

Repair of a Calvarial Defect With Biofactor and Stem Cell–Embedded Polyethylene Glycol Scaffold

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Objective: Segmental bony defects resulting from congenital facial anomalies, facial trauma, infection, or oncologic surgical resection represent a common and significant clinical problem. Currently, these defects are reconstructed with autologous or allogeneic bone grafts or prosthetic devices. These options are limited by bone supply for grafting, donor site morbidity, risk of infection, and extrusion. This study investigated the in vivo osteogenic capability of polyethylene glycol-diacrylate (PEG-DA) and a protease-sensitive PEG matrix metalloproteinases (PEG-MMP), photoencapsulated with mesenchymal stem cells (MSCs) and bone morphogenetic protein (BMP)-2, in healing a critical-size rat calvarial defect.

Methods: Both PEG-DA and PEG-MMP scaffolds photoencapsulated with rat MSCs (rMSCs) and/or BMP-2 were implanted into a critical-size defect. Microcomputed-

tomographic (micro-CT) analysis was completed 1, 4, and 8 weeks after implantation. Bone growth was histologically evaluated. The micro-CT data were analyzed using ASPIProVM software to calculate the percentage of closure of cranial defects.

Results: Both PEG-MMP and PEG-MMP + BMP2 showed significantly enhanced bone compared with controls. Polyethylene glycol-diacrylate seemed to inhibit bone growth regardless of biofactor and rMSCs. The addition of rMSCs did not enhance bone regeneration.

Conclusion: Polyethylene glycol sensitive to proteolysis significantly improved bone repair in a critical-size calvarial defect.

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SEGMENTAL BONY DEFECTS resulting from congenital facial anomalies, facial trauma, infection, or oncologic surgical resection represent a common and significant clinical problem. Data from the US Health Cost and Utilization project show that 12 700 craniotomies and craniectomies were performed in 2001, and procedures to correct defects associated with facial trauma numbered 20 616.¹ The national costs for these procedures are estimated to be approximately \$549 million and \$400 million, respectively.¹

Currently, these tissue defects are reconstructed with autologous bone or rib grafts, allogeneic bone grafts, or biocompatible prosthetic devices. While these solutions have been shown to improve bone regeneration, each method has a unique set of drawbacks. Autologous grafting is limited by the supply of suitable bone or rib and complications arising from donor site morbidity. Allogeneic bone grafts from donors or cadavers can present a risk of disease transmission. Prosthetic devices are plagued by the potential risk of infection and extrusion.²

Tissue engineering attempts to repair or regenerate damaged tissue by using engineered tissue substitutes that can sustain functionality during regeneration and eventually integrate with the host tissue or resorb. With tissue-engineered grafts, the appropriate signals (eg, osteoconductive surface, growth factors, and osteoprogenitor cells) can be delivered in a controlled fashion. Growth factors associated with osteoinduction are bone morphogenetic proteins (BMPs), transforming growth factor β , fibroblast growth factor 2, vascular endothelial growth factor, and insulinlike growth factor 1.³

Most research has focused on the use of the BMPs, and in particular BMP-2, because they have been shown to stimulate mesenchymal cell chemotaxis and proliferation and promote the differentiation of these cells into chondrocytes and osteoblasts.⁴ These osteoinductive capabilities are primarily evident by the induction of new bone formation via a process of endochondral ossification when implanted at ectopic sites.⁵

Bone-derived mesenchymal stem cells (MSCs) may be an ideal cell type for bone regeneration applications. Mesenchymal

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stem cells can be cultured in vitro, allowing for the rapid expansion of multipotent cells that are capable of differentiating into several distinct cell types, including osteoblasts, chondrocytes, adipocytes, and myocytes.⁶ Given this potential, experiments have been initiated to test the efficacy of MSCs to induce in vivo bone regeneration in animals.

To facilitate retention of BMP-2 and MSCs at the treatment site and reduce the effective dose, an appropriate carrier is required. The preferred carrier consists of a scaffold that is both biocompatible and resorbable in order to limit tissue rejection and allow for bone growth.

A calvarial wound model has many similarities to the maxillofacial region. Morphologically and embryologically, the calvarium develops from a membrane precursor and thus resembles the membranous bones of the face. Tagaki and Urist⁷ determined that an 8-mm diameter defect created in the calvaria of 6-month-old Sprague-Dawley rats reduced to 5 mm in 4 weeks, but no further healing of the defect was noted at 12 weeks. This work supported the 8-mm calvarial wound as a critical-size defect in this species. Using this model, the osteogenic potential of an implant may be considered unequivocal.

This preliminary project was directed at optimizing bone regrowth in a critically sized, nonhealing calvarial defect. We tested the osteoconductive and osteoinductive capability of 2 polyethylene glycol (PEG) scaffolds embedded with bioactive growth factors and MSCs. We hypothesized that PEG scaffolds would be osteoconductive and that the presence of biofactors and MSCs would provide increased osteoinduction and thus improved bone regrowth when compared with negative control.

METHODS

PREPARATION OF PEG MONOMERS

Formation of PEG-diacrylate (DA) was prepared as previously described.⁸ The PEG-matrix metalloproteinases (MMP) consisted of a 4-arm 20 000 Da PEG-(tetra) norbornene hub and an enzymatically degradable dicysteine peptide linker.

SYNTHESIS OF NORBORNENE ANHYDRIDE

Under inert atmosphere, 5-norbornene-2-carboxylic acid (1.38 g; Sigma, St Louis, Missouri) was dissolved in 30 mL of dichloromethane (DCM); di-isopropylcarbanoamide (DIC) (1.26 g; Sigma) was added slowly to the reaction mixture and stirred for 30 minutes. The reaction mixture was filtered under inert conditions; filtrate was reacted with PEG mixture as described in the next subsection.

SYNTHESIS OF PEG-TETRA-NORBORNENE

Four-arm PEG 20 000 Da (10 g; JenKem USA, Allen, Texas) was dissolved in 100 mL of DCM and cooled to 0°C under an inert atmosphere. Pyridine (0.8 g; Sigma) and a catalytic amount of dimethylaminopyridine (DMAP) (0.012 g; Sigma) were added to the reaction flask under an inert atmosphere and stirred. The symmetric norbornene anhydride (2.5 eq; 1.38 g prepared as described in the previous subsection) was added to the reaction mixture under inert atmosphere and stirred at 0°C overnight. The reaction mixture was filtered and

subsequently PEG-tetra-norbornene was precipitated in ethyl ether (1 L) at 0°C. The filter cake was placed in a Soxhlet extractor; remaining impurities were extracted with ethyl ether (for 48 hours). The purified product was confirmed by ¹H NMR (CDCl₃) (a type of proton nuclear magnetic resonance [deuterated chloroform]).

RAT MSC CULTURE

Rat MSCs (rMSCs) were obtained from Tulane University Health Science Center, New Orleans, Louisiana, and cultures were successfully grown in the Anseth Laboratory at the University of Colorado.

PHOTOENCAPSULATION OF rMSCs

Photoencapsulation of rMSCs has been tested on rMSC cultures, and the encapsulation process showed no abnormal effects on cell viability. For PEG-DA hydrogels, a solution containing 4600 kDa PEG-DA (PEG4600DA) at 10% wt/vol was prepared in phosphate-buffered saline (PBS) containing the photoinitiator 4-(2-hydroxyethoxy) phenyl-(2-hydroxyl-2-propyl) ketone (I2959; Ciba, Florham Park, New Jersey). I2959 is a water-soluble photoinitiator that initiates the polymerization of acrylate functionalities in the presence of long-wavelength UV light and has been shown to exhibit low toxicity to cells under these photoencapsulation conditions. The CRGDS (cysteine, arginine, glycine, aspartic acid, serine) peptide serves as an extracellular matrix adhesion mimic that cross-links into the network via the thiol contained within the cysteine residue and allows integrin-mediated cell attachment to the scaffold. Rat MSCs were trypsinized from cell culture and centrifuged to pellet the cells. The cells were then resuspended in the PEG monomer solution at a concentration of 5 million cells/mL. Approximately 40 µL of cell/polymer solution was then placed into sterile polytetrafluoroethylene wells that were 7 mm in diameter. The wells were placed under UV light for 10 minutes at room temperature to polymerize the samples. On polymerization, the disk-shaped constructs were removed from the wells and placed into tissue culture media and incubated at 37°C in 5% carbon dioxide until the time of implantation. After 24 hours, Live/Dead assays (Molecular Probes, Eugene, Oregon) were performed on the hydrogel constructs to ensure that the rMSCs remained viable throughout the encapsulation process.

A similar process was performed for MMP-degradable scaffolds (PEG-MMP). For these gels, 20 000 Da-PEG-norbornene was resuspended in PBS at a concentration of 10% wt/vol with 0.25 mM CRGDS, 0.05% I2959, and the degradable peptide cross-linker (KKCGGPQGIAGQGCKK) containing 2 cysteine residues flanking an MMP-cleavable sequence.

Bone morphogenetic protein 2 was purchased from R&D Systems (Minneapolis, Minnesota) and included in monomer solutions before polymerization at a concentration of 5 ng/80 µL for PEG-DA-based scaffolds. To account for volume changes resulting from free swelling of PEG-MMP hydrogels after polymerization, a concentration of 15 ng/80 µL BMP-2 was used.

RAT CRANIAL SURGERY

Operations were performed on albino male Sprague-Dawley rats aged 10 to 11 weeks (weight, 300-350 g). General anesthesia was administered by intraperitoneal injection of ketamine hydrochloride (40 mg/kg) mixed with xylazine (10 mg/kg). After induction of anesthesia, the surgical site was shaved of fur. The head was placed in a stereotaxic frame. An incision was made along the sagittal suture, and the periosteum was el-

Table 1. PEG-DA Scaffold: Microcomputed Tomography: Percentage of Reduction in Bony Wound Volume^a

Time	Animal Group				
	Control (n=6)	PEG-DA (n=6)	PEG-DA + rMSCs (n=6)	PEG-DA + BMP-2 (n=6)	PEG-DA + rMSCs + BMP-2 (n=6)
Week 4	32 (10.57)	27 (7.56)	20 (5.45)	8 (5.20)	5 (4.19)
Week 8	50 (5.63)	42 (10.88)	35 (4.84)	42 (4.38)	23 (8.44)

Abbreviations: BMP-2, bone morphogenetic protein 2; PEG-DA, polyethylene glycol–diacrylate; PEG-MMP, polyethylene glycol–matrix metalloproteinases; rMSCs, rat mesenchymal stem cells.

^aData are given as the mean of the percentage of reduction in the defect (SE).

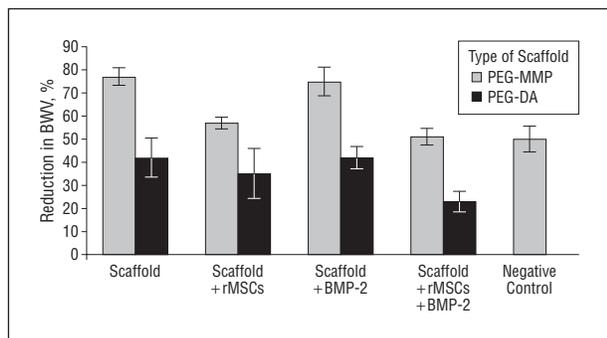


Figure 1. Percentage of reduction in bony wound volume (BWV) by scaffold type. Standard error bars are shown. BMP-2 indicates bone morphogenetic protein 2; PEG-DA, polyethylene glycol–diacrylate; PEG-MMP, polyethylene glycol–matrix metalloproteinases; rMSCs, rat mesenchymal stem cells.

evated. A full-thickness calvarial bone defect 8 mm in diameter was created without dura perforation using a surgical microdrill fitted with a diamond burr. The wound was thoroughly irrigated with warmed saline to remove residual bone dust. After implantation of the appropriate scaffold, the periosteum was closed with 4.0 Vicryl suture, and the skin was closed with staples. The animals were killed by carbon dioxide asphyxiation 9 weeks after scaffold implantation.

A total of 54 animals were randomly assigned to 9 groups, testing 2 scaffold chemistries, PEG-DA and PEG-MMP. The total number of animals per group was determined by power calculation analysis. There were 6 animals in each of the following groups: (1) surgical defect, (2) PEG-DA scaffold, (3) PEG-MMP, (4) PEG-DA + BMP-2 (5 ng/80 μ L), (5) PEG-MMP + BMP-2 (5 ng/80 μ L), (6) PEG-DA + rMSCs (1×10^7 cells/mL), (7) PEG-MMP + rMSCs (1×10^7 cells/mL), (8) PEG-DA + rMSCs + BMP-2, and (9) PEG-MMP + rMSCs + BMP-2.

MICROCOMPUTED TOMOGRAPHY

Microcomputed tomography (micro-CT) was applied to longitudinally assess bone formation in the time frame of 8 weeks. Each animal underwent micro-CT with a spatial resolution of 70 μ m at 1 week, 4 weeks, and 8 weeks after implantation. Using ASPIProVM software (Siemens Medical Solutions USA Inc, Hoffman Estates, Illinois) to analyze the micro-CT data, the percentage of new bone formation was presented as the ratio of new bone volume vs total defect volume.

HISTOLOGIC FINDINGS

After the animals were killed, the skulls were fixed in 10% formalin overnight, then dehydrated in 70% ethanol, decalcified in EDTA solution, and embedded in paraffin. For histochemical analysis, paraffin sections were made in the midline of the calvarial defect. Three representative animals in each group were

evaluated in this way. A Masson trichrome stain was used for detection of cells and bone regrowth. Digital images from stained sections were taken by means of a transmission and polarized light Axioskop Microscope (Carl Zeiss MicroImaging Inc, Thornwood, New York). Defect volume data were analyzed using the SAS statistical package (SAS Inc, Cary, North Carolina). This study protocol was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Colorado at Denver Health Sciences Center.

RESULTS

We were successfully able to prefabricate PEG-DA and PEG-MMP scaffolds. Live/Dead assays performed on the hydrogel constructs showed that the rMSCs remained viable throughout the encapsulation process. Rats serving as a negative control illustrated a mean closure of defect of 32% at 4 weeks and 50% at 8 weeks. No animals in the control group had complete closure of their defects.

PEG-DA SCAFFOLD

Microcomputed tomographic analysis at 4 weeks after implantation with PEG-DA, PEG-DA + rMSCs, PEG-DA + BMP-2, and PEG-DA + rMSCs + BMP-2 illustrated mean reductions in defect volume of 27%, 20%, 8%, and 5%, respectively. At 8 weeks the mean reduction in defect volume had increased to 42%, 35%, 42%, and 23% (**Table 1**, **Figure 1**, and **Figure 2**).

On gross examination, there was no resorption of the PEG-DA scaffold. Microscopic evaluation showed sharp demarcation of the bony wound and PEG-DA interface. There were no islands of ossification. Bone regrowth was limited deep to the scaffold and immediately adjacent to dura mater. A representative image is shown in **Figure 3**.

PEG-MMP SCAFFOLD

Microcomputed tomographic analysis at 4 weeks after implantation with PEG-MMP, PEG-MMP + rMSCs, PEG-MMP + BMP-2, and PEG-MMP + rMSCs + BMP-2 illustrated mean reductions in defect volume of 59%, 32%, 55%, and 26%, respectively. At 8 weeks, mean reductions of 77%, 57%, 75%, and 51% were present (**Table 2**, **Figure 1**, and **Figure 4**).

On gross examination, there was complete resorption of the PEG-MMP scaffold. Microscopic evaluation showed good infiltration of bone with near-complete coverage of the defect. Bone regrowth was present along the

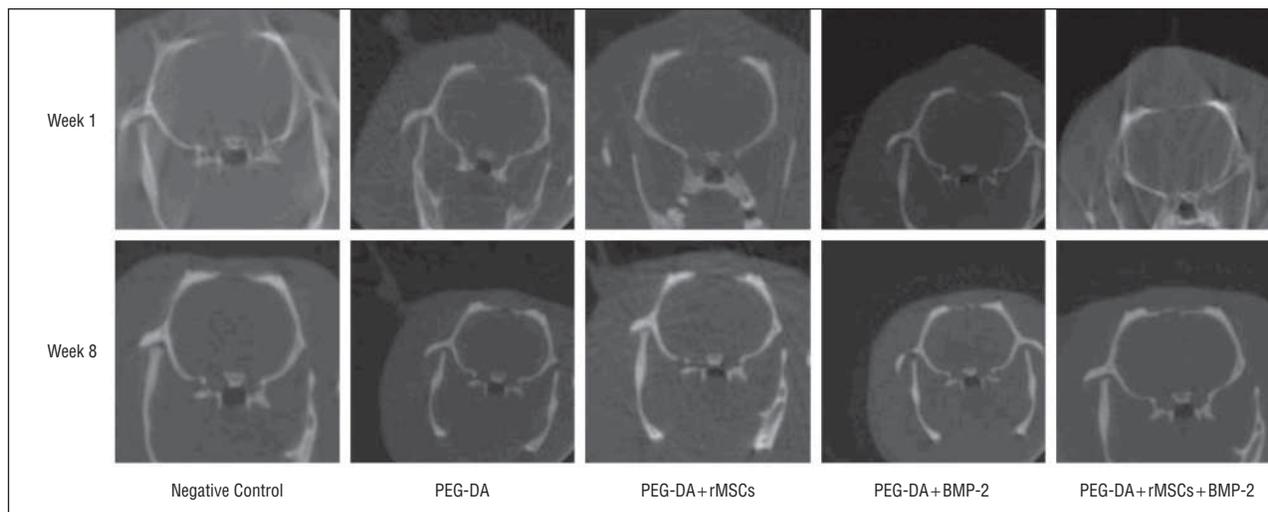


Figure 2. Representative microcomputed tomographic images of animals implanted with polyethylene glycol–diacrylate (PEG-DA) scaffold. Scans taken at 1 week and 8 week are shown. See Figure 1 for expansions of abbreviations.

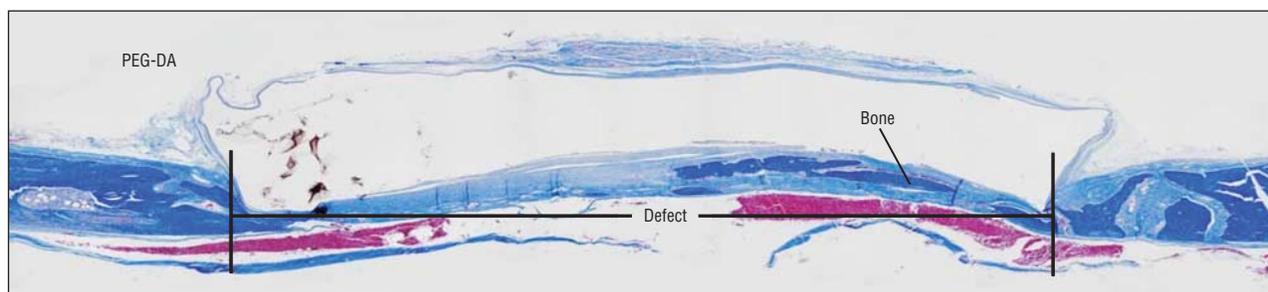


Figure 3. Representative animal implanted with a polyethylene glycol–diacrylate (PEG-DA) scaffold, 9 weeks after implantation. The specimen was stained with Masson trichrome (original magnification $\times 10$). A fibrous capsule is seen surrounding the nondegraded PEG-DA scaffold. There is sharp demarcation at the wound-PEG interface and no islands of ossification within the scaffold. Limited bone regrowth is present immediately adjacent to the dura.

Table 2. PEG-MMP Scaffold: Microcomputed Tomography: Percentage of Reduction in Bony Wound Volume^a

Time	Animal Group				
	Control (n=6)	PEG-MMP (n=6)	PEG-MMP + rMSCs (n=6)	PEG-MMP + BMP-2 (n=6)	PEG-MMP + rMSCs + BMP-2 (n=6)
Week 4	32 (10.57)	59 (4.90)	32 (6.78)	55 (2.01)	26 (8.72)
Week 8	50 (5.63)	77 (7.73)	57 (6.04)	75 (2.44)	51 (3.69)

Abbreviations: See Table 1.

^aData are given as the mean of the percentage of reduction in the defect (SE).

periphery and centrally. A histologic section of a representative sample is shown in **Figure 5**.

STATISTICAL ANALYSIS

Mean values of the percentage of reduction in initial defect volume were calculated using a cell means model (a form of linear model useful for analyzing categorical data). Mean values for each treatment category were calculated, as were differences between treatment and the control group for the 8-week time point. Statistical tests were performed to determine if the difference between each treatment group and the control group were statistically significant. The model indicates that taken as a whole, there is a significant difference in means for the groups ($P < .001$).

For each paired difference, the difference, test statistic, and P value are listed in **Table 3**. The P value represents the calculated probability of observing a difference in means between the control and the comparison group as or more extreme than the one observed, based on the experimental design. The PEG-MMP and PEG-MMP + BMP-2 seem to be statistically greater in reducing the initial defect volume than control, with an estimated mean difference of 27.3% and 25.0% ($P = .004$ and $P = .008$, respectively).

COMMENT

We attempted to use PEG scaffolds as osteoconductive moieties and as an effective delivery vehicle for the cytokine

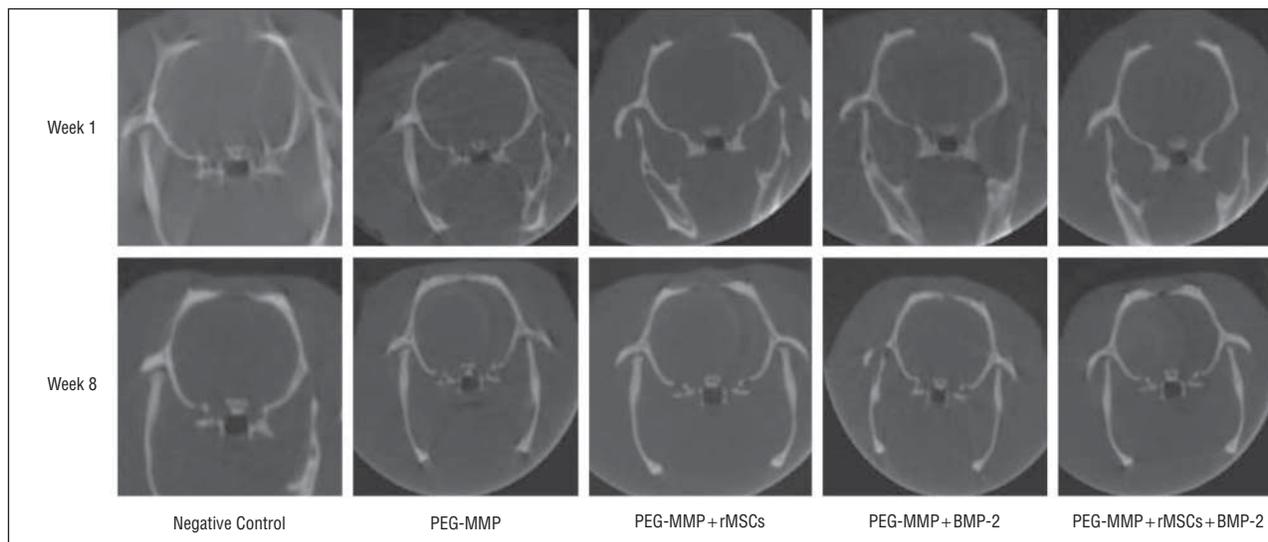


Figure 4. Representative microcomputed tomographic images of animals implanted with polyethylene glycol-matrix metalloproteinases (PEG-MMP) scaffold. One-week and 8-week scans are shown. See Figure 1 for expansions of abbreviations.

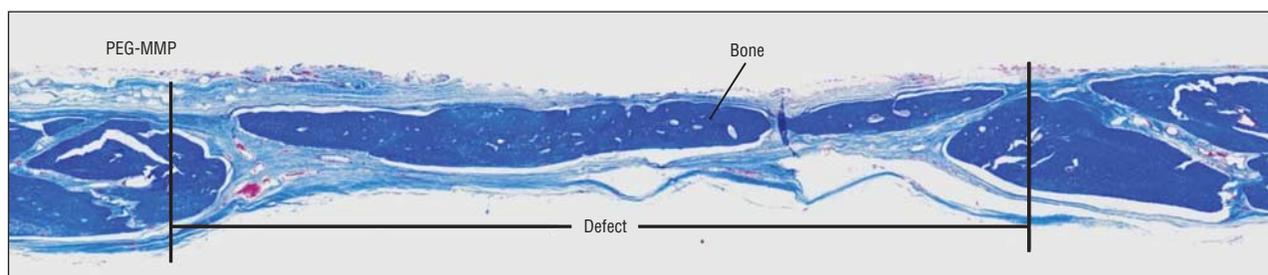


Figure 5. Representative animal implanted with a polyethylene glycol-matrix metalloproteinases (PEG-MMP) scaffold, 9 weeks after implantation. The specimen was stained with Masson trichrome (original magnification $\times 10$). The scaffold is completely resorbed. Peripheral and central bone regrowth is present within the area previously occupied by the scaffold.

Table 3. Differences in Mean Bony Wound Volume (BWV) and Control

Comparison Group	Difference in Mean BWV and Control ^a	t Value	P Value
PEG-DA	-0.08	-0.86	.39
PEG-DA + rMSC	-0.14	-1.72	.09
PEGDA + BMP-2	-0.08	-0.88	.38
PEG-DA + rMSC + BMP-2	-0.27	-2.99	.005
PEG-MMP	0.27	3.02	.004
PEG-MMP + rMSC	0.07	0.79	.43
PEG-MMP + BMP2	0.25	2.77	.008
PEG-MMP + rMSC + BMP2	0.01	0.10	.92

Abbreviations: See Table 1.

^aThe standard error was 0.09 in all cases.

BMP-2. By encapsulating MSCs, which have the potential to commit to an osteoblastic lineage, we aimed to establish new centers of bone formation within a defect. In theory, combining scaffold, cytokines, and cells to generate ex vivo tissue-engineered constructs should provide more effective bone regeneration in vivo in comparison with biomaterial matrices alone⁹ or negative control.

We were able to successfully prefabricate PEG-DA and PEG-MMP and maintain cell viability following photopolymerization in PEG. Negative control animals illus-

trated a mean reduction in defect volume of approximately 50% at 8 weeks, and none had complete closure of their defects. This reconfirms the validity of this calvarial critical-size defect model.

Our PEG-DA scaffolds were nondegradable by cell initiated proteolysis and did not enhance osteoconduction in this model. In fact, we showed a trend toward decreased bone growth regardless of encapsulated biofactor or stem cell. These findings were clear by micro-CT, gross post mortem evaluation, and on histologic analysis. Sharp demarcation of the bony wound edge and a lack of islands of ossification were seen radiographically and histologically (Figure 1). When bone regrowth occurred, it was deep to the scaffold and immediately adjacent to the dura mater. This is not surprising given the large body of evidence supporting the osteogenic potential of dura mater.¹⁰

By comparison, PEG scaffolding susceptible to cell-triggered proteolysis (PEG-MMP) showed substantially more bone formation than negative control and thus appeared osteoconductive. This increased bone formation was seen in both peripheral new bone (in continuity with the native calvaria) and central new bone (not in continuity with the calvarial edges) (Figure 3).

It is clear that osteoconduction was dependent on cell-triggered proteolysis. Within a nondegradable scaffold,

cell migration can occur only by amoeboid cell migration, and, thus, pore size must be larger than the migrating cell's diameter. However, the porosity of our PEG-DA scaffold is well below the critical porosity by which migration is possible. Conversely, PEG-MMP enables cell-initiated proteolytic migration, and thus porosity is less critical.

Bone morphogenetic protein-2 at a concentration of (5 ng/80 μ L) was not osteoinductive in vivo and showed no increased osteogenesis beyond that seen with PEG-MMP alone. Previous in vitro work has shown improved bone regeneration using significantly higher concentrations (5 μ g/100 μ L) of BMP-2.¹¹ Our decision to use a low concentration was based on our previous in vitro cell culture studies. In addition, high concentrations of BMP-2 have been shown to give rise to heterotrophic ossification.¹²

The addition of rMSCs did not increase osteoinduction, as was hypothesized, and in fact seemed to be inhibitory (Table 3). There is literature suggesting that rMSCs in vitro are largely unresponsive to BMP-2 without dexamethasone in the culture medium.¹³ Also, BMP-2 used in isolation with MSCs has shown very low osteogenic potential in some reports.¹³ Some in vitro and in vivo studies suggest that early treatment of rMSCs with basic fibroblast growth factor (bFGF) and dexamethasone induced BMP-2 responsive osteoprogenitor cells that when treated with BMP-2 differentiated into fully mature osteoblasts.¹³ Our protocol did not use dexamethasone or bFGF.

In conclusion, this calvarial critical-size defect model seemed valid and enabled evaluation of the osteogenic potential of our scaffold. The micro-CT imaging techniques allowed longitudinal analysis of bone regrowth. Polyethylene glycol scaffold sensitive to cell-triggered proteolysis was necessary for improving bone regrowth. The BMP-2 and rMSCs in the concentration and conditions of our project did not further increase bone regrowth. This was likely owing to low BMP-2 concentration and MSCs that despite good viability were not fully responsive to the osteogenic differentiating influence of BMP-2.

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