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In Vivo Evaluation of Stem Cell Aggregates on Osteochondral Regeneration

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ABSTRACT: To date, many osteochondral regenerative approaches have utilized varied combinations of biocompatible materials and cells to engineer cartilage. Even in cell-based approaches, to date, no study has utilized stem cell aggregates alone for regenerating articular cartilage. Thus, the purpose of this study was to evaluate the performance of a novel stem cell-based aggregate approach in a fibrin carrier to regenerate osteochondral defects in the Sprague-Dawley rat trochlear groove model. Two different densities of rat bone marrow mesenchymal stem cell (rBMSC) aggregates were fabricated by the hanging drop technique. At 8 weeks, the cell aggregates supported the defects and served as a catalyst for neo-cartilage synthesis, and the experimental groups may have been beneficial for bone and cartilage regeneration compared to the fibrin-only control and sham groups, as evidenced by histological assessment. The cell density of rBMSC aggregates may thus directly impact chondrogenesis. The usage of cell aggregates with fibrin as a cell-based technology is a promising and translational new treatment strategy for repair of cartilage defects. © 2016 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 35:1606–1616, 2017.

Keywords: osteochondral; trochlear groove; rBMSC; aggregates; fibrin

In the United States, articular defects and aging-related arthritis is a growing concern and there is an unmet need to develop long-term and substantial cost-effective treatments for osteoarthritis.1–3 Over the last couple of decades, numerous strategies have been developed in an effort to regenerate the osteochondral interface4–10 that employ autologous cell suspensions of either differentiated chondrocytes or undifferentiated bone marrow-derived mesenchymal stem cells (BMSCs).9,11,12 Several review papers discuss various types of cell-based approaches and the relative advantages they impose compared to other material-based strategies.13–16 Current techniques typically engage MSC monolayers or sheets that are directly implanted into the periosteal space. Cellular monolayers are not only mechanically unstable, but also may not adhere to the defect region, but posing a serious concern of cellular retention. In the present study, the fibrin coating of the aggregates was leveraged to secure the cells to the defect region by providing anchorage and mechanical support. Apart from superior survival and retention properties, cellular aggregates may recapitulate embryonic events during skeletal tissue formation, thus providing an appropriate microenvironment for cellular differentiation. In addition, the current study exploited the regenerative capacity by placing several aggregates in close proximity to maximize opportunities for co-differentiation. It is commonly known that culturing cells in pellets is preferred for chondrogenesis, and ultimately it is our hope that aggregate-based approaches to cell-based cartilage therapies can overcome the long-standing challenge of regenerating true hyaline cartilage. Despite the success of cell-based techniques, clinical outcomes are significantly affected by the formation of fibrocartilage after long-term implantation, thus requiring multiple surgeries that, in turn, may give rise to donor site morbidity and increase the overall cost of healthcare.17,18 To overcome the above listed disadvantages, investigators have resorted to employing cells in combination with biocompatible natural materials such as alginate, agarose, collagen, and silk fibroin, and polymers such as poly(lactic-co-glycolic acid), polyglycolic acid, and polycaprolactone, which under certain fabrication conditions may possess mechanical properties similar to the native cartilage.19–22

While there have been several advances for in vivo approaches for cartilage tissue engineering using cell-based strategies, the number of cell aggregate approaches are limited.9,23–27 Commonly, aggregates have been explored for pancreatic islet regeneration, targeted drug delivery approaches for cancer therapy, and generally they serve as representative three-dimensional cellular model for drug testing.9,28–31 In our previous study, aggregates and cell suspensions were explored in vitro and compared for cartilage tissue engineering applications.32 For the purpose of translational application, the aggregate group that demonstrated superior chondrogenesis compared to the cell suspension group was chosen for further in vivo studies that are described here. Translating the aggregate technology to regenerate articular cartilage is starting to gain interest.31,35–35
During the last decade, fibrin has been increasingly used as a gel, by itself or as a combination delivery system along with cells and other bioactive factors in a cartilage tissue-engineering context.36–39 The current model may serve as a basis for evaluating the role of fibrin with aggregate delivery and fill the gap in the literature that may help the iteration of aggregate-based approaches.

Looking at material-based approaches in rat models, a steady number of studies use external chemical stimuli such as raw materials, growth factors, and other bioactive molecules in their scaffolds.40–42 Specifically, TGF-β1 and TGF-β3 are known to promote chondrogenesis, and BMP-2 and BMP-7 are known to promote osteogenesis.43–45 Cellular aggregates employed in the current study, however, provide a more translational approach by exploiting known intercellular communication from cell pellet cultures in chondrogenesis as an inherent cue to differentiate MSCs toward the cartilage phenotype.

Small animal models such as rodents are considered valid as proof of concept and preliminary research models.46,47 Since this is the first time we are seeking to employ cell aggregates with fibrin for articular cartilage regeneration, a critical sized defect of the trochlear groove was selected as a reasonable defect area based on established precedent in the literature.47–49 The current study investigated cartilage regeneration in a critical sized trochlear groove defect in Sprague-Dawley rats with cell aggregates of different densities, and compared the performance with a fibrin-only control group and a sham negative control group. Regeneration was evaluated at 8 weeks with gross morphological scoring and histological staining. The goal of this pilot study was to determine whether the cell aggregates would facilitate neo-cartilage formation in the rat trochlear groove as a foundation for future in vivo studies.

MATERIALS AND METHODS

Cell Harvest and Culture

Rat bone marrow-derived mesenchymal stem cells (rBMSC) were harvested from the femurs of five young male Sprague-Dawley rats (7 weeks old, 200–250 g, Charles River) following a University of Kansas approved Institutional Animal Care and Use Committee (IACUC) protocol (175–08). The IACUC approved the use of rBMSC cells for use in this particular study. The cells were isolated according to our previously reported protocol.40,50 Briefly, isolated cells were cultured in rBMSC media (αMEM supplemented with 10% MSC-qualified FBS and 1% Penicillin-Streptomycin [Invitrogen Life Technologies, Carlsbad, CA]), and passaged at 80% confluence until P4 at standard condition (37°C and 5% CO2). All cells from different femurs were pooled together, frozen at P2 and later expanded to P4, and used for the study.

Cellular Aggregate Preparation

Aggregates for the current study were generated by the hanging drop technique.51,52 Cellular suspensions (rBMSC) were prepared at two concentrations, either 10.0 × 10^6 or 20.0 × 10^6 cells/ml, in rBMSC media. The cellular concentration was confirmed using a Cellometer automatic T4 cell viability counter (Nexcelom Bioscience, Lawrence, MA). Droplets of cell suspensions at a controlled volume of 10 μl that had 10,000 or 20,000 cells were pipetted in an array onto the inside surface of a sterile petri dish lid using a 10 μl pipette (Eppendorf, Hauppauge, NY), making sure that the droplets were at safe distance from each other to prevent mixing. The cells were allowed to aggregate overnight, sized by gravity when the petri dish was reversed. The sterile petri dish bottom was then filled with sterile PBS to prevent drying of these droplets. After 24 h, the cell aggregates were carefully collected from the dish with a 1 ml pipette and collected in Eppendorf tubes for the study. Aggregates were placed in defects within 3 h of harvest, as noted below.

Description of Experimental Groups

Four different treatment groups were investigated: (i) Group A, where the defect was created and filled with 75 rBMSC aggregates, each with a cell density of 10 million/ml (note: Density based on volume of initial 10 μl suspension); (ii) Group B, where the defect was created and filled with 75 rBMSC aggregates each with cell density of 20 million/ml; (iii) sham surgery, in which a defect was created, but no implant was placed; and (iv) fibrin group where the defect was created and only fibrin was used to fill the defects (Supplemental Fig. S1). For group (i) and (ii), each petri-dish used to make the hanging drop aggregates had exactly 75 drops and care was taken to harvest all of the aggregates, thus making sure we had 75 aggregates for each knee.

Surgical Procedure

Surgical procedures were conducted under an approved IACUC protocol at the University of Kansas (Animal Use Statement #175-20), utilizing a total of 10 male Sprague-Dawley rats (200–250 g, Charles River). The IACUC approved the protocol to be used for this particular study. Following stable general anesthesia, hair was shaved from the area around each rat knee. The knee was then disinfect with three borone scrubs of Betadine and 70% ethanol, and then draped so as to expose only the knee area. Care was taken to use only strict aseptic techniques and sterile instruments; and the surgeon wore sterile gowns, masks, and head covers. All surgical tools, including drills and stoppers, were sterilized prior to surgery. A midline knee incision was made for an intra-articular lateral parapatellar arthrotomy sufficient enough to allow exposure of the trochlear groove. The osteochondral defect was drilled to a depth of 2.0 mm and 2.0 mm diameter using a drill with a stopper attached to the bit, thereby breaching the osteochondral plate. There was mild bleeding into the defect site. Defects were then filled with either the aggregate groups (Group A or B), or fibrin (Tissel®10, Baxter, Deerfield, IL) (Supplemental Fig. S1). The aggregates were harvested 3 h before the surgery, as noted in the “Cellular aggregate preparation” subsection above, and immersed in rBMSC media until implantation. At the time of implantation, aggregates were carefully pipetted into the defect area. Sham defects were created, in which a hole was drilled, but no implant was placed. Following implantation of the aggregate groups, 50 μl of fibrin was added on top of the aggregates to seal the clot with the surrounding tissue. Although 6 μl was enough to cover the defect, extra fibrin was added to secure the clot with the surrounding tissue as it hardened over time. For the fibrin group, 50 μl of fibrin was added to the defect similar to the procedure followed for group
A and B. The sealing time was approximately 2 min. The joint was then washed with sterile pharmaceutical grade saline water and the bursa was closed with absorbable suture. The skin was then closed with polysorb 3.0, a non-absorbable suture. The procedure was followed on the contralateral knee with a different group to ensure independence of knees within groups (Supplemental Fig. S2). After the surgery was completed, the rats were administered carprofen subcutaneously, and returned to be caged individually.

Post-Surgical Care
The rats were continuously monitored for the first 3 days and any signs of limping or unease was treated with carprofen injection administered subcutaneously only once a day. Three times a week, the rats were monitored for general mobility, specific mobility of hind legs, response to touch, and signs of inflammation at the surgical area. No adverse events were observed and all the rats progressed at a healthy rate during the entire period of 8 weeks.

Morphological Analysis of the Retrieved Implants
At 8 weeks, the rat knees were retrieved and immediately placed in 10% neutral buffered formalin (Fisher Scientific, Rockville, NJ) for 72 h, changed every 24 h. After fixation, the joints were rinsed in distilled water and decalcified (Cal Rite, Richard-Allan Scientific, Kalamazoo, MI) for 2 weeks and the solution was changed every 3 days. After decalcification, the knees were rinsed briefly and dehydrated in graded ethanol. For paraffin embedding, the samples were cleared with xylene, then infiltrated with paraffin, with the infiltrate being changed every 3 h.

After three infiltrations, the trochlear grooves were embedded in paraffin tissue cassettes and allowed to cool down before sectioning. Sagittal sections were taken on a microtome (Thermo Scientific; Microm HM 355S) using a tungsten carbide blade with a sample thickness of 7 μm, placed on coated glass slides (Superfrost coated slides, Thermo Scientific, NJ) and dried for 24 h at 44°C. The glass slides were cleaved in xylene and slowly hydrated in series of ethanol according to a procedure previously reported.4 After incubating the slides in ddH2O for 5 min, the slides were stained with either Safranin-O/Fast green stain for glycosaminoglycans (GAGs) or hematoxylin and eosin to look at overall structure. Slides were briefly dehydrated and cleared in xylene for mounting. Furthermore, additional slides were processed for immunohistochemistry (collagen I, II, and aggrecan) discussed in the next section. All staining reagents were purchased from Sigma–Aldrich (St Louis, MO).

Immunohistochemistry
The slides were processed similar to basic histology as described above up through hydration in distilled water. The sections were then exposed to 3% hydrogen peroxide in methanol for 10 min to suppress endogenous peroxidase activity, and the slides were immediately incubated in proteinase K (HICWORLD IW-1101, Woodstock, MD) at 37°C for 10 min. Sections were blocked with 3% blocking horse serum (Vector Laboratories S-2012, Burlingame, CA) for 30 min preceding primary antibody incubation. The primary antibodies used in the current study were obtained from Abcam (Cambridge, MA): collagen I (catalog no: AB24133, dilution 1:200), collagen II (catalog no: AB116142, dilution 1:200), and aggrecan (catalog no: AB36861, dilution 1:100). Following primary antibody incubation, slides were exposed to biotinylated secondary antibody (horse anti-rabbit IgG) and ABC reagent (Vectastain ABC kit PK-6200, Burlingame, CA) for 30 min each. Visualization was accomplished with ImmPact DAB peroxidase substrate (Vector laboratories SK-4105, Burlingame, CA) before rinsing with distilled water and counter-stained with VECTOR hematoxylin QS stain. Following staining, slides were rinsed in tap water, dehydrated in ethanol, cleared in xylene for mounting (Permout SP15-500 Fair Lawn, NJ), and viewed under an upright microscope (Zeiss, Axiomanager 2.0, Thornwood, NY).

Histology Scoring
A simple scoring system (Table S1) was modified from the previously reported system by O’Driscoll et al.54 to evaluate the cartilage and bone regeneration of this pilot study. Three-blinded observers performed all scoring and the assignment of the scores was aided by evaluating structure using histological and immunohistological images.

Statistical Analyses
Wherever applicable, all data are expressed as mean ± standard deviation. Statistical analyses were performed using one-way ANOVA (Minitab 15, Minitab Incorporated, State College, PA), followed by a Tukey’s post hoc comparison test for repeated measurements. The statistical significance threshold was set at 0.05 for all tests (with p < 0.05).

RESULTS
Gross Morphological Observations
All of the rats continued to exhibit normal movement and gait in their hind legs during the 8-week period except three rats (R03, R05, and R07) that had signs of limping/uneasiness in their walking gait during the first week alone. Gross signs of inflammation (swelling or reddening of the joint) or infection were not evident upon visual inspection of the joint surface at the time of tissue retrieval. Figure 1 shows representative gross morphology images of the rat trochlear grooves at 8 weeks. For Group B, the repair tissue was completely flush to the surface and had a smooth texture surrounding the defect area with color similar to the native cartilage, whereas all of the other groups had an opaque, slightly depressed, or overgrown surface with either rough or intermediate texture at 8 weeks. Figure 2a represents the mean morphological score and Figure 2b represents the percent defect area fill occupied by the regenerated cartilage with respect
to the total defect area was determined macroscopically for each sample at 8 weeks. The percent area values were between 80% and 95% for Group A and the sham and Fibrin group. Group B values were between 95% and 100%. Group B received a statistically significantly higher morphological score (Fig. 2a) than Group A alone \((p < 0.05)\). Group B did not have a statistically significantly higher score compared to sham and fibrin group. The difference in mean morphological score between Group A and fibrin group and sham group was not statistically significant.

**Histomorphometric Observations**

Figure 3 shows representative sections stained with H&E, Safranin O for GAGs, collagen I, II, and aggrecan immunostaining, for Group A. Rat #2 (i.e., R02) (Fig. 2) had intense Saf-O staining, whereas R04 and R09 did not stain very intensely.

Figure 4b represents the morphometric score distribution of Saf-O stained sections of the defect area, and we observed that 40% of the knees were slightly stained, 20% were moderately stained, and the remaining 40% was normally stained. Collagen II staining was prominent in the defect region for R03 and R04, and aggrecan immunostaining was intense for R04 and R09 cartilage region. There was no intense staining of collagen I around the defect area, but overall there was a mild staining throughout the subchondral bone region. Sixty percent of Group A knees received a score of 2 (75–100% of adjacent cartilage) and the remaining 40% received a score of 3 (100% thickness of the adjacent cartilage) (Fig. 4a). Additionally, 60% of the Group A knees showed normal subchondral bone regeneration with smooth and intact edge integration of the defect with the surrounding native cartilage. Upon closer observation at the defect region, Group A aggregates (R01 and R04) showed columnar arrangement and are surrounded by tiny lacunae spaces. The lacunae spaces increased in size at the osteochondral junction. R03

**Figure 1.** Representative gross morphological images of all the experimental and control Groups. They are divided into three categories based on the relative morphological appearance. The repair tissue was opaque, completely flush to the surface, and had smooth texture in Group B implants, whereas all of the other groups had opaque, slightly depressed, or overgrown surface, with either a rough or intermediate texture at 8 weeks. (Circled region points to the defect area on the trochlear groove on the retrieved knees). Scale bar = 2 mm.

**Figure 2.** (a) Average morphological score of the rat knees. (b) Percentage defect repair area coverage evaluating the percentage of repair tissue filled at the defect site in for all the groups. Three independent co-authors compiled morphological scores based on parameters in Table S1. Average scores are represented as average ± standard deviation. The maximum possible score a healthy cartilage can receive is 10/100 correspondingly. All groups had a sample size of \(n = 5\). \((*)\) Indicates statistically significant difference from Group A.
and R04 showed intense collagen II and aggrecan pericellular staining. Apart from R02, there was no presence of cellular clusters in any of the Group A defects. There were small aggregates at the defect site, but it was not possible to say whether they were the implanted rBMSC aggregates or host-cell aggregates.

Figure 5 displays representative sections stained with H&E, Saf-O for GAGs, collagen I, II, and aggrecan immunostaining for Group B. R06 and R08 sections did not display any Saf-O staining, but intense staining for R07 and staining around the defect area for R05. Sixty percent of the samples had a Saf-O score of 1 (slight staining) and the remaining 40% a score of 3 (nearly normal staining) (Fig. 4a). As indicated by Figure 4, 80% of the knees exhibited 75–100% cartilage thickness and reduced subchondral bone reduction. Sixty percent of the knees had smooth and intact edge integration of the regenerated cartilage with the surrounding tissue. There were no distinct lacunae observed except for R05 and R08, where the lacunae spaces increased at the osteochondral junction. R06 showed intense collagen II and aggrecan pericellular staining. For R06, the cells in the defect region had more intense pericellular collagen I and II staining compared to the surrounding tissue. H&E and Saf-O staining of R07 revealed aggregates at the osteochondral interface.

Figure 6 shows representative sections stained with H&E, Saf-O for GAGs, collagen I, II, and aggrecan immunostaining for the fibrin-only group. Saf-O staining was prevalent in the defect area for R07 and R09. The staining of Saf-O in the native tissue was evident for R02 and R05. Eighty percent of the fibrin group showed moderate staining of Saf-O (Fig. 6). Collagen I staining was intense in the defect area for R05 and stained deeper in the bone defect region for R02 and
R03. One hundred percent cartilage thickness was observed in 60% of the knees. Just like the sham defect group, 80% of the samples had reduced subchondral bone regeneration and only 60% showed superficial horizontal lamination. The regenerated tissue of the fibrin group did not show any columnar organization of cells. The cells in the regenerated tissue for R07 showed intense collagen I staining compared to the surrounding cartilage tissue and, similarly, the defect area tissue had increased aggrecan around the cells compared to the surrounding tissue. In general, there was no appearance of cell clusters in any of the images.

Figure 7 displays representative sections stained with H&E, Safranin-O for GAGs, collagen I, II, and aggrecan immunostaining for the sham knee surgeries. R01 demonstrated distributed Safranin-O staining, but the regenerated tissue looked weak with a visible fissure in the bone-cartilage interface. R08 and R10 showed minimal to no Safranin-O staining at the defect region. R04 and R06 on the other hand had Safranin-O staining at the regenerated cartilage surface. As indicated by the morphometric analysis, 80% of the group had moderate Safranin-O staining and the remaining 20% had regular Safranin-O staining, comparable to that of native cartilage (Fig. 4). Collagen II staining was evident for the regenerated surface for R01, R08, and R10, and aggrecan immunostaining was intense for R08 and R10. Collagen I was not evident for any other group except R08. Forty percent of the samples showed 100% of normal cartilage thickness and the remaining 60% had 50–75% of native tissue thickness (Fig. 4). Eighty percent of the samples had reduced subchondral bone regeneration and the remaining 20% showed normal bone regeneration without any sclerosis or loss of bone tissue. Concerning edge integration with the native tissue, 60% showed superficial horizontal lamination and 40% showed smooth and intact integration. The regenerated tissue of the sham group did not show any columnar organization. Only R04 showed intense aggrecan staining of cells in the defect area compared to the surrounding tissue. There were no cell clusters in any of the images.

**DISCUSSION**

In the current pilot study, we have introduced a new method to incorporate cellular aggregates with fibrin into an animal model as a proof of concept study. The rat trochlear groove is an established animal model that provided a great insight into both the potential and the limitations to the aggregate-based approach.\(^5\text{5,56}\) As noted at 8 weeks, the fibrin-only control group demonstrated extensive tissue regeneration, marked by full thickness tissue, presence of GAGs, dense collagen II, and aggrecan immunostaining. In the experimental groups, Group B had the highest average morphological score and the highest percentage filling of the defect area compared to all other groups. From the data presented here, there is evidence that dense rBMSC aggregates (Group B) may be beneficial for osteochondral tissue engineering. The cells were regular cartilage-like cells and did not exhibit any hypercellularity. Since there was no strong collagen I staining, it may be indicative of a hyaline-like cartilage. The lack of intense collagen II staining may additionally indicate the absence of mature hyaline cartilage but may be indicative of a maturing phase of the tissue.
A potential limitation with respect to the use of rat trochlear grooves for a cartilage regeneration study is that the negative control group may not be ideal. A primary observation was the spontaneous healing of all of the sham groups. Previous studies by other groups that had looked at sham surgery in the rodent trochlear groove region (rat and rabbit) had also observed that this may be a common trend for a typical small animal model.52,53 Additionally, in the current study the starting weights of the rats were comparable; however, variation in the ages of the rats might have contributed toward a slightly different result owing to different regeneration rates of both the sham and experimental groups.

The defect size was deemed critical and selected based on previous studies that are cited in the manuscript. As a next step, to distinguish between spontaneous and focused regeneration, load-bearing regions of the knee such as the medial and femoral condyle will be explored in rodents and other animal models. The trochlear groove in a lower weight-bearing region is considered an appropriate model for proof of concept studies.57,58 Earlier studies that employed biomaterials on rat trochlear groove regeneration for critical sized defects have shown similar rates of regeneration.59,60 Although previous rat studies have not explicitly provided aggrecan immunohistochemistry, based on morphological scoring and basic histology (H&E, Safranin O), similar rates of regeneration may be inferred.42,61 Munirah et al.62 have demonstrated that fibrin mixed with autologous cells resulted in osteochondral regeneration in a sheep model. Similarly, Deponti et al.63 demonstrated that fibrin mixed with porcine cells possessed enhanced in vivo properties, compared to in vitro conditions, for increased cell viability and activity.

Overall, the current study adequately established proof of concept. The cellular aggregates did not necessarily demonstrate conclusive outperformance of

Figure 5. Histology (H&E, Safranin-O) and immunohistochemistry (collagen I, II, and aggrecan antibody staining) for Group B. Black arrowheads indicate defect region. Numbers on the top refer to the rat number (see Supplemental Fig. S2). Note the more intense stain for collagen II in R06. Scale bar = 200 μm. Below the histology image is a stacked column plot showing the histological score distribution for cartilage thickness Safranin O staining reconstruction of subchondral bone, and edge integration for the newly regenerated tissue at the defect area for 8-week implants (Group B).
the fibrin group. The results of the current study, with differences between Groups A and B, suggested that there may be more effective ways to combine fibrin with cell aggregates. For example, exploring different cell densities for the aggregates, larger numbers of aggregates, aggregates of other cell types (e.g., Wharton’s jelly cells\(^{61-63}\)), pre-treatment of aggregates, or differences in surgical placement procedure may be warranted for future investigation in an attempt to more conclusively prove the hypothesis that aggregates provide better quality and a more consistent cartilage regeneration compared to the controls.

Now that the feasibility and the potential of the aggregate technology has been demonstrated, experimental designs will be expanded in future studies to include higher load-bearing regions, larger defect sizes, bigger animal models, larger aggregate sizes, higher aggregate numbers and cell density of the aggregates, and more sophisticated techniques to differentiate between the implanted and host cells even after several months of implantation. From a clinical standpoint, comparison of cell suspensions with fibrin versus cell aggregates with fibrin would yield a meaningful and more direct implication of the efficacy of aggregate technology over conventional cell suspension techniques. Furthermore, aggregate technology may augment current existing methods such as autologous chondrocyte implantation (ACI) to produce a new class of products for treating focal cartilage lesions. While ACI employs autologous cells that require a long culture time and limited cell viability from older patients, allogeneic cells can be readily

Figure 6. Histology (H&E, Safranin-O) and immunohistochemistry (collagen I, II, and, aggrecan antibody staining) for the fibrin-only Group. Black arrowheads indicate defect region. Numbers on the top refer to the rat number (see Supplemental Fig. S2). Note that the R05 section showed almost zero staining for Safranin-O and the bone region for R09 was deeply stained for Safranin-O, suggesting neo-cartilage formation. Scale bar –200 µm. Below the histology image is a stacked column plot showing the histological score distribution for cartilage thickness Safranin O staining reconstruction of subchondral bone, and edge integration for the newly regenerated tissue at the defect area for 8-week implants (Group C).
available in an off-the-shelf format. The current study looked at allogeneic aggregates, and, although long-term implications were not evaluated, the model provides a method for reducing time constraints and provides a better model compared to ACI, thus preventing multiple surgeries and reducing the overall healthcare costs.

The current study has clearly established a proof of concept for a novel approach of incorporating aggregates of rBMSCs with two different cell densities for osteochondral defect repair.

CONCLUSIONS
The current study demonstrated a cell-based approach where stem cell aggregates employed with fibrin were used for regenerating the osteochondral interface tissue in a rat knee defect model. This is the first in vivo study that employed dense stem cell aggregates with fibrin in a rat trochlear knee defect model. Stem cells presented in a 3D aggregate form along with fibrin seemed to enhance regeneration.

In conclusion, the inclusion of fibrin showed some degree of regeneration that may have been enhanced with the higher aggregate density group. Between the two experimental groups, the higher cell density aggregate group (Group B) demonstrated superior cartilage regeneration compared to all other groups and arguably had the most complete defect filling. The complete filling was believed to be the result of denser cellular aggregates in Group B that enabled better cell-to-cell interaction, filled the defect and remained intact due to its relative density. The merit of the study design shows potential in that the cellular density of the aggregates is an important parameter in osteochondral tissue regeneration, which may further be facilitated by the priming of rBMSC aggregates and exploration of even higher cell densities or larger numbers of aggregates, which is certainly worthy of long-term systematic evaluation in the future.
AUTHORS’ CONTRIBUTIONS
B.S, D.M.P, and M.D designed the overall structure and flow of the experiment. B.S compiled the manuscript and A.D.L, and M.A.H helped with data collection and analysis. D.M.P and N.K.W performed the knee arthroscopy on the rats. B.S, A.D.L, and M.A.H assisted during the surgery, performed post-op assessment, histology, and gross morphological testing. All authors have read and approved the final submitted manuscript.

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