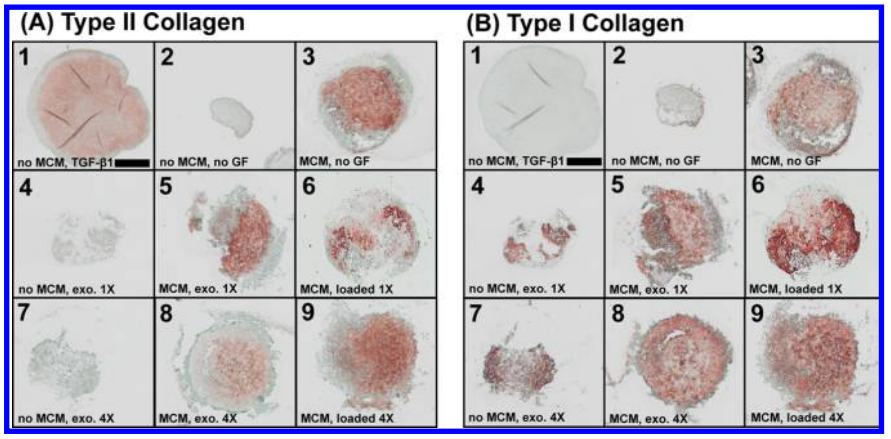


Figure 5. Photomicrographs of types (A) II and (B) I collagen immunohistochemical staining of week 5 aggregates from donor A. Scale bar = 500 μm. All images are at the same scale.

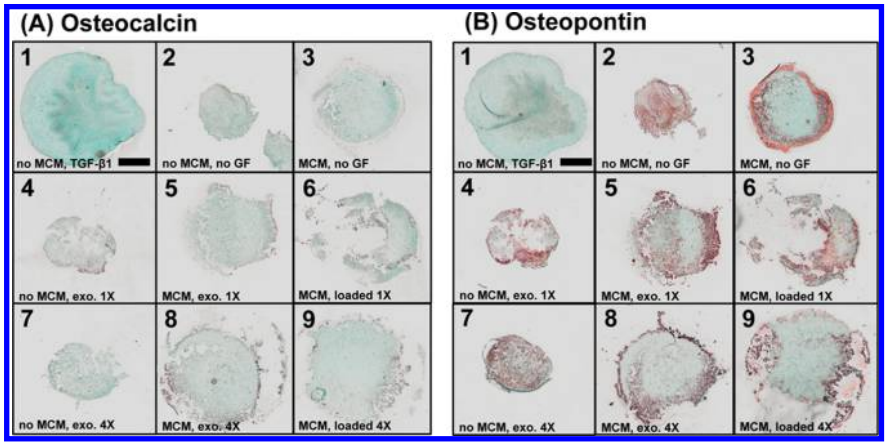


Figure 6. Photomicrographs of (A) osteocalcin and (B) osteopontin immunohistochemical staining of week 5 aggregates from donor A. Scale bar = 500 μm. All images are at the same scale.

7), mineralization took place as early as week 2 in group 4 (1× BMP-2) for donor A and group 7 (4× BMP-2) for donors A and B. By week 5, calcium content significantly increased in group 4 for donors A and B and in group 7 for all three donors. When aggregates were treated with MCM alone (group 3), only the amount of calcium that was initially incorporated was detected at week 2. By week 5, this level significantly increased for donor A but stayed the same for donors B and C.

At 1× BMP-2, MCM-incorporated aggregates receiving exo. treatment (group 5) had significantly higher calcium content than the MCM-treated group 3 at week 2 for donor A and week 5 for donors B and C, and group 4 at both time points for all donors. Calcium content in group 6 with MCM loaded with 1× BMP-2 was higher than that of group 3 at week 2 for donor A, week 5 for donor B, and both time points for donor C, and group 4 at week 2 for donor B and at both time points for donors A and C. Group 6 for donor B had a level similar to that of group 4 at week 5.

When BMP-2 concentration was increased to 4×, MCM-incorporated groups 8 and 9 receiving exo. and loaded BMP-2, respectively, exhibited significantly higher mineralization than groups 3, 4, and 7 at week 2 for donor A and at both time points for donor C. At week 5, group 8 remained higher than these same groups, and calcium content in group 9 was higher than that of group 3 for donor B. For all donors, no differences in mineralization were found between 1× BMP-2 and 4× BMP-2, exogenous or loaded, in MCM-treated groups.

Histological Analysis of hMSC Aggregates. To verify the quantitative GAG/DNA and calcium data, histological sections of week 5 aggregates were stained for GAG and calcium with Safranin O/Fast Green and Alizarin Red S, respectively. Immunohistochemistry against types I and II collagen, osteopontin, and osteocalin were also performed. Representative sections from donor A are shown (Figures 4–6).

GAG staining was most intense for the chondrogenic control group 1, while the other cells-only aggregates (groups 2, 4, and 7) did not stain (Figure 4A). Importantly, MCM-incorporated aggregates (groups 3, 5, 6, 8, and 9) stained positively, albeit to a lesser extent than group 1. Histology resulting from staining for calcium in week 5 aggregates confirmed the biochemical data (Figure 4B). Cells-only aggregates treated with no growth factor (group 2) or with TGF-β1 (group 1) did not stain for calcium. While aggregates treated with BMP-2 alone (groups 4 and 7) stained positively for calcium, staining was not as extensive as with the aggregates treated with either MCM alone (group 3) or with both MCM and BMP-2 (groups 5, 6, 8, and 9). Consistent with the biochemical data, groups 5, 6, 8, and 9, which were treated with BMP-2 exogenously or loaded, respectively, stained similarly with substantial calcium content.

Quantification of ARS staining in all aggregates from this donor revealed significantly higher staining intensity in MCM-incorporated groups compared to that of cells-only groups treated with TGF-β1 (group 1), no growth factor (group 2),

DOI: 10.1021/acsbiomaterials.5b00277
ACS Biomater. Sci. Eng. 2016, 2, 30–42

and exo. 1× BMP-2 (group 4) (Figure 4C). However, only groups 5, 6, and 9 exhibited significantly more ARS staining than group 4. Importantly, ARS staining intensity in group 9 was significantly higher than that of group 3.

Immunohistochemical staining for type II collagen provided additional evidence for the presence of neocartilage in MCM-treated groups (Figure 5A). In general, regions positive for GAG staining also stained for type II collagen with intense staining in groups 1, 3, 5, 6, 8, and 9 and negative staining in groups 2, 4, and 7. Immunohistochemistry was also performed for type I collagen, osteopontin, and osteocalcin to assess the amount and distribution of these bone extracellular matrix molecules within the constructs (Figures 5B and 6). The TGF-β1-treated group 1 did not stain for any of these bone markers. While group 2, which was not treated with any growth factor, lacked staining for osteocalcin, it stained lightly for type I collagen and quite intensely for osteopontin. In general, positive staining for type I collagen (Figure 5B) and osteopontin (Figure 6) were localized to regions of mineralized tissue in groups 3−9. Interestingly, some staining for type I collagen appeared in nonmineralized areas that also stained positively for GAG and type II collagen in MCM-treated groups 3, 4, 5, 8, and 9 (Figures 4A,B and 5). Although osteocalcin staining was weak compared to that of the other markers, positive staining was observed in the outer mineralized regions of groups 4, 5, 6, 8, and 9 (Figure 6A).

Similar to donor A, GAG and calcium staining corroborated the respective biochemical data and staining for cartilage and bone markers were localized for donors B and C. A notable difference between these donors and donor A is that staining for calcium in group 3 aggregates was much less extensive in donors B and C (Supporting Information, Figure S2). The addition of BMP-2, either at 1× or 4×, resulted in a substantial increase in ARS staining for donors B and C. As for cartilage markers, staining for GAG and type II collagen for donor B was weaker than that in donor A, while, with the exception of group 1, no staining for either marker was observed the other groups for donor C.

DISCUSSION

With the goal to partially recapitulate endochondral ossification to engineer bone, the effects of mineral-coated HAp and BMP-2 on chondrogenesis and osteogenesis were investigated in high-density hMSC aggregates. Additionally, the challenges of exogenous growth factor supplementation were addressed by employing a tailorable growth factor delivery system consisting of MCM to locally present BMP-2 within hMSC aggregates in a sustained manner. Without a growth factor delivery system incorporated within these scaffold-free cellular constructs, the primary method of growth factor presentation to these constructs would be to supplement the culture media with growth factor(s). Thus, lengthy *in vitro* culture may be necessary prior to construct implantation. In previous work aimed to promote endochondral bone formation *in vivo* by first forming a cartilaginous anlage, cell constructs were primed *in vitro* in media containing chondrogenic signals for at least 3 weeks to as long as 12 weeks before being implanted.[19,20,49,50]

Delivery of BMP-2 from MCM Was Controlled. We have recently demonstrated the tunable release of various growth factors including BMP-2 from MCM by modifying the stability of the mineral coating layer.[35] By simply varying the concentration of carbonate or fluoride in the mSBF, the mineral coating morphology and therefore its dissolution rate can be altered to regulate release kinetics. Carbonate substitution in the mineral coating accelerated coating dissolution, whereas fluoride substitution delayed it. These mechanisms allow for the controlled release of growth factors bound to MCM. Here, a specific mSBF formulation containing 4.2 mM $NaHCO_3$ was utilized to uniformly coat HAp microparticles that released BMP-2 for over 60 days with minimal initial burst release (Figure 1C). Total amount released expressed in terms of BMP-2 mass correlated with amount of loaded BMP-2, providing evidence that the amount of BMP-2 delivered to the cells can be regulated in this tunable system by simply changing growth factor loading concentration.

MCM May Have Aided Cell Survival within hMSC Aggregates. When hMSC aggregates were cultured for 5 weeks in serum-free osteogenic media, the inclusion of MCM usually resulted in significantly higher DNA content at both time points compared to that of cells-only aggregates not treated with TGF-β1 (groups 2, 4, and 7), suggesting that the presence of MCM may have aided cell survival (Figure 2). It has been shown that aggregates cultured in medium containing osteogenic factors shrink over time potentially due to decreased cell viability. For example, Burns et al. reported an ∼50% decrease in aggregate diameter after 21 days in medium containing dexamethasone, ascorbic acid, and β-glycerophosphate.[16] Histology of week 5 aggregates revealed noticeably smaller aggregates in cells-only groups 2, 4, and 7 than in MCM-treated groups 3, 5, 6, 8, and 9. Therefore, the presence of MCM may have reduced total cell death and/or enhanced cell proliferation, potentially due to their high affinity for serum-contained or cell-secreted proteins.

In addition, compared to TGF-β1-treated group 1, DNA content in most other groups was significantly lower at week 5. Furthermore, group 1 had increased DNA content from week 2 to 5 with donors A and C having higher DNA content than 2 μg/aggregate, the theoretical amount of DNA in the number of cells used per aggregate assuming ∼8 pg of DNA per cell.[51] This may be attributed to the role of TGF-β1 in enhancing cell proliferation in the presence of ITS+, a major component of the culture medium.[52]

BMP-2 Induced Osteogenesis in a Concentration-Dependent Manner. BMP-2 promoted hMSC osteogenesis through ALP expression and subsequent mineralization (Figure 3). There is a strong trend in which exo. BMP-2 treatment promoted ALP activity in cells-only aggregates with higher expression at a higher concentration at both time points (donors A and B: groups 2, 4, and 7, which were treated with exo. BMP-2 at 0, 25, and 100 ng/mL, respectively). BMP-2 also induced mineralization as early as week 2 in groups 4 (donor A) and 7 (donors A and B), which significantly increased by week 5 in groups 4 and 7 for both donors. Some mineralization was also observed in groups 4 and 7 for donor C at 5 weeks. In contrast, no calcium was measured in group 2, which did not receive BMP-2 treatment, for all three donors. Together, this data indicates a concentration dependent osteogenic response to exogenous BMP-2 delivery.

BMP-2 May Have Accelerated MCM-Induced Chondrogenesis for Donors A and B. Increasing GAG/DNA content in MCM-treated groups from week 2 to 5 was observed for donors A and B. Aggregates treated with MCM alone (group 3) exhibited significant increases in GAG/DNA content from week 2 to week 5, providing strong evidence for the chondroinductive nature of MCM in this system (Figure 2: donors A and B). At week 2, aggregates treated with both

DOI: 10.1021/acsbiomaterials.5b00277
ACS Biomater. Sci. Eng. 2016, 2, 30−42

MCM and BMP-2 at 1× and 4× had higher GAG/DNA than group 3 for both donors, suggesting that BMP-2 may have accelerated chondrogenesis in the presence of MCM.

MCM Were Chondroinductive but Less than TGF-β1. While GAG production was observed in MCM-treated aggregates by week 5, it was not to the same extent as the positive chondrogenic control (group 1). However, all MCM-containing groups except group 3 for donors B and C exhibited a higher degree of mineralization by week 5, while no calcium content was detected in group 1 (Figure 3), suggesting that some of the cartilage tissue in aggregates treated with both MCM and BMP-2 (groups 5, 6, 8, and 9) may have been replaced by mineralized tissue by week 5. Therefore, the role of MCM in promoting cartilage remodeling and tissue mineralization may be the cause for its lower potency in chondrogenic induction compared to that of exogenous TGF-β1 supplementation.

TGF-β1 Promoted ALP Activity but Not Mineralization. For all three donors, ALP activity was detected in group 1 at both time points (Figure 3), suggesting that mature cartilage may be present in these aggregates as ALP expression has been observed in this tissue type.[48] Consistent with this finding, Mueller et al. observed increasing ALP activity in hMSC aggregates cultured in TGF-β1-containing chondrogenic media after 4 weeks.[53] However, in our study, no calcium content was measured in this group at either time point, indicating that even though TGF-β1 promoted ALP expression, it did not induce mineralization.

BMP-2 Enhanced ALP Activity and Mineralization in the Presence of MCM. For donor A, ALP expression in groups 5, 8, and 9, which were exposed to both MCM and BMP-2, was significantly higher at week 2 compared to that of groups 2 and 3, which were not treated with BMP-2 (Figure 3). While ALP activity in aggregates treated with both MCM and BMP-2 (groups 5, 6, 8, and 9) was similar to that of the cells-only group 4, which was treated with 1× BMP-2, they were significantly lower at both time points than cells-only group 7, which received 4× BMP-2 treatment. It is possible that group 7 aggregates directly underwent osteogenic differentiation upon exposure to this higher concentration of BMP-2, while chondrogenesis was first induced in MCM-incorporated aggregates resulting in more delayed expression of ALP. However, tissue mineralization occurred as early as week 2 in MCM- and BMP-2-treated groups 5, 6, 8, and 9. Since only the amount of calcium that was initially incorporated was measured in MCM-incorporated group 3, the presence of BMP-2 may have accelerated mineralization demonstrated by the significantly higher calcium content in MCM- and BMP-2-treated groups 5, 6, 8, and 9 compared to that in the group treated with MCM alone (group 3). Cells-only aggregates receiving exogenous BMP-2 treatment (groups 4 and 7) also exhibited mineralization by week 2, but their calcium contents were significantly lower than those of groups 5, 6, 8, and 9 by week 5, providing strong evidence that the presence of MCM and BMP-2 enhanced mineralization.

For donor B, MCM- and BMP-2-treated groups 5, 6, 8, and 9 had higher ALP activity than MCM-treated group 3 at both time points, corroborating the augmentation of ALP expression by BMP-2 in the presence of MCM. Increasing BMP-2 concentration from 1× to 4× led to higher ALP expression with groups 8 and 9 exhibiting higher ALP activity at week 2 than groups 4, 5, and 6 and group 5, respectively. By week 5, group 9 was significantly higher than all other groups except group 8, further demonstrating a BMP-2 concentration dependence response of ALP activity. The presence of both MCM and BMP-2 also promoted increased mineralization (Figures 3 and S2). With the exception of cells-only group 7 treated with exo. 4× BMP-2, aggregates treated with MCM alone (group 3) and BMP-2 alone (group 4) had significantly lower calcium content at week 5 than groups 5, 6, 8, and 9 and groups 5 and 8, respectively.

Lastly, the enhancing effects of BMP-2 in the presence of MCM on ALP expression and mineralization were much more apparent for donor C. Although groups 6 and 9, in which BMP-2 were delivered from MCM, had similar levels of ALP activity compared to that of groups 3 and 5 for donor C, mineralization occurred in the BMP-2-loaded groups 6 and 9 as early as week 2, while only the baseline calcium level was measured in groups 3 and 5. By week 5, groups 5, 6, 8, and 9 had significantly higher calcium content compared to that of all other groups, which further verifies the augmentation of mineralization in the presence of both MCM and BMP-2 (Figures 3 and S2).

Mode of Growth Factor Delivery Influenced Cellular Response. Compared to BMP-2 exogenously supplemented in the media, BMP-2 released from incorporated MCM may have improved chondrogenesis in aggregates from donors A and B. When aggregates were treated with 1× BMP-2, those with incorporated MCM (groups 5 and 6) had higher GAG/DNA content than cells-only aggregates (group 4) at both time points for donor A and at week 5 for donor B. For these two donors, group 6, in which 1× BMP-2 was delivered from incorporated MCM, exhibited significantly higher GAG production than MCM-incorporated group 5, which was treated with exo. 1× BMP-2. When BMP-2 concentration was increased to 4×, GAG content in cells-only group 7 was significantly lower than that of MCM-incorporated groups at both time points for donor A (groups 8 and 9) and at week 5 for donor B (groups 8 and 9). The lower GAG production in exo. 1× BMP-2 groups 4 and 5 and exo. 4× BMP-2 group 7 compared to BMP-2-loaded groups 6 and 9, respectively, indicates the potential importance of the mode of BMP-2 presentation on chondrogenic differentiation.

The mode of growth factor delivery may have also influenced ALP activity and mineralization. While no difference in response of donor A to varying BMP-2 concentrations and modes of delivery was observed as ALP activity and calcium content were similar among groups 5, 6, 8, and 9, several differences were found for donors B and C (Figure 3). For donor B, week 5 ALP activity in group 9 was significantly higher than that of all other groups except group 8 (Figure 3). Group 9 was higher than group 8, but no significance was found. Nevertheless, the increased expression in group 9 may lead to additional mineralization at a later time point especially since the incorporated MCM are capable of sustained release of BMP-2 even after 60 days (Figure 1C). The continuing presentation of BMP-2 within group 9 aggregates may further augment bone formation.

For donor C, ALP activity at week 2 was highest in group 8, suggesting that BMP-2 delivered exogenously at the higher concentration is optimal for enhancing ALP expression in MCM-treated aggregates for this donor. It is interesting that the high ALP activity at week 2 did not result in a higher degree of mineralization in group 8 by week 5. In fact, groups 6 and 9, in which BMP-2 was delivered from MCM, had similar calcium levels to group 8 despite having much lower ALP activity. It may be that ALP expression in the BMP-2-loaded groups

DOI: 10.1021/acsbiomaterials.5b00277
ACS Biomater. Sci. Eng. 2016, 2, 30−42

peaked before week 2 or between the observed time points, and the local delivery of BMP-2 from the MCM contributed to promoting mineralization despite the lower ALP expression in these groups.

Additionally for donor C, calcium content in group 6, which had MCM loaded with 1× BMP-2, was significantly higher at week 2 than group 5, which received MCM and exo. BMP-2 treatment. The average calcium content in group 6 was also higher but not significantly at week 5. The difference between these groups signifies the importance of how BMP-2 is presented to the cells, exogenously versus delivery from incorporated microparticles, at this lower concentration (1× BMP-2) in enhancing mineralization.

Donor-to-Donor Variability Was Observed. Differences in response to the timing and degree of chondrogenic and osteogenic induction were found among the donors, most likely due to donor-to-donor variability. Unlike donors A and B, little to no GAG content was detected at week 5 for donor C (Figure 2). Comparing the chondrogenic positive control (group 1) results among all 3 donors, donor C appears to be considerably less chondrogenic than donors A and B with much lower GAG/DNA content at both time points. Thus, the minimal chondrogenic response to MCM and BMP-2 by hMSCs from donor C may be due to the lower intrinsic chondrogenic potential of these cells. It is also possible that in aggregates treated with both MCM and BMP-2, higher GAG/DNA production occurred between weeks 2 and 5 and that most of the cartilage had already been replaced by bone by week 5. In fact, significantly higher degrees of mineralization in these groups were observed for this donor, and the resulting calcium levels were similar to those in donor A and higher than that of donor B (Figure 3).

Also for donor C, ALP activity at week 2 was highest in group 8, in which MCM-containing aggregates received exo. 4× BMP-2. This observation greatly differs from what was observed in donor A, for which aggregates treated with both MCM and BMP-2 had significantly lower ALP activity than that of the cells-only group 7 receiving 4× BMP-2. Comparing the GAG content of both donors may explain the difference in ALP expression. While MCM-treated groups exhibited greater GAG production as early as week 2 for donor A, GAG content was much lower for donor C, which as discussed earlier is the least chondrogenic. Thus, it is possible that the higher ALP expression promoted by the presence of MCM and BMP-2 in group 8 at week 2 was not delayed by the occurrence of chondrogenesis for donor C as it was for donor A.

Additionally, MCM incorporation (group 3) promoted ALP expression and mineralization by week 5 for donor A but did not for the other donors (Figures 3 and S2). The strong osteogenic potential of cells from donor A may have been enough for MCM alone to promote mineralization. The very low or lack of ALP activity and absence of cell-secreted calcium in aggregates treated with only MCM for donors B and C provide strong evidence for the strong bioactivity of BMP-2 during the culture period and the importance of BMP-2 on osteogenesis in these donors. Furthermore, the absence of additional calcium beyond the amount initially incorporated in group 3 demonstrates that mineralization observed in MCM- and BMP-2-treated groups was cell-mediated since each group was treated with the same amount of MCM.

The observed donor-to-donor variability confirms previous reports demonstrating variations in hMSC osteogenesis among different donors.[54−56] The tunability of this novel system enables customization by varying MCM formulation and BMP-2 dose to achieve optimal results for hMSCs from various donors. In the future, it may be possible to correlate specific MCM formulations and BMP-2 concentrations to the performance of hMSCs from donors with varying degrees of chondrogenic and osteogenic potential.

Histological Staining Confirmed Biochemical Data. In general, the histological results for donor A corroborated the corresponding biochemical data. TGF-β1-treated group 1 only stained positively for GAG and type II collagen, which was expected given the role of TGF-β1 in inducing articular cartilage formation. Group 2 did not stain for any cartilage markers but stained for osteopontin and lightly for type I collagen, suggesting that the serum-free osteogenic media without BMP-2 could not induce chondrogenesis or bone formation via intramembranous ossification. For MCM-treated groups 3, 5, 6, 8, and 9, staining for cartilage markers were colocalized with intense GAG and type II collagen staining in the same regions, and positive staining for bone markers were colocalized with type I collagen, osteopontin, and osteocalcin observed in calcium-stained regions. Notably, calcium and type I collagen stained intensely throughout constructs receiving local delivery of BMP-2 within the aggregates (groups 6 and 9), corroborating the importance of the mode of BMP-2 delivery on bone formation. Staining of aggregates for donors B and C also supported the respective biochemical data.

Effects of Varying BMP-2 Concentration on Aggregate Histology. Cells-only groups that were not treated with TGF-β1 did not stain for any cartilage markers (Figures 4−5). Group 7 stained more intensely for calcium, type I collagen, and osteopontin than groups 2 and 4 (Figures 4−6), corroborating the biochemical data that showed a concentration-dependent osteogenic response of hMSC aggregates to exo. BMP-2. For MCM-incorporated groups, no major differences were observed in staining for GAG and calcium between groups 8 and 9 compared to groups 5 and 6, respectively, with the exception of more extensive GAG and calcium staining in group 9 compared to group 6. However, the difference in ARS staining intensity was not significant (Figure 4C). For type I collagen staining, group 6 stained most intensely compared to that of all other groups. Type II collagen staining was weaker in group 8 than in group 5, and more extensive staining was observed in group 9 compared to that in group 6. Despite differences in collagen staining, osteocalcin and osteopontin staining were similar among groups that were treated with both MCM and BMP-2 at both concentrations (Figure 6).

Similar to donor A, cells-only aggregates treated with varying exo. BMP-2 concentrations did not stain positively for cartilage markers for donors B and C, and bone markers calcium and type I collagen stained more intensely in group 7 compared to that in groups 2 and 4. No major differences were observed between MCM-incorporated aggregates treated with 1× BMP-2 and 4× BMP-2. Overall, varying BMP-2 concentration significantly affected staining for calcium and type I collagen in cells-only aggregates, while the effect was less apparent for MCM-treated aggregates.

Overall, the effects of BMP-2 and MCM within hMSC aggregates on chondrogenesis and osteogenesis were demonstrated. Notably, the use of serum-free media for culturing the constructs enabled elucidation of the roles of these bioactive factors on chondrogenesis and osteogenesis without interference from the rich variety of proteins present in serum. Despite

DOI: 10.1021/acsbiomaterials.5b00277
ACS Biomater. Sci. Eng. 2016, 2, 30−42

some donor-to-donor variability in responses with regard to the timing and degree of chondrogenic and osteogenic induction, similar trends were found. When aggregates were treated with BMP-2 alone without MCM, BMP-2-induced osteogenic response appeared to be concentration-dependent. At both BMP-2 concentrations, the importance of BMP-2 presentation strategy (i.e., exogenously in the media versus delivery from incorporated microparticles) on chondrogenic differentiation was evident for donors A and B. Treatment of aggregates with MCM alone affected cell viability and chondrogenesis. In general, the presence of MCM improved cell number throughout the culture period. For donors A and B, MCM incorporation was shown to induce chondrogenesis, and the addition of BMP-2 may have accelerated the chondroinductive stimulation of MCM. For donors B and C, BMP-2 was necessary to promote mineralization in MCM-incorporated aggregates as shown by a lack of increase in calcium content in MCM-only aggregates (group 3). The presence of both MCM and BMP-2 may have improved mineralization and induced bone formation via endochondral ossification as demonstrated by early cartilage formation followed by mineralized bone-like tissue growth. Importantly, the released BMP-2 from MCM in general had similar or higher levels of bioactivity compared to that of exo. BMP-2 in inducing chondrogenesis, osteogenesis, and mineralization. Further, no differences in mineralization alone were found between 1× BMP-2 and 4× BMP-2, exogenous or loaded, in MCM-treated groups for all donors, suggesting that the lower BMP-2 concentration may be as effective in enhancing tissue mineralization in this MCM-incorporated system. These results provide strong evidence in support of incorporating BMP-2-loaded MCM within aggregates for bone regeneration via endochondral ossification to overcome the challenges and inefficiencies of exogenous growth factor supplementation.

CONCLUSIONS

The individual roles and interactions of BMP-2 and mineral-coated HAp microparticles on chondrogenesis and osteogenesis within hMSC aggregates were investigated. In addition, the diffusion limitations and practical inefficiencies associated with traditional growth factor supplementation in the media were addressed by employing MCM to locally deliver BMP-2 to cells within the aggregates in a controlled and sustained manner. In general, the BMP-2 released from incorporated MCM in our self-sustaining system induced GAG production, ALP expression, and mineralization at levels higher than or similar to exogenously supplemented BMP-2. In groups treated with both MCM and BMP-2 for all three donors, cartilage formation occurring as early as week 2 may have promoted the additional mineralization at week 5, suggesting that the presence of MCM and BMP-2 may have induced endochondral ossification and enhanced mineralization. To our knowledge, this is the first report of guided chondrogenesis and osteogenesis via the controlled delivery of bioactive signals using mineral-coated microparticles within hMSC aggregates. This MCM-incorporated high-density hMSC system shows great promise as a tissue engineering therapy that can be readily injected to repair critical-sized defects without prior long-term culture. From a clinical perspective, long-term culture is not ideal due to the time and high costs involved. While there are scaffold-based approaches delivering cells and growth factors that can be readily implanted, they require the precise control of scaffold properties such as degradation. For example, if scaffold degradation is not synchronized with the rate of new bone formation, various processes including cell migration and proliferation, extracellular matrix production, tissue remodeling, and construct integration can be adversely affected, potentially compromising the integrity of the regenerated bone. This system has potential as a scalable therapy that can be customized to repair critical-sized bone defects of different shapes and sizes, and its efficacy may be optimized by varying MCM and/or BMP-2 concentration and tuning the BMP-2 delivery profile through modification of the MCM mineral coating composition.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsbiomaterials.5b00277.

Photomicrographs of representative Alizarin Red S histology of MCM-incorporated hMSC aggregate after 2 days of culture; of week 5 aggregates for all three donors; and of immunohistochemistry negative controls in which aggregate sections were treated with rabbit IgG instead of primary antibody (PDF)

AUTHOR INFORMATION

Corresponding Author

*Tel: 216-368-6425. Fax: 216-368-4969. E-mail: exa46@case.edu.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Amad Awadallah for technical assistance and Nathan Morris for statistical analysis consultation. We gratefully acknowledge funding from the National Institutes of Health (R01AR063194), the AO Foundation, and a National Science Foundation Graduate Research Fellowship (to P.N.D.).

REFERENCES

(1) Hildebrand, F.; van Griensven, M.; Giannoudis, P.; Schreiber, T.; Frink, M.; Probst, C.; Grotz, M.; Krettek, C.; Pape, H. C. Impact of hypothermia on the immunologic response after trauma and elective surgery. *Surgical technology international* **2005**, *14*, 41−50.

(2) Habal, M. B.; Reddi, A. H. Bone grafts and bone induction substitutes. *Clin. Plast. Surg.* **1994**, *21* (4), 525−42.

(3) Brinker, M. R.; O'Connor, D. P. Outcomes of tibial nonunion in older adults following treatment using the Ilizarov method. *Journal of orthopaedic trauma* **2007**, *21* (9), 634−42.

(4) Schmidmaier, G.; Capanna, R.; Wildemann, B.; Beque, T.; Lowenberg, D. Bone morphogenetic proteins in critical-size bone defects: what are the options? *Injury* **2009**, *40* (Suppl 3), S39−S43.

(5) Ronga, M.; Fagetti, A.; Canton, G.; Paiusco, E.; Surace, M. F.; Cherubino, P. Clinical applications of growth factors in bone injuries: experience with BMPs. *Injury* **2013**, *44* (Suppl 1), S34−S39.

(6) Steinert, A. F.; Rackwitz, L.; Gilbert, F.; Noth, U.; Tuan, R. S. Concise review: the clinical application of mesenchymal stem cells for musculoskeletal regeneration: current status and perspectives. *Stem Cells Transl. Med.* **2012**, *1* (3), 237−47.

(7) Johnstone, B.; Hering, T. M.; Caplan, A. I.; Goldberg, V. M.; Yoo, J. U. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp. Cell Res.* **1998**, *238* (1), 265−72.

(8) Tuan, R. S.; Boland, G.; Tuli, R. Adult mesenchymal stem cells and cell-based tissue engineering. *Arthritis research & therapy* **2003**, *5* (1), 32−45.

DOI: 10.1021/acsbiomaterials.5b00277
ACS Biomater. Sci. Eng. 2016, 2, 30−42

(9) Welter, J. F.; Solchaga, L. A.; Penick, K. J. Simplification of aggregate culture of human mesenchymal stem cells as a chondrogenic screening assay. *BioTechniques* **2007**, *42* (6), 732 734−7.
(10) Athanasiou, K. A.; Eswaramoorthy, R.; Hadidi, P.; Hu, J. C. Self-organization and the self-assembling process in tissue engineering. *Annu. Rev. Biomed. Eng.* **2013**, *15*, 115−36.
(11) Lin, R. Z.; Chang, H. Y. Recent advances in three-dimensional multicellular spheroid culture for biomedical research. *Biotechnol. J.* **2008**, *3* (9−10), 1172−84.
(12) Langenbach, F.; Naujoks, C.; Smeets, R.; Berr, K.; Depprich, R.; Kubler, N.; Handschel, J. Scaffold-free microtissues: differences from monolayer cultures and their potential in bone tissue engineering. *Clinical oral investigations* **2013**, *17* (1), 9−17.
(13) Solorio, L. D.; Vieregge, E. L.; Dhami, C. D.; Alsberg, E. High-density cell systems incorporating polymer microspheres as micro-environmental regulators in engineered cartilage tissues. *Tissue Eng., Part B* **2013**, *19* (3), 209−20.
(14) Hildebrandt, C.; Buth, H.; Thielecke, H. A scaffold-free in vitro model for osteogenesis of human mesenchymal stem cells. *Tissue Cell* **2011**, *43* (2), 91−100.
(15) Ferro, F.; Falini, G.; Spelat, R.; D'Aurizio, F.; Puppato, E.; Pandolfi, M.; Beltrami, A. P.; Cesselli, D.; Beltrami, C. A.; Impiombato, F. S.; Curcio, F. Biochemical and biophysical analyses of tissue-engineered bone obtained from three-dimensional culture of a subset of bone marrow mesenchymal stem cells. *Tissue Eng., Part A* **2010**, *16* (12), 3657−67.
(16) Burns, J. S.; Rasmussen, P. L.; Larsen, K. H.; Schroder, H. D.; Kassem, M. Parameters in three-dimensional osteospheroids of telomerized human mesenchymal (stromal) stem cells grown on osteoconductive scaffolds that predict in vivo bone-forming potential. *Tissue Eng., Part A* **2010**, *16* (7), 2331−42.
(17) Wang, W.; Itaka, K.; Ohba, S.; Nishiyama, N.; Chung, U. I.; Yamasaki, Y.; Kataoka, K. 3D spheroid culture system on micro-patterned substrates for improved differentiation efficiency of multipotent mesenchymal stem cells. *Biomaterials* **2009**, *30* (14), 2705−15.
(18) Muraglia, A.; Corsi, A.; Riminucci, M.; Mastrogiacomo, M.; Cancedda, R.; Bianco, P.; Quarto, R. Formation of a chondro-osseous rudiment in micromass cultures of human bone-marrow stromal cells. *J. Cell Sci.* **2003**, *116* (Pt 14), 2949−55.
(19) Farrell, E.; Both, S. K.; Odorfer, K. I.; Koevoet, W.; Kops, N.; O'Brien, F. J.; Baatenburg de Jong, R. J.; Verhaar, J. A.; Cuijpers, V.; Jansen, J.; Erben, R. G.; van Osch, G. J. In-vivo generation of bone via endochondral ossification by in-vitro chondrogenic priming of adult human and rat mesenchymal stem cells. *BMC Musculoskeletal Disord.* **2011**, *12*, 31.
(20) Scotti, C.; Tonnarelli, B.; Papadimitropoulos, A.; Scherberich, A.; Schaeren, S.; Schauerte, A.; Lopez-Rios, J.; Zeller, R.; Barbero, A.; Martin, I. Recapitulation of endochondral bone formation using human adult mesenchymal stem cells as a paradigm for developmental engineering. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107* (16), 7251−6.
(21) Govender, S.; Csimma, C.; Genant, H. K.; Valentin-Opran, A.; Amit, Y.; Arbel, R.; Aro, H.; Atar, D.; Bishay, M.; Borner, M. G.; Chiron, P.; Choong, P.; Cinats, J.; Courtenay, B.; Feibel, R.; Geulette, B.; Gravel, C.; Haas, N.; Raschke, M.; Hammacher, E.; van der Velde, D.; Hardy, P.; Holt, M.; Josten, C.; Ketterl, R. L.; Lindeque, B.; Lob, G.; Mathevon, H.; McCoy, G.; Marsh, D.; Miller, R.; Munting, E.; Oevre, S.; Nordsletten, L.; Patel, A.; Pohl, A.; Rennie, W.; Reynders, P.; Rommens, P. M.; Rondia, J.; Rossouw, W. C.; Daneel, P. J.; Ruff, S.; Ruter, A.; Santavirta, S.; Schildhauer, T. A.; Gekle, C.; Schnettler, R.; Segal, D.; Seiler, H.; Snowdowne, R. B.; Stapert, J.; Taglang, G.; Verdonk, R.; Vogels, L.; Weckbach, A.; Wentzensen, A.; Wisniewski, T. Recombinant human bone morphogenetic protein-2 for treatment of open tibial fractures: a prospective, controlled, randomized study of four hundred and fifty patients. *Journal of bone and joint surgery. American volume* **2002**, *84-A* (12), 2123−34.
(22) Wozney, J. M.; Rosen, V.; Celeste, A. J.; Mitsock, L. M.; Whitters, M. J.; Kriz, R. W.; Hewick, R. M.; Wang, E. A. Novel regulators of bone formation: molecular clones and activities. *Science* **1988**, *242* (4885), 1528−34.
(23) Wang, E. A.; Rosen, V.; D'Alessandro, J. S.; Bauduy, M.; Cordes, P.; Harada, T.; Israel, D. I.; Hewick, R. M.; Kerns, K. M.; LaPan, P.; et al. Recombinant human bone morphogenetic protein induces bone formation. *Proc. Natl. Acad. Sci. U. S. A.* **1990**, *87* (6), 2220−4.
(24) Schmitt, B.; Ringe, J.; Haupl, T.; Notter, M.; Manz, R.; Burmester, G. R.; Sittinger, M.; Kaps, C. BMP2 initiates chondrogenic lineage development of adult human mesenchymal stem cells in high-density culture. *Differentiation* **2003**, *71* (9−10), 567−77.
(25) Leboy, P. S.; Sullivan, T. A.; Nooreyazdan, M.; Venezian, R. A. Rapid chondrocyte maturation by serum-free culture with BMP-2 and ascorbic acid. *J. Cell. Biochem.* **1997**, *66* (3), 394−403.
(26) Soballe, K.; Overgaard, S.; Hansen, E. S.; Brokstedt-Rasmussen, H.; Lind, M.; Bunger, C. A review of ceramic coatings for implant fixation. *J. Long-Term Eff. Med. Implants* **1999**, *9* (1−2), 131−51.
(27) Dumbleton, J.; Manley, M. T. Hydroxyapatite-coated prostheses in total hip and knee arthroplasty. *Journal of bone and joint surgery. American volume* **2004**, *86-A* (11), 2526−40.
(28) He, P.; Sahoo, S.; Ng, K. S.; Chen, K.; Toh, S. L.; Goh, J. C. Enhanced osteoinductivity and osteoconductivity through hydroxyapatite coating of silk-based tissue-engineered ligament scaffold. *J. Biomed. Mater. Res., Part A* **2013**, *101A* (2), 555−66.
(29) Chiroff, R. T.; White, R. A.; White, E. W.; Weber, J. N.; Roy, D. The restoration of the articular surfaces overlying Replamineform porous biomaterials. *J. Biomed. Mater. Res.* **1977**, *11* (2), 165−78.
(30) Suominen, E.; Aho, A. J.; Vedel, E.; Kangasniemi, I.; Uusipaikka, E.; Yli-Urpo, A. Subchondral bone and cartilage repair with bioactive glasses, hydroxyapatite, and hydroxyapatite-glass composite. *J. Biomed. Mater. Res.* **1996**, *32* (4), 543−51.
(31) Sotoudeh, A.; Jahanshahi, A.; Takhtfooladi, M. A.; Bazazan, A.; Ganjali, A.; Harati, M. P. Study on nano-structured hydroxyapatite/zirconia stabilized yttria on healing of articular cartilage defect in rabbit. *Acta cirurgica brasileira/Sociedade Brasileira para Desenvolvimento Pesquisa em Cirurgia* **2013**, *28* (5), 340−5.
(32) Sumner, D. R.; Turner, T. M.; Urban, R. M.; Virdi, A. S.; Inoue, N. Additive enhancement of implant fixation following combined treatment with rhTGF-beta2 and rhBMP-2 in a canine model. *Journal of bone and joint surgery. American volume* **2006**, *88* (4), 806−17.
(33) Alt, V.; Pfefferle, H. J.; Kreuter, J.; Stahl, J. P.; Pavlidis, T.; Meyer, C.; Mockwitz, J.; Wenisch, S.; Schnettler, R. Effect of glycerol-L-lactide coating polymer on bone ingrowth of bFGF-coated hydroxyapatite implants. *J. Controlled Release* **2004**, *99* (1), 103−11.
(34) Hossain, M.; Irwin, R.; Baumann, M. J.; McCabe, L. R. Hepatocyte growth factor (HGF) adsorption kinetics and enhancement of osteoblast differentiation on hydroxyapatite surfaces. *Biomaterials* **2005**, *26* (15), 2595−602.
(35) Yu, X.; Khalil, A.; Dang, P. N.; Alsberg, E.; Murphy, W. L. Multilayered Inorganic Microparticles for Tunable Dual Growth Factor Delivery. *Adv. Funct. Mater.* **2014**, *24* (20), 3082−3093.
(36) Jongpaiboonkit, L.; Franklin-Ford, T.; Murphy, W. L. Growth of hydroxyapatite coatings on biodegradable polymer microspheres. *ACS Appl. Mater. Interfaces* **2009**, *1* (7), 1504−11.
(37) Ishaug-Riley, S. L.; Crane, G. M.; Gurlek, A.; Miller, M. J.; Yasko, A. W.; Yaszemski, M. J.; Mikos, A. G. Ectopic bone formation by marrow stromal osteoblast transplantation using poly(DL-lactic-co-glycolic acid) foams implanted into the rat mesentery. *J. Biomed. Mater. Res.* **1997**, *36* (1), 1−8.
(38) Ishaug, S. L.; Crane, G. M.; Miller, M. J.; Yasko, A. W.; Yaszemski, M. J.; Mikos, A. G. Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds. *J. Biomed. Mater. Res.* **1997**, *36* (1), 17−28.
(39) Coyle, C. H.; Izzo, N. J.; Chu, C. R. Sustained hypoxia enhances chondrocyte matrix synthesis. *J. Orthop. Res.* **2009**, *27* (6), 793−9.
(40) Schipani, E. Hypoxia and HIF-1alpha in chondrogenesis. *Ann. N. Y. Acad. Sci.* **2006**, *1068*, 66−73.
(41) Petersen, W.; Tsokos, M.; Pufe, T. Expression of VEGF121 and VEGF165 in hypertrophic chondrocytes of the human growth plate and epiphyseal cartilage. *J. Anat.* **2002**, *201* (2), 153−7.

DOI: 10.1021/acsbiomaterials.5b00277
ACS Biomater. Sci. Eng. 2016, 2, 30−42

(42) Haynesworth, S. E.; Goshima, J.; Goldberg, V. M.; Caplan, A. I. Characterization of cells with osteogenic potential from human marrow. *Bone* **1992**, *13* (1), 81−8.
(43) Solorio, L. D.; Dhami, C. D.; Dang, P. N.; Vieregge, E. L.; Alsberg, E. Spatiotemporal regulation of chondrogenic differentiation with controlled delivery of transforming growth factor-beta1 from gelatin microspheres in mesenchymal stem cell aggregates. *Stem Cells Transl. Med.* **2012**, *1* (8), 632−9.
(44) Honda, Y.; Ding, X.; Mussano, F.; Wiberg, A.; Ho, C. M.; Nishimura, I. Guiding the osteogenic fate of mouse and human mesenchymal stem cells through feedback system control. *Sci. Rep.* **2013**, *3*, 3420.
(45) Farndale, R. W.; Buttle, D. J.; Barrett, A. J. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim. Biophys. Acta, Gen. Subj.* **1986**, *883* (2), 173−7.
(46) Estes, B. T.; Diekman, B. O.; Gimble, J. M.; Guilak, F. Isolation of adipose-derived stem cells and their induction to a chondrogenic phenotype. *Nat. Protoc.* **2010**, *5* (7), 1294−311.
(47) Egan, K. P.; Brennan, T. A.; Pignolo, R. J. Bone histomorphometry using free and commonly available software. *Histopathology* **2012**, *61* (6), 1168−73.
(48) Miao, D.; Scutt, A. Histochemical localization of alkaline phosphatase activity in decalcified bone and cartilage. *J. Histochem. Cytochem.* **2002**, *50* (3), 333−40.
(49) van der Stok, J.; Koolen, M. K.; Jahr, H.; Kops, N.; Waarsing, J. H.; Weinans, H.; van der Jagt, O. P. Chondrogenically differentiated mesenchymal stromal cell pellets stimulate endochondral bone regeneration in critical-sized bone defects. *Eur. Cell. Mater.* **2014**, *27*, 137−48 discussion 148.
(50) Liu, K.; Zhou, G. D.; Liu, W.; Zhang, W. J.; Cui, L.; Liu, X.; Liu, T. Y.; Cao, Y. The dependence of in vivo stable ectopic chondrogenesis by human mesenchymal stem cells on chondrogenic differentiation in vitro. *Biomaterials* **2008**, *29* (14), 2183−92.
(51) Kim, Y. J.; Sah, R. L.; Doong, J. Y.; Grodzinsky, A. J. Fluorometric assay of DNA in cartilage explants using Hoechst 33258. *Anal. Biochem.* **1988**, *174* (1), 168−76.
(52) Awad, H. A.; Halvorsen, Y. D.; Gimble, J. M.; Guilak, F. Effects of transforming growth factor beta1 and dexamethasone on the growth and chondrogenic differentiation of adipose-derived stromal cells. *Tissue Eng.* **2003**, *9* (6), 1301−12.
(53) Mueller, M. B.; Fischer, M.; Zellner, J.; Berner, A.; Dienstknecht, T.; Prantl, L.; Kujat, R.; Nerlich, M.; Tuan, R. S.; Angele, P. Hypertrophy in mesenchymal stem cell chondrogenesis: effect of TGF-beta isoforms and chondrogenic conditioning. *Cells Tissues Organs* **2010**, *192* (3), 158−66.
(54) Siddappa, R.; Licht, R.; van Blitterswijk, C.; de Boer, J. Donor variation and loss of multipotency during in vitro expansion of human mesenchymal stem cells for bone tissue engineering. *J. Orthop. Res.* **2007**, *25* (8), 1029−41.
(55) Mentink, A.; Hulsman, M.; Groen, N.; Licht, R.; Dechering, K. J.; van der Stok, J.; Alves, H. A.; Dhert, W. J.; van Someren, E. P.; Reinders, M. J.; van Blitterswijk, C. A.; de Boer, J. Predicting the therapeutic efficacy of MSC in bone tissue engineering using the molecular marker CADM1. *Biomaterials* **2013**, *34* (19), 4592−601.
(56) Phinney, D. G.; Kopen, G.; Righter, W.; Webster, S.; Tremain, N.; Prockop, D. J. Donor variation in the growth properties and osteogenic potential of human marrow stromal cells. *J. Cell. Biochem.* **1999**, *75* (3), 424−36.

DOI: 10.1021/acsbiomaterials.5b00277
ACS Biomater. Sci. Eng. 2016, 2, 30−42