Sustained Release of Bone Morphogenetic Protein 2 via Coacervate Improves the Osteogenic Potential of Muscle-Derived Stem Cells

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ABSTRACT

Muscle-derived stem cells (MDSCs) isolated from mouse skeletal muscle by a modified preplate technique exhibit long-term proliferation, high self-renewal, and multipotent differentiation capabilities in vitro. MDSCs retrovirally transduced to express bone morphogenetic proteins (BMPs) can differentiate into osteocytes and chondrocytes and enhance bone and articular cartilage repair in vivo, a feature that is not observed with nontransduced MDSCs. These results emphasize that MDSCs require prolonged exposure to BMPs to undergo osteogenic and chondrogenic differentiation. A sustained BMP protein delivery approach provides a viable and potentially more clinically translatable alternative to genetic manipulation of the cells. A unique growth factor delivery platform comprised of native heparin and a synthetic polycation, poly(ethylene argininylaspartate diglyceride) (PEAD), was used to bind, protect, and sustain the release of bone morphogenetic protein-2 (BMP2) in a temporally and spatially controlled manner. Prolonged exposure to BMP2 released by the PEAD:heparin delivery system promoted the differentiation of MDSCs to an osteogenic lineage in vitro and induced the formation of viable bone at an ectopic site in vivo. This new strategy represents an alternative approach for bone repair mediated by MDSCs while bypassing the need for gene therapy. Stem Cells Translational Medicine 2013;2:667–677

INTRODUCTION

The existence of osteoprogenitor cells in the skeletal muscle has long been postulated [1–5]. A number of candidates have been proposed, such as satellite cells [6] and primary myoblasts [6, 7]. Our group has also isolated and characterized a slowly adherent cell population (muscle-derived stem cells [MDSCs]) from skeletal muscle via a modified preplate technique [8–11]; however, their exact relationship to satellite cells and blood vessel-derived progenitor cells remains unclear [12]. When provided with osteogenic stimuli such as bone morphogenetic proteins (BMPs), they can produce osseous-like tissue in vitro [6, 8] and, when retrovirally transduced to express BMP2 or BMP4, have been shown to differentiate into osteocytes and chondrocytes and enhance bone and articular cartilage repair in vivo [9, 13–17]. More importantly, BMP-transduced MDSCs show superior bone healing capabilities compared with other similarly transduced muscle-derived cells [18, 19]. These reports suggest that progenitor cells derived from skeletal muscle, especially MDSCs, could represent a promising alternative cell source to bone marrow-derived mesenchymal stem cells for bone tissue engineering.

Despite the promise that MDSCs hold for bone regeneration, the absence of BMPs they do not differentiate toward an osteogenic lineage; rather, they form myotubes and myofibers when injected alone into a muscle pocket [5, 20, 21]. Applying the proper stimuli to create an osteoinductive environment in vivo is therefore important for harnessing the osteogenic potential of MDSCs. BMP2 and BMP4 have been shown to produce the most successful results using gene transduction [9, 15–17]; however, from a translational point of view, a nonviral approach is preferred and thus far has received limited exploration.

It is known that prolonged exposure to BMPs at an appropriate concentration is necessary to induce MDSC differentiation and subsequent osteochondrogenesis in vivo; however, BMPs have very short half-lives in the body [22, 23], and maintaining an adequate local BMP concentration is challenging. A controlled delivery method that can protect and sustain the release of BMPs would be particularly attractive for muscle cell-based bone engineering. To this end, we used a...
The BMP2 coacervate formed immediately upon addition of PEAD, visible as a turbid solution. The BMP2 coacervate was then added by pipet to cell culture medium for in vitro testing or suspended in fibrinogen solution for in vivo implantation.

**BMP2 Loading and Release Assays**

The BMP2-coacervate was formed with 100 μg of heparin, 100 ng of BMP2, and 500 μg of PEAD in Dulbecco’s phosphate-buffered saline (DPBS) and centrifuged at 12,100 g for 5 minutes to pellet the coacervate. For determination of loading efficiency, Western blot was performed by mixing the supernatant and pellet with sample buffer followed by denaturation at 95°C for 5 minutes. The proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred to a polyvinylidene difluoride membrane. Rabbit anti-human BMP2 primary antibody (PeproTech, Rocky Hill, NJ, http://www.peprotech.com) and horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) were used, followed by the addition of a chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA, http://www.thermofisher.com). Band intensities were quantified using ImageLab software (Bio-Rad, Hercules, CA, http://www.bio-rad.com) and compared with a standard band of 100 ng of free BMP2 in DPBS for calculation of loading efficiency. To determine the release profile, media supernatant was aspirated at various time points, replaced with fresh DPBS, and incubated at 37°C. BMP2 levels in the supernatant were quantified by BMP2 enzyme-linked immunosorbent assay (ELISA) kit (PeproTech).

**Materials and Methods**

**Preparation of the BMP2 Coacervate**

PEAD was synthesized as previously described [26, 27]. Clinical-grade heparin sodium, USP from porcine intestine (Scientific Protein Labs, Waunakee, WI, http://www.spl-pharma.com), and PEAD were each dissolved in saline at 0.125 mg/ml and filter-sterilized at 0.22 μm. Heparin and recombinant human BMP2 (Medtronic, Fridley, MN, http://www.medtronic.com) were combined initially and pipetted to mix and allow for growth factor binding. PEAD was added at a 5:1 PEAD:heparin mass ratio, previously shown to produce an overall neutrally charged solution [27]. The BMP2 coacervate formed immediately upon addition of PEAD, visible as a turbid solution. The BMP2 coacervate was then added by pipet to cell culture medium for in vitro testing or suspended in fibrinogen solution for in vivo implantation.

**Isolation, Culture, and Transduction of Mouse MDSCs**

MDSCs were isolated from the hind limb skeletal muscle of 3-week-old C57/BL10J mice (Jackson Laboratory, Bar Harbor, ME, http://www.jax.org) via a modified preplate technique that has been described previously [8–10]. MDSCs were cultured on collagen I-coated flasks in MDSC basal medium, defined as Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum, 10% horse serum, 1% penicillin/streptomycin (all from Invitrogen, Carlsbad, CA, http://www.invitrogen.com), and...
0.5% chick embryo extract (Accurate Chemical, Westbury, NY, http://www.accuratechemical.com). The cells were trypsinized and reseeded to a density of 250 cells per cm² until a sufficient number of cells were available for the assays. For in vivo cell tracking, MDSCs were retrovirally transduced with a green fluorescent protein (GFP) vector as described previously [28]. The transduced cells were sorted for GFP signal by fluorescence-activated cell sorting (FACSAria; BD Biosciences, San Diego, CA, http://www.bdbiosciences.com) and cultured for two passages prior to use in the experiments.

**In Vitro Assays of Osteogenic Potential of C2C12 Cells and MDSCs**

The bioactivity of delivered BMP2 was determined by its ability to stimulate alkaline phosphatase (ALP) production by a mouse myoblast cell line, C2C12 (CRL-1772; American Type Culture Collection, Manassas, VA, http://www.atcc.org), in monolayer culture. The osteogenic effects of delivered BMP2 were also evaluated in vitro by ALP production of the MDSCs in both monolayer and threedimensional (3D) culture within a fibrin gel. Briefly, for monolayer culture the cells were seeded in a 24-well plate in MDSC basal medium, and a cell culture insert (BD Biosciences) was overlaid on each gel. After 5 days of culture, fibrin gels were frozen/thawed twice. Cell debris was removed by centrifugation, and 10^5 MDSCs were seeded in 10 μl of MDSC basal medium and incubated over-night. The next day, the medium was replaced with MDSC basal medium supplemented with 0 (control), 0.125, 0.25, or 0.5 mg/ml coacervate (PEAD:heparin). The coacervate was prepared by dissolving PEAD and heparin in DPBS and then combining them at a 5:1 PEAD:heparin mass ratio. Appropriate volumes of preformed coacervate were then added to the media at the desired concentrations. On days 1, 3, and 5, cell lysates were prepared by the addition of 0.1% Triton X-100 (Sigma-Aldrich) followed by three freeze-thaw cycles. Double-stranded DNA (dsDNA) content of the cell lysate was measured using a Quant-iT dsDNA high-sensitivity assay kit (Invitrogen).

**In Vitro MDSC Proliferation Assay**

Cell growth over time was assessed by DNA quantification assay as described previously [29]. Briefly, 2 × 10^5 MDSCs were seeded in a 48-well plate in MDSC basal medium and incubated overnight. The next day, the medium was replaced with MDSC basal medium supplemented with 0 (control), 0.125, 0.25, or 0.5 mg/ml coacervate (PEAD:heparin). The coacervate was prepared by dissolving PEAD and heparin in DPBS and then combining them at a 5:1 PEAD:heparin mass ratio. Appropriate volumes of preformed coacervate were then added to the media at the desired concentrations. On days 1, 3, and 5, cell lysates were prepared by the addition of 0.1% Triton X-100 (Sigma-Aldrich) followed by three freeze-thaw cycles. Double-stranded DNA (dsDNA) content of the cell lysate was measured using a Quant-iT dsDNA high-sensitivity assay kit (Invitrogen).

**In Vivo Bone Formation at a Heterotopic Site**

University of Pittsburgh Institutional Animal Care and Use Committee approval was obtained prior to any animal studies being performed. Power analysis was based on a pilot study performed under identical conditions but with different treatment groups, all of which contained MDSCs and BMP2 in a fibrin gel. The pooled standard deviation of bone volume after 4 weeks of healing was 1.032 mm^3 for this pilot study (unpublished data). A power analysis calculation indicated that in order to detect 1.5 standard deviations of the variable means (α-error = 0.05, β-error = 0.2), six ectopic sites per group were required (Minitab Inc., State College, PA, http://www.minitab.com). Thus, fifteen 6–8-week-old C57/BL10J mice were randomly allocated into five groups: control (no cells, no coacervate), MDSCs only, BMP-2 coacervate only, free BMP-2 + MDSCs, and BMP-2 coacervate + MDSCs. Mice were anesthetized with isoflurane (Henry Schein Animal Health, Dublin, OH, https://www.henryscheinvet.com), and a 1-cm incision was made on the lateral aspect of both thighs (six thighs per group). The quadriceps muscles were identified, and a small incision was made and held open with forceps. Immediately before implantation, 2 μg of free BMP2 or BMP2 coacervate (2 μg of BMP2, 40 μg of heparin, and 200 μg of PEAD) and 5 × 10^5 MDSCs were suspended in 40 μl of 10 mg/ml fibrinogen solution and combined with 9.5 NIH U/ml thrombin. The solution was pipetted into the open muscle pocket where it conformed to the pocket space and was allowed to solidify briefly, forming the fibrin gel. The incision was then closed with 4-0 Prolene suture (Cardinal Health, Dublin, OH, http://www.cardinal.com). The animals were examined radiographically 2 and 4 weeks after implantation and histologically at week 4. No immune-suppressive treatment was given as the MDSCs were isolated from C57/BL10J mice from the same inbred colony. Furthermore, our previous work demonstrated the efficacy of BMP4-expressing MDSCs for induction of de novo bone formation in immunocompetent mice despite the presence of an immune reaction [14].
Radiographical and Histological Analysis
At each time point, the animals were anesthetized by inhalation of isoflurane. Both thighs were scanned with a micro-computed tomography (micro-CT) scanner (VivaCT 40; Scanco, Brüttschellen, Switzerland, www.scanco.ch), and the mineralized bone tissue within the muscle pocket was quantified. The micro-CT scanning and quantification of the mineralized bone volume were performed by a specialist in the laboratory who was blinded to the group design. Following sacrifice, quadriceps muscles were removed, fixed in 4% paraformaldehyde, embedded in sodium carboxy methyl cellulose (NEG50; Richard-Allan Scientific, Kalama-zoo, MI), and frozen in liquid nitrogen, and 5-μm nondecalcified cryosections were obtained using a cryostat (HM505E; Microm, Walldorf, Germany, www.microm.de). Sections were stained with 2% alizarin red for calcium deposition and 2% silver nitrate for phosphate detection (von Kossa staining), and immunostained for GFP to detect the transplanted MDSCs. For GFP immunostaining, nondecalcified sections were rinsed in phosphate-buffered saline (PBS), blocked in 10% horse serum, and incubated overnight at 4°C with rabbit anti-GFP antibody (ab290; Abcam, Cambridge, U.K., http://www.abcam.com) in 5% horse serum. After washing, sections were incubated for 1 hour at room temperature with biotin-labeled horse anti-rabbit IgG antibody. Biotin-immune complexes were detected using the Avidin/Biotin technique (ABC) and diaminobenzidine peroxidase substrate kit (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com). All of the images were acquired using an up-right bright-field microscope (Eclipse E800; Nikon, Tokyo, Japan, http://www.nikon.com). The number of GFP-positive cells within and at the edge of the osteoid were also counted and normalized to bone surface (mm²) using NIH ImageJ software, version 1.46r.

Statistical Analysis
Statistical tests were performed using SPSS 16.0 (SPSS, Chicago, IL, http://www.spss.com). The data were tested for normality and equal variance before analysis. Statistical differences were calculated using analysis of variance (or analysis of variance on ranks if equal variance testing failed). The differences were considered significant at p < .05.

RESULTS
Loading and Controlled Release of BMP2
To determine loading efficiency, the amount of BMP2 in the supernatant and coacervate pellet after centrifugation was determined by Western blotting. Of the BMP2, 98.2% was detected in the pellet, indicating that the coacervate loaded the BMP2 with high efficiency, and the remaining unloaded BMP2 was detected in the supernatant (Fig. 2A). The heparin: BMP2 molar ratio used for the ectopic bone formation experiments in vivo was approximately 20:1. Assuming a 1:1 binding interaction between BMP2 and heparin, the coacervate has a theoretical loading potential of 20 times the amount of BMP2 used for in vivo experiments. The release profile of BMP2 from the coacervate into DPBS was determined by pelleting the BMP2 coacervate by centrifugation and measuring the concentration of free BMP2 in the supernatant at various time points using ELISA. Less than 1% of the BMP2 was detected in the supernatant immediately after forming the coacervate, confirming the high loading efficiency determined by Western blot. A minimal burst release of only 1.3% after 1 day was observed (Fig. 2B). Thereafter, release was linear until day 10 when the release rate slowed and then sustained through the end of the experiment. After 28 days, approximately 25% of the BMP2 had been released from the coacervate. The data indicated that the coacervate can efficiently control the release of incorporated BMP2.

Effect of the Coacervate on MDSC Proliferation
The influence of the coacervate (PEAD:heparin) on the proliferation of MDSCs was tested by adding different concentrations of coacervate to the culture media and assessing cell proliferation after 1, 3, and 5 days by DNA quantification (Fig. 3). No significant differences were seen among the groups at days 1 and 3; however, at day 5, the coacervate inhibited the growth of the MDSCs in a dose-dependent manner at concentrations above 0.125 mg/ml. The coacervate at 0.125 mg/ml showed no difference from the control and therefore had no inhibitory effects on MDSC growth. This concentration was consequently used for all the following experiments, including the in vivo tests.

Bioactivity of BMP2 Released From the Coacervate
One important feature of a growth factor delivery vehicle is its ability to maintain growth factor bioactivity. We assessed this capability of the coacervate by stimulating ALP expression of the myogenic cell line, C2C12, with coacervate-released BMP2. Evaluating the expression of this preosteoblast marker in C2C12 cells is a common method of determining the activity of BMPs [30–32]. We compared 100 ng of BMP2 coacervate with an equal amount of free BMP2, and control groups included DPBS and the
delivery vehicle (coacervate) alone (Fig. 4B). After 5 days of culture, BMP2 released from the coacervate induced significant expression of ALP by the C2C12 cells compared with the control. Furthermore, the ALP expression level induced by the BMP2 coacervate was significantly greater than induction with the same amount of free BMP2. As expected, the delivery vehicle alone had no effect on ALP expression by the C2C12 cells compared with the DPBS control, and ALP staining showed similar results (Fig. 4A). The data suggest that the coacervate delivery system can preserve or even enhance the bioactivity of delivered BMP2 compared with free BMP2.

Effects of BMP2 Coacervate on the Osteogenesis of MDSCs In Vitro

Upon stimulation with BMP2, MDSCs undergo osteogenic differentiation, which can be evaluated by their expression of ALP [8, 11]. The efficacy of sustained delivery of BMP2 for inducing osteogenesis of MDSC was tested both in monolayer and in a 3D fibrin gel, representing a potential scaffold for cell delivery to a bone defect. After 5 days in monolayer culture, the ALP activity of the MDSCs was effectively stimulated with 100 ng of BMP2 coacervate, whereas an equal single dose of free BMP2 showed no difference from the negative control and the coacervate-only groups (Fig. 5A). A multidose free-BMP2 group was also included to mimic sustained release conditions with an equal dose of free BMP2 being added every 2 days. This amounted to a threefold higher total dosage of BMP2 (300 ng) compared with the BMP2 coacervate group (100 ng). The multidose free-BMP2 group did stimulate ALP activity, indicating that MDSCs require more than just a single exposure to free BMP2 to be effective; however, the ALP expression of this multidose BMP2 group showed no statistical difference from the BMP2 coacervate group, nor was there a difference between the BMP2 coacervate group and the BMP4-transduced MDSC group (positive control), which was also supported by positive ALP staining (Fig. 5C). RT-PCR analysis showed the mRNA expression level of Runx.2 was slightly higher in MDSCs that were stimulated by free BMP2 and BMP2 coacervate when compared with the control; however, no significant differences were found among groups. The mRNA expression level of collagen type I significantly increased in MDSCs that were stimulated by BMP2 coacervate when compared with the MDSCs in the control and single-dose free-BMP2 groups. No difference was observed between the multidose free-BMP2 and BMP2 coacervate groups (Fig. 5D). This further confirms the notion that the coacervate delivery system can potentiate BMP2 activity with a lower dose, producing a similar effect to threefold amounts of free BMP2.

To better mimic the in vivo environment, the effects that BMP2 coacervate had on the osteogenesis of MDSCs were also examined in a 3D fibrin gel culture system (Fig. 5B). Contrary to the results from the monolayer cultures, after 5 days of culture in fibrin gel, free BMP2 induced ALP expression above that of the control groups, possibly attributable to growth factor protection from degradation afforded by fibrin gel itself. Even so, BMP2 coacervate stimulated significantly more (approximately twofold) ALP activity compared with an equal amount of free BMP2 (Fig. 5B). It should also be noted that the amount of BMP2 available to the cells in the BMP2 coacervate group was lower because of the nature of controlled release. The release assay indicated that less than 10% of the BMP2 was released by the coacervate after 5 days in vitro. These data indicate that sustained release of BMP2 by the coacervate was more efficient.

Figure 4. Bioactivity of delivered BMP2. C2C12 cells were cultured in medium supplemented with DPBS as a control, delivery vehicle (coacervate only), free BMP2, or BMP2 coacervate. BMP2 dosage in the free-BMP2 and BMP2 coacervate groups was equal. (A): Representative images of ALP staining of cultured cells at day 5 (magnification, ×10). (B): ALP activity was evaluated at day 5 and normalized by DNA content. The data represent the means ± SD. *p < .05 (n = 4). Abbreviations: ALP, alkaline phosphatase; BMP2, bone morphogenetic protein-2; DPBS, Dulbecco’s phosphate-buffered saline.
Figure 5. Osteogenic potential of MDSCs in monolayer and three-dimensional (3D) culture in vitro. (A): ALP expression of MDSCs after 5 days of monolayer culture, exposed to a single dose of 100 ng of free BMP2 or BMP2 coacervate or to 100 ng of BMP2 at three different time points to mimic controlled release (multidose free BMP2). Control groups were exposed to Dulbecco’s phosphate-buffered saline (DPBS) only (control) or the coacervate only without BMP2. (B): ALP expression by the MDSCs after 5 days of culture in a 3D fibrin gel, exposed to 100 ng of free BMP2 or BMP2 coacervate or DPBS only as a control. The data represent the means ± SD. *p < .05 (n = 4). (C): Representative images of ALP staining of monolayer cultured MDSCs at day 5 (magnification, ×10). (D): Reverse transcription-polymerase chain reaction analysis of the expression of Runx.2 and COL1A1 in MDSCs after a 5-day culture. mRNA expression of Runx.2 and COL1A1 were normalized to β-actin and then scaled according to the control sample. This value was set to 1. The data represent the means ± SD. *p < .05 (n = 3). Abbreviations: ALP, alkaline phosphatase; BMP2, bone morphogenetic protein-2; BMP4, bone morphogenetic protein-4; MDSC, muscle-derived stem cell.
Figure 6. In vivo effects of BMP2 coacervate on the osteogenesis of MDSCs. (A): Representative micro-computed tomography (micro-CT) images of ectopic bone formation after 2 and 4 weeks. Groups were transplanted with a fibrin gel containing 2 μg of free BMP2 or BMP2 coacervate alongside MDSCs, the BMP2 coacervate alone, MDSCs alone, or fibrin gel alone (control). (B): Ectopic bone volume quantification of micro-CT images after 2 and 4 weeks. The data represent the means ± SD. *, p < .05 (n = 6). (C): Representative images of newly formed bone with alizarin red staining (red; scale bars = 200 μm), von Kossa staining (black; scale bars = 200 μm) of calcified osteoid matrix, and IHC staining for transplanted MDSCs (GFP-positive, brown; scale bars = 50 μm). (D): Quantification of the number of GFP-positive cell within and at the edge of the osteoid per mm² of bone surface; *, p < .01 (n = 6). Abbreviations: BMP2, bone morphogenetic protein-2; GFP, green fluorescent protein; IHC, immunohistochemical; MDSC, muscle-derived stem cell; W, week.
than free BMP2 at stimulating osteogenesis of MDSCs in a 3D fibrin gel environment.

**Effects of BMP2 Coacervate on the Osteogenesis of MDSCs In Vivo**

We next examined the ability of BMP2 coacervate to stimulate the osteogenic potential of MDSCs in a mouse ectopic bone formation model. Free BMP2 or BMP2 coacervate, with or without MDSCs, was suspended in fibrin glue to keep the cells and coacervate localized and then implanted into a skeletal muscle pocket created in the hind limbs of mice. All mice survived the experiment, and no complications such as infection or peripheral nerve damage were observed. Quantification of calcified tissue (bone volume) by 3D micro-CT analysis revealed extensive bone formation by the MDSCs stimulated with BMP2 coacervate 2 weeks after implantation, and the amount of bone increased at 4 weeks (Fig. 6A, 6B). MDSCs with free BMP2 showed minimal bone formation at week 2 and also increased in volume by week 4 (Fig. 6A, 6B). BMP2 coacervate alone showed some bone formation at week 2 but no increase by week 4. MDSCs alone showed no significant bone formation at either time point, similar to the control group of fibrin gel alone (Fig. 6A, 6B). Significantly greater bone volume was formed by the MDSCs stimulated by BMP2 coacervate compared with all other groups at both time points; bone volume was 9-fold greater at week 2 and 4.5-fold greater at week 4 compared with the free BMP2 + MDSC group (Fig. 6B).

Histological analysis included both alizarin red staining and von Kossa staining to determine the calcium phosphate deposition and immunohistochemical staining for GFP to determine the location of GFP-labeled MDSCs. Alizarin red and von Kossa stainings revealed calcified osteoid matrix at the implantation site in the BMP2 coacervate-only, free-BMP2 + MDSC, and BMP2 coacervate + MDSC groups, which is consistent with the micro-CT data (Fig. 6C). This newly formed osteoid was abutting and expanding within the normal muscle tissue, and the interface between the muscle and newly formed bone was clearly detectable. The myofibers adjacent to the ectopic bone formation appeared morphologically normal with a slight compression in the transition zone (Fig. 6C). Calcified osteoid matrix was found mostly at the edge of the transplantation area in the BMP2 coacervate-only group but was found throughout the transplantation area in the free BMP2-MDSC group and in the BMP2 coacervate + MDSC group, which also displayed a more mature trabecular bone structure (Fig. 6C). To determine whether the newly formed bone was derived from the transplanted MDSCs or from local host cells, MDSCs were labeled with GFP and then stained immunohistochemically. In the BMP2 coacervate-only group, no GFP was detected in the newly formed bone osteoid, which must therefore be derived from the host cells (Fig. 6C). In the free-BMP2 + MDSC and BMP2 coacervate + MDSC groups, cells expressing GFP were identified throughout and at the edge of the osteoid with significantly more GFP-positive cells observed in the BMP2 coacervate + MDSC group (Fig. 6C). The transplanted MDSCs were closely associated with the newly formed osteoid, consistent with the hypothesis that the MDSCs were not only present within the osteoid but also active in the bone formation. Quantification of GFP-positive cells within and at the edge of the osteoid showed significantly higher numbers in the BMP2 coacervate + MDSC group than in the free-BMP2 + MDSC group (Fig. 6D).

**DISCUSSION**

Skeletal muscle is a good source of progenitor cells with potential musculoskeletal therapeutic applications [3, 4, 8]. MDSCs are a population of cells that can be isolated by a modified preplate technique from mouse skeletal muscle and that display a superior regeneration capacity in various musculoskeletal tissues, including skeletal and cardiac muscles, bone, and articular cartilage, when compared with myoblasts [9, 15–17]. MDSCs demonstrate the capacity for self-renewal, long-term proliferation, and multipotent differentiation and have a superior ability for survival because of their increased resistance to oxidative and inflammatory stresses [33]. MDSCs are therefore a good alternative cell source to bone marrow- or fat tissue-derived mesenchymal stem cells, for bone tissue engineering. Since local stimulation of MDSCs by BMPs following transplantation is essential for osteogenic differentiation, maintaining an adequate concentration of stable, active BMPs at the transplantation site is necessary. Unfortunately, for most clinical applications requiring cell transplantation such as bone nonunion, delayed nonunion, and large bone defects, local BMP shortage is a primary cause for insufficient healing. Without supplementation of exogenous BMPs, most transplanted progenitor cells, such as mesenchymal stem cells, fail to undergo osteogenic differentiation and eventually form fibrous tissue instead of functional bone [34, 35]. More importantly for the current study, nontransduced MDSCs do not undergo osteogenic and chondrogenic differentiation when transplanted into bone and articular cartilage defects in vivo, rather they undergo default myogenic differentiation [20, 21]. Simply incorporating recombinant BMP protein within a commonly used soluble scaffold such as fibrin glue fails to sufficiently stimulate MDSCs to undergo osteogenesis [21]. Additionally, the half-lives of BMPs in the body are very short, on the order of minutes to hours [22, 23]. As a result, large doses of BMP are required to obtain the desired osteogenic effect [36]. Many studies use doses on the order of 1,000 times greater than can be found in the entire human body, drawing concerns with regard to cost and safety [37, 38]. With such short half-lives and rapid dilution in the body, even these large doses may not provide adequate local concentrations at critical times to optimally induce bone formation by the transplanted cells. As a result, controlled delivery systems that supply active BMPs alongside the transplanted progenitor cells to promote osteogenesis are highly important for the future of cell-based bone regeneration strategies.

Developing suitable carriers for BMPs that can preserve their bioactivity and sustain their release over time presents a great challenge. To date, a number of systems have been designed and evaluated for the delivery of BMPs, including gelatin/β-tricalcium phosphate [39], collagen gel [40], poly-l-lactic acid scaffolds [41], a porous hydroxyapatite composite [42], hyaluronic acid [43], and fibrin gel [43]. Bovine type I collagen is currently used in the clinical setting as a carrier and has been approved by the Food and Drug Administration for spine fusion in humans [44, 45]. These delivery systems have been shown to enhance bone repair and accelerate fracture healing to some degree; however, they often suffer issues inherent to poor control over release rate such as low loading efficiency and a large initial burst release of the protein [39–41]. Furthermore, the use of harsh organic solvents in the synthesis of many polymeric delivery systems may denature
loaded growth factor proteins and reduce their bioactivity [46]. Clearly, a controlled release system that avoids these common setbacks could greatly advance the field of growth factor delivery for bone regeneration.

In the current study, a PEAD:heparin growth factor delivery system was designed and used to provide continual release of BMP2 alongside the transplanted MDSCs. Heparan sulfate glycosaminoglycans are a natural component of the extracellular matrix with a high affinity for many growth factors and cytokines. Heparin has a similar structure and is commonly incorporated into delivery systems for heparin-binding growth factors. Heparin binds BMP2 with high affinity [47] and has been previously shown to retain its bioactivity well [22, 48, 49]. In the presence of heparin, degradation of BMP2 is reduced, and its half-life in culture media is prolonged 20-fold [22]. To best preserve heparin in its intact form and maintain its full functionality, we used a polycation that complexes with heparin by charge interactions. PEAD is a synthetic polycation with a high charge density, easy synthesis, and good biocompatibility [26]. Heparin and bound growth factors precipitate out of the aqueous solution upon interaction with PEAD to form a self-assembled coacervate. Once incorporated in the coacervate, growth factors are protected from degradation and slowly released over time based on disassembly of the complex in an ionic environment and hydrolytic degradation of PEAD [26]. Our data using both C2C12 cells and MDSCs demonstrated the retained bioactivity of BMP2 released from the coacervate. Furthermore, BMP2 coacervate stimulated higher cellular ALP activity than an equal dose of free BMP2 and showed similar results to a threefold higher total dose of free BMP2 added at multiple times during the experiment. These results indicate that the coacervate may directly potentiate the activity of BMP2 beyond what is inherent to sustained release. Indeed, heparin has been shown to modulate the interactions between heparin-binding growth factors and their receptors [50, 51] and likely plays a similar role in promoting the bioactivity of BMP2 [49]. Moreover, heparin has also been shown to protect BMP2 from the inhibitory actions of noggin, which is expressed as part of the negative feedback loop in response to BMP2 expression [22].

Pharmacokinetic models of BMP release from collagen sponges and other common delivery vehicles show rapid initial effluxes, during which the carrier can lose 30% or more of its loaded BMP [52, 53]. This initial burst release spikes the BMP concentration in the surrounding tissue to supraphysiological levels, which can result in severe complications such as ectopic bone formation in the spinal canal, hematomas in soft tissue, and bone resorption around implants [54–57]. Clearly such systems are inefficient and potentially harmful. Conversely, the PEAD-heparin delivery system loaded BMP2 with high efficiency (98.2% loading), showed a negligible initial burst release (1.3% after 1 day), and thereafter produced slow and sustained release for at least 4 weeks. Our in vivo results showed extensive bone formation by the transplanted MDSCs and minimal participation of surrounding host tissue cells, which indicates the local preservation of BMP2 activity and the efficacy of sustained release of BMP2 on the osteogenesis of the donor MDSCs. The released BMP2 did not appear to have any significant effect on the surrounding tissue, indicating that the coacervate localizes the BMP2 to the site of implantation. Our in vitro data showed that only 25% of the BMP2 was released after 28 days; however, release is expected to be accelerated in vivo where degradation of the polyester backbone of PEAD is catalyzed in the presence of hydrolases [58]. Hydrolases may also be secreted by the MDSCs, which could contribute to the targeting of BMP2 release on the transplanted cells.

In the present study, the coacervate served as a multifunctional vehicle for growth factor delivery. The coacervate naturally loads any heparin-binding growth factor and could enable the codeelivery of other factors alongside BMP2 in the future. Of particular importance, angiogenic growth factors to stimulate the formation of a supportive vascular network to enhance MDSC survival and osteogenesis will be further investigated. Additionally, the strongly polyvalent nature and many free functional groups of the PEAD-heparin coacervate enable easy coating onto a wide range of materials. Held by relatively weak Coulombic forces and hydrogen bonding, the release functionality of the delivery system is unlikely to be affected. This could be useful for stimulating host cell seeding of osteoinductive scaffolds in situ or to improve the osteogenesis of cells seeded into scaffolds ex vivo.

**CONCLUSION**

In the current study, a polycation-heparin coacervate delivery system was used to bind, protect, and provide prolonged release of BMP2. We demonstrated that the controlled delivery of BMP2 from this system can effectively stimulate MDSCs to differentiate into an osteogenic lineage both in vitro and in vivo. When transplanted along with BMP2 coacervate, MDSCs produced substantial bone formation at an ectopic site. These data suggest that a dual MDSC and BMP2 delivery approach may serve as a viable treatment for bone repair without the requirement of gene therapy.

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**AUTHOR CONTRIBUTIONS**

H.L. and N.R.J.: conception and design, collection and assembly of data, data analysis and interpretation, writing and editing of manuscript; A.U.: collection and assembly of data; M.P.: stem cell isolation, collection and assembly of data; A.L.: conception and design, administrative support; J.H.: conception and design, financial support, writing and editing of manuscript, administrative support, final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

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