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Combinatorial cassettes to systematically evaluate tissue-engineered constructs in recipient mice

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ABSTRACT

Ectopic bone formation in mice is the gold standard for evaluation of osteogenic constructs. By regular procedures, usually only 4 constructs can be accommodated per mouse, limiting screening power. Combinatorial cassettes (combi-cassettes) hold up to 19 small, uniform constructs from the time of surgery, through time in vivo, and subsequent evaluation. Two types of bone tissue engineering constructs were tested in the combi-cassettes: i) a cell-scaffold construct containing primary human bone marrow stromal cells with hydroxyapatite/tricalcium phosphate particles (hBMSCs + HA/TCP) and ii) a growth factor-scaffold construct containing bone morphogenetic protein 2 in a gelatin sponge (BMP2 + GS). Measurements of bone formation by histology, bone formation by X-ray microcomputed tomography (μCT) and gene expression by quantitative polymerase chain reaction (qPCR) showed that constructs in combi-cassettes were similar to those created by regular procedures. Combi-cassettes afford placement of multiple replicates of multiple formulations into the same animal, which enables, for the first time, rigorous statistical assessment of: 1) the variability for a given formulation within an animal (intra-animal variability), 2) differences between different tissue-engineered formulations within the same animal and 3) the variability for a given formulation in different animals (inter-animal variability). Combi-cassettes enable a more high-throughput, systematic approach to in vivo studies of tissue engineering constructs.

1. Introduction

Formation of ectopic ossicles (bone and associated soft tissue) in the subcutaneous compartment of mice is the biologically relevant assay for measuring osteogenic capacity of constructs (cells or osteogenic inducers with scaffolds [1–5]). When screening osteogenic formulations, simultaneous assessment of different constructs is preferable. Cell products used in osteogenic constructs can be inconsistent, a recognized hurdle for the cell therapy industry [6–9]. Moreover, components such as scaffolds, structural proteins (fibrinogen, gelatin) and growth factors can impact assay replicability [10–12]. Usually only 4 constructs can be placed per mouse. Furthermore, there is mouse to mouse variation in outcomes, even when using gender-matched congenic recipients. These issues are addressed by including an osteogenic positive control construct in each mouse, thereby reducing the number of experimental constructs from 4 to 3. In vivo assays take 3–4 months from start to finish. Regular constructs do not exhibit a uniform shape and often fuse or disappear. Harvested constructs are processed and evaluated as individual entities, which is cumbersome and adds variability. These issues limit the practicality of animal models to screen large numbers of osteogenic constructs for tissue engineering. There is a need for improved quantitative rigor in animal models for assessing the ability to tissue-engineered constructs to regenerate tissue [13–16].

To address these limitations, a combinatorial cassette ("combi-cassette") was constructed for a more systematic screening of osteogenic constructs in the ectopic bone formation assay (Suppl. Fig. 1). Combi-cassettes are planar, hexagonal structures, made from polytetrafluoroethylene (PTFE) and have small holes. Construct formulations are loaded into the holes, and the loaded combi-cassettes are placed subcutaneously into mice. To assess the suitability of combi-cassettes to

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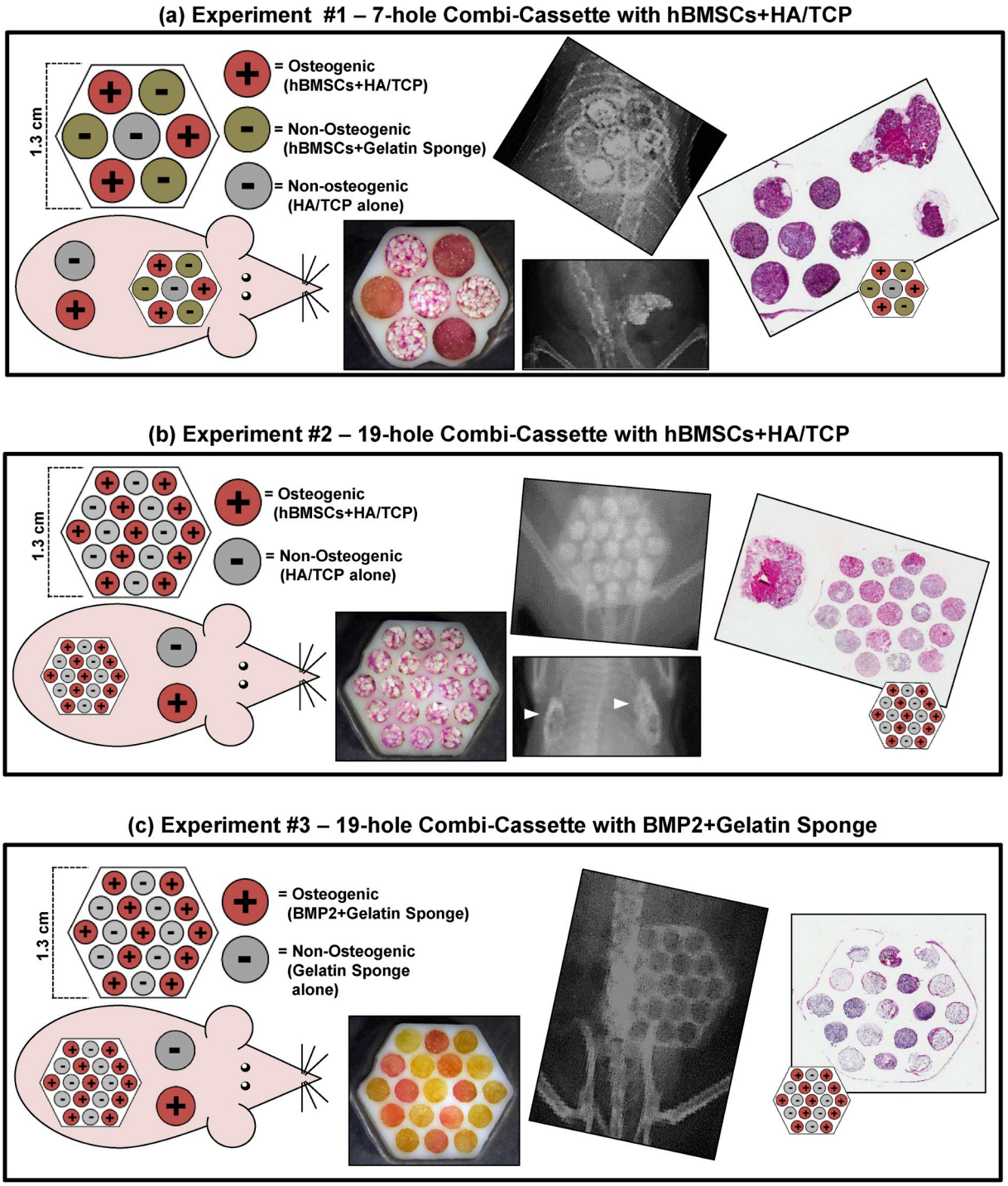

Fig. 1. Illustration of the 3 experiments conducted: (a) Experiment #1 – 7-hole Combi-Cassette with hBMSCs + HA/TCP, hBMSCs + Gelatin Sponges or HA/TCP along (b) Experiment #2–19-hole Combi-Cassette with hBMSCs + HA/TCP or HA/TCP alone, and (c) Experiment #3–19-hole Combi-Cassette with BMP2 + Gelatin Sponges or Gelatin Sponges alone. HBSS contains phenol red. The BMP2 solutions is acidic, causing the gelatin sponge to appear yellow. Each panel contains a description of the osteogenic and non-osteogenic constructs, the layout of the holes for the combi-cassettes, a photograph of the combi-cassette after loading but prior to placement in mice, an X-ray micrograph of the combi-cassette within the mouse and a scan of a histological section (H&E staining) of the Combi-Cassette after 8 weeks in vivo. Note the "legend" at the bottom left of the H&E panel depicting layout of the constructs in the holes of the combi-cassette. Note: do not try to assess bone formation in the histological sections in this figure since the resolution is not high enough. The histological sections shown in this figure are for informational purposes to help the reader to understand the combi-cassette system. Note that some images were rotated so that the positions of the formulations are consistent for each panel. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

test osteogenic constructs, three experiments were performed using two well-characterized osteogenic formulations (summarized in Fig. 1 and Suppl. Fig. 3).

In regard to the experimental design, the goal was not to identify an optimal bone graft formulation. The goal was to assess the reliability of the combi-cassette system for screening tissue engineering constructs. This was done by testing the combi-cassette using known osteogenic formulations and known non-osteogenic formulations. We chose to

independently assess two different types of constructs in the cassette in order to show that the combi-cassettes could work with different types of constructs. One of the types of constructs was cell-based (primary human bone marrow stromal cells, hBMSCs, also known as bone marrow-derived mesenchymal stem cells, MSCs) and the other was growth factor-based (bone morphogenetic protein 2, BMP2). hBMSCs were selected for the cell-based system because hBMSCs are known to be osteogenic in the mouse ectopic bone formation model [17]. hBMSCs also have clinical relevance since there is intense interest in using hBMSCs as a therapeutic [18]. BMP2 was selected for the growth factor-based system because BMP2 is known to be osteogenic in the mouse ectopic bone formation model [4]. BMP2 also has clinical relevance since it is used in a collagen product to treat bone defects [19].

For the cell-based hBMSC system, we tested a known osteogenic formulation that contained cells, hBMSCs with HA/TCP (hBMSCs + HA/TCP). HA/TCP supports osteogenesis and is used clinically [20]. For a known non-osteogenic formulation, we used the HA/TCP without cells (HA/TCP alone). We also used a known non-osteogenic formulation that contained cells, hBMSCs, in a gelatin sponge (hBMSCs + Gelatin Sponge). For the growth-factor based system, we tested a known osteogenic formulation that contained a growth factor (BMP2 + Gelatin Sponge). For a known non-osteogenic formulation, we used the gelatin sponge without BMP2 (Gelatin Sponge alone). The overall goal was to determine if formulations that were known to be osteogenic were indeed osteogenic when used in the combi-cassette; and if formulations that were known to be non-osteogenic were indeed non-osteogenic when used in the combi-cassette. The goal was not to test many different formulations to try to identify the best formulation.

2. Results

In Experiment #1, 7-hole combi-cassettes were loaded with human bone marrow stromal cells (hBMSCs, also known as bone marrow-derived mesenchymal stem cells) attached to hydroxyapatite/tricalcium phosphate particles (HA/TCP) (Suppl. Fig. 2). For non-osteogenic constructs, combi-cassettes were: i) loaded with hBMSCs in gelatin sponges (GS) (hBMSCs in GS do not form bone) or ii) with HA/TCP without cells (HA/TCP alone is not osteoinductive [1]). Two regular constructs outside of the cassette consisted of hBMSCs + HA/TCP and hBMSCs + GS. For histological processing, the retrieved, loaded combi-cassettes could be fixed, demineralized, embedded, sectioned (PTFE can be sectioned via microtome), mounted, stained and imaged in one piece, without manipulation of each construct individually (Fig. 1). In addition to saving time, results from sections of intact combi-cassette specimens are directly comparable.

New bone formation was observed in H&E-stained (hematoxylin and eosin) histological sections of osteogenic constructs (hBMSCs + HA/TCP) for both the combi-cassettes and regular constructs (Fig. 2a and b). The new bone was both autofluorescent and birefringent [21]. The non-osteogenic constructs (hBMSCs + GS or HA/TCP alone) from combi-cassettes or regular constructs lacked new bone and contained fibrous tissue that was neither autofluorescent nor birefringent. Bone scoring revealed that osteogenic constructs from combi-cassettes and regular constructs were not statistically different ($P = 0.420$) (Suppl. Fig. 7). Non-osteogenic constructs were also not statistically different when comparing combi-cassette to regular constructs ($P = 0.989$). Bone scores for osteogenic constructs were significantly different from non-osteogenic constructs ($P = 0.007$). The increased number of replicates in combi-cassettes allowed variability between the bone scores for osteogenic constructs to be determined [42% for the 3 mice (coefficient of variation, $n = 3$)]. Immunostaining for human mitochondria demonstrated the presence of human cells in the new bone that was formed in both the combi-cassettes and regular constructs (Suppl. Fig. 4). Human cells were not detected in the non-osteogenic constructs.

In Experiment #2, 19-hole combi-cassettes were loaded with hBMSCs + HA/TCP or HA/TCP alone as the non-osteogenic construct. As in Experiment #1, new bone was observed that was autofluorescent and birefringent for the osteogenic constructs (hBMSCs + HA/TCP) from combi-cassettes and regular constructs (Fig. 3). The non-osteogenic constructs (HA/TCP alone) for combi-cassettes and regular constructs lacked new bone. For the combined bone scores from all the mice (Fig. 3c), combi-cassettes and regular constructs were not statistically different for either the osteogenic constructs ($P = 0.998$) or for the non-osteogenic constructs ($P = 0.946$) (Suppl. Fig. 7). The 95% confidence interval for the difference between osteogenic constructs in regular versus combi-cassette was -0.36 to 0.61 for bone scoring. Bone scores for osteogenic constructs were statistically different from non-osteogenic constructs for both combi-cassettes and regular ($P < 0.01$).

Since combi-cassettes enable multiple replicates in each mouse, the results within individual mice can be assessed (Fig. 3d). For combi-cassettes, the osteogenic constructs were significantly different from the non-osteogenic constructs for all three mice ($P < 0.27$) (Suppl. Fig. 7). In addition, the variability between mice can be assessed (Fig. 3e). A statistical difference between Mouse 1 and Mouse 3 ($P = 0.027$) was detected for the combi-cassette osteogenic constructs. Congenic mice may exhibit mouse-to-mouse variability due to environmental influences. In addition, the variability of the mean bone scores for the 3 mice was 27% (coefficient of variation, $n = 3$). The results for regular constructs for individual mice could not be assessed with statistical tests because each mouse only received 1 replicate for each type of regular construct. Human cells in new bone (combi-cassettes and regular constructs) stained positively with anti-human mitochondrial antibody, but human cells were not detected in non-osteogenic constructs (data not shown).

In Experiment #3, 19-hole combi-cassettes and regular constructs were loaded with BMP2 in GS (BMP2 + GS) or with GS alone (non-osteogenic construct). H&E staining of all BMP2 + GS-containing constructs revealed new bone that was autofluorescent and birerefringent, whereas all constructs with GS alone did not (Fig. 4). Because GS is radio-lucent, bone was quantified via microcomputed tomography (μCT). Three-dimensional reconstructions showed that bone volume in the osteogenic constructs in combi-cassettes and regular constructs were not statistically different ($P = 0.2512$) (Fig. 4c and d, Suppl. Fig. 5a and b), and both had significantly more than the non-osteogenic constructs ($P < 0.0001$) (Suppl. Fig. 7). The osteogenic and non-osteogenic constructs were loaded into the combi-cassettes in alternating rows so that the potential for BMP2 to diffuse out of its hole and into a neighboring hole could be assessed. In Suppl. Fig. 5a, some spillover can be seen in one of the combi-cassette holes for Mouse 3 (see red arrowheads). The 95% confidence interval for the difference between osteogenic constructs in regular versus combi-cassette was -0.0171 to 0.0418 for BV/TV. Bone quality as assessed by the TMD metric (total mineral density) for the combi-cassettes and regular constructs was not statistically different ($P = 0.877$) (Suppl. Fig. 6 and 7).

Mouse-to-mouse variability was also assessed (Fig. 4e, coefficient of variation = 35%, $n = 6$ mice). There was significantly more new bone formed in Mouse 6 than in Mouse 1 ($P = 0.011$). Statistics could not be used to analyze the mouse to mouse variability for regular, osteogenic constructs since there was only 1 replicate per mouse. GS alone in regular constructs is almost completely resorbed with little fibrous tissue, making retrieval and further analysis problematic. In contrast, combi-cassettes provided a defined volume for GS alone constructs, which were filled with fibrous tissue that could be analyzed by μCT and qPCR to enable statistical comparisons.

Expression of three osteogenic genes [*Runx2* (Runt-related transcription factor 2), *Ibsp* (bone sialoprotein, Bsp), *Bglap* (osteocalcin, Ocn)] was assessed by qPCR (quantitative polymerase chain reaction) for Experiment #3 (Fig. 5). All three genes were expressed at statistically higher levels in the combi-cassette osteogenic constructs (BMP2 + GS) compared with combi-cassette non-osteogenic constructs

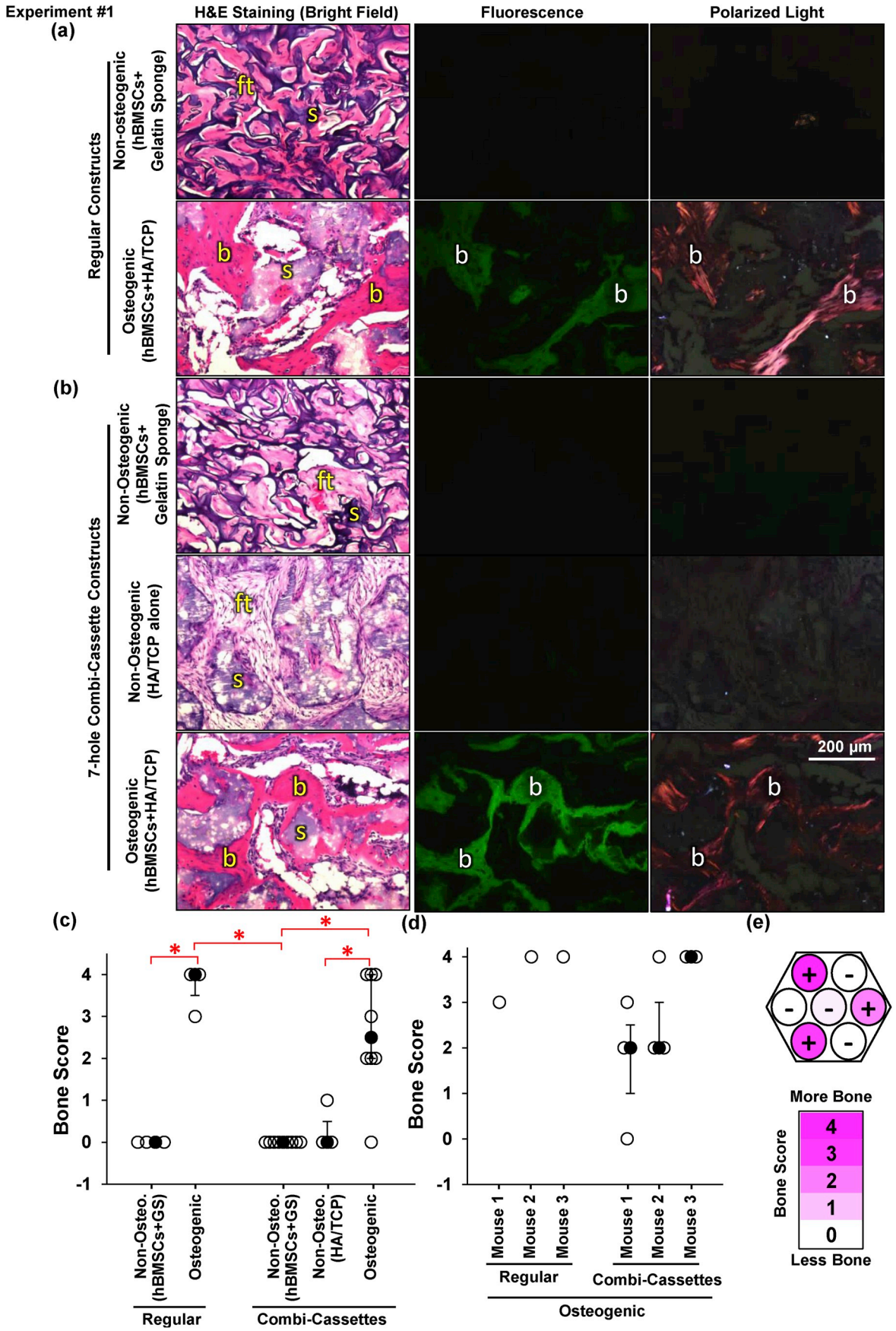

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Fig. 2. Experiment #1 – 7-hole Combi-cassette/hBMSCs experiment after 8 weeks in vivo. The osteogenic construct was hBMSCs + HA/TCP. There were two non-osteogenic constructs in this experiment: 1) hBMSCs + Gelatin Sponge (GS), and 2) HA/TCP alone. (a,b) *Left Column:* Bright field images of H&E stained sections. *Middle Column:* Autofluorescence images (bone is autofluorescent). *Right Column:* Polarized light micrographs showing birefringence (due to collagen fibers) as light areas. All images are at 200× magnification. Images in the same row are of the same field of view (b – bone, ft – fibrous tissue, s – gelatin sponge or HA/TCP scaffold). (c) Bone scores for 7-hole combi-cassettes (n = 3, 3, 8, 3, 8 constructs, left to right). All the osteogenic constructs were significantly different from all the non-osteogenic constructs (1-way ANOVA with Tukey's test, P = 0.007). There were no significant differences between combi-cassette and regular osteogenic constructs, or between combi-cassettes and regular non-osteogenic constructs (P = 0.42). (d) Mouse-to-mouse variability in bone scores (n = 1, 1, 1, 3, 3 and 2 constructs, left to right). There were no significant differences between combi-cassette osteogenic constructs for the 3 mice (1-way ANOVA with Tukey's test, P = 0.18). (c,d) Open circles are individual data points, closed circles are medians (error bars are first and third quartiles). (e) Heat map (n = 3 mice) variability in bone scores.

(GS alone) (P ≤ 0.006) (Suppl. Fig. 7). There were no significant differences in gene expression between combi-cassettes and regular constructs for the osteogenic constructs (P = 0.44) or for the non-osteogenic constructs (P = 0.98).

3. Discussion

Here, it was determined that the combi-cassette approach can be used for higher-throughput screening of osteogenic constructs in the mouse subcutaneous osteogenesis model. For both cell-based (hBMSCs + HA/TCP) and growth factor-based (BMP2 + GS) osteogenic graft formulations, combi-cassettes were compared with the regular approach using histology, μCT measurements and qPCR gene expression. By all measures, bone formation in combi-cassettes was similar to that generated by regular osteogenic constructs.

In Experiments 2 and 3, each animal tested received 11 replicates of the osteogenic formulation and 8 replicates of the non-osteogenic formulation. To our knowledge, an animal experiment with this much statistical power has not been conducted for a tissue engineering application. Combi-cassettes afford placement of multiple replicates of multiple formulations into the same animal, which enables rigorous statistical assessment of: 1) the variability for a given formulation within an animal (intra-animal variability), 2) differences between different formulations within the same animal and 3) the variability for a given formulation in different animals (inter-animal variability). The error bars on the data points in Fig. 3d may be the first time that the within-animal variability has been quantified for a tissue engineered construct. The data in Fig. 3d may also be the first time that two different tissue engineering formulations have been statistically compared within the same animal. Fig. 3e may be the first demonstration of a statistically significant difference for the same formulation placed into 2 different animals (i.e., demonstration of animal to animal variability). These rigorous statistical analyses have not been possible previously, since it has not previously been possible to place multiple replicates of multiple formulations into the same animal. These data are valuable for researchers as they design their experiments and can help determine how many animals they will need to detect an effect of a given size [22].

Combi-cassettes enable a high-throughput, systematic approach to in vivo studies of that could have broad appeal. Herein, combi-cassettes were used test osteogenic tissue engineering constructs, but they could also be used to test other types of constructs including chondrogenic, myogenic, vasculogenic, angiogenic, adipogenic or neurogenic constructs.

A comparison of combi-cassettes to the regular approach is given in Table 1. Combi-cassettes can be used to increase the number of replicates (~4.75-fold increase, 19 vs. 4) for each construct to improve confidence in results and to enable statistical testing. The regular approach is not sufficient for: i) delivery of multiple replicates of a construct formulation, or ii) placement of osteogenic positive controls and non-osteogenic negative controls into each mouse. Combi-cassettes provide a defined cylindrical volume for constructs so that tissue can be retrieved when the construct is fully resorbed, as was the case for gelatin sponge. This defined volume also improves comparability since the same volume of tissue can be retrieved and analyzed for different formulations in a combi-cassette. The combi-cassettes hold all the constructs in place throughout processing so that histology results are more comparable between constructs. The regular approach may have some advantages over the combi-cassettes. The regular constructs have access to the in vivo environment on all sides whereas constructs in combi-cassettes have access from the top and bottom only. Constructs in the combi-cassette are closer to one another than in the regular approach, which means that constructs in neighboring holes could influence one another as cells may migrate and growth factors may diffuse.

Fig. 5 shows that the osteogenic constructs from the combi-cassette had greater variability than the regular osteogenic constructs. This may be a result of the experimental design, whereby the regular constructs were used as a process control to determine if an experimental run was successful (detailed in the Methods section). Although this strategy saves time, the downside is that the results are biased in favor of the regular constructs. The combi-cassettes are in a statistically disadvantageous position since they are compared only to "regular constructs that showed good bone formation". By default, regular construct results should have lower variability, since all runs were selected on the basis of good bone formation in the regular constructs. Thus, the experimental design may explain why, in Fig. 5, the combi-cassettes have higher variability than the regular constructs. Having the deck stacked against the combi-cassettes makes it even more surprising that the combi-cassettes were not significantly different from the regular approach.

Although "false positive" bone is rarely formed subcutaneously, non-osteogenic negative controls are key for statistical analysis. When a construct undergoes resorption, the combi-cassette defines the tissue volume for analysis. If osteogenic-positive controls are omitted and poor bone formation is observed, then it is not clear if the failure was caused by differences between mouse strains, a poor batch of cells, variability in the scaffold or other factors. While the "false negative" rate for the subcutaneous osteogenesis model is unknown, a conservative estimate would be 30% for a single replicate. By using a combi-cassette to deliver 3 replicates per formulation, the false negative rate may be decreased to 3%, a 10-fold reduction (Suppl. Fig. 8). Based on the current results, combi-cassettes provide the opportunity to directly compare multiple replicates of each test osteogenic composition within the same mouse to facilitate acquisition of data that is amenable to rigorous statistical analysis.

4. Methods

4.1. Fabrication of combi-cassettes

Polytetrafluoroethylene (PTFE, Teflon) was used to fabricate combinatorial cassettes ("combi-cassettes"). PTFE was chosen since it is biocompatible and compatible with histological processing (soft enough for paraffin microtome sectioning, solvent resistant). Two configurations of the combi-cassettes were used, a 7-hole and a 19-hole arrangement (Suppl. Fig. 1). Cassettes were cut from a PTFE sheet (3.3 mm thickness) using a laser-cutter (Class 2 CO2 laser cutter, EPILOG Legend, 36 inch × 24 inch EXT) at 5000 Hz laser frequency, 100% laser scan speed and 80% laser power. Combi-cassettes were cut into hexagons (15 mm width by 13 mm length). This size of combi-

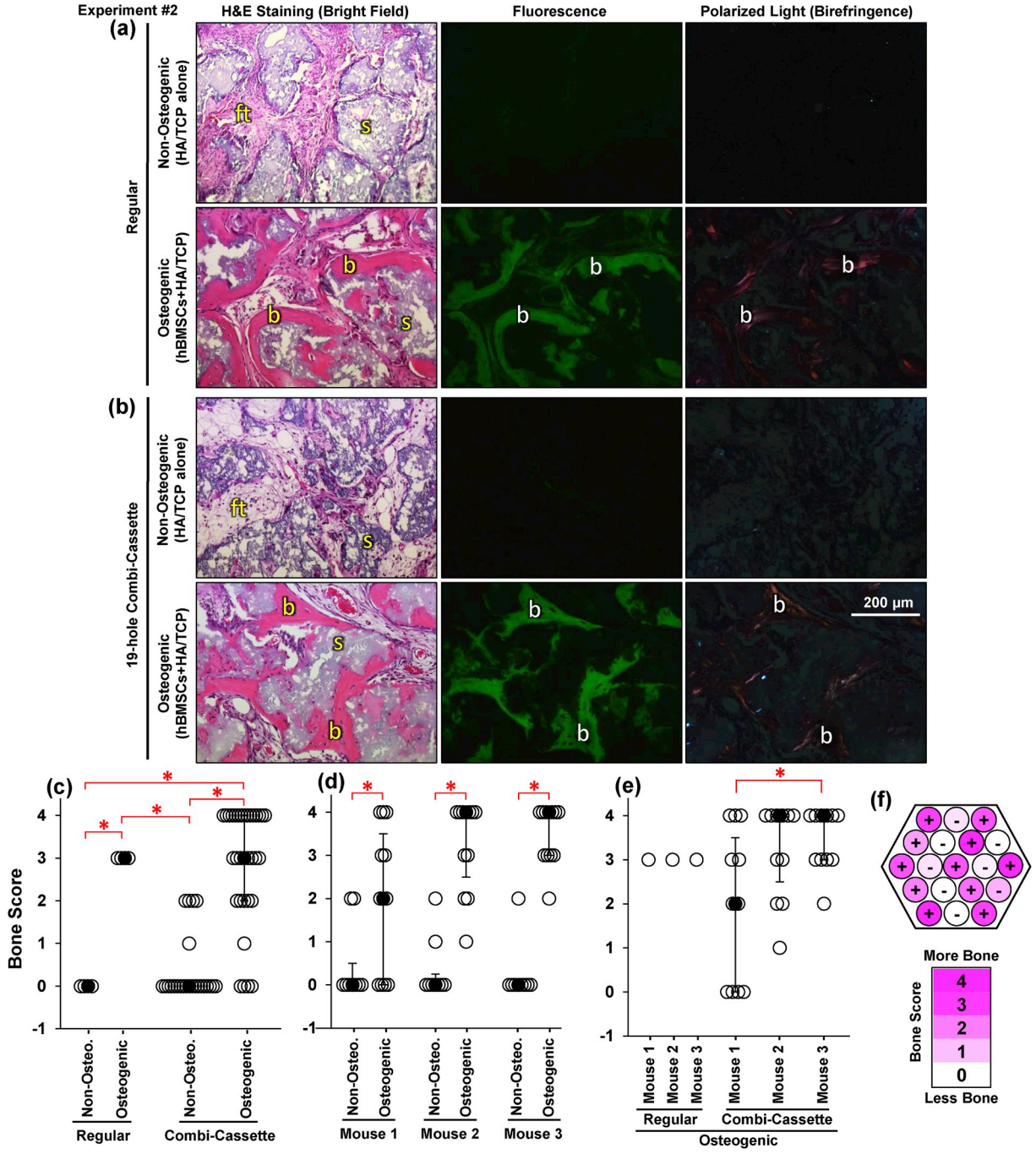

Fig. 3. Experiment #2–19 hole Combi-Cassette experiment using hBMSCs + HA/TCP after 8 weeks in vivo. (a,b) *Left Column:* Bright field images of H&E stained sections. *Middle Column:* Autofluorescence images (bone is autofluorescent). *Right Column:* Polarized light micrographs showing birefringence (due to collagen fibers) as light areas. All images are at 200× magnification. Images in the same row are of the same field of view (b – bone, ft – fibrous tissue, s – HA/TCP scaffold). (c) Combined bone scores from all mice (n = 3, 3, 24 and 33 constructs, left to right). All osteogenic constructs were significantly different from all non-osteogenic constructs (1-way ANOVA with Tukey's test, $P < 0.009$). There were no significant differences between combi-cassette osteogenic and regular osteogenic constructs or between combi-cassette non-osteogenic and regular non-osteogenic constructs ($P > 0.95$). (d) Osteogenic versus non-osteogenic constructs for individual mice. Osteogenic and non-osteogenic constructs were significantly different from one another for all three mice (*t*-test, $P < 0.03$). (e) Mouse-to-mouse variability (n = 11 constructs/mouse) in bone scores (n = 1, 1, 1, 11, 11 and 11 constructs, left to right). For combi-cassette constructs, Mouse 1 was significantly different from Mouse 3 (1-way ANOVA with Tukey's test, $P < 0.03$). (c,d,e) Open circles are individual data points, closed circles are medians and error bars are first and third quartiles. (f) Heat map to demonstrate variability in bone scores (n = 3 mice).

cassette was selected because it fit comfortably within the subcutaneous pocket of the mice. The combi-cassettes were cut with 4.3 mm-diameter holes for the 7-hole cassette (volume 47.9 μL/hole) and with 2.4-mm diameter holes (volume 14.9 μL/hole) for the 19-hole cassette (Suppl. Fig. 1). The holes are open on the top and bottom to allow nutrient/waste diffusion and vascularization. The center-to-center distance between two adjacent holes was 0.48 cm and 0.29 cm for 7-hole and 19-hole combi-cassettes, respectively. Fiduciary cuts were made on each cassette to keep track of the different formulations that were loaded. Edges of the combi-cassettes were smoothed with 1200 grit SiC sand paper to minimize irritation. A color photograph of the combi-cassettes was captured with a cell phone camera (1457 pixels by 837 pixels, 32-

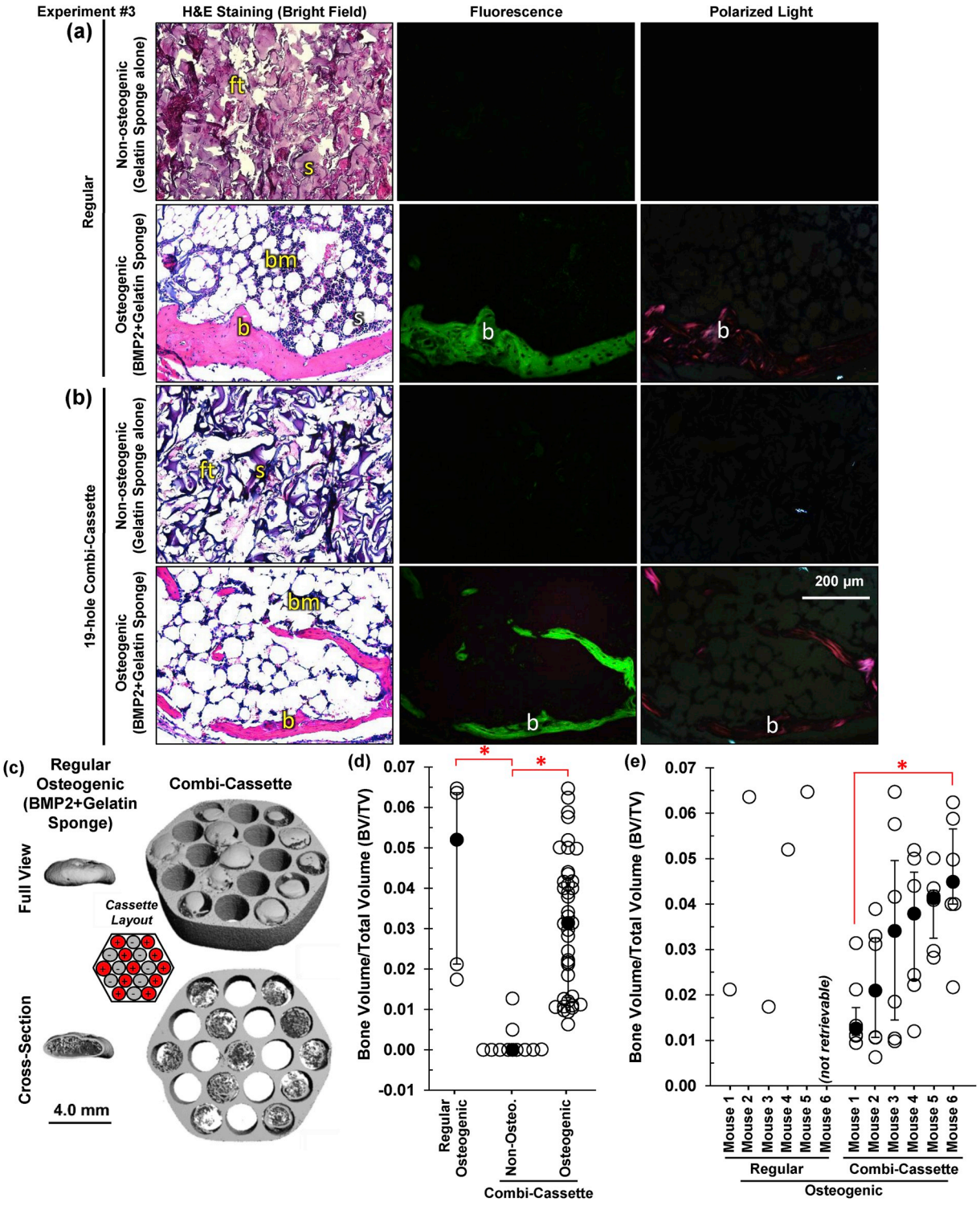

Fig. 4. Experiment #3 – 19-hole Combi-Cassette experiment using BMP2 + Gelatin Sponges after 8 weeks in vivo. (a,b) *Left Column:* Bright field images of H&E stained sections. *Middle Column:* Autofluorescence images (bone is autofluorescent). *Right Column:* Polarized light images showing birefringence (due to collagen fibers) as light areas. All images are 200× magnification. Images in the same row are from the same field of view. (b – bone, bm – bone marrow, ft – fibrous tissue, s – gelatin sponge). (a) Regular constructs (Gelatin Sponge alone, BMP2 + Gelatin Sponge). (b) Combi-cassette constructs (gelatin sponge alone, BMP2 + Gelatin Sponge). (c) 3D reconstructions from µCT from Mouse 4 [layout – Osteogenic (BMP2 + Gelatin Sponge), red; Non-Osteogenic (Gelatin Sponge alone), grey]. (d) Bone volume measurements from µCT (n = 5, 11, and 39 constructs, left to right). There were no significant differences between combi-cassette osteogenic and regular osteogenic constructs ($P < 0.00004$). (e) Mouse-to-mouse variability in bone volume (n = 1, 1, 1, 1, 1, 0, 7, 6, 7, 7, 6 and 6 constructs, left to right). For combi-cassette osteogenic constructs, Mouse 1 was significantly different from Mouse 6 (1-way ANOVA with Tukey's test, $P = 0.01$). (d,e) Open circles are individual data points, closed circles are medians, and error bars are first and third quartiles. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

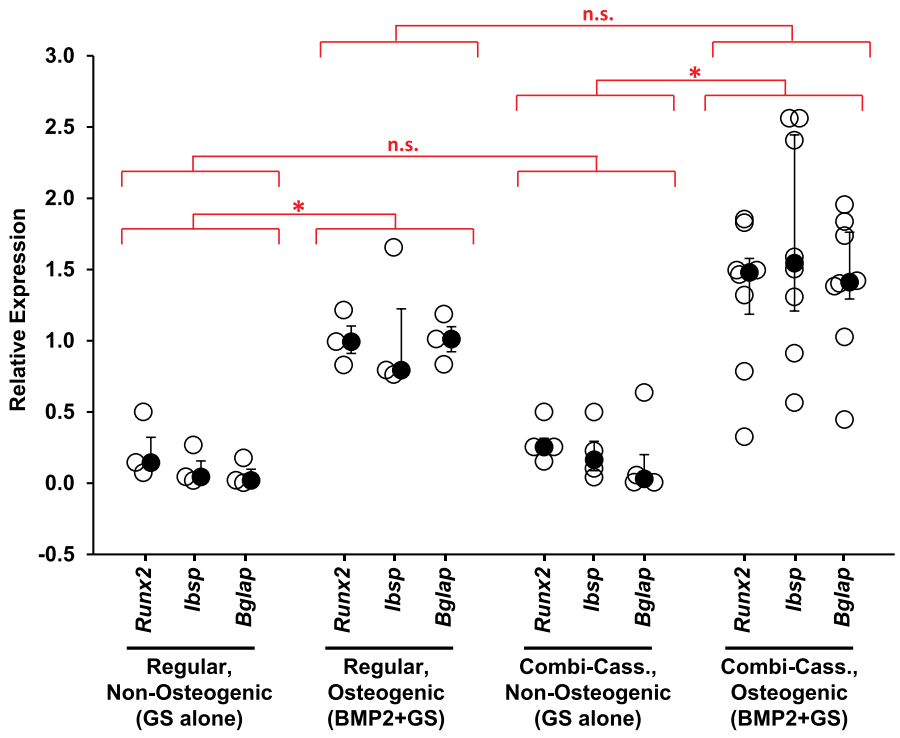

Fig. 5. Experiment #3 – Gene expression was measured by RT-PCR for the 19-hole combi-cassette experiment using BMP2 + GS after 8 weeks in vivo. *Runx2* (runt related transcription factor 2), *Ibsp* (bone sialoprotein) and *Bglap* (osteocalcin) and were assessed in 3 mice. Open circles are individual data points: n = 3 for regular constructs; n = 4 for combi-cassette non-osteogenic constructs; n = 8 for combi-cassette osteogenic constructs. Closed circles are median and error bars are first and third quartiles. Combi-cassette osteogenic constructs were significantly different from non-osteogenic constructs for all three genes (1-way ANOVA with Tukey's test, P = 0.005). For all three genes, there were no significant differences between combi-cassette osteogenic versus regular osteogenic constructs (1-way ANOVA with Tukey's test, P = 0.44).

bit image depth). The image was not processed. Using an ultra-sonic bath, combi-cassettes were cleaned by sequential immersion in acetone (5 min), ethanol (10 min) and deionized water (10 min). Combi-cassettes were autoclaved. Next, in order to reduce leaking during loading, plastic paraffin film (Parafilm, folded twice into a 3 cm by 3 cm square) was placed into Petri dishes and heated to 60 °C. Combi-cassettes were pressed into the softened film to seal the bottom (the film was removed prior to placement into the mouse). Before use, the plastic paraffin film was washed with 70% by volume ethanol and exposed to ultraviolet light for sterilization.

4.2. Materials

4.2.1. Gelatin sponges (GS)

Sterile porous gelatin sponges (GelfoamTM, GS) were obtained from Pfizer (New York, NY). For imaging the pore structure, GS were sputtercoated with gold for 60 s and imaged by scanning electron microscopy (SEM, Hitachi S-4700-II FE-SEM, 3 kV, image resolution 1280 pixels by 960 pixels, 8-bit pixel depth). Pores of approximate diameter of 100 μm were visible in SEMs of the GS (Suppl. Fig. 2a).

4.2.2. Hydroxyapatite/β-tricalcium phosphate particles (HA/TCP)

HA/TCP particles (65:35 by mass hydroxyapatite/β-tricalcium phosphate particles, 0.5 mm–1.0 mm nominal particle size) were obtained from Zimmer, Inc. To confirm the composition of HA/TCP particles, X-ray diffraction (XRD, DMAX 220, Rigaku Denki) was performed using copper Kα radiation (wavelength 0.1541874 nm) in the 2θ range 10°–60° (Suppl. Fig. 2b). Scanning electron microscopy (SEM, Hitachi S-4700-II FE-SEM, 10 kV, image resolution 1280 pixels by 960 pixels, 8-bit pixel depth) verified the HA/TCP particle nominal size range of 0.5 mm–1 mm diameter.

HA/TCP particles were heat-sterilized at 200 °C for 2 h. For regular constructs and 7-hole combi-cassettes, 40 mg of HA/TCP particles were placed in round-bottomed 2 mL polypropylene cryotubes (Nunc) and vortexed with 1 mL of culture medium. The particles were allowed to settle by gravity and the supernatant was aspirated to remove ceramic dust. For 19-hole combi-cassettes, 120 mg of HA/TCP particles were washed by the same procedure with 2 mL of medium.

4.2.3. Recombinant human bone morphogenetic protein 2 (BMP2)

Recombinant human bone morphogenetic protein 2 (BMP2) was obtained from eBioscience (San Diego, CA) and reconstituted in sterile water to yield a 10× solution (5 mg/mL), and then diluted 1:10 with HBSS (Hank's balanced salt solution, Invitrogen) to a final concentration of 0.5 mg/mL.

4.3. Primary human bone marrow stromal cells (hBMSCs)

A bank of primary human bone marrow stromal cells (hBMSCs) was established previously in our lab as described [23]. Cells were prepared according to NIH ethical guidelines (NIH OHSRP exemption #373). Cells from a single donor were used for all studies (orthopedic surgical waste, spinal correction, congenital scoliosis, 11 years old, female).

A single cell suspension of bone marrow was prepared as described previously [24–26]. Briefly, fragments of human trabecular bone and marrow were scraped sterilely into culture medium [α-minimum essential medium (α -MEM), 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (all from Invitrogen, Carlsbad, CA), and 20% non-heat inactivated fetal bovine serum (FBS) of a pre-selected[5] lot (Atlanta Biologicals, Inc., Norcross, GA)] with a steel blade. This marrow preparation was repeatedly pipetted and consecutively passed through the 16 and 19-gauge needles to break up cell aggregates. The resulting cell suspension was filtered through a 70 μm nylon cell strainer (352350, Becton Dickinson, Franklin Lakes, NJ) to remove remaining cell aggregates. The single cell suspension of bone marrow was then plated into 75 cm^2 filter cap tissue culture flasks (Corning, Inc., Corning, NY) at 5 × 106 nucleated cells per flask (6.7 × 104 cells/cm^2) in 30 mL of culture medium. Cultivation was conducted at 37 °C in a humidified atmosphere of 5% by volume CO2 in air. Full medium replacements were performed two days after plating, and three times a week thereafter. Fourteen days after plating, when hBMSCs were approaching confluence, the cultures were washed with HBSS, and cells were detached with two consecutive portions of trypsin [0.05% by mass trypsin with 1 mmol/L ethylenediaminetetraacetate (EDTA), Invitrogen]. The effect of trypsin was stopped by the addition of cold culture medium containing 1% FBS, and total cell numbers were determined. hBMSCs of passage 1, were pelleted (10 min at 406 × g_n) and

Table 1
Comparison of Combi-Cassettes vs. Regular Constructs*.

Regular Constructs	Combi-Cassettes
Up to 4 formulations can be tested in a single mouse.	19 formulations can be tested in a single mouse, which can improve confidence in results & reduce the number of animals used.
Typically, only a single replicate of each formulation is placed into each mouse, which prevents a statistical analysis within one mouse.	Multiple replicates of a formulation can be tested in each mouse, which enables statistical analyses of: i) mouse-to-mouse variability, & ii) differences between formulations placed into the same mouse.
When a construct is mostly resorbed, fibrous tissue may not be visible, making retrieval and further analysis problematic.	When a construct is resorbed, the volume within the holes provides a defined volume that can be analyzed to provide data for statistical analyses.
Histological analysis is less consistent, since each construct must be individually fixed, demineralized, embedded, sectioned, mounted, stained & imaged.	More consistent histological analysis, since an entire cassette can be fixed, demineralized, embedded, sectioned, mounted, stained & imaged in one piece, without requiring individual manipulations of each construct. This is especially useful for immunohistochemistry studies.
Due to the inconsistent shape of the of construct, the volume of interest for analysis is subjective & may vary from construct to construct; for osteogenic constructs, the volume of interest will be defined by the presence or absence of bone, which biases results.	The volume of interest for analysis is defined by the walls of the hole, making results less biased, more comparable and more quantitative.
Constructs under the skin can be displaced after surgery due to the tightness of the skin of some mice and due to mechanical activity of the mice, which complicates construct retrieval & analysis.	Holds constructs in place in the mouse after surgery.
Uses a larger amount of osteogenic construct, which is disadvantageous when material is limited.	Uses a smaller dose of the osteogenic construct, which is advantageous when material is limited.
Uses a larger amount of scaffolding material, which may increase assay sensitivity.	Uses a smaller dose of the osteogenic construct, which may reduce assay sensitivity.
Constructs are separated by a longer distance which may reduce cross-contamination	Constructs in neighboring holes may influence one another: cells may migrate to an adjacent hole, growth factors may diffuse.
All sides of the regular constructs are exposed to the subcutaneous microenvironment.	Shields the sides of constructs from the subcutaneous microenvironment, which could hinder osteogenesis or vascularization.
Constructs cannot “fall out.”	Some constructs may fall out of the cassette prior to surgery: hydrogels & sponges do not fall out, loose particles not held together with fibrin gel may fall out.
*Grey shading indicates an advantage	

frozen at 1 × 106 cells per cryotube in 1 mL of medium consisting of 50% by volume culture medium and 50% by volume of 2× freezing medium [60% α-MEM, 20% by volume FBS, and 20% by volume dimethyl sulphoxide (Hybri-Max, Sigma, St. Louis, MO)]. The frozen hBMSCs were stored in liquid nitrogen until further use. The cells expressed all of the cell surface markers representative of human bone marrow stromal cells [23].

4.4. Culture of bone marrow stromal cells for generation of constructs

hBMSCs at passage 1 (1×10^6 cells) were cultured in 20 mL of growth medium [α-MEM with 20% by volume lot-selected FBS (Atlanta Biologicals), 2 mmol/L L-glutamine (Invitrogen), 1 U/mL penicillin and 1 μg/mL streptomycin (Cellgro)] in a T-175 (175 cm^2) cell culture flask (5.7×103 cells per cm^2) (Corning, Sigma-Aldrich) [23]. Culture

medium was replaced every 2 d. Upon approaching 80% confluency, hBMSCs were trypsin-released [0.25% by mass containing 1 mmol/L ethylenediaminetetraacetate (EDTA) (Invitrogen)] and transferred into new T-175 cell culture flasks. Passage 3 hBMSCs were used for combi-cassettes and for regular constructs for placement into mice.

4.5. Preparation of osteogenic and non-osteogenic constructs

Three experiments were performed to assess combi-cassettes for bone formation by osteogenic constructs (Fig. 2 and Suppl. Fig. 3).

4.5.1. Experiment #1: 7-hole combi-cassette with hBMSCs

Regular non-osteogenic (hBMSCs + GS): Sterile GS (8 mm × 8 mm x 4 mm) were immersed in culture medium, squeezed with forceps with sterile filter paper, placed in a cryotube containing hBMSCs (2 million cells in 25 μL of culture medium) and placed into the mouse.

Regular osteogenic: hBMSCs (2 million hBMSCs in 1 mL of medium) were added to the washed HA/TCP particles (40 mg) in cryotubes and incubated for 90 min on a rotating platform inside a cell culture incubator. After incubation, the hBMSCs + HA/TCP mixture was centrifuged for 60 s at 406 × g_n to pellet the cells that did not attach and the supernatant was discarded. The hBMSCs + HA/TCP constructs were gently removed from the cryotubes using a sterile spatula and placed into the mouse subcutaneously.

Combi-cassette non-osteogenic (hBMSCs + GS): Sterile GS (5 mm × 5 mm x 4 mm) was prepared as described above and placed in a 2 mL cryotube containing hBMSCs (0.75 million cells in 25 μL of medium) and loaded into the 7-hole combi-cassettes. Three such hBMSCs + GS constructs were placed in each 7-hole combi-cassette.

Combi-cassette non-osteogenic (HA/TCP alone): In order to form a cohesive mixture of HA/TCP, fibrinogen (15 μL) and thrombin (15 μL) were added to the washed particles (40 mg) and mixed by gentle tapping of the tubes (a fibrin gel formed within 1 min). One cryotube with gelled HA/TCP-fibrin was prepared. The construct was gently removed from the cryotube using a spatula, placed into a 1 mL pipet tip that had been cut in half (along the long axis) and scraped into the 7-hole combi-cassette using a needle. Each hole received ~12 mg of HA/TCP particles and ~9 μL of fibrin gel. One such construct was loaded into each 7-hole combi-cassette.

Combi-cassette osteogenic: hBMSCs (2 million cells in 1 mL of medium) were added to the washed HA/TCP particles (40 mg) and incubated, and then mixed with fibrinogen and thrombin as described above. Three such cryotubes were prepared with hBMSCs-HA/TCP-fibrin constructs to occupy 3 of the holes in the 7-hole combi-cassette as described above. Each hole received ~0.60 million hBMSCs, ~12 mg of HA/TCP particles and ~9 μL of fibrin gel.

4.5.2. Experiment #2: 19-hole combi-cassette with hBMSCs

Regular non-osteogenic: The washed HA/TCP particles (40 mg) were gently removed from the cryotubes using a spatula and placed into the mouse.

Regular osteogenic: These were prepared as described above for Experiment #1.

Combi-cassette non-osteogenic (HA/TCP alone): Fibrinogen (45 μL) and thrombin (45 μL) were added to 120 mg HA/TCP particles and allowed to gel, and one cryotube was prepared to occupy 8 of the holes in the 19-hole combi-cassette. Using a dissecting microscope, the gelled HA/TCP-fibrin construct was removed from the cryotube as described above and loaded into the 19-hole combi-cassette using a needle. Each hole received ~10 mg of HA/TCP particles and ~8 μL of fibrin gel.

Combi-cassette osteogenic: hBMSCs (6 million hBMSCs in 1.5 mL of medium on 120 mg HA/TCP particles with fibrin gel) were prepared as described above. One cryotube was prepared with gelled hBMSCs-HA/TCP-fibrin to occupy 11 of the holes in the 19-hole combi-cassette as described above. Each hole received ~0.5 million hBMSCs, ~10 mg of HA/TCP and ~8 μL of fibrin gel.

4.5.3. Experiment #3: 19-hole combi-cassette with BMP2

Gelatin sponges (GS): GS sections for regular constructs (8 mm × 8 mm x 4 mm) and for 19-hole combi-cassettes (4 × 4 × 4 mm) were prepared as described above.

Regular non-osteogenic: The GS block was loaded with 10 μL of HBSS (Hank's balanced salt solution, Invitrogen) and placed into the mouse (no BMP2).

Regular osteogenic: The GS were loaded with 10 μL of BMP2 (5 μg/scaffold), which is known to recruit and induce local cells into an osteogenic fate [4,5].

Combi-cassette non-osteogenic: GS were loaded into the 19-hole combi-cassettes and then loaded with 8 μL of HBSS. Eight such constructs were loaded into each 19-hole cassette.

Combi-cassette osteogenic: GS were loaded into the 19-hole combi-cassettes and then loaded with 8 μL of BMP2 (4 μg/scaffold). Eleven such constructs were loaded into each 19-hole combi-cassette.

4.5.4. Photographs and storage

After loading the cassettes, color photographs were captured with a Zeiss STEMI SV6 stereo microscope using a Nikon DS-Fi2-L3 camera (2090 pixels by 1829 pixels, 24-bit pixel depth). The contrast was adjusted equally for all three images. To avoid construct dehydration during mouse preparations, the parafilm was removed from the bottom of the combi-cassettes, the combi-cassettes were placed in a Petri dish and a piece of sterile gauze soaked in PBS was placed on each side of the combi-cassettes inside the petri dish. The dishes were stored at 4 °C until placement under the microscope (up to 2 h).

4.6. In vivo procedures

Mouse studies were conducted according to NIH ethical guidelines under an animal protocol that was approved by the NIDCR ACUC (ASP 13-694). Female mice (*Mus musculus*, 6 weeks old) were received from the supplier and allowed to recover for 2 wks. Mice were housed one per cage in a conventional veterinary facility (with quarantine requirements, and exclusion of specific pathogens) with a 12 h/12 h light-dark cycle and fed ad libitum with NIH 07 (autoclavable) hard diet (Envigo, Frederick, MD).

For Experiment #1 and Experiment #2, 8-week old female NOD.SCID-gamma immunocompromised mice (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ, catalog # 005557, The Jackson Laboratory, Bar Harbor, ME) were used to prevent immune rejection. Three mice were used for each experiment. For Experiment #3, six 8-week old female FVB/NHsd mice (FVB/NHsd, catalog # 118, Harlan-Sprague Dawley, Envigo, Indianapolis, IN) were used. For all experiments: i) each mouse received one combi-cassette and two regular constructs, and ii) an 8-week incubation time was used (Fig. 1 and Suppl. Fig. 3).

The incision area was shaved 24 h before surgery. After constructs were prepared, mice were anesthetized with isoflurane (2%–5% by volume in oxygen) and Buprenorphine (0.1 mg/kg) was administered before surgery by subcutaneous injection. The skin was disinfected sequentially with an iodine-containing detergent (Wescodyne) and with 70% by volume ethanol. A horizontal incision of 2 cm was made across the dorsal midline approximately overlying L1-L2 vertebral bodies. Subcutaneous pockets were created by introducing closed blunted surgical scissors and opening them inside to separate the dermis from the muscles. For Experiment #1, the 7-hole combi-cassettes were placed over the T4-T5 vertebral bodies and regular constructs were placed at both lateral flanks at the level of L5-L6. For Experiment #2 and Experiment #3, the 19-hole combi-cassettes were placed over the L5-L6 vertebral bodies and regular constructs were placed at both lateral flanks at the level of T4-T5.

Combi-cassettes were placed into the mice through the incision using sterile forceps. The constructs did not fall out of the cassettes during placement into the mice. The constructs with HA/TCP scaffolds included fibrin which gelled and held the constructs in place. The

constructs with GS did not fall out because the GS fit snugly in the holes of the combi-cassettes. Regular constructs containing HA/TCP particles were placed with a sterile spatula, while regular constructs containing GS were placed with sterile forceps. Incisions were closed with surgical clips. When necessary, air pockets surrounding the cassette were removed by suctioning with a syringe with a 23-gauge needle. Buprenorphine (0.1 mg/kg) was administered after surgery by subcutaneous injection. No signs of discomfort from the combi-cassettes were observed in the mice following surgeries: their water and food intake was normal, there were no signs of chafing and the incisions healed normally.

4.7. X-ray radiography

Mice were periodically imaged by radiography after surgery to assess the position of the constructs. X-ray radiographs (872 by 722 pixels, 24-bit pixel depth) of mice were captured using an IVIS Lumina X-ray Imaging System (Living Image Version 4.1.0.11858, X-ray tube voltage 35 kV, exposure time 2 s, binning factor 2, f number 2).

4.8. Construct recovery and analysis

At 8 weeks postoperatively, mice were euthanized by CO2 asphyxiation and constructs were harvested for analysis. For histology and μCT, combi-cassettes containing the constructs were fixed in 4% neutral-buffered formaldehyde at 4 °C overnight and stored in PBS with 0.025% sodium azide at 4 °C. Constructs assigned for qPCR were pushed out of the holes in the combi-cassettes, snap frozen in liquid nitrogen and stored at −80 °C before RNA extraction.

Due to the complexity, lengthy timelines and high variability of animal studies, and because we were unsure if the combi-cassettes would work at the time that we started this project, we used the results from the regular constructs as a “process control” for each run of the experiment. After placing the combi-cassettes and regular constructs in mice for 8 weeks, the retrieved regular constructs were assessed before analyzing the combi-cassettes. The preliminary results from the regular constructs were used to determine if a run of the experiment was acceptable for further analysis. If the regular osteogenic constructs showed good bone formation (bone score ≥ 3 for hBMSC-based constructs or significant radiopacity in X-ray radiography for BMP2-based constructs), then a full analysis of both the regular constructs and combi-cassette constructs was conducted. This strategy was implemented because we did not want to invest months of analytical effort on a questionable run.

4.9. Histology

Constructs were decalcified in a 0.25 mmol/L EDTA (in PBS, pH 8.0) at 4 °C for 14 d. After 14 days, decalcification was confirmed by X-ray radiography. Constructs were dehydrated using a tissue processor, embedded in paraffin, sectioned using a microtome (5 μm), mounted on glass slides, deparaffinized, re-hydrated and stained with hematoxylin and eosin (H&E). Constructs were not removed from the combi-cassettes for processing or sectioning. The whole combi-cassettes with loaded grafts in place were sectioned with the microtome so that all combi-cassette constructs could be mounted, stained and imaged on the same slide. When slides were immersed in xylene during hydration, the 5 μm sheet of PTFE from each section naturally detached from the slide, leaving the array of constructs attached to the slides. H&E-stained sections were imaged with a Zeiss AX-10 microscope via three channels, brightfield, fluorescence and crossed-polarizers, using an AxioCam HRc camera (Carl Zeiss, Jena, Germany, 2584 by 1936 pixels, 24-bit image depth). Brightfield was used to assess H&E staining and bone formation. Fluorescence microscopy was used to confirm the presence of bone matrix by autoflourescence (excitation 450 nm–490 nm, emission 500 nm–550 nm). In addition, sections were observed through crossed-polarizers to confirm the presence of woven or lamellar bone by collagen birefringence [21]. Representative brightfield, fluorescence and polarized images were captured for the figures. The only processing that was done to images was adjustment of brightness and contrast, which were applied evenly to all images in a treatment group. In addition, low magnification images of the slides showing the whole mounts of the combi-cassettes were captured using a slide scanner (Aperio CS2 Scanscope, full sample 200× scan, Leica Biosystems, Nussloch, Germany, 1712 by 952 pixels, 24-bit pixel depth). None of the images were processed.

4.10. Bone scoring

Three sections from each construct (the entire slide was reviewed) were blindly scored for bone formation by 3 independent expert observers. It has been demonstrated that semi-quantitative bone scoring by expert observers correlates (r = 0.86) with histomorphometry measurements [27]. H&E stained sections were observed under brightfield microscopy by each observer and assigned a score of 0–4: score 0, no bone formation; score 1, minimal bone formation, just a single or a few bone trabeculae in one or a few sections; score 2, low bone formation, multiple bone trabeculae are in several parts of some sections but bone occupies only a small portion of the sections; score 3, moderate bone formation, bone occupies a significant portion but less than one half of most sections; score 4, abundant bone formation, bone occupies greater than one half of each section [27].

4.11. Human mitochondrial staining

In order to assess the human origin of the ossified tissue formed in the constructs containing hBMSCs, sections were stained for the presence of human mitochondria. Sections mounted on slides were deparaffinized and hydrated. Endogenous peroxidase activity was blocked using 3% by volume H_2O_2 in PBS for 5 min at room temperature. To avoid the recognition of the tissue endogenous mouse antibodies by the secondary antibody, a mouse IgG blocking kit was used following the manufacturer's instructions (MOM kit, Vector Laboratories, Burlingame, CA). Non-specific primary antibody binding was blocked with 2.5% by volume normal horse serum in PBS for 1 h at room temperature. The primary human mitochondria-specific mouse antibody (MAB1273, anti-human mitochondria antibody, surface of intact mitochondria, clone 113-1, EMD Millipore) or negative control IgG (normal mouse IgG, sc-2025, Santa Cruz, Dallas, TX) were incubated at a 1:100 dilution (by volume) in the blocking buffer overnight at 4 °C in a humidified chamber. After washing with PBS, sections were incubated in a horseradish peroxidase-conjugated secondary anti-mouse IgG antibody for 10 min at room temperature. The secondary antibody was detected using aminoethyl carbazole (AEC) solution (Life Technologies, Carlsbad CA).

Two sections each from combi-cassettes and regular non-combi constructs from each mouse were visually examined. The stained sections were mounted with aqueous mounting medium (VectaMount AQ, Vector Laboratories, Inc., Burlingame, CA). Images were captured at 2 magnifications (200× and 1000×) on two channels (brightfield and fluorescence) using a Zeiss AX-10 microscope with an AxioCam HRc camera (Carl Zeiss, Jena, Germany, 1388 by 2040 pixels, 24-bit image depth). Brightfield was used to identify human mitochondrial staining (red) while fluorescence was used to determine the location of bone by autoflourescence (excitation 450 nm–490 nm, emission 500 nm–550 nm). For display images in the manuscript, two images (magnification 100×) of the same field of view at different focal planes were combined using Photoshop CS6 so that all of the objects present in the images were in focus.

The antibody (MAB1273) was validated by: i) staining a section of human bone (positive), ii) staining the non-osteogenic constructs from Exp. 1 and Exp. 2 (HA/TCP alone), which contained mouse fibroblasts

(negative), and iii) by staining the osteogenic constructs in Exp. 1 and Exp. 2 (hBMSCs + HA/TCP) with a non-immune immunoglobulin IgG, often called an “IgG Control” or an “Isotype Control” (negative) (Suppl. Fig. 4).

4.12. X-ray microcomputed tomography (μCT)

For Experiment 3 (BMP2 + GS), the fixed, non-decalcified constructs, in PBS were imaged by X-ray microcomputed tomography (μCT, 70 kVp, 85 μA, 0.3 s integration, 10 μm^3 voxel resolution, Scanco μCT 50, Brütisellen, Switzerland). For generating the combi-cassette three-dimensional (3D) binarized reconstructions, constructs were segmented using a relative threshold of 212/1000 (sigma 0.8/5, support 1/9) using Scanco software. These settings allow the visualization of both the PTFE combi-cassettes and the constructs. For quantitative analysis, cylindrical volumes of interest (VOI) were delineated within the boundaries of each hole (3.3 mm height, 2.4 mm dia.) and the VOI were binarized using a threshold of 250/1000 (sigma 0.8/5, support 1/9), which excluded PTFE from the analysis (only bone was visualized). For regular osteogenic constructs, an irregularly-shaped VOI was traced around the construct bone tissue. Regular non-osteogenic constructs could not be assessed by μCT since GS (without BMP2) were resorbed by 8 weeks. “Bone Volume per Total Volume” (BV/TV) and “Tissue Mineral Density” (TMD) were determined for each VOI. TMD is a measure of the bone quality that assesses the radiopacity (mineral density) of the voxels whose intensities were above the threshold.

4.13. qRT-PCR gene expression analysis

Gene expression measurements were conducted for Experiment 3 (BMP2 + GS). Immediately upon euthanizing mice at 8 weeks, some of the constructs were removed from the combi-cassette and snap frozen in liquid nitrogen for analysis by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Frozen constructs were ground into a fine powder with a mortar and pestle in liquid nitrogen and mixed with Trizol reagent (LifeTechnologies). RNA was extracted with an RNA extraction kit (Qiagen) for analysis. A kit was used for reverse transcription using 0.5 μg RNA (iScript cDNA Synthesis Kit, Bio-Rad). qPCR was performed using a CFX-96 Real Time System paired with a C1000 Thermal Cycler (Bio-Rad, Hercules, CA). qPCR reactions were set up using iQ SYBR Green Supermix (Bio-Rad #170-8882) according to the kit's instructions. Gene expression levels of three osteogenic markers, *Runx2 (Runt-related transcription factor 2)*, *Ibsp (Bone Sialoprotein, Bsp)*, *Bglap (Osteocalcin, Ocn)* were evaluated, and *Rps29 (Ribosomal protein S29)* expression was used as a control. The following primers were used:

- mouse *Runx2* (AF010284)-F: 5′-GCAGTTCCCAAGCATTTCAT-3′, R: 5′-CACTCTGGCTTTGGGAAGAG-3’;
- mouse *Ibsp* (NM_008318.3)-F: 5′-AAGTGAAGGAAAGCGACGAGGAAG-3′, R: 5′-GTTGGTGCTGGTGCCGTTGAC-3’;
- mouse *Bglap* (NM_001032298)-F: 5′-CCAAGCAGGAGGGCAATAAGGTAG-3′, R: 5′ CTCGTCACAAGCAGGGTCAAGC-3’;
- mouse *Rps29* (NM_009093.2)-F: 5′-GGAGTCACCCACGGAAGTTCGG-3′, R: 5′-GGAAGCACTGGCGGCACATG-3’.

Three technical replicates were prepared for each construct. A total of 18 constructs were analyzed: 3 regular osteogenic, 3 regular non-osteogenic, 4 combi-cassette non-osteogenic (1 hole each from 2 of the mice, plus 2 holes from one mouse) and 8 combi-cassette osteogenic (3 holes each from 2 of the mice, plus 2 holes from 1 mouse). qPCR results, expressed as critical threshold (CT) values, were normalized to the levels of Rsp29, generating ΔCT values; levels of relative expression were calculated as $2^{-\Delta CT}$.

4.14. Statistical analysis

Data are presented as medians with first and third quartiles. Statistical analysis was performed using t-tests or 1-way analysis of variance (ANOVA) with Tukey's test for multiple comparisons (two-sided). P-value below 0.05 was considered significant. Minitab (version 17.3.1) was used for statistical analysis.

Author contributions

S.B., L.F.D.C., S.A.K., P.G.R. and C.G.S. conceived the project, analyzed the data and wrote the paper. S.B., L.F.D.C., S.A.K., A.M. and D.B. conducted the experiments.

Competing financial interests

The authors declare no competing financial interests.

Data availability statement

All data from the paper are provided in the Supplementary Data File 1.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biomaterials.2018.09.035.

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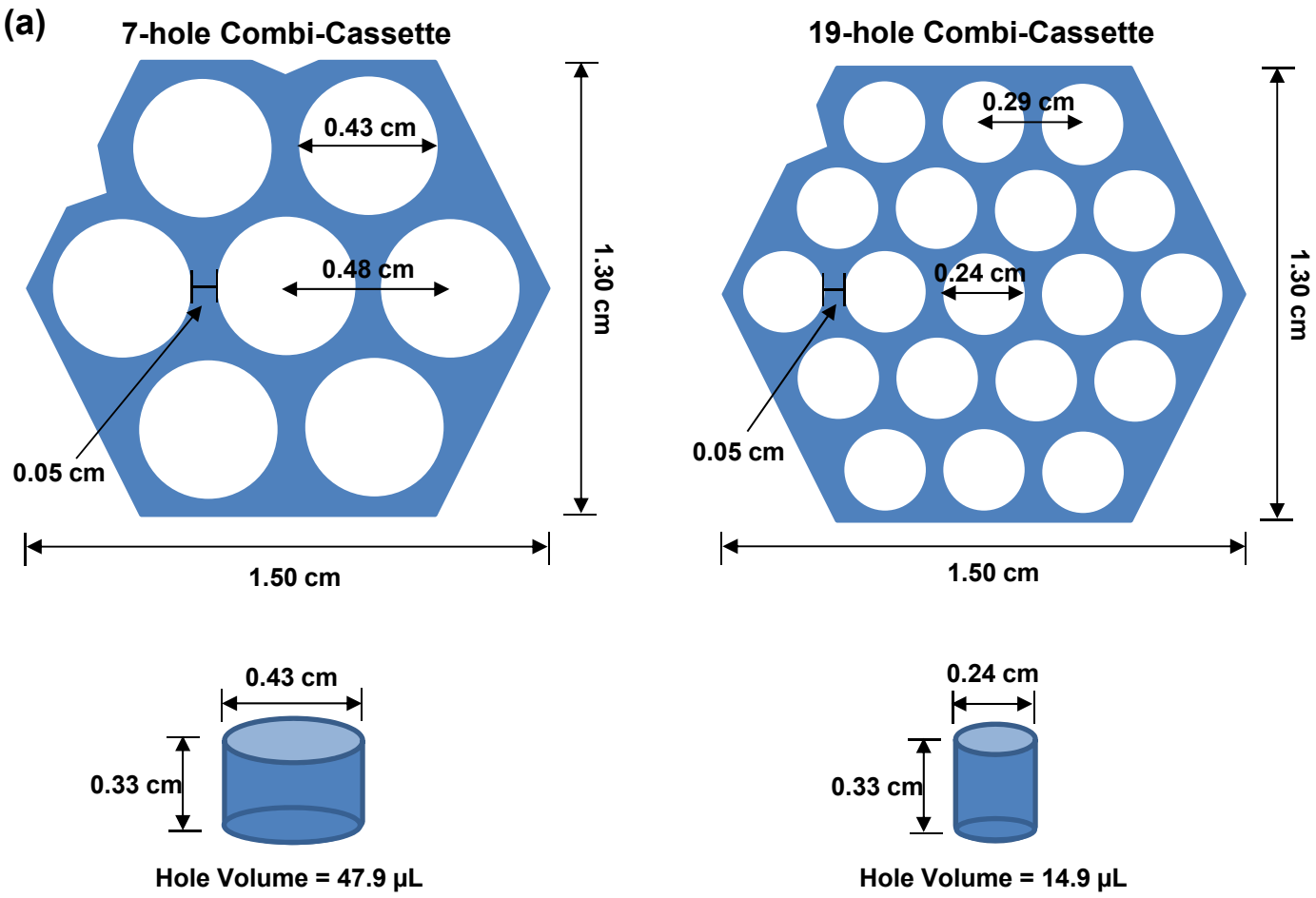
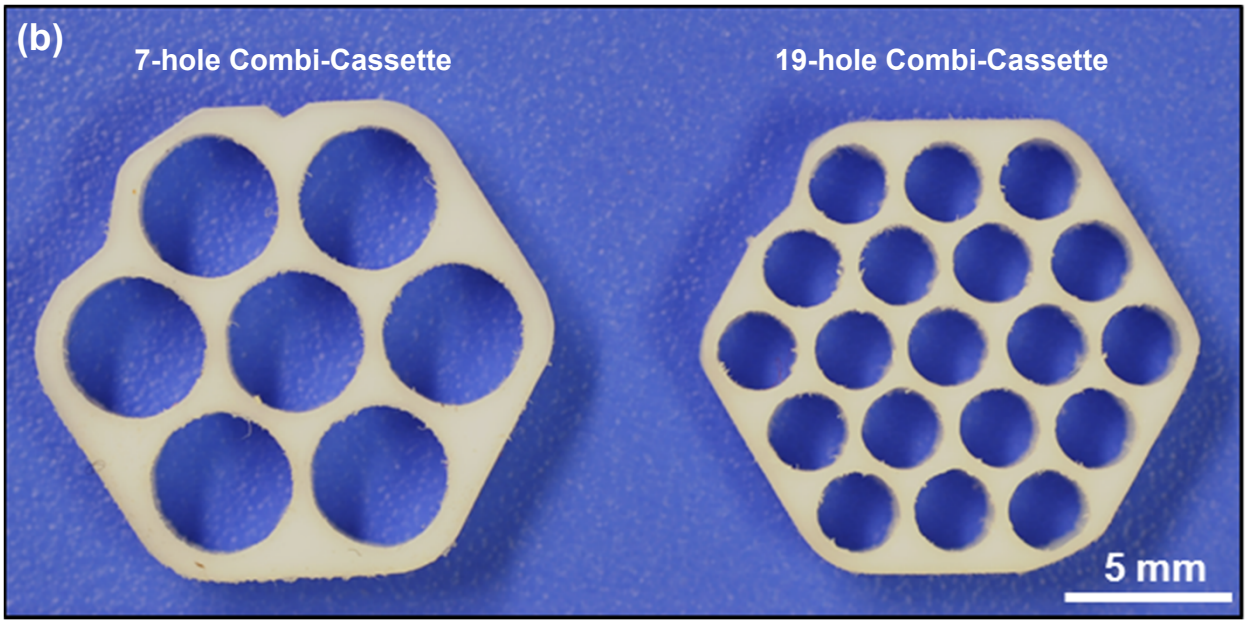
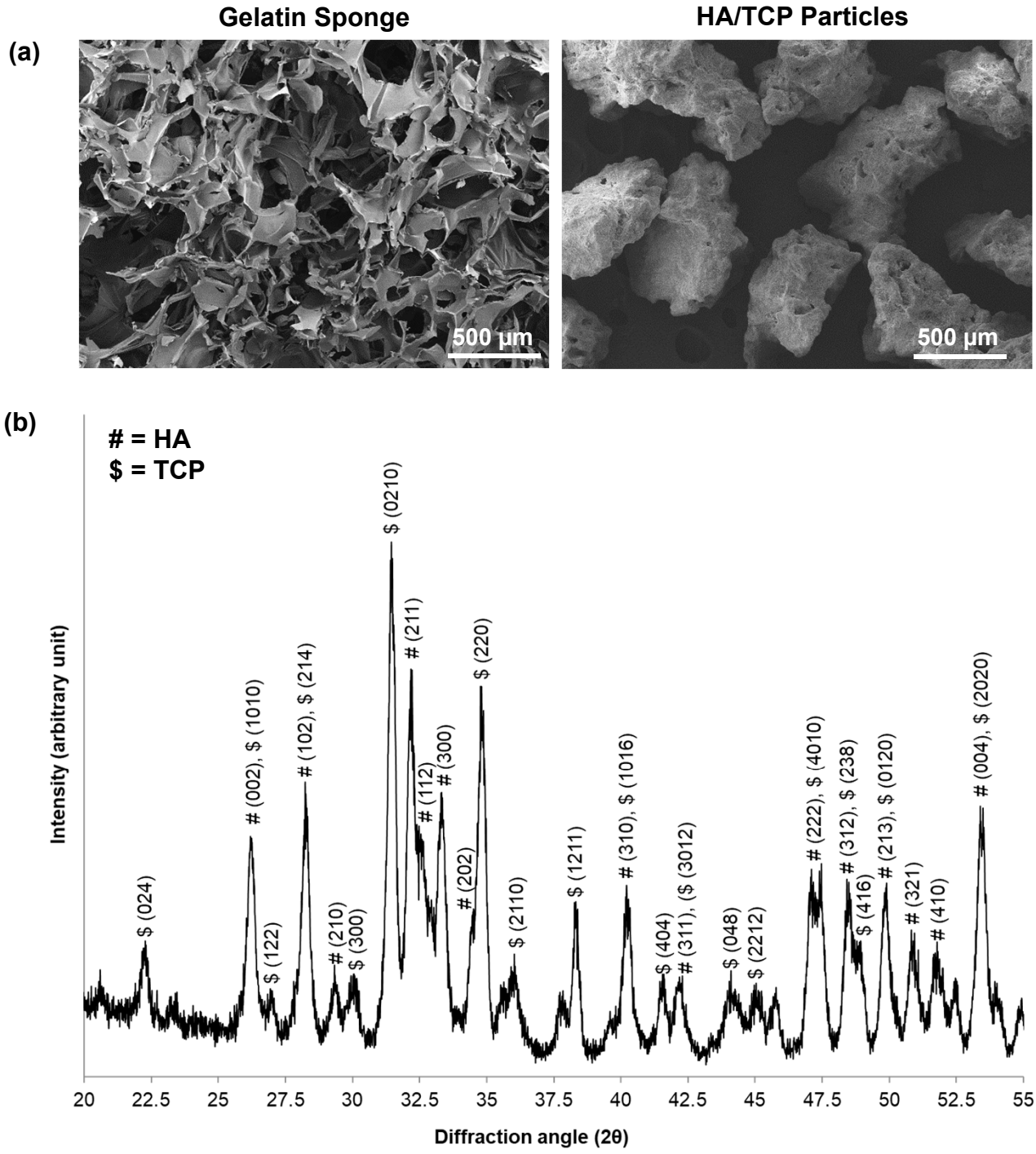




Supplementary Figure 1. (a) Illustration of dimensions of the 7-hole and 19-hole Combi-Cassettes. (b) Photograph of PTFE Combi-Cassettes as fabricated using a laser cutter.



Supplementary Figure 2. (a) Scanning electron micrographs showing microstructure of gelatin sponges and biphasic hydroxyapatite/tricalcium phosphate (HA/TCP) particles. (b) X-ray diffraction pattern of HA/TCP particles. The peaks at 32.18^{o} [plane (211)], 32.48^{o} [plane (112)], and 33.30^{o} [plane (300)] establish the presence of HA in the biphasic HA/TCP powder [as per Joint Committee on Powder Diffraction Standards (JCPDS) card number 09-0432]. The peaks at 28.24^{o} [plane (214)], 31.45^{o} [plane (0210)], and 34.77^{o} [plane (220)] establish the presence of TCP in the biphasic HA/TCP powder (as per JCPDS card number 09-0169).

Supplementary Figure 3. Experimental Summary

Experiment #1 – 7-Hole Combi-Cassettes with hBMSCs+HA/TCP

	Combi-Cassettes			Regular Constructs	
Constructs/Mouse	7-hole Combi-Cassette, 1 Cassette per Mouse			2 Regular Constructs per Mouse	
Type of Constructs	Osteogenic	Non-Osteogenic	Non-Osteogenic	Osteogenic	Non-Osteogenic
Construct Components	hBMSCs+HA/TCP +Fibrin Gel*	hBMSCs+ Gelatin Sponge	HA/TCP alone +Fibrin Gel*	hBMSCs+ HA/TCP	hBMSCs+ Gelatin Sponge
# of Constructs	3/Cassette	3/Cassette	1/Cassette	1/Mouse	1/Mouse
Scaffold Weight or Size[a]	HA/TCP 40 mg per Batch (≈12 mg per Hole)	Gelatin Sponge 5 x 5 x 4 mm	HA/TCP 40 mg per Batch (≈12 mg per Hole)	40 mg HA/TCP	Gelatin Sponge 8 mm x 8 mm x 4 mm
hBMSCs[b]	2.0 x 10^6 per Batch (≈600,000 per Hole)	≈0.75 x 10^6	0	≈2.0 x 10^6	≈2.0 x 10^6
Fibrin Gel[c]	≈30 µL Gel per Batch (15 µL Fibrinogen + 15 µL Thrombin) (≈9 µL Gel per Hole)	0	≈30 µL Gel per Batch (15 µL Fibrinogen + 15 µL Thrombin) (≈9 µL Gel per Hole)	0	0
Mice & Time Point	3 NSG Mice for 8 Weeks				

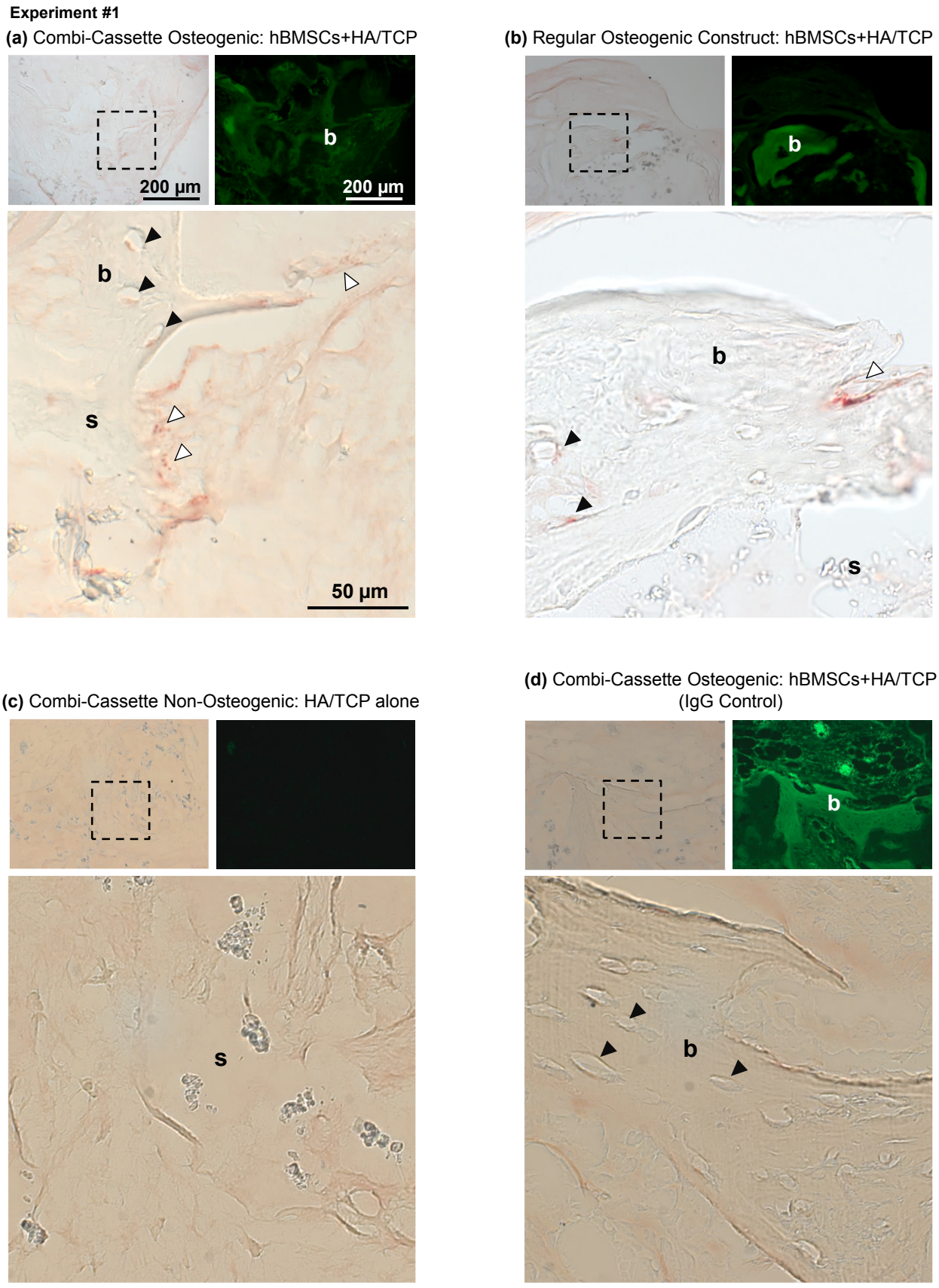
Experiment #2 – 19-Hole Combi-Cassettes with hBMSCs+HA/TCP

	Combi-Cassettes		Regular Constructs	
Constructs/Mouse	19-hole Combi-Cassette, 1 Cassette per Mouse		2 Regular Constructs per Mouse	
Type of Constructs	Osteogenic	Non-Osteogenic	Osteogenic	Non-Osteogenic
Construct Components	hBMSCs + HA/TCP + Fibrin Gel	HA/TCP + Fibrin Gel	hBMSCs + HA/TCP	HA/TCP alone
# of Constructs	11/Cassette	8/Cassette	1/Mouse	1/Mouse
Scaffold Weight or Size[a]	120 mg per Batch (≈10 mg HA/TCP per Hole)	120 mg per Batch (≈10 mg HA/TCP per Hole)	40 mg HA/TCP	40 mg HA/TCP
hBMSCs[b]	6 x 10^6 per Batch (≈500,000 per Hole)	0	≈2.0 x 10^6	0
Fibrin Gel[c]	≈90 µL per Batch (45 µL Fibrinogen + 45 µL Thrombin) (≈8 µL per Hole)	≈90 µL per Batch (45 µL Fibrinogen + 45 µL Thrombin) (≈8 µL per Hole)	0	0
Mice & Time Point	3 NSG Mice for 8 Weeks			

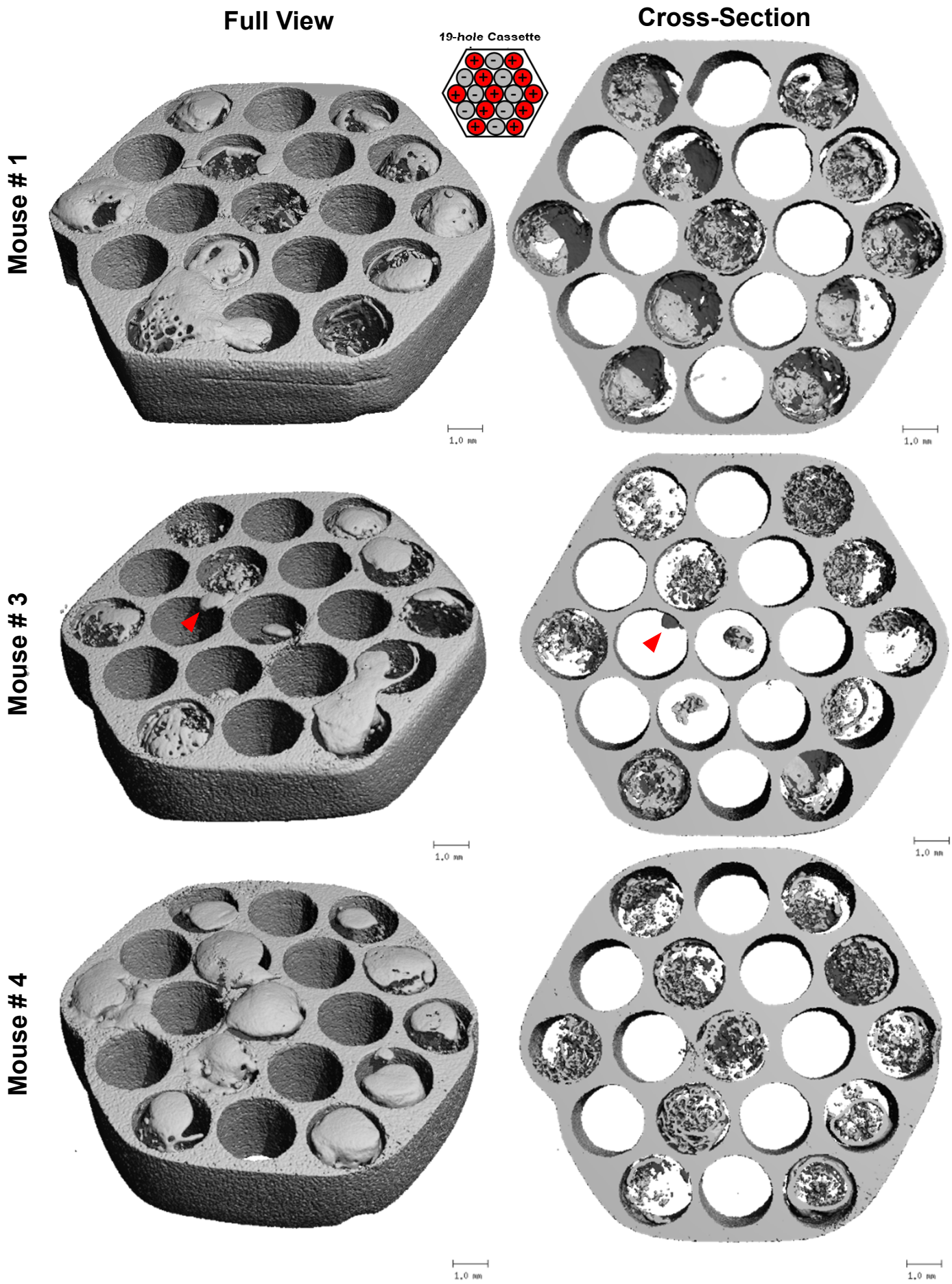
Experiment #3 – 19-Hole Combi-Cassettes with BMP2+GS

	Combi-Cassettes		Regular Construct	
Constructs/Mouse	19-hole Combi-Cassette, 1 Cassette per Mouse		2 Regular Constructs per Mouse	
Type of Constructs	Osteogenic	Non-Osteogenic	Osteogenic	Non-Osteogenic
Construct Components	BMP2 + Gelatin Sponge	Gelatin Sponge Alone	BMP2 + Gelatin Sponge	Gelatin Sponge Alone
# of Construct	11 per Combi-Cassette	8 per Combi-Cassette	1 per Mouse	1 per Mouse
Scaffold Size	4 mm x 4 mm x 4 mm Gelatin Sponge	4 mm x 4 mm x 4 mm Gelatin Sponge	8 mm x 8 mm x 4 mm Gelatin Sponge	8 mm x 8 mm x 4 mm Gelatin Sponge
BMP2	4 µg per Scaffold (8 µL of BMP2 at 0.5 µg/µL)	0 µg per Scaffold (8 µL of Vehicle)	5 µg per Scaffold (10 µL of BMP2 at 0.5 µg/µL)	0 µg per Scaffold (10 µL of Vehicle)
Mice & Time Point	6 FVB Mice for 8 Weeks			

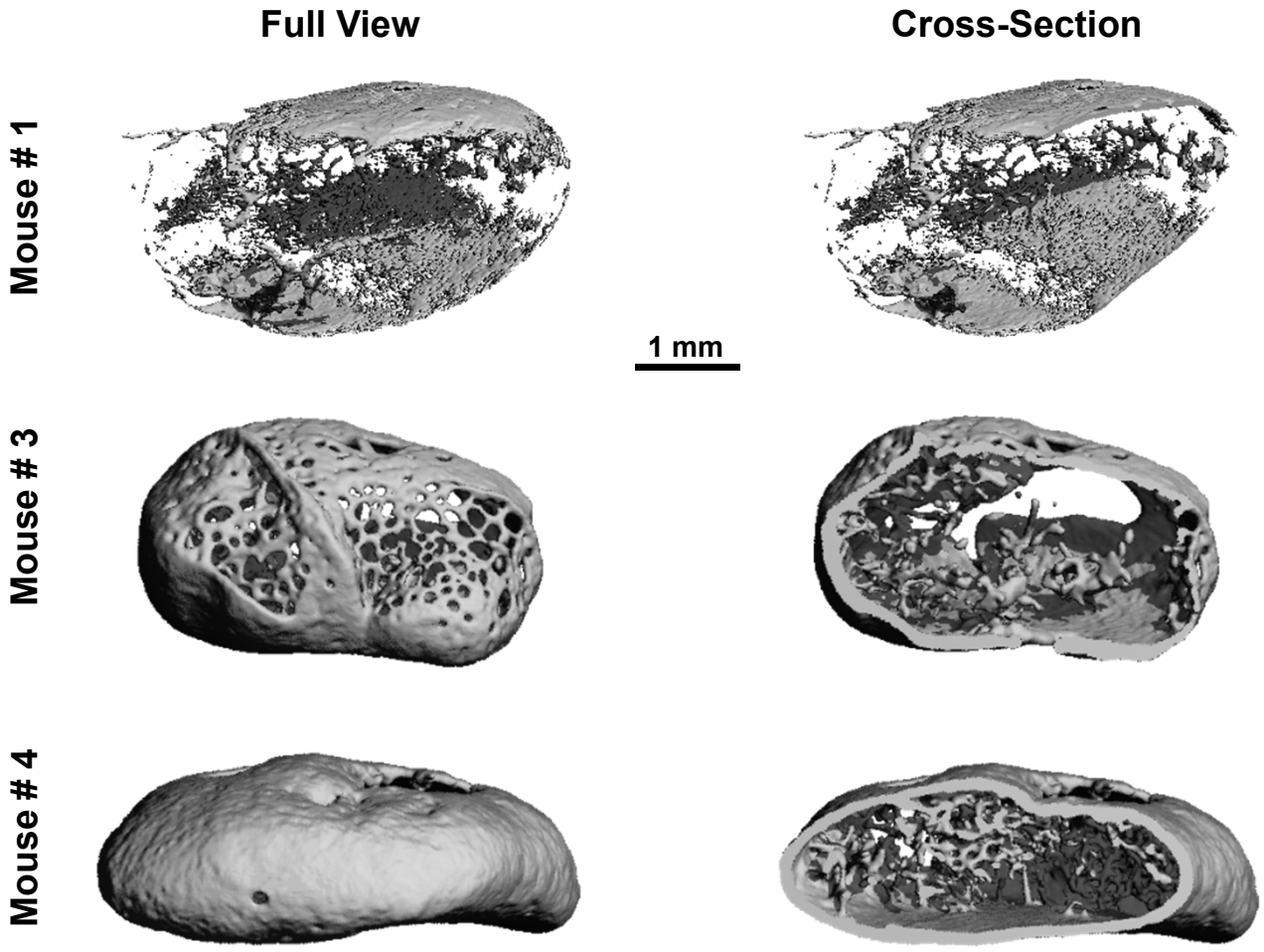
[a] For combi-cassettes, constructs were made in batches and then distributed amongst the holes. Thus, the amount of HA/TCP per hole is approximate.
[b] The number of hBMSCs per hole is approximate since it depends upon the fraction of the hBMSCs that adhere to the HA/TCP particles.
[c] Fibrin gel swells during polymerization so final volumes are approximate.



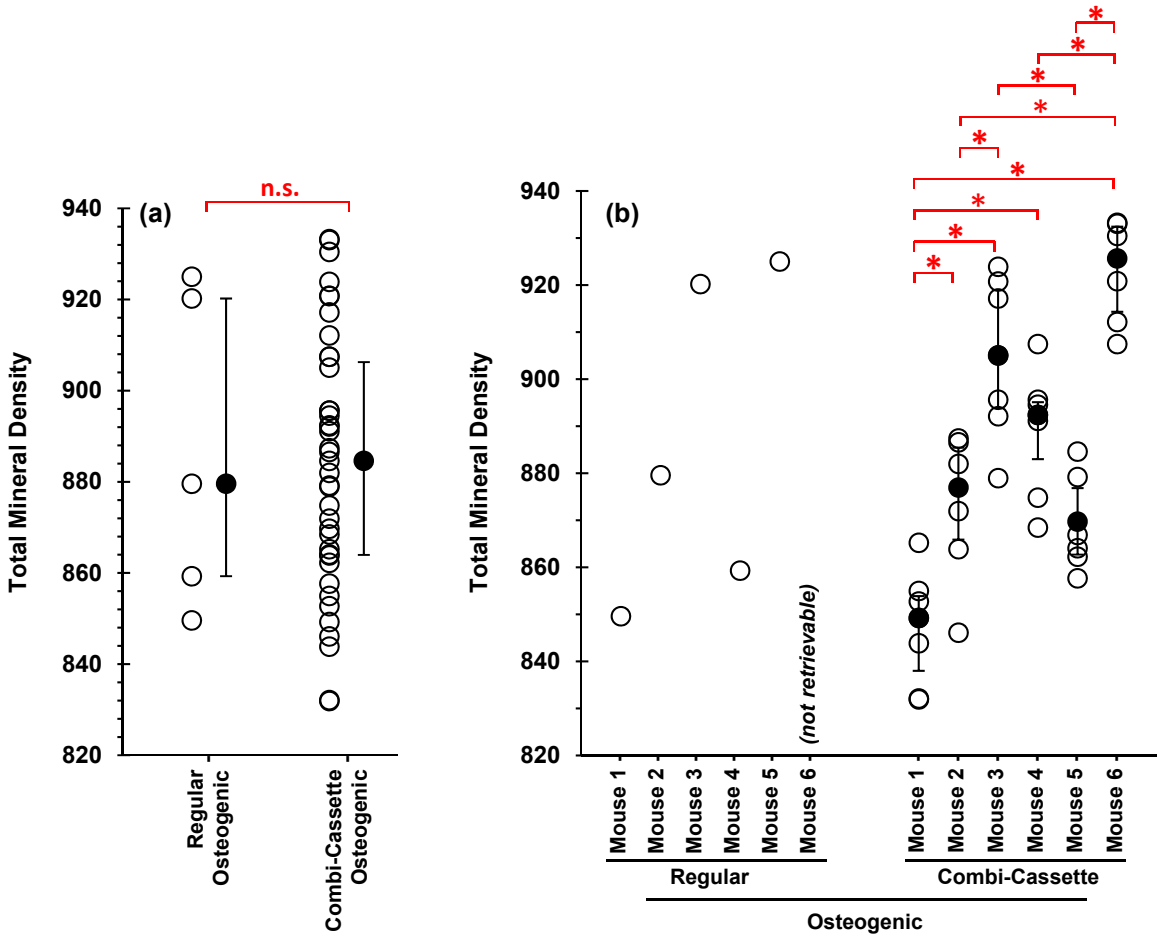
Supplementary Figure 4. Experiment #1 – Human mitochondrial staining (brown) for the 7-hole experiment (hBMSCs+HA/TCP) after 8 weeks in vivo. For each panel, the upper-left image is brightfield (200X), the upper-right image is the same field of view for auto-fluorescence to show bone and the bottom panel is a magnified brightfield view (400X) of the outlined area from the upper-left panel. Panel (d) is negative control IgG staining, where a non-immune immunoglobulin IgG was used. *Labels:* black arrowheads – osteocytes; white arrowheads – osteoblasts; b – bone; s – scaffold (HA/TCP). Brown staining visible in panels (a) and (b) indicates cells of human origin.



Supplementary Figure 5a. Experiment #3 - 19-hole combi-cassette experiment using BMP2+Gelatin Sponges after 8 weeks in vivo. X-ray microcomputed tomography 3D reconstructions of 19-hole combi-cassettes from three mice. Osteogenic – BMP2+Gelatin Sponge. Non-Osteogenic – Gelatin Sponge. 19-hole layout of osteogenic (red) and non-osteogenic constructs (grey) is shown at the top of the figure. The red arrowheads show some spillover of BMP2 from a hole that was loaded with an osteogenic construct into a hole that was loaded with a non-osteogenic construct.



Supplementary Figure 5b. Experiment #3 – 19-hole Combi-Cassette experiment using BMP2+Gelatin Sponges after 8 weeks in vivo. X-ray microcomputed tomography 3D reconstructions of regular osteogenic constructs (BMP2+Gelatin Sponge) from 3 mice.



Supplementary Figure 6. Experiment #3 - "Total mineral density" (TMD) determined by μCT for the 19-hole Combi-Cassette experiment using BMP2+Gelatin Sponges after 8 weeks in vivo. (a) TMD values (n= 5 and 39 constructs, left to right). For osteogenic constructs, there was no significant difference in TMD for the bone formed in combi-cassettes osteogenic and in regular osteogenic constructs (t-test, P=0.88). (b) Mouse-to-mouse variability in TMD (n=1, 1, 1, 1, 1, 0, 7, 6, 7, 7, 6 and 6 constructs, left to right). For combi-cassette osteogenic constructs, Mouse 1 was significantly different from all other mice except Mouse 5, Mouse 2 was significantly different from Mouse 3, and Mouse 6, and Mouse 3 was significantly different from Mouse 5, and Mouse 4 was significantly different from Mouse 6 (1-way ANOVA with Tukey's test, P=0.02). (a,b) Open circles are individual data points and closed circles are medians (error bars are first and third quartiles).

Experiment #1 – 7-hole Combi-Cassette: hBMSCs+HA/TCP

Bone Scores (1-Way ANOVA with Tukey's)			
Comparisons			P-Value
Combi-Cassette Osteogenic	vs	Combi-Cassette Non-Osteogenic (Gelatin Sponge)	<0.001
Combi-Cassette Osteogenic	vs	Combi-Cassette Non-Osteogenic (HA/TCP alone)	0.007
Combi-Cassette Osteogenic	vs	Regular Osteogenic	0.420
Combi-Cassette Osteogenic	vs	Regular Non-Osteogenic (HA/TCP alone)	0.002
Combi-Cassette Non-Osteogenic (Gelatin Sponge)	vs	Combi-Cassette Non-Osteogenic (HA/TCP alone)	0.979
Combi-Cassette Non-Osteogenic (Gelatin Sponge)	vs	Regular Osteogenic	<0.0001
Combi-Cassette Non-Osteogenic (Gelatin Sponge)	vs	Regular Non-Osteogenic (HA/TCP alone)	1.000
Combi-Cassette Non-Osteogenic (HA/TCP alone)	vs	Regular Osteogenic	0.001
Combi-Cassette Non-Osteogenic (HA/TCP alone)	vs	Regular Non-Osteogenic (HA/TCP alone)	0.989
Regular Osteogenic	vs	Regular Non-Osteogenic (HA/TCP alone)	<0.001

Bone Scores: Mouse-to-Mouse Differences in Combi-Cassette Osteogenic (1-Way ANOVA with Tukey's)			
Comparisons			P-Value
Mouse 1	vs	Mouse 2	0.602
Mouse 1	vs	Mouse 3	0.182
Mouse 2	vs	Mouse 3	0.500

Experiment #2 – 19-hole Combi-Cassette: hBMSCs+HA/TCP

Bone Scores (1-Way ANOVA with Tukey's)			
Comparisons			P-Value
Combi-Cass. Osteogenic	vs	Combi-Cass. Non-Osteo.	<0.001
Combi-Cass. Osteogenic	vs	Regular Osteogenic	0.998
Combi-Cass. Osteogenic	vs	Regular Non-Osteo.	<0.001
Combi-Cass. Non-Osteo.	vs	Regular Osteogenic	0.002
Combi-Cass. Non-Osteo.	vs	Regular Non-Osteo.	0.946
Regular Osteogenic	vs	Regular Non-Osteo.	0.009

Bone Scores (T-Test)				
Comparisons				P-Value
Mouse 1	Combi-Cassette Osteogenic	vs	Combi-Cassette Non-Osteogenic	0.027
Mouse 2	Combi-Cassette Osteogenic	vs	Combi-Cassette Non-Osteogenic	<0.0001
Mouse 3	Combi-Cassette Osteogenic	vs	Combi-Cassette Non-Osteogenic	<0.0001

Bone Scores: Mouse-to-Mouse Differences in Combi-Cassette Osteogenic (1-Way ANOVA with Tukey's)			
Comparison			P-Value
Mouse 1	vs	Mouse 2	0.865
Mouse 1	vs	Mouse 3	0.027
Mouse 2	vs	Mouse 3	0.082

TMD Values from µCT (T-Test)			
Comparison			P-Value
Combi-Cass. Osteogenic	vs	Regular Osteogenic	0.877

Experiment #3 – 19-hole Combi-Cassette: BMP2+Gelatin Sponge

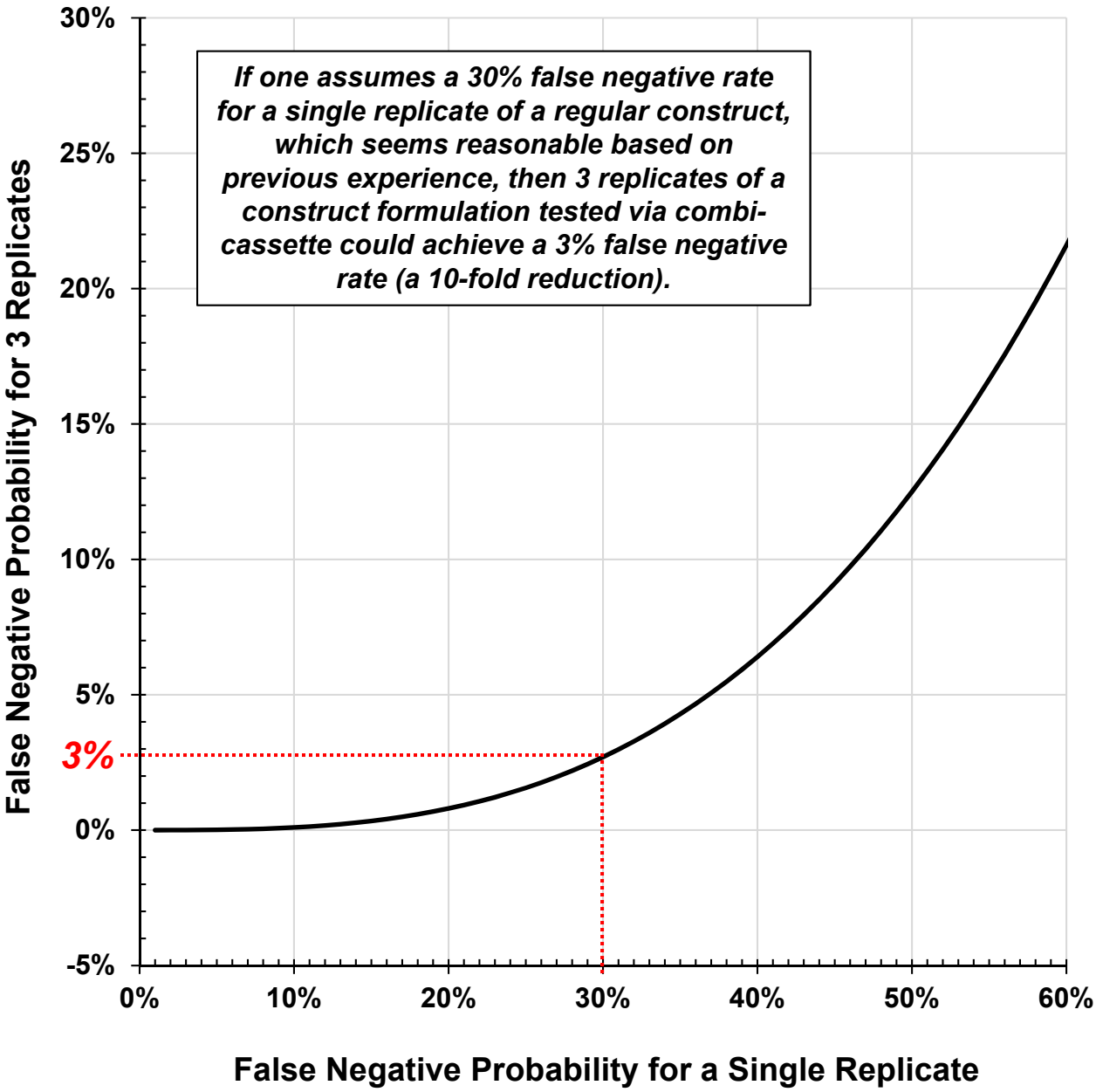
BV/TV Values from µCT (1-Way ANOVA with Tukey's)			
Comparison			P-Value
Combi-Cassette Osteogenic	vs	Combi-Cassette Non-Osteogenic	<0.0001
Combi-Cassette Osteogenic	vs	Regular Osteogenic	0.2512
Combi-Cassette Non-Osteogenic	vs	Regular Osteogenic	<0.0001

BV/TV Values from µCT: Mouse-to-Mouse Differences in Combi-Cassette Osteogenic (1-Way ANOVA with Tukey's)			
Comparison			P-Value
Mouse 1	vs	Mouse 2	0.972
Mouse 1	vs	Mouse 3	0.210
Mouse 1	vs	Mouse 4	0.184
Mouse 1	vs	Mouse 5	0.071
Mouse 1	vs	Mouse 6	0.011
Mouse 2	vs	Mouse 3	0.679
Mouse 2	vs	Mouse 4	0.636
Mouse 2	vs	Mouse 5	0.347
Mouse 2	vs	Mouse 6	0.087
Mouse 3	vs	Mouse 4	1.000
Mouse 3	vs	Mouse 5	0.988
Mouse 3	vs	Mouse 6	0.727
Mouse 4	vs	Mouse 5	0.993
Mouse 5	vs	Mouse 6	0.767
Mouse 5	vs	Mouse 6	0.975

TMD Values from µCT: Mouse-to-Mouse Differences in Combi-Cassette Osteogenic (1-Way ANOVA with Tukey's)			
Comparison			P-Value
Mouse 1	vs	Mouse 2	0.027
Mouse 1	vs	Mouse 3	<0.001
Mouse 1	vs	Mouse 4	<0.001
Mouse 1	vs	Mouse 5	0.075
Mouse 1	vs	Mouse 6	<0.001
Mouse 2	vs	Mouse 3	0.008
Mouse 2	vs	Mouse 4	0.135
Mouse 2	vs	Mouse 5	0.998
Mouse 2	vs	Mouse 6	<0.001
Mouse 3	vs	Mouse 4	0.815
Mouse 3	vs	Mouse 5	0.002
Mouse 3	vs	Mouse 6	0.103
Mouse 4	vs	Mouse 5	0.053
Mouse 4	vs	Mouse 6	0.005
Mouse 5	vs	Mouse 6	<0.001

PCR Relative Gene Expression: 1-Way ANOVA with Tukey's					
Comparisons			P-Value		
			Runx2	*Ibsp*	*Bglap*
Combi-Cassette Osteogenic	vs	Combi-Cassette Non-Osteogenic	0.0036	0.0055	0.0006
Combi-Cassette Osteogenic	vs	Regular Osteogenic	0.6584	0.4458	0.4554
Combi-Cassette Osteogenic	vs	Regular Non-Osteogenic	0.0055	0.0069	0.0007
Combi-Cassette Non-Osteogenic	vs	Regular Osteogenic	0.1193	0.2667	0.0531
Combi-Cassette Non-Osteogenic	vs	Regular Non-Osteogenic	0.9982	0.9948	0.9810
Regular Osteogenic	vs	Regular Non-Osteogenic	0.1189	0.2289	0.0391

Supplementary Figure 7. Results from statistical analyses. Grey shading indicates P<0.05.



Supplementary Figure 8. Plot of "false negative" rate for a single replicate versus three replicates. A conservative estimate of the false negative rate for the mouse subcutaneous construct test is 30%. In other words, when using a single replicate, 30% of the time the test may misidentify an Osteogenic bone graft formulation as "Non-Osteogenic" (for a myriad of reasons), which is represented by the red dotted line on the x-axis. If Combi-Cassettes are used to test graft formulations in triplicate, then the false negative rate drops to approximately 3% (30% × 30% × 30% ≈ 3%), which is represented by the red dotted line on the y-axis. Thus, a 10-fold reduction in the false negative rate may be achieved by increasing from 1 replicate to 3 replicates.