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OSTEOCHONDRAL INTERFACE REGENERATION OF THE RABBIT KNEE WITH MACROSCOPIC GRADIENTS OF BIOACTIVE SIGNALS

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Abstract

To date, most interfacial tissue engineering approaches have utilized stratified designs, in which there are two or more discrete layers comprising the interface. Continuously-graded interfacial designs, where there is no discrete transition from one tissue type to another, are gaining attention as an alternative to stratified designs. Given that osteochondral regeneration holds the potential to enhance cartilage regeneration by leveraging the healing capacity of the underlying bone, we endeavored to introduce a continuously graded approach to osteochondral regeneration. The purpose of this study was thus to evaluate the performance of a novel gradient-based scaffolding approach to regenerate osteochondral defects in the New Zealand White rabbit femoral condyle. Bioactive plugs were constructed from poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres with a continuous gradient transition between cartilage-promoting and bone-promoting growth factors. At six and 12 weeks of healing, results suggested that the implants provided support for the neo-synthesized tissue, and the gradient in bioactive signaling may have been beneficial for bone and cartilage regeneration compared to the blank control implant, as evidenced by histology. In addition, the effects of pre-seeding gradient scaffolds with umbilical cord mesenchymal stromal cells (UCMSCs) from the Wharton's jelly of New Zealand White rabbits were evaluated. Results indicated that there may be regenerative benefits to pre-localizing UCMSCs within scaffold interiors. The inclusion of bioactive factors in a gradient-based scaffolding design is a promising new treatment strategy for defect repair in the femoral condyle.

Keywords

Osteochondral; Interface; Gradient; Microsphere; Umbilical Cord Stem Cells; PLGA; BMP-2; TGF-β_1

INTRODUCTION

Interface tissue engineering has emerged as a type of functional tissue engineering, which involves hierarchical examination of native tissues and their structures. Simply, the interface

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is where two tissue types adjoin. Most approaches directly targeting tissue engineering of interfaces have been stratified in nature, with multiple discrete layers of differing physical or chemical properties.[1-15] The approach of using continuously-graded 3D designs for interface tissue engineering, as opposed to stratified designs, is gaining attention.[16]

Many groups[4,16-19] have compiled comprehensive reviews on state of the art applications for incorporating continuous gradients in tissue engineering, illustrating numerous current and potential strategies for interfacial tissue engineering. Concepts for formulating multiple tissue systems[14] rely on one or more forms of physical or chemical stimuli, which affect cell-specific movement, substrate affinity, or tissue formation.[17] Furthermore, most two-dimensional (2D) continuous gradient applications have been intended for simply characterizing tissue engineering phenomena in the form of high-throughput screening, and have not always been translated to 3D interfacial tissue applications.

While there have been many recent *in vivo* approaches for interface tissue engineering using stratified designs,[20-23] the number of continuously-graded approaches are limited.[16] *In vitro*, continuous gradients for interface tissue engineering thus far have demonstrated continuous gradients of tissue-specific matrix.[16] Specifically, a gradient approach might provide more regenerative control to nature, using a continuous transition of signals, directing the interface to form in a specified region. This ultimately may be conducive to patient-specific regenerative designs. Interestingly, techniques for creating seamless transitions between tissue regions are not necessarily more elaborate, or time-consuming, than analogous stratified approaches.[16] While gradient designs have been implemented for many tissue interfaces (such as tendon/ligament-bone, muscle-tendon, and *tunicae* of the vasculature), the cartilage-bone interface is of particular interest. With arthritis as one of the leading causes of disability in the world, treatments for degenerative conditions of cartilage surfaces and/or subchondral bone are urgently needed. Moreover, osteochondral regeneration has strong potential to emerge as the preferred strategy over regeneration of cartilage in isolation for the treatment of focal cartilage injuries, which is because by intentionally creating a defect in the underlying bone, the implant can leverage the underlying bone for a) an anchoring site to facilitate early integration with the host tissue, and b) a wealthy reservoir of autologous marrow cells to infiltrate the biomaterial. Thus, for *in situ* regeneration of cartilage and bone simultaneously, chemical stimuli for each tissue type may be beneficial. In this regard, transforming growth factors (TGFs) and bone morphogenetic proteins (BMPs) have been shown to stimulate stem cell differentiation into cartilage and bone, respectively. Specifically, TGF-β_1 and TGF-β_3 promote chondrogenesis,[24-27] which may serve to mitigate a common problem with fibrous tissue formation in lieu of hyaline cartilage formation in cartilage defect repair, whilst BMP-2 and BMP-7 promote osteogenesis.[28-30] Beyond the stimulatory nature of the aforementioned proteins, a physical arrangement of stimuli in a gradient fashion might provide a seamless transition in neo-synthesized tissue properties, as opposed to a discrete change between cartilage-like and bone-like tissue, as seen in some stratified interfacial tissue engineering solutions. Our hypothesis was that scaffolds with a gradient in chemical signals would regenerate cartilage and bone with a seamless interface.

The present study investigated bone and cartilage regeneration in femoral condyle defects in New Zealand White rabbits with microsphere-based scaffolds containing gradients of bioactive signals, a design that previously had only been characterized *in vitro*.[31-33] The major findings of these *in vitro* characterization were that gradients in signaling could increase biochemical production (such as collagen and glycosaminoglycans), upregulate expression of osteogenic and chondrogenic differentiation markers, and produce regionalized tissue formation within a single construct after 6 weeks. The scaffolds utilized a continuous transition from cartilage-promoting, (TGF-β_1-loaded microspheres) to bone-

promoting (BMP-2-loaded microspheres) regions. The induced defect size was approximately 3.0 mm in diameter and 3.0 mm in depth. Regeneration was evaluated at six and 12 weeks with histological staining. The goal was to determine whether the continuously-graded design would facilitate osteochondral defect regeneration in the rabbit femoral condyle as a foundation for future *in vivo* studies.

MATERIALS AND METHODS

Scaffolding Materials

Poly(D,L-lactide-co-glycolic acid) copolymer (PLGA; 50:50 lactic acid: glycolic acid, acid end group, M_W ~80,000 Da) of intrinsic viscosity (i.v.) 0.55 dL/g was purchased from Lakeshore Biomaterials (Birmingham, AL). Poly(vinyl alcohol) (PVA; 88% hydrolyzed, 25,000 Da) was obtained from Polysciences, Inc. (Warrington, PA). Transforming growth factor (TGF)-β_1 and bone morphogenetic protein (BMP)-2 were purchased from Peprotech, Inc. (Rocky Hill, NJ). Ten New Zealand White rabbits were obtained from Myrtle's Rabbitry (Thompson Station, TN) in accordance with the University of Kansas IUCAC procedures.

Preparation of Protein-loaded Microspheres

BMP-2 was reconstituted in 0.1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) (both from Sigma, St. Louis, MO). TGF-β_1 was reconstituted in 0.01% BSA in PBS. The reconstituted protein solutions were individually mixed with PLGA dissolved in dichloromethane (DCM) (20% w/v) to reach a final loading ratio of 30 ng TGF-β_1 or 60 ng BMP-2 per 1.0 mg of PLGA. The final mixtures were then sonicated over ice (50% amplitude, 20 seconds) with a Sonic Dismembrator 500 (Fisher Scientific, Pittsburgh, PA). Using PLGA-protein emulsions, uniform protein-loaded PLGA microspheres were prepared using technology from our previous reports.[31-34] Briefly, using acoustic excitation produced by an ultrasonic transducer, regular jet instabilities were created in the polymer stream that produced uniform polymer droplets. An annular carrier non-solvent stream (0.5% w/v PVA in ddH_2O) surrounding the droplets was produced using a nozzle coaxial to the needle. The emanated polymer/carrier streams flowed into a beaker containing the non-solvent. Incipient polymer droplets were stirred for 3-4 hours to allow solvent evaporation, which were then filtered and rinsed with ddH_2O to remove residual PVA, and stored at -20 °C (Fig. 1A). Blank control microspheres were prepared in a similar manner, where the protein solution was replaced with an equivalent volume of BSA solution (1 mg/mL). Following 48 hours of lyophilization, the size distribution of microsphere preparations was determined using a Coulter Multisizer 3 (Beckman Coulter Inc., Fullerton, CA) equipped with a 560-μm aperture. Blank control microspheres were prepared in a similar manner without growth factors. All groups utilized microspheres with a nominal diameter of approximately 165 μm, obtained in the same manner as previous reports.[31-34]

Scaffold Fabrication

Gradient scaffolds were prepared using our previously reported technology.[31-33] Briefly, lyophilized protein-loaded microspheres were dispersed in ddH_2O at a concentration of 2.5% w/v, and separately loaded into two syringes. Each construct in total contained ~30 mg of microspheres. The suspensions were pumped into a cylindrical glass mold (3.2 mm diameter, 3.0 cm in height) in a controlled manner using programmable infusion syringe pumps (PHD 22/2000, Harvard Apparatus, Inc., Holliston, MA). Using a filter (particle retention > 3 μm) at the bottom of the mold, ddH_2O was filtered, while the microparticles stacked in the mold until a height of 3.0 mm was reached. The profile for Gradient constructs was linear, where the transition region from TGF-β_1 to BMP-2 constituted the second quarter of the scaffold volume, and the top quarter and bottom half contained all

TGF-β_1- or BMP-2-loaded microspheres, respectively (Fig. 1B). Using an additional infusion syringe pump and a vacuum pump, a constant level of distilled water was maintained in the mold. The stacked microspheres were then sintered using a 100% ethanol treatment for 2 hours.[33] A longer sintering time, compared to previous investigations,[31,33] was necessary for microsphere fusion due to the higher molecular weight polymer. The molds (containing the scaffolds) were freeze-dried for 48 hours, then the gradient scaffolds were retrieved and stored at −20 °C. Blank scaffolds were prepared in a similar manner. Scaffolds were packaged and sterilized with ethylene oxide for 12 hours prior to implantation.

Description of Experimental Groups

Four different treatment groups were investigated: (i) Sham surgeries, in which a defect was made, but no implant was placed, (ii) Blank scaffolds with no growth factors encapsulated, (iii) Gradient scaffolds with a transition between BMP-2- and TGF-β_1-loaded microspheres, and (iv) Gradient scaffolds pre-cultured with rabbit umbilical cord mesenchymal stromal cells (rUCMSCs) before surgical implantation (Fig. 1C).

Cell Harvest and Seeding

Following an IACUC-approved protocol at the University of Kansas (Animal Use Statement #175-02), rUCMSCs were harvested from rabbit umbilical cords. Briefly, near-term New Zealand White rabbits were euthanized, the fetuses were retrieved and decapitated with scissors, and cord segments were minced and incubated in 0.75 mg/mL type II collagenase (Worthington Biochemical, Lakewood NJ) at 37 °C. After a 5-hour incubation, the resulting homogenous gelatinous solution was obtained and diluted (1:8) in sterile PBS. The solution was centrifuged, the supernatant was discarded, and cells were plated for 24 hours to allow viable cell attachment. Adhered cells were then trypsinized and frozen for future use. Frozen rUCMSCs (P1, i.e., plated once) were thawed and resuspended at a concentration of 20×10^6 cells/mL in a solution of low glucose DMEM and 1% penicillin/streptomycin (P/S) (all from Invitrogen Life Technologies, Carlsbad, CA). Approximately 15 μL (50% of the scaffold volume, approximately corresponding to the pore volume[33]) of cell solution was placed directly onto the top of the scaffold, which was soaked into the scaffold via capillary action. Cells were allowed to attach to the scaffolds for 12 hours, before surgical implantation.

Surgical Procedure

Surgical procedures were conducted under an approved IACUC protocol at the University of Kansas (Animal Use Statement #175-01), utilizing a total of 10 rabbits. Following stable general anesthesia, hair was shaved from the area around each rabbit knee. The bare knee was disinfected with three alternate scrubs of Betadine and 70% ethanol, and then draped. Only strict aseptic techniques and sterile instruments were used, and the surgeon wore sterile gowns, masks, and head covers. All surgical tools, including drill, were sterilized prior to surgery. A medial parapatellar incision was made, sufficient to allow exposure of the medial condyle. The tibia was lightly pushed to displace it laterally to allow the exposure of the medial femoral condyle. A pilot notch was created with a scalpel blade on the superficial cartilage in the central load-bearing region of the medial condyle. The defect was enlarged to 3.0 mm diameter and to the depth of 3.0 mm using a drill with a depth gauge attached to the bit. Defects were then filled by press-fitting one of three engineered plugs (either Blank, Gradient-only, or Gradient with precultured rUCMSCs) into the defect (Fig. 2). Sham defects were also created, in which a hole was drilled, but no implant was placed. The joint was then washed of debris, the patella and femur were relocated, the articular capsule and bursae were closed with an absorbable suture, and the skin was closed with a non-absorbable suture. Then the same procedure was performed on the contralateral knee following the

same procedure but with the alternative plug construct implanted (Table 1). After the procedure was finished, rabbits were administered buprenorphine subcutaneously and returned to their cage. The knee joints were allowed unconstrained movement postoperatively.

Histological Preparation and Staining

At 6 and 12 weeks, rabbit femoral condyles were retrieved and immediately placed in 10% neutral buffered formalin (Fisher Scientific) for at least three days. After fixation, the condyles were rinsed and dehydrated in graded ethanol. For plastic embedding, the samples were first infiltrated for 4 days with a solution consisting of 85 v/v% methylmethacrylate, 14 v/v% dibutyl phthalate, 1 v/v% poly(ethylene glycol) 400, and 7 mg/mL benzoyl peroxide (BPO) (all reagents from Sigma Aldrich). The infiltrate was changed every 24 hours. Following infiltration, condyles were embedded in 20 mL glass scintillation vials with a solution identical to the infiltrate, with the exception of using 4 mg/mL BPO and an additional 33 μL of N,N-dimethyl-p-toluidine (reagents from Sigma). The vials were placed at 4 °C for one week to polymerize. After polymerization, the glass vials were broken and the samples were removed.

Following plastic embedding, the samples were cut into blocks with a Buehler Isomet 1000 precision saw (Lake Bluff, IL). Sagittal sections were taken on a Microm HM 355S microtome using a tungsten carbide blade with a sample thickness of 10 μm. Sections were placed on gelatin-coated slides, and dried for 48 hours at 44 °C (materials from Fisher Scientific). After drying, the plastic resin was removed from the slides using a series of 2-methoxyethylacetate (20 minutes, three times), acetone (5 minutes, two times), and ddH_2O (5 minutes, two times). Safranin-O/Fast Green staining for glycosaminoglycans (GAGs), Alizarin Red staining for calcium deposition, and von Kossa staining for calcium phosphate was done as described elsewhere.[35] Slides were then dehydrated in graded ethanol and cleared in xylene for mounting. Sudan Black staining for residual polymer[36] was performed according to the manufacturer's instructions (a positive control PLGA section was stained, image not shown). All materials were from Sigma Aldrich.

Histology Scoring

To evaluate results of this pilot study, a simple scoring system (Table 2) was developed to evaluate cartilage and bone regeneration, where points were assigned for new tissue growth across the diameter of the original defect, and throughout the depth of the original defect, compared to adjacent tissue. All scoring was performed blinded. The assignment of histological scores was aided by evaluating staining intensity for GAGs, calcium ions, and calcium phosphate to contextualize the maturity of the neo-synthesized tissue. No statistical analysis was implemented to the scoring system, as the sample number for the entire study was not sufficient to determine significance among groups. Thus, scoring was used as reference for guiding future studies.

RESULTS

Post-surgical Course

At three weeks, one rabbit (with a Blank implant in one knee, and a Gradient-only implant in the contralateral knee) died prematurely. A necropsy later determined the death was due to gut stasis, which was unrelated to the implant. This brought the sample number for the six-week rabbits down to $n = 3$ for Blank and Gradient-only groups. During histology, a Gradient-only sample for another six-week rabbit was lost in processing, bringing the sample number for Gradient-only scaffolds at 6 weeks to $n = 2$. Table 3 lists the final sample number.

Cartilage Histomorphological Evaluation

As indicated by histological staining at 6 weeks, there were small differences in cartilage regeneration between the experimental groups (Fig. 3). The Sham group exhibited full filling of the articular cartilage and subchondral bone spaces with a continuous tissue, indicated by an un-calcified, non-specific staining. Animals with Blank treatments demonstrated, on average, very little regeneration of a cartilage layer, with minimal polymer still remaining. In one Blank sample, however, the cartilage layer was thick. Histology indicated that the Gradient-only groups seemed to perform better than the Blank treatments with respect to width of defect filling and neo-cartilage depth. Gradient treatments with pre-cultured rUCMSCs did not seem to out-perform Gradient-only treatments in the context of cartilage formation at 6 weeks (Table 3). Both Gradient groups (with and without cells) also had remaining polymer, albeit a lesser amount than the Blank group. In all groups at 6 weeks, Safranin-O staining was faint or not visible at 6 weeks.

By 12 weeks, all experimental groups demonstrated more cartilage regeneration than at 6 weeks, as indicated by histology (Fig 4). Safranin-O staining for GAGs at 12 weeks was intense for all groups at the cartilage surface, with the exception of the Blank group, which had staining mostly in the middle of the defect. The Sham group exhibited full thickness cartilage, which closed the diameter of the original defects. Blank treatments had only a fraction of the closure and thickness that the Sham treatment did. The Gradient-only group and the Gradient group with pre-cultured rUCMSCs were almost identical in appearance to the Sham treatment, earning greater histological scores than the Blank group. In all groups, there were no signs of remaining polymer in the cartilage layer after 12 weeks. Respective histological ratings can be viewed in Table 3. In all groups at 6 and 12 weeks, Sudan Black staining confirmed that there was little, if any, residual polymer in the neo-synthesized cartilage tissue.

Bone Histomorphological Evaluation

At 6 weeks, bone regeneration in all experimental groups was incomplete (Fig. 3). Sham animals showed bone apposition from the defect perimeter inward, which was complementary to the fibrocartilage tissue. The Blank group exhibited the poorest apposition with minimal tissue formation in the microsphere matrix. Gradient-only animals had slightly more bone apposition than Blank groups from the bottom and sides of the original defect wall, but the defect centers still were void of any mineralization. At 6 weeks, overall bone regeneration in the two Gradient groups was perhaps equivalent in volume to the Sham treatment, but with a different pattern; bone growth was taking place from the perimeter of the defect in the Sham group, and in the center of the Gradient samples. The extent of bone growth of Gradient-only samples at 6 weeks was also highly variable. Bone regeneration in the Gradient sample with pre-cultured rUCMSCs was not easily distinguishable from the Gradient-only group (Table 3). Where bone growth had occurred, Alizarin Red and von Kossa staining was intense. In areas that had not experienced mineralization or closure with mature tissue, Alizarin Red and von Kossa staining was faint.

By 12 weeks, Sham-treated animals had complete filling of the defect volume with extensive mineralization (Fig. 4). Blank treatments exhibited much more new bone formation than they had at 6 weeks, but overall, the healing was inferior to the Sham treatment. Gradient-only samples appeared to have a greater degree of mineralization than Blank samples, on average. Subchondral trabeculae in Sham samples, however, were much thicker than in Gradient-only groups. In the Gradient sample with rUCMSCs, the area of regeneration appeared to be at least equivalent to the Gradient-only group, but overall mineralization was still inferior to the Sham treatment. Mineralization, as evidenced by von Kossa staining, had greatly improved in all treatments between 6 and 12 weeks, with the

exception of the Blank group. Respective histological ratings can be viewed in Table 3. In all groups at 6 and 12 weeks, Sudan Black staining confirmed that there was little, if any, residual polymer in the neo-synthesized bone tissue.

DISCUSSION

In taking this continuously-graded scaffold design beyond *in vitro* evaluation,[31] the present study provided great insight to both the potential and limitations to this microsphere-based gradient scaffolding technique.

As noted, by 12 weeks, the Sham treatment demonstrated extensive tissue regeneration, marked by full thickness tissue and dense mineralization of the subchondral bone. In the Gradient groups, bone growth and mineralization occurred within the scaffold architecture, whereas growth in Sham animals progressed inward from the perimeter of the implants. The pattern of tissue regeneration seen in the Gradient groups suggested that the bioactive implant may have provided architecture for infiltrating progenitor cells to attach, and a protein signal to induce differentiation at various points within the defect, not just around the perimeter. At 12 weeks, however, regeneration in the Gradient-only group was still inferior to the Sham group, with narrower subchondral trabeculae. The less dense mineralization in the Gradient-only group suggested that the polymer presence might have delayed the overall progress of new bone formation. Despite such a delay in bone healing, differences in cartilage regeneration between Sham and Gradient groups was almost negligible.

Gradient samples with pre-cultured rUCMSCs appeared to have slightly accelerated healing compared to Gradient-only samples with regard to bone regeneration. At 6 weeks, advantages of pre-culturing with rUCMSCs were not noticeable, but at 12 weeks there were distinct differences in GAG staining and mineral deposition between the Gradient groups. Specifically, at 12 weeks, the type of tissue in the bone region of the Gradient-only treatment contained a considerable amount of GAG and exhibited only diffuse mineralization near the osteochondral interface, compared to the rUCMSC-Gradient treatment. No definitive conclusions could be made due to low sample number. It should also be noted that using non-autologous stem cells in the implants could induce an unfavorable immune response, but in this specific instance, no adverse effects were observed. This may have been due to the relatively small amount of the cells used, or the insignificance of cellular presence compared to the inflammation and healing of the animal from to the surgical procedure itself. Using autologous cells would be ideal for patients whose UCMSCs have been preserved after birth, but for the majority of patients who do not have their own cells preserved, the burgeoning field of cord cell banking could eventually provide a tremendously diverse source of cells from which HLA/MHC matching could be done, thus minimizing the immune response.

Without bioactive signals, however, bone and cartilage growth appeared to be inhibited in the earliest stages, as demonstrated by Blank treatments at 6 weeks. By 12 weeks, the scaffold architecture in Blank-treated animals was lost, but the volume of new bone formation and cartilage thickness was inferior to the Sham and Gradient-only treatments. Thus, growth factor presence was most likely a crucial component for modulation of osteoblastic activity by week 12. The differences in regeneration between the Blank and Sham treatments suggested that polymer presence served as a great physiological “hurdle” for regeneration.

From the data presented here, there is evidence that gradients in bioactive signaling may be beneficial for osteochondral tissue regeneration. The potential benefits of a gradient are supported by our previous *in vitro* investigation,[31] where there were statistically significant

increases in cell number, glycosaminoglycan content, collagen production, and alkaline phosphatase activity in Gradient scaffolds compared to Blank scaffolds. Most notably, Gradient scaffolds produced twice as much collagen as Blank and Biphasic scaffolds.[31]

A caveat, however, does exist with respect to optimal use of this microparticle-based technology. A primary observation was the delayed healing of all experimental groups with respect to the Sham surgery. The reason for slower healing might be related to the molecular weight of the polymer, which was approximately twice that of the initial *in vitro* investigation.[31] Comparison of *in vitro* and *in vivo* degradation rates of PLGA and other biocompatible polymers have shown that *in vivo* degradation can be more than twice as fast as *in vitro* degradation,[37,38] and the degradation time increases proportionally with an increase in molecular weight.[39] Thus, it was speculated that a higher molecular weight would allow for prolonged mechanical integrity of the defect and extended release of growth factor throughout healing, catering to a continual influx of progenitor cells from the marrow space. The current design also utilized a protein loading concentration three times of that used in the first *in vitro* investigation,[31] to counteract growth factor diffusion away from the healing site with interstitial fluid flow. Future investigations should consider a lower molecular weight PLGA with rabbit osteochondral defects, although Sudan Black staining indicated that there was minimal residual polymer in the defects at 6 or 12 weeks. A slower degradation time with a higher molecular weight polymer may be beneficial for larger animals and humans.

Now that the feasibility and potential of the gradient scaffold technology has been demonstrated, experimental designs will be expanded in future studies to include larger sample sizes, larger animal models, larger defects, and more detailed histological analyses. Specifically, a true critical size defect would provide the circumstances under which a Sham treatment does not spontaneously regenerate. While the histological ratings in this study can be used only for reference, an appropriate conclusion would be that the Blank constructs cannot be expected to provide a desirable outcome. These factors will more effectively illustrate the potential advantages of the Gradient design over Sham and Blank procedures, especially in defects that are of critical diameter, which will be of crucial importance in advancing to pre-clinical and clinical studies. Given that rabbits are well-known for regeneration of sham osteochondral defects, the study has clearly established a proof of concept for a novel approach of both incorporating opposing gradients of growth factor release and incorporating umbilical cord mesenchymal stromal cells from Wharton's Jelly for osteochondral defect repair.

CONCLUSION

This investigation utilized a microparticle-based technology that employed continuous gradients in bioactive signals for simultaneous bone and cartilage regeneration in the rabbit knee. The key advantage of this design is spatiotemporal release of growth factors for osteochondral regeneration. As a pilot *in vivo* study using this design, the Gradient design was compared to Blank implants, a Sham procedure, and a highly experimental group: Gradient implants with pre-seeded rUCMSCs. While Gradient groups demonstrated bone regeneration at 12 weeks that was possibly superior to Blank groups, the Sham treatment arguably had the most complete defect filling. This was believed to be a result of a polymer molecular weight that did not exhibit a degradation rate commensurate with bone growth or cartilage growth in the rabbit knee. The merit of this gradient design, however, shows potential in that growth factor incorporation may have a positive effect on osteochondral tissue regeneration, which may be further facilitated by the inclusion of UCMSCs, which is certainly worthy of expanded evaluation in the future.

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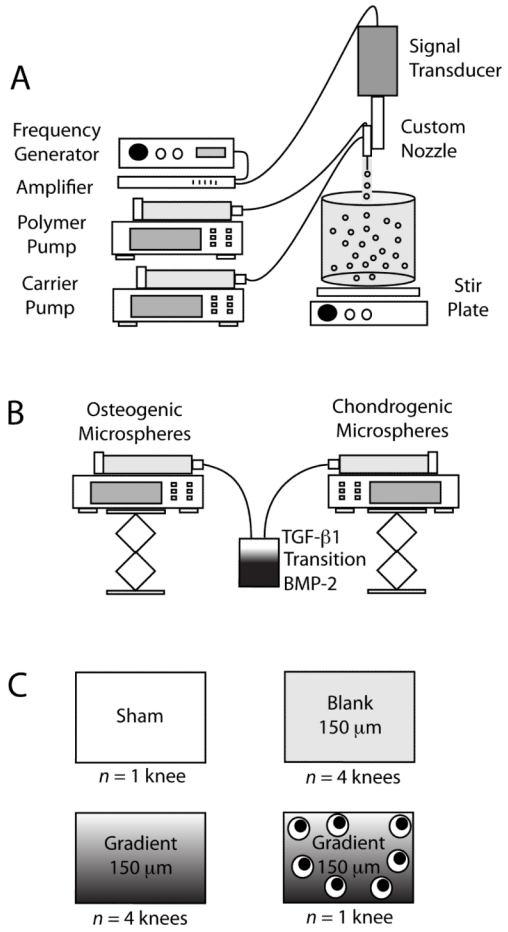

Figure 1.

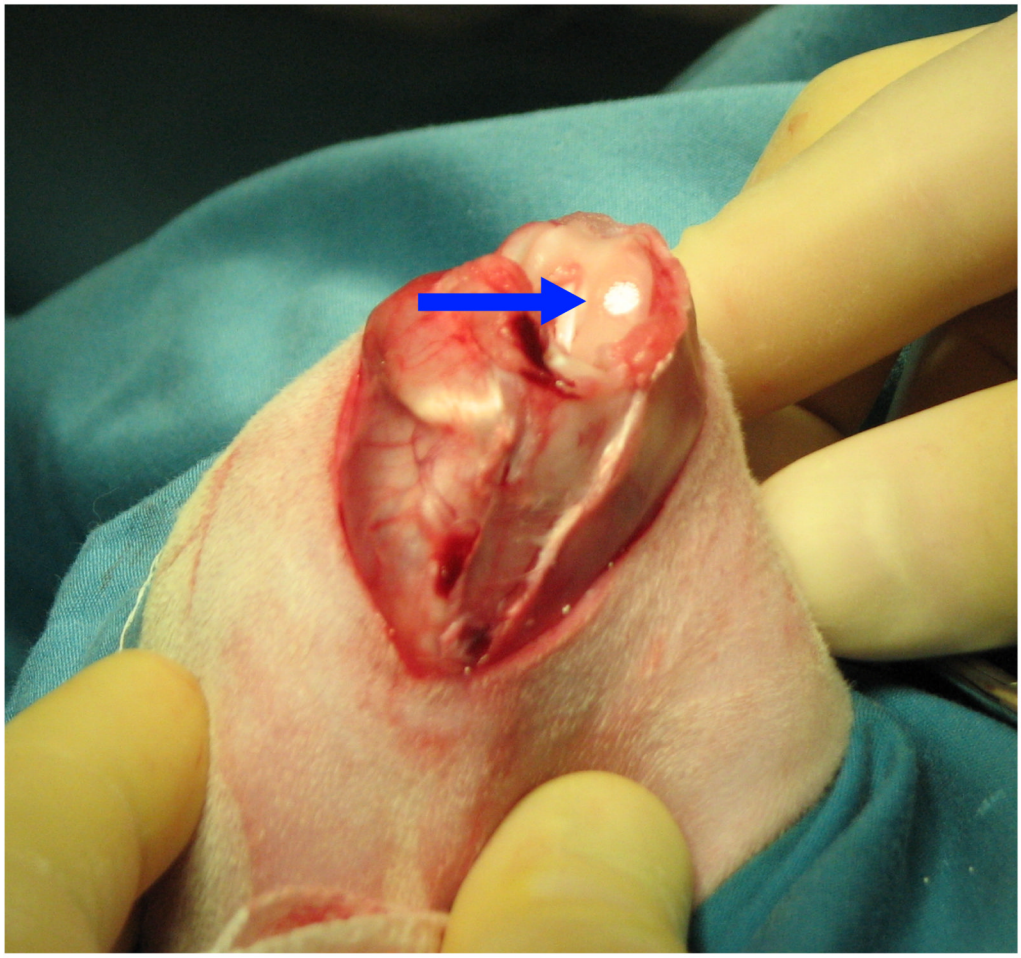

Figure 2.

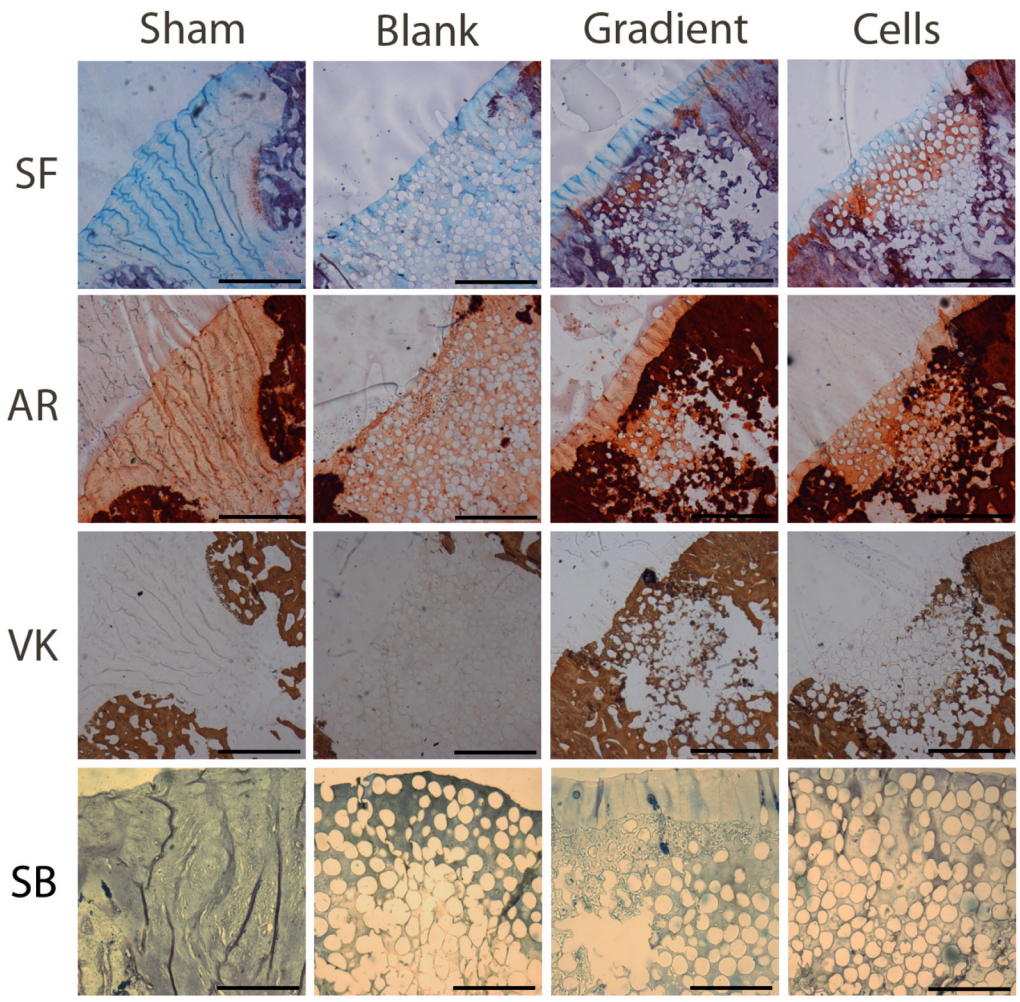

Figure 3.

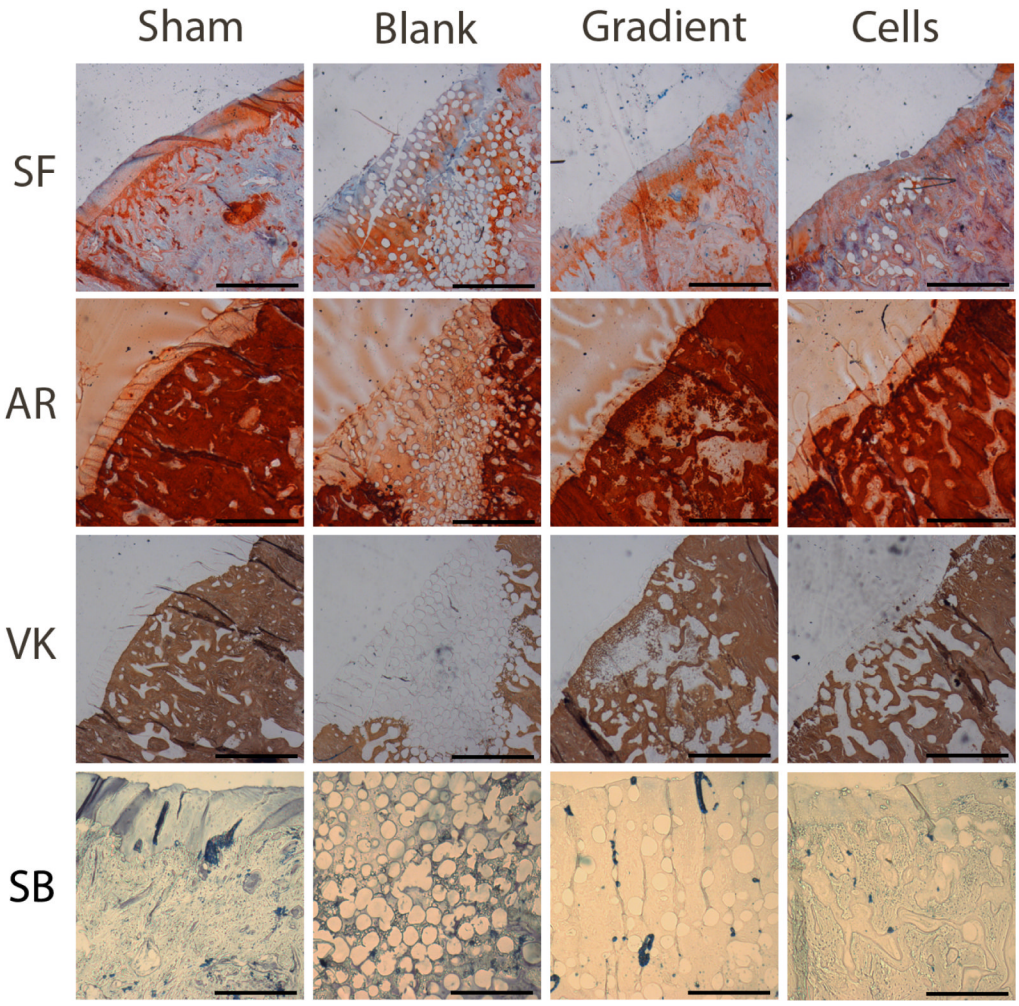

Figure 4.

Table 1

List of treatments for each animal.

	Time (wks)	Left Knee	Right Knee
Rabbit 1 *	6	Blank	Gradient
Rabbit 2	6	Blank	Gradient
Rabbit 3 **	6	Blank	Gradient
Rabbit 4	12	Blank	Gradient
Rabbit 5	12	Blank	Gradient
Rabbit 6	12	Blank	Gradient
Rabbit 7	6	Sham	Blank
Rabbit 8	12	Sham	Blank
Rabbit 9	6	Gradient	Gradient w/ Cells
Rabbit 10	12	Gradient	Gradient w/ Cells

* Died prematurely from causes unrelated to the implant.

** Gradient sample lost in processing.

Table 2

Rating system for cartilage regeneration and bone apposition.

Cartilage Scorecard	**Bone Scorecard**
Closure Across Diameter	**Apposition Across Diameter**
No closure (1)	No apposition (1)
25% Closed (2)	25% Apposition (2)
50% Closed (3)	50% Apposition (3)
75% Closed (4)	75% Apposition (4)
Full closure (5)	Full Apposition (5)
Thickness of Cartilage	**Apposition Toward Surface**
No Thickness (1)	No apposition (1)
25% Thickness (2)	25% Apposition (2)
50% Thickness (3)	50% Apposition (3)
75% Thickness (4)	75% Apposition (4)
Full Thickness (5)	Full Apposition (5)
Total Possible = 10	Total Possible = 10

Table 3

Histological ratings of experimental groups at 6 and 12 weeks.

	6 weeks	
Group (n)	**Cartilage**	**Bone**
Sham (1)	10.0	5.0
Blank (3)	4.3 ± 4.0	2.7 ± 1.2
Gradient (2)	8.5 ± 0.7	5.0 ± 1.4
Gradient with Cells (1)	7.0	6.0

	12 weeks	
Group (n)	**Cartilage**	**Bone**
Sham (1)	10.0	10.0
Blank (4)	6.3 ± 1.3	5.0 ± 0.8
Gradient (4)	9.0 ± 1.2	7.5 ± 0.6
Gradient with Cells (1)	9.0	9.0