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Endochondral Bone Formation from Hydrogel Carriers Loaded with BMP2-transduced Cells

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Abstract—The success of *ex vivo* viral gene therapy systems for promoting bone formation could be improved through the development of systems to spatially localize gene expression. Towards this goal, we have encapsulated adenovirus-transduced human diploid fetal lung fibroblasts (MRC-5) expressing bone morphogenetic protein-type 2 (BMP-2) within non-degradable poly(ethylene glycol)-diacrylate (PEG-DA) hydrogels and implanted these intramuscularly to promote endochondral bone formation. To optimize BMP-2 secretion, the molecular weight of the polymers and cell densities were varied. Polymers with molecular weights of 6, 10, and 20 kDa were used to prepare hydrogels containing 1, 5, or 10 million transduced cells. The results showed that 10 million transduced fibroblasts that was the maximum number of cells feasible for encapsulation within PEG-DA 10 and 20 kDa hydrogels produced the highest amount of secreted BMP-2 protein. Encapsulation of MRC-5 and transduced fibroblasts resulted in 71 and 58% cell viability, respectively. The bioactivity of secreted BMP-2 protein from the hydrogels was confirmed with an alkaline phosphatase assay. Micro-CT of the lower limb muscles of NOD/SCID mice following implantation with hydrogels showed 39.5 ± 25.0 mm^3 mineralized tissue and 31.8 ± 7.8 mm^3 for the cell-injected mice, and the bone was localized to the hydrogel surfaces. Histology revealed bone as well as cartilage for both hydrogel implanted and cell-injected animals.

Keywords—Gene therapy, Adenovirus, Bone, Hydrogel, Cell encapsulation, Biomaterials, Bone morphogenetic protein, Tissue engineering, Tissue repair.

INTRODUCTION

There is an overwhelming need to develop safe and efficacious systems for bone regeneration for the treatment of fractures, osteoporosis, tumor resection, spinal arthrodesis, maxillofacial surgery, and implant fixation.[4,12,14,26,28,30] In many of these cases, there are large defects that remain relatively unrepaired. Therefore, bone regeneration is needed in order to restore the structure and function to the bone. Current treatments for these defects include autografts, allografts, and xenografts. However, one of the major limitations of bone regeneration using these types of treatment is the source of graft material. The benchmark for most orthopedic applications is autologous bone graft obtained from the patient's iliac crest, which is not only limited but requires an additional surgical procedure.[1,47] Thus, allogeneic and xenogeneic bone grafts have been developed but issues related to immunogenicity, disease transmission, immunological rejection, and unfavorable biomechanical properties have limited their use.[10,24,25,45]

Therefore, in an effort to overcome the hurdles associated with bone graft materials, research has focused on the induction of bone with proteins such as bone morphogenetic proteins (BMPs). These cytokine proteins have been shown to induce the formation of new cartilage and bone tissues as well as to act as potent morphogens in pattern formation and development of the embryonic skeleton.[18,19,23,27,41,44] As a result, BMP-2 protein was commercially introduced as a bone graft substitute for spinal fusion and was subsequently applied to a collagen sponge as the protein carrier.[16] However, the use of a collagen sponge reduces the protein's bioavailability, so that large amounts of protein are required to produce a clinical response.[17] Therefore, gene therapy may be an alternative to direct protein delivery.

Presently, there have been several promising studies utilizing viral vectors encoding BMP-2 for promoting heterotopic bone formation.[29,48,49] These studies

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include the use of retroviruses such as lentiviruses,[37] adeno-associated viruses (AAV),[13] and adenoviruses.[35] For example, using a chimeric adenovirus, Ad5F35-BMP2, containing the cDNA for human BMP-2, injection of *ex vivo* transduced cells expressing high levels of BMP-2 has been shown to induce endochondral bone formation.[15,31] The recombinant chimeric adenoviral BMP-2 vector was constructed from adenovirus type 5 (Ad5) containing a fiber gene from adenovirus type 35 (Ad35), which facilitates entry into cells in a coxsackie-adenovirus receptor (CAR) independent fashion. However, the localization of BMP-2-transduced cells at a delivery site needs to be improved in order to circumvent uncontrolled gene expression in undesirable places such as vital organs and blood vessels. An alternative to direct administration of *ex vivo* transduced cells is the encapsulation of cells within a protective barrier that can restrict cell migration from an injected site and in doing so effectively localize gene expression. Currently, there are many types of materials that can be used for cell encapsulation including silk fibroin hydrogels,[11] oligo(poly(ethylene glycol) fumarate) (OPF) hydrogels,[38] sodium alginate hydrogels,[2] hydroxypropylmethylcellulose hydrogels,[40] and poly(ethylene glycol)-diacrylate (PEG-DA) hydrogels.[46] Hubbell *et al.* has previously shown that pancreatic islets of Langerhans encapsulated within PEG-DA hydrogels were able to allow the rapid diffusion of nutrients and insulin. Moreover, they demonstrated that encapsulation within the PEG-DA hydrogels provided immunoprotection since the hydrogels were not permeable to antibodies or complement proteins and did not interact with inflammatory cells.[6,7,22]

PEG-DA hydrogels are hydrophilic, polymeric networks that imbibe large quantities of water without dissolution and in doing so impart the physical characteristics of soft tissues. Moreover, the pores or mesh sizes of the hydrogel can be controlled by varying the molecular weight of the PEG-DA polymers so as to mediate the diffusion of gases, nutrients, and metabolites throughout the network.[32] In addition, these hydrogels can be photopolymerized in any conformation with the use of photoinitiators using visible or long wavelength ultraviolet (UV) light. Also, PEG-DA hydrogels have been widely used for tissue engineering applications such as in the development of biocompatible barriers to prevent thrombosis and restenosis[20,21] and for cell encsapsulation.[6,7,22,34]

Therefore, the objective in this paper is to encapsulate adenovirus-transduced cells expressing BMP-2 within non-degradable PEG-DA hydrogels for endochondral bone formation. Our central hypothesis is that encapsulation of cells expressing BMP-2 will lead to spatial control of gene expression that will limit bone formation to a desired area. Thus, we have encapsulated human diploid fetal lung fibroblasts (MRC-5), which have been transduced *ex vivo* with the adenovirus, Ad5F35BMP2, within non-degradable PEG-DA hydrogels. The molecular weight of the PEG-DA polymers as well as the cell densities within the hydrogels will be varied so as to optimize both BMP-2 protein secretion from the hydrogels and the mechanical properties of the hydrogels.

MATERIALS AND METHODS

Cell Culture Maintenance

Human diploid fetal lung fibroblasts (MRC-5) were obtained from American Type Culture Collection (ATCC; Manassas, VA). The cells were propagated in Eagle's Minimum Essential Media (EMEM; ATCC) supplemented with 10% fetal bovine serum (FBS; BioWhittaker, Walkerrsville, MD), 1000 U/L penicillin, 100 mg/L streptomycin, and 0.25 μg/ml amphotericin B (Sigma; St. Louis, MO), and grown in a humidified incubator at 37°C and 5% CO_2.

Fibroblast Transduction with Adenoviral Vectors

MRC-5 cells were transduced by replacing the media from the cultured cells with fresh EMEM supplemented with 10% FBS and the antibiotic–antimycotic. Ad5F35BMP2 adenoviral vectors at a previously optimized concentration of 2500 viral particles per cell were then added to the flask, which was incubated overnight prior to use.[31] The transduced cells were then rinsed with sterile phosphate buffered saline (PBS), trypsinized, and counted with a hemacytometer.[15]

Hydrogel Preparation

The molecular weight of the PEG-DA polymers and hence the mesh size of the hydrogels can affect the diffusion of gases and nutrients throughout the scaffold as well as efficient diffusion of BMP-2 protein from the hydrogels. Additionally, the molecular weight of the polymers can also influence the mechanical properties of the hydrogels. Thus, in order to determine the optimum PEG-DA hydrogel for cell encapsulation and hence gene expression, hydrogels of varying molecular weight were prepared. PEG-DA (6000, 10,000, 20,000 Da) was synthesized by reaction of PEG (Fluka, Milwaukee, WI) with acryloyl chloride as previously described.[9] The dried polymers were then dissolved in 10 ml of ultrapure water and purified by dialysis (MWCO 3500 Da; Fisher

Scientific, Pittsburg, PA) against deionized water for 3 days. The purified polymers were then lyophilized and stored at −20 °C. Polymer solutions were sterilized via filtration (0.2 μm filter; Gelman Sciences, Ann Arbor, MI) prior to use. Hydrogel disks (11.5 mm (d) × 0.5 mm (h)) were photopolymerized by combining 0.1 g/ml PEG-DA (10% w/v) with 1.5% (v/v) triethanolamine/HEPES buffered saline (HBS, pH 7.4), 37 mM 1-vinyl-2-pyrrolidinone, 10 mM eosin Y, and MRC-5 or MRC-5 cells transduced with Ad5F35BMP-2 viral vectors for hydrogels with cells for a final concentration of 1, 5, or 10 million cells/disk, which was the maximum number of cells that were feasible for encapsulation. The pre-polymer solution was formulated with and without cells as described above to a final volume of 150 μl, which was then pipetted into the wells of a 48-well cell culture dish that was exposed to visible light for 2 min. The hydrogels were then immediately transferred to 150 mm × 25 mm cell culture dishes (Corning Inc., Corning, NY) to which 35 ml cell culture media was added. Control cells were seeded in tissue culture flasks (Fisher Scientific; Pittsburg, PA) that were pre-treated with a 1% (w/v) gelatin solution (Sigma, St. Louis, MO).

Hydrogel Characterization

PEG-DA hydrogels prepared from varying molecular weight precursors were fabricated as described above. The hydrogels were then dried in a vacuum oven for 1 week, after which the dry weights were recorded. To determine the swelling ratios (swelling ratio = W_s/W_d where W_s and W_d are the weight of water in the swollen gel after equlibrium and the dry weight of the gel, respectively), the hydrogels were reswollen in PBS and the swollen weights were recorded.[50] The water content, molecular weight between crosslinks (M_c), and the mesh sizes of the prepared hydrogels were then determined as previously described.[5,8] The compressive moduli of the different hydrogels with and without transduced fibroblasts were determined using an Instron® 3342 (Canton, MA) mechanical tester. Briefly, the hydrogel precursors were formulated with and without 10 million transduced fibroblasts as described above. The solutions were then placed in a rectangular glass mold (~1.4 mm thickness) and exposed to visible light for 2 min. The faceplate was removed and a cork borer was used to cut out 11.5 mm diameter disks that corresponded to a final volume of 150 μl. The disks were then transferred to cell culture plates to which 35 ml of complete media was added and the plates were incubated overnight. Prior to analysis, the hydrogels were dabbed with a Kimwipe to remove excess surface media and placed between two parallel platens mounted on the Instron® mechanical tester. The compressive modulus of the hydrogels was determined from the slope of the calculated stress (Pa) versus strain curves using a 10 N load cell at a crosshead speed of 1 mm/min.

ELISA for BMP-2

BMP-2 levels were measured with a Quantikine® BMP-2 immunoassay kit (R & D Systems Inc.; Minneapolis, MN) as per manufacturer's instructions. Transduced and non-transduced cells were encapsulated and maintained in 35 ml of media as described above. Control cells were seeded at a density of 1, 5, and 10 million cells per flask and also maintained in 35 ml of media as described above. Samples of 1.0 ml of the conditioned media from both the cultured flasks and hydrogel disks were then taken at 1, 3, 5, 7, 9, and 15 days followed by media change and the samples were then assayed for secreted BMP-2 protein. Levels of BMP-2 protein were normalized to total protein content determined with a BCA™ protein assay kit (Pierce; Rockford, IL) and reported as pg BMP-2/mg protein. The PEG-DA hydrogels with encapsulated transduced fibroblasts that produced the highest amounts of secreted BMP-2 protein compared with their plated counterparts as well as the hydrogels with best mechanical properties were then selected as the optimal molecular weight for further analysis.

Cell Viability

Viability of the MRC-5 and transduced fibroblasts encapsulated within the optimized PEG-DA (10 kDa) hydrogels were determined with a LIVE/DEAD® Viability/Cytotoxicity kit (Molecular Probes; Eugene, OR). Briefly, hydrogel disks with either 10 million MRC-5 or transduced fibroblasts were formulated and photopolymerized in 48-well cell culture plates as described above. The hydrogels were then transferred to cell culture dishes to which 35 ml of media was added. On days 1 and 7, the media was removed and the hydrogels were transferred to 6-well plates. The disks were washed with sterile tissue culture grade PBS three times for 10 min each. The fluorophore solution was then prepared by adding 20 μl ethidium homodimer (2 mM) and 5 μl calcein AM (4 mM) to 10 ml PBS. The resulting solution was vortexed and 2 ml was then added to each well containing the hydrogels. The plate was incubated for 45 min at 37 °C after which the hydrogels were washed as previously described and the fluorescence of the live and dead cells were analyzed using a Zeiss LSM 510 confocal microscope (Thornwood, NY) with LSM 5 Image Browser software (Version 1.0). Briefly, three sections of each of the

hydrogel were viewed and within each section, three independent areas of the section were analyzed in which all fluorescently labeled cells within the view were taken into account. The amount of fluorescently labeled live and dead cells was reported as a percent of the total cells counted.

Alkaline Phosphatase Activity

Conditioned media from the optimized PEG-DA (10 kDa) hydrogel constructs with 10 million transduced fibroblasts as well as the plated controls were used to assess the ability of the secreted BMP-2 to stimulate alkaline phosphatase activity in W20-17 cells.[31] W20-17 cells are bone marrow stromal cells that were originally isolated from murine bone marrow by Thies *et al.*,[39] and further demonstrated that these cells respond to BMP-2 by up-regulation of the alkaline phosphatase activity. Briefly, W20-17 cells at a cell density of 5×10^4 cells/well were seeded in 24-well plates. After 24 h, the cell culture media was replaced and 200 μl of conditioned media was added to the wells of the plates that were then incubated for 3 days. The cellular alkaline phosphatase was extracted by three freeze–thaw cycles in 100 μl/cm^2 of 25 mM Tris–HCl (pH 8.0) with 0.5% Triton X-100. After extraction, 100 μl of CSPD® Ready-to-use solution with Sapphire II enhancer (Tropix, Inc., Bedford, MA) was added to the samples. The alkaline phosphatase activity was then measured by chemiluminescence in which the output from each sample was integrated for 10 s after a 2 s delay by a luminometer (TD-20/20, Turner Designs, Sunnyvale, CA).

Western Blot Detection

Conditioned media from the optimized PEG-DA (10 kDa) hydrogels with 10 million transduced fibroblasts were precipitated by addition of five volumes of cold acetone and the resulting solution was stored overnight at −20 °C.[31] The proteins were pelleted by centrifugation for 10 min at 4000 rpm, resuspended in sample buffer (0.5 M Tris–HCl, pH 6.8, 10% (w/v) sodium dodecyl sulfate, 25% glycerol, 0.5% (w/v) bromophenol blue, and 5% (v/v) β-mercaptoethanol, and heated at 95 °C for 4 min. The samples were cooled on ice and the proteins were separated on a Tris–HCl 4–15% Ready gel (Bio-rad; Hercules, CA) under denaturing conditions and transferred to a supported nitrocellulose membrane (Bio-rad; Hercules, CA). Purified recombinant BMP-2 protein (Research Diagnostics, Inc.; Flanders, NJ) was used as the positive control. BMP-2 protein was detected with an Opti-4CN substrate (Bio-rad; Hercules, CA) kit with an anti-BMP-2 monoclonal antibody (Genetics Institute; Cambridge, MA) and a secondary anti-mouse IgG antibody conjugated with horseradish peroxidase (Bio-rad; Hercules, CA).

In vivo Formation of Endochondral Bone

To facilitate implantation into the muscle, hydrogel beads were employed rather then disks. PEG-DA (10 kDa) hydrogel solution with 10 million transduced fibroblasts were formulated as previously described. Aliquots of 25 μl hydrogel solution were pipetted along surgical suture (CP Medical, Portland, OR) on the surface of a Teflon® sheet (Boedeker Plastics; Shiner, TX) to form 6 hydrogel microbeads, which represented a single sample having a total volume of 150 μl (Fig. 6). The hydrogels were photopolymerized and added to cell culture medium prior to implantation the same day. Female NOD/SCID mice (8–12 weeks old; Charles River Laboratories; Wilmington, MA) were placed five per cage and fed with an *ad libitum* diet and tap water in a 12 h day/night cycle according to Baylor College of Medicine Institutional Animal Care and Use Committee (IACUC) protocols. Prior to surgery, the animals were separated into groups of three and each hind leg of the mouse was shaved and cleansed with alcohol and then a skin incision was made to reveal the muscles of the upper long bone. Blunt dissection of the muscles of both hind legs created pockets for the hydrogel constructs. The PEG-DA (10 kDa) hydrogel beads (6 beads with total volume = 150 μl) containing 10 million transduced fibroblasts were then inserted into the quadricep muscles of each of the two hind legs of the mouse. Control hydrogels were prepared with MRC-5 cells transduced with the Ad5F35-HM4 empty cassette viral vector and these control hydrogels were implanted into each of the two hind legs of the designated control animals. The muscles of the animals were then sutured and the skin was stapled. The control animals were injected intramuscularly into each of the two hind legs of the mouse with either 10 million transduced fibroblasts or 10 million MRC-5 cells transduced with the empty Ad5F35-HM4 viral vector. Two weeks after implantation, one mouse was sacrificed and the hydrogels were removed, washed with PBS and analyzed for cell viability as described above. After 3 weeks, the remaining mice were sacrificed and both lower limbs were removed and fixed in formaldehyde solution (VWR; Sugar Land; TX). The overlying skin of each of the lower limbs of the formaldehyde preserved NOD/SCID mice were removed and scanned at 14 μm resolution with a commercial micro-CT system (GE Locus SP, GE Heathcare, London, Ontario). Three-dimensional reconstructions of the lower limb bones and any mineralized tissue in the surrounding muscle

were created at 29 μm resolution to visualize endochondral mineralized tissues. A volume of interest was defined for each specimen, and a threshold was chosen to exclude any non-mineralized tissue. The total volume of endochondral bone was then measured (eXplore MicroView, v. 2.0, GE Healthcare, London, Ontario) and the femurs and tibias of the animals were not taken into account for the measurements of mineralized tissue in the muscles of the animals. The formaldehyde fixed lower limbs of the NOD/SCID mice were then decalcified in EDTA, embedded in paraffin, and sectioned at a thickness of 5 μm. The sections were stained with hematoxylin and eosin and observed under light microscopy.

Statistics

Statistical analysis was performed with Graph-Pad Prism® version 3.02 software using one-way ANOVA with Tukey's *post-hoc* test for p-values ≤ 0.05. Data represented with error bars indicates sample group mean $\pm$ standard deviation of the mean (σ).

RESULTS

Characterization of PEG-DA Hydrogels

The 6, 10, and 20 kDa hydrogels were characterized with respect to swelling ratios and water content as determined from the dried and swollen hydrogel weights for a sample size of five ($n = 5$). The swelling ratio for the 6, 10, and 20 kDa hydrogels was found to be 8 ± 0.7, 10 ± 0.4, and 16 ± 0.2, respectively. The water content of the hydrogels was 89 ± 0.7, 91 ± 0.3, and $94 \pm 0.1\%$ for the 6, 10, and 20 kDa, respectively. In addition, the molecular weight between crosslinks, M_c, of the hydrogels was 617 ± 98, 780 ± 54, 1556 ± 24 g/mol for the 6, 10, and 20 kDa PEG-DA hydrogels, respectively, in which the hydrogels had mesh sizes of 34 ± 3, 39 ± 2, and 65 ± 1 Å, respectively.

In Vitro Optimization of BMP-2 Expression

In addition to optimizing the molecular weight of the PEG-DA hydrogels, different cell densities were encapsulated within the hydrogels to investigate optimum loading and gene expression. As can be seen from the ELISA data for a sample size of five ($n = 5$) for each group, encapsulation of 1 million cells within any of the hydrogels resulted in ~20-fold reduction in detected BMP-2 protein compared with the plated controls ($p < 0.001$) (Fig. 1a) and the expression was biphasic over the 15-day period with highest protein detected (~600 pg/ml) on day 5. In contrast, BMP-2 protein detected for 5 and 10 million encapsulated cells was found to be ~4600 and ~7000 pg/ml, respectively up to day 7 regardless of the molecular weights of the polymers in which peak BMP-2 protein

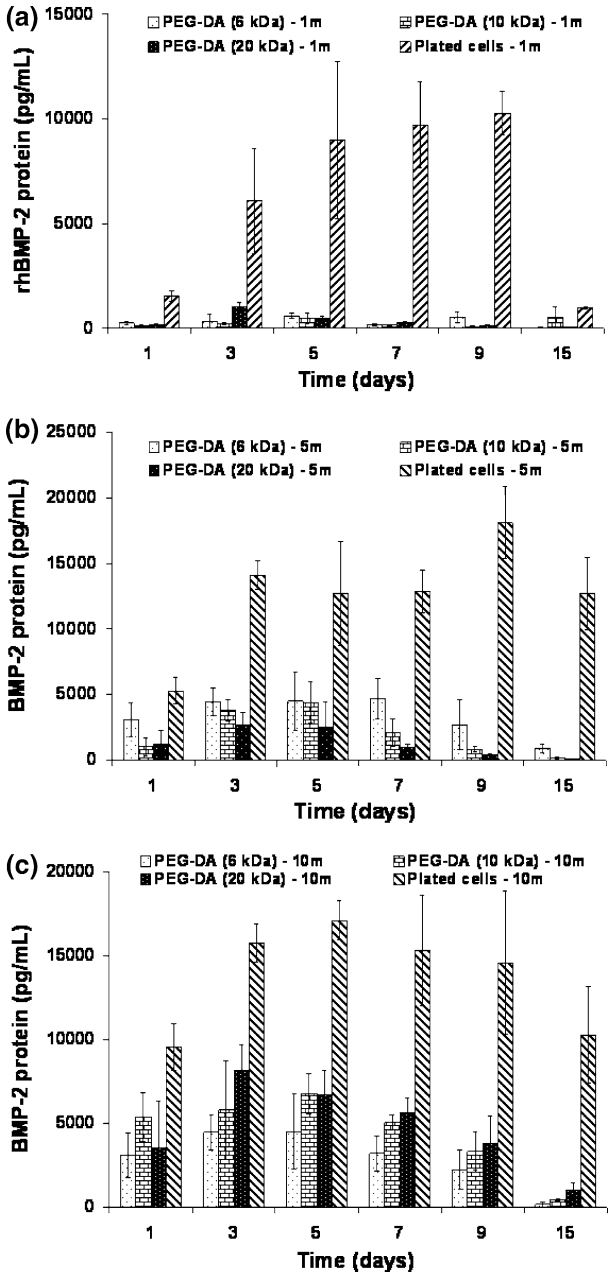

FIGURE 1. (a) Evaluation of BMP-2 protein secretion from 1 million adenovirus transduced MRC-5 cells (tMRC-5) encapsulated within PEG-DA (6, 10, 20 kDa) hydrogels with ELISA assay. (b) Evaluation of BMP-2 secretion from 5 million adenovirus transduced MRC-5 cells (tMRC-5) encapsulated within PEG-DA (6, 10, 20 kDa) hydrogels with ELISA assay. (c) Evaluation of BMP-2 secretion from 10 million adenovirus transduced MRC-5 cells (tMRC-5) encapsulated within PEG-DA (6, 10, 20 kDa) hydrogels with ELISA assay. Negative hydrogel and plated controls were less than 100 pg/ml BMP-2 detected. Data reported as mean ± SD, $n = 5$.

secretion occurred between days 3–7 for the 5 million cells within 6 kDa hydrogels and day 3 for the 10 million cells within the 20 kDa hydrogels (Fig. 1b and c).

Mechanical Testing of PEG-DA Hydrogels

To determine the compressive moduli of the PEG-DA hydrogels after encapsulation with transduced fibroblasts, we performed compressive testing with the PEG-DA hydrogels of various molecular weights containing a cell concentration of 10 million transduced fibroblasts for a sample size of five ($n = 5$). The compressive modulus of the PEG-DA hydrogel only was 454 ± 63, 395 ± 55, and 155 ± 19 kPa for the 6, 10, and 20 kDa hydrogels, respectively (Fig. 2). The maximum compressive strength was found to be 0.231 ± 0.19, 0.220 ± 0.063, and 0.076 ± 0.042 kN for the 6, 10, and 20 kDa hydrogels, respectively. Subsequent encapsulation of the hydrogels with 10 million transduced fibroblasts resulted in a significant decrease in compressive modulus of the hydrogels to 153 ± 93 kPa ($p < 0.001$), 236 ± 48 kPa ($p < 0.01$), and 90 ± 10 kPa ($p < 0.05$) for PEG-DA 6, 10, and 20 kDa, respectively. The maximum compressive strength was found to be 0.011 ± 0.003, 0.259 ± 0.206, and 0.009 ± 0.001 kN for the 6, 10, and 20 kDa hydrogels, respectively. In addition, even though there was no difference in compressive modulus for the 6 and 10 kDa PEG-DA hydrogels containing fibroblasts, there was a significant decrease in compressive modulus of the encapsulated 20 kDa hydrogels in comparison with both the 6 and 10 kDa hydrogels ($p < 0.001$).

Viability of Encapsulated Cells

The viability of 10 million non-transduced and transduced fibroblasts was determined following encapsulation of cells within the PEG-DA 10 kDa hydrogels after 1 and 7 days via staining with calcein AM and ethidium homodimer, which causes live cells to fluoresce green and dead cells to fluoresce red. The data showed that encapsulation of cells after 24 h resulted in 71 ± 9% viable non-transduced fibroblasts, whereas PEG-DA hydrogels with transduced fibroblasts had 58 ± 7% viable cells. However, even though there was a decrease in cell viability following encapsulation, the cell viability after 7 days in culture was maintained at 69 ± 8% viable for non-transduced fibroblasts and 60 ± 14% viable for transduced fibroblasts, respectively. The sample size for all data collected was five ($n = 5$).

Characterization of Secreted BMP-2

The Western blot showed that only the plated transduced cells (Fig. 3a, lanes 5–8) and hydrogels containing cells transduced with the Ad5F35BMP2 vector (Fig. 3a, lanes 10–17) produced detectable amounts of BMP-2 protein. In contrast, no BMP-2 protein was detected from the control hydrogel only (Fig. 3a, lane 2) or from the conditioned medium from non-transduced cells (Fig. 3a, lanes 3 & 4). In addition, an alkaline phosphatase assay was performed with the cultured hydrogel media to investigate the biological activity of secreted BMP-2 protein, since BMP-2 has been shown to induce alkaline phosphatase activity in W20-17 cells.[39] The alkaline phosphatase assay showed that conditioned media from the encapsulated

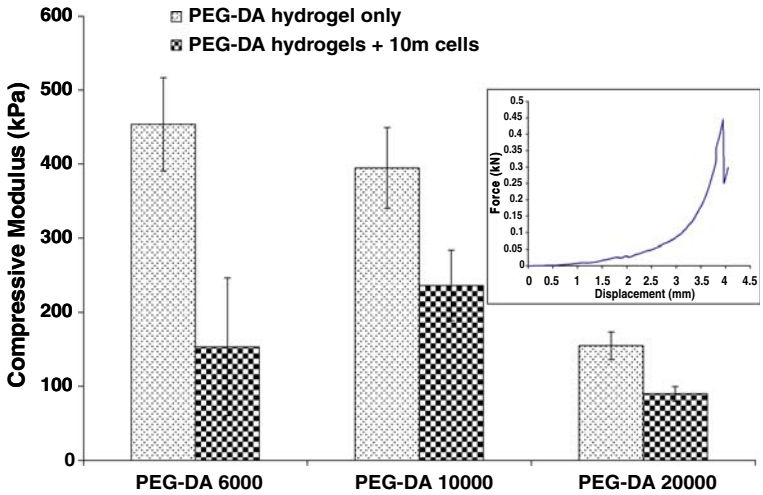

FIGURE 2. Compressive modulus for prepared PEG-DA hydrogels of varying molecular weights with and without 10 million encapsulated transduced fibroblasts after 24 h. Inset shows representative load versus displacement curve for the raw data. Data reported as mean ± SD, $n = 5$.

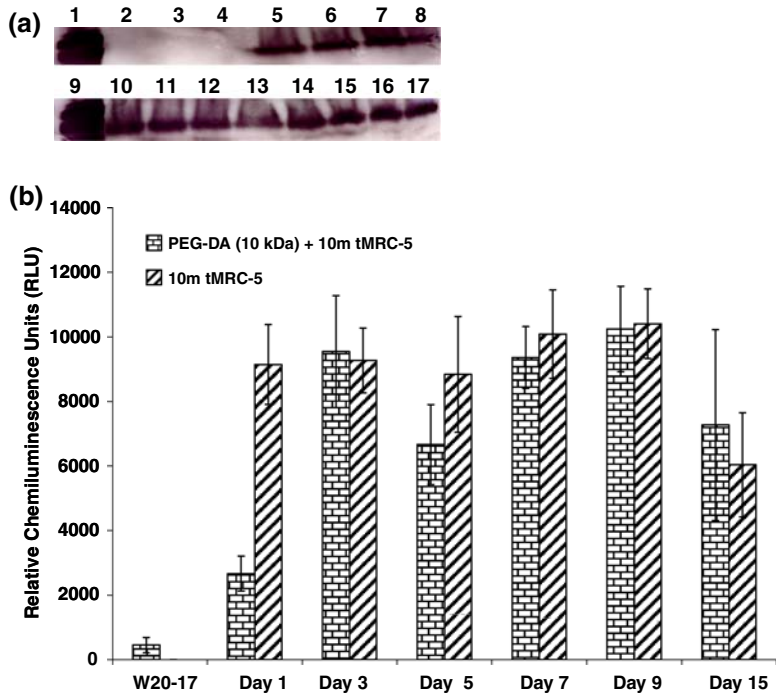

FIGURE 3. Western blot analysis for the detection of secreted BMP-2 protein. (a) Human recombinant BMP2 (lane 1), conditioned medium from PEG-DA (10 kDa) hydrogels only (lane 2), conditioned medium from 10 million MRC-5 cells encapsulated within PEG-DA (10 kDa) hydrogels (lanes 3 and 4), conditioned medium from 10 million transduced fibroblasts control (lanes 5–8), human recombinant BMP-2 (lane 9), conditioned medium containing secreted BMP-2 protein from 10 million transduced fibroblasts encapsulated within PEG-DA (10 kDa) hydrogels (lanes 10–17). (b) Alkaline phosphatase activity in W20-17 cells without the addition of conditioned medium (W20-17) and after addition of conditioned media from PEG-DA (10 kDa) hydrogels with 10 million transduced fibroblasts and control plated transduced fibroblasts (Days 1–15). Data reported as mean ± SD, $n = 5$.

transduced fibroblasts contained active BMP-2 protein, which produced high levels of alkaline phosphatase activity in W20-17 cells that was comparable to the levels of alkaline phosphatase activity obtained from the medium collected from the control plated transduced fibroblasts (Fig. 3b).

Endochondral Bone Formation

To evaluate endochondral bone formation, PEG-DA (10 kDa) hydrogels containing a total of 10 million transduced fibroblasts were implanted intra-muscularly in each hind limb of three NOD/SCID mice per group ($n = 6$), and microCT was used to measure the volume of bone formed after 3 weeks. The mean amount of mineralized tissue in the muscle of the mice was 31.8 ± 7.8 mm^3 for mice injected with transduced fibroblasts only, 39.5 ± 25.0 mm^3 for mice implanted with the PEG-DA hydrogels encapsulated with transduced fibroblasts, respectively ($p = 0.47$), and no bone was detected in any of the control animals (0.0 mm^3). More importantly, the mineralized tissue that was formed in the muscle of animals implanted with the hydrogels was found to be localized to the hydrogel surfaces. Representative images are shown in Fig. 4. Moreover, there was no endochondral bone detected in the mice that were administered control cells transduced with the Ad5F35-HM4 control empty cassette vector either as a cell suspension or in hydrogel constructs. In addition, calcein AM/ethidium homodimer staining of transduced fibroblasts within the implanted hydrogel constructs showed that $68 \pm 19\%$ of the cells were still viable 2 weeks after surgical implantation. Histological analysis confirmed the presence of bone as well as cartilage for both groups of animals implanted with the hydrogels and those receiving transduced fibroblasts only. In contrast, there was no bone and/or cartilage observed for either animals injected with *ex vivo* transduced Ad5F35-HM4 cells or for the animals that were implanted with PEG-DA hydrogels containing *ex vivo* transduced Ad5F35-HM4 cells. Representative images for each group are shown in Fig. 5.

DISCUSSION

In this study, we have demonstrated that adenovirus-transduced BMP-2 expressing fibroblasts can be localized within PEG-DA hydrogels, which allowed

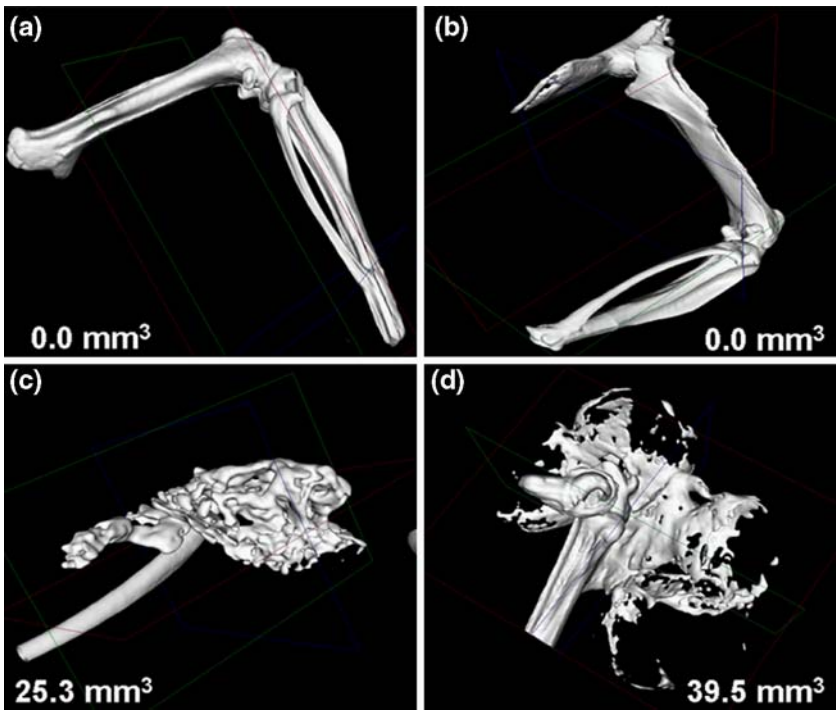

FIGURE 4. Micro-CT analysis of endochondral bone formation at 3 weeks. (a) Representative image of control 10 million MRC-5 cells transduced with HM4-1 adenovirus, (b) representative image of control PEG-DA (10 kDa) hydrogels with 10 million MRC-5 cells transduced with HM4-1 adenovirus, (c) representative image of bone formation from 10 million transduced fibroblasts expressing BMP-2 administered via intramuscular (i.m.) injection, and (d) representative image of bone formation from PEG-DA (10 kDa) constructs with 10 million transduced fibroblasts expressing BMP-2 following surgical implantation. Measurements for the femur and tibia shown in panels were not included in any of the volume calculations and the values of mineralized tissues shown in figure are only for the depicted image. Data reported as mean ± SD, *n* = 6.

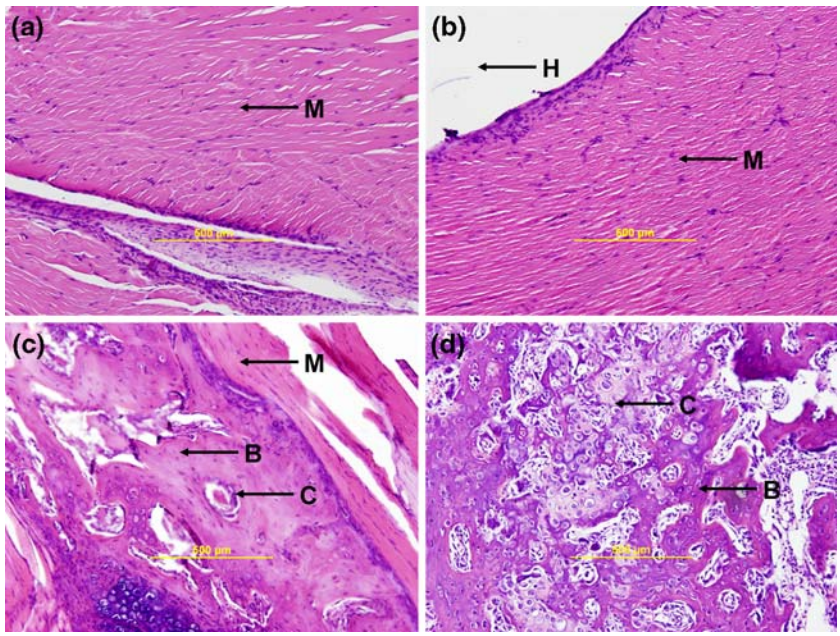

FIGURE 5. Histological evaluation of endochondral bone formation at 3 weeks. (a) 10 million MRC-5 cells transduced with HM4-1 adenovirus, (b) PEG-DA (10 kDa) hydrogels with 10 million MRC-5 cells transduced with HM4-1 adenovirus, (c) 10 million transduced fibroblasts expressing BMP-2, (d) PEG-DA (10 kDa) hydrogels with 10 million transduced fibroblasts expressing BMP-2 where M, H, B, C represents muscle, hydrogel, bone, and cartilage, respectively.

the diffusion of sufficient amounts of BMP-2 protein from the cell-seeded hydrogels to induce bone formation *in vivo*. The data from the ELISA assays suggested that cell densities within the hydrogels are very important for BMP-2 production by the transduced cells, suggesting that maybe cell–cell contact is an important consideration. Investigation of cell densities and BMP-2 production for this specific cell line on tissue culture plates of varying sizes showed that the same number of cells plated on different flasks of varying sizes produced significantly different gene expression (data not shown). Thus, even though specific cell–cell contact measurements were not made, this data suggests that cell densities for this particular cell line in a 3D polymeric network that would increase cell–cell distances may affect gene expression and hence suggest that maybe cell–cell contact is an important consideration. This observation will be further investigated in future studies. The molecular weight of the hydrogel precursor polymer also plays an important role, impacting both diffusion and mechanical properties. Prior studies have shown that the molecular weights of PEG precursors were directly proportional to the hydrogel's permeability to oligonucleotides and proteins.[43] However, even though diffusion of BMP-2 [MW 26 kDa] protein from the hydrogels may be impeded by the mesh sizes of the polymeric network, the localized encapsulation of high cell densities that constitutively express BMP-2 protein can be utilized to produce bone *in vivo*.

The compressive moduli of the PEG-DA hydrogels of varying molecular weights were determined in order to select the hydrogel with the best mechanical properties following cell encapsulation. The data showed that the compressive moduli of the PEG-DA hydrogels only decreased with increasing molecular weight, which was probably due to an increase in the molecular weight of the hydrogel crosslinks, M_c, and hence an increase in mesh size of the hydrogels resulting in a less rigid structure (Fig. 2). Subsequent encapsulation of cells resulted in a significant decrease in compressive moduli of the hydrogels, which was due to the formation of a composite material. In addition, the significant decrease in compressive modulus for the cell-encapsulated 20 kDa hydrogels in comparison with 6 and 10 kDa cell-encapsulated hydrogels was probably due to the increase in M_c (1556 ± 24 g/mol) and mesh size of (65 ± 1 Å) of these hydrogels as well as to the formation of a composite material, which resulted in very soft hydrogels. Our concern for the integrity of the cell encapsulated hydrogels following implantation into the hindlimb muscles of the animals led us to select the PEG-DA (10 kDa) hydrogels for further studies because of their better mechanical properties.

The BMP-2 protein secreted from the cell-seeded PEG-DA hydrogels was characterized with Western blot analysis, which confirmed that the mature form of the BMP-2 protein from the hydrogels was the same as the mature form of BMP-2 protein secreted from the plated transduced fibroblasts. Also, no BMP-2 protein was detected in any of the negative controls, which were not transduced with the Ad5F35BMP2 viral vector. In addition, the data from the alkaline phosphatase assay indicated that the secreted BMP-2 protein from the hydrogels was biologically active, since the media from the cultured hydrogels were able to stimulate alkaline phosphatase activity in W20-17 cells. The similar levels of alkaline phosphatase activity that was observed for the plated control as well as for the conditioned media suggested that a saturation limit at very high BMP-2 concentrations was achieved due to the sensitivity of the chemiluminescence substrate.[3]

For surgical implantation, hydrogels were formed as microbeads (Fig. 6) to allow localized deployment of BMP-2 production in complex geometries. An ELISA assay was performed with conditioned media from the microbeads, which indicated that the shape of the hydrogels could be successfully modified from large disks to small beads without any change in BMP-2 secretion (data not shown). The data from the microCT analysis, which showed the formation of bone in the muscle tissue following implantation of the PEG-DA hydrogel beads indicated that the transduced cells within the hydrogels were able to produce sufficient levels of BMP-2 protein, despite lower BMP-2

FIGURE 6. PEG-DA (10 kDa) hydrogel microbeads synthesized on surgical suture for *in vivo* implantation into NOD/SCID mice. Scale measure depicted represents mm increments.

secretion from the encapsulated cells. Therefore, the micro-CT data suggest that lower amounts of BMP-2 protein expressed over time may be adequate for bone formation. Moreover, the formation of bone that was found to be localized to the hydrogel construct suggests that this may be a unique method of spatially controlling gene expression to only a desired area. This spatial control of gene expression is extremely important for the expression of BMP-2 since uncontrolled bone formation in vital organs could lead to deleterious effects. Tantamount to this is that the formation of bone localized to the tiny hydrogel surface will be less likely to hinder the quality of bone formed within a desired area. Specifically, in spinal fusions the standard treatments are quite invasive and involves decortication of the traverse processes of the vertebrae followed by insertion of bone grafting materials either free or within cages.[33,42] In addition, the process is augmented with pedicle screw instrumentation or intervertebral joint screw-fixation, which is used to provide mechanical stability during the fusion process but in doing so occupies valuable surface area that could be utilized to enhance bone fusion.[36] Thus, the use of these tiny hydrogel constructs represents a significant improvement over the current spinal hardware, in that it utilizes much less surface area in comparison with joint screw-fixation in which placement of the hydrogels next to the defect [not within the defect] and periosteum may be sufficient to induce strong bone formation despite deposition of some bone onto the hydrogel surface. In addition, the data obtained from the stained sections of the muscle tissue corroborated the presence of bone formed in the muscle and the sections also showed the presence of cartilage in both the hydrogel-implanted animals and the animals injected with the transduced fibroblasts. Thus, the presence of cartilage in the muscles shows that bone formation may be occurring via an endochondral pathway. Moreover, the successful use of several microbead constructs in producing similar levels of mineralized tissue compared to the cell injected animals instead of one large hydrogel disk for the localization of BMP-2 protein expression suggests that smaller beads may improve the diffusion of gases and nutrients throughout the hydrogels as well as BMP-2 diffusion by decreasing the diffusional distances within the hydrogels. This observation is supported by the experimental cell viability study for hydrogel beads implanted into the hindlimb muscles of the animals in which we found that there was a higher fraction of live to dead cells retrieved from the implanted hydrogels in comparison with cultured cell encapsulated hydrogel disks. Thus, the sum of the smaller parts may be more effective than the whole and this observation can be used to improve the delivery of BMP-2 protein.

CONCLUSION

In summary, we have shown that encapsulation of adenovirus-transduced cells expressing BMP-2 within non-degradable PEG-DA hydrogel carriers can be used to spatially control the production and the release of BMP-2 protein. In addition, these PEG-DA hydrogels can facilitate the encapsulation of high cell densities that can produce sufficient amounts of BMP-2 protein to elicit bone formation *in vivo*, even though lower amounts of BMP-2 protein was detected from the hydrogel carriers in comparison to the same number of plated transduced fibroblasts. Thus, these results illustrate the benefits of localized gene therapy, which can significantly limit unwanted side effects and may have suitable applications for many orthopedic procedures such as spinal fusion.

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