Published in final edited form as:

Wound Repair Regen. 2018 September ; 26(5): 359–365. doi:10.1111/wrr.12658.

Involvement of Calvarial Stem Cells in Healing: A Regional Analysis of Large Cranial Defects

Emily L Durham, M.A.¹, Rebecca N. Howie, PhD¹, Reed Houck¹, Brayden Oakes¹, Zachary Grey¹, SarahRose Hall¹, Martin Steed, DDS², Amanda LaRue, PhD^{3,4}, Robin Muise-Helmericks, PhD⁵, and James Cray, PhD^{1,3,5,6}

¹Department of Oral Health Sciences, MUSC, Charleston, SC

²Department of Oral and Maxillofacial Surgery, MUSC, Charleston, SC

³Department of Pathology and Laboratory Medicine, MUSC, Charleston, SC

⁴Ralph H. Johnson VA Medical Center, Charleston, SC

⁵Department of Regenerative Medicine and Cellular Biology MUSC, Charleston, SC

⁶Division of Anatomy, College of Medicine, The Ohio State University, Columbus OH

Abstract

Large craniofacial defects present a substantial clinical challenge that often requires the use of osteoconductive matrices and osteoinductive cues (i.e. Bone Morphogenetic Proteins (BMP2)) to augment healing. While these methods have improved clinical outcomes, a better understanding of how the osteogenic fronts surrounding the defect, the underlying dura mater, and the cranial suture area contribute to healing may lead to more targeted therapies to enhance bone regeneration. We hypothesized that healing within a large bone defect will be precipitated from cells within the remaining or available suture mesenchyme abutting the edges of a murine critical sized defect. To investigate this hypothesis, 39 adult, wild-type mice were randomly arranged into groups (9 or 10 per group) by time (4 and 8 weeks), and treatment (control, acellular collagen sponge (ACS) alone, or ACS loaded with a clinically relevant scaled dosage of BMP2). The skulls were then subjected to μ CT and histological analysis to assess bone regeneration in regions of interest within the defect area. A regional assessment of healing indicated that BMP2 drives greater healing than control and that healing emanates from the surgical margin, particularly from the margin associated with undisrupted suture mesenchyme. Though BMP2 treatment drove an increase in cell presence within the healing defect, there was no regional orientation of craniofacial stem cells or vascularity. Overall, these data reinforce that osteoconductive matrices in conjunction with osteoinductive peptides result in better healing of large calvarial defects. This healing is characterized as emanating from the surgical margin where there is an abundant supply of vasculature and progenitor cells.

Corresponding Author: James Cray Jr., Ph.D., Associate Professor, The Ohio State University, College of Medicine, Division of Anatomy, 1645 Neil Ave., 279 Hamilton Hall, Columbus, OH 43210, 614-292-4831, cray.30@osu.edu.

None of the authors has a financial interest in any of the products or devices mentioned in this article.

Keywords

Stem Cell; Bone Wound; Cranial Defect

Introduction:

Bone is a dynamic and highly vascularized tissue that continues to remodel throughout life and has the innate capacity for healing upon damage. However, bone injuries and defects resulting from trauma or iatrogenic (e.g. tumor removal) sources present a major problem in craniofacial surgery, as the regenerative process often fails and surgical intervention to augment natural bone wound repair is required (1). For clinicians, knowing which anatomical tissues play key roles in healing can help shape a treatment plan. While autologous bone grafting remains the standard of care due to its consistent clinical outcome (2), its use is severely hampered by short supply and considerable donor site morbidity associated with the harvest (3, 4). The field of bone tissue engineering provides alternative treatment options; (5) however, there are still gaps in knowledge concerning mechanisms by which defects fail to heal natively and how engineered scaffolds augment the healing process.

The composite tissue environment surrounding a cranial defect contributes to a cascade of effects to allow for the regenerative capabilities inherent to bone. The osteogenic fronts surrounding the defect aid healing likely by donating progenitor cells and endogenous blood supply (6–8). Retention of a craniofacial suture near the area of defect also appears to contribute to greater healing. This may be due to the sutures containing certain mesenchymal cell types, such as Gli1⁺ cells that have been hypothesized to contribute to cranial bone healing (9, 10). Additionally, the periosteum overlying the cranial bone and the dura mater underlying calvarial bone contain key cell populations and growth factors that participate in bone regeneration (11, 12).

Studies have shown that the use of osteoconductive matrices can increase the healing of a bone defect by creating an artificial gap between the bony edges that can be slowly turned over simulating indirect healing (13–15). One current option for augmentation of bone healing utilized heavily in the fields of orthopaedic and craniofacial surgery is the use of an acellular collagen sponge (ACS) (16) in combination with FDA approved Bone Morphogenic Protein 2 (BMP2) (INFUSE® Bone Graft (Medtronic, Minneapolis, MN). BMP2 is an osteogenic peptide appearing to have a synergistic effect with endogenous osteogenic signaling (17–19). In this study, we aimed to determine what factors play a role in the healing process by using a regional analysis of a murine critical sized defect treated with an osteoconductive scaffold (ASC) and a clinically relevant dose of BMP2 (20, 21). As calvarial suture derived stem cells have been hypothesized to contribute to bone wound healing, we posit that healing will be greater in approximation to undisturbed suture mesenchyme at the edge of the defect and that stem cells will play a vital role in the healing process.

Materials and Methods:

Animals

Adult (8 weeks old) C57BL6 wildtype male and female mice were randomly assigned 5 per group into one of 2 treatment groups: A) Control, implanted with ACS matrix with PBS and B) BMP2, implanted with ACS matrix soak loaded with 325 ng BMP2. This sub-clinical dosage of BMP2 was used as it has previously been identified to precipitate quality bone regenerate in this model (22, 23). A 5mm critical sized calvarial defect was created according to established protocol(22–24). Briefly, the mice were anesthetized using isoflurane (Bethlehem, PA, USA), their scalps were shaved, and a midline 5 mm craniectomy defect was trephined using a slow-speed hand drill. Care was taken to leave the dura mater intact, however the periosteum was necessarily removed overlying the defect site. The surgical sites were then treated as detailed above and the incision was closed with a 6×0 polypropylene suture. Mice were monitored for 48 hours post-surgery for signs of inflammation and distress including over grooming, altered water intake, and wound dehiscence. Half of the mice (n=10 control, n=9 BMP) were euthanized 4 weeks post-surgery, and the remaining mice (n=10 control, n=10 BMP) were euthanized 8 weeks post-surgery using CO₂ gas followed by a cervical spine dislocation. Following sacrifice, skulls were collected and bisected from the occipital protuberance to nasale and the surgical sites were subjected to micro computed tomography (μCT) analyses.

Animal Use Protocols were approved by the Medical University of South Carolina Institutional Animal Care and Use Committee (AR#3452). All procedures were carried out in an Association for Assessment and Accreditation of Laboratory Animal Care International accredited facility where husbandry and related services are provided by the Division of Laboratory Animal Resources. Procedures and reporting thereof are in compliance with the Animal Research: Reporting in Vivo Experiments (ARRIVE) guidelines (25).

Micro Computed Tomography Analysis

μCT images of the defects were obtained via *ex vivo* Skyscan 1176 (Skyscan, Aartlesaar, Belgium) utilizing a 0.5 mm thick aluminum filter at a voltage of 50 kV and current of 500 μA. Data were acquired at a resolution of 18 μm isotropic voxels, with a rotation step of 0.5°, and 180° rotation. Representative images were created using CTvol software v 2.3.2.0 r810 (Skyscan). Standard morphometric parameters were determined for all samples (26). Bone volume/tissue volume using a standardized volume of interest at four different regions within the 5 mm defect site: Margin; surgical margin not associated with calvarial suture, Posterior; surgical margin associated with the abutment of the sagittal and lambdoid sutures, Anterior; surgical margin associated with the abutment of the sagittal and coronal sutures, and Center: midline of the surgical defect where the sagittal suture was removed, were analyzed (Figure 1a).

Histological Staining and Cell Counts

After μCT scanning, representative samples (n=4/time/group) from the control and BMP2 treated groups at each timepoint were bisected in the coronal plane through the center for the

surgical defect and prepared for paraffin sectioning. Briefly, samples were placed in 3.7% formaldehyde for two days then decalcified in 0.25 M EDTA at pH 7.4 for 21 days. Samples were then washed, dehydrated in graded ethanol (70–100%), cleared in xylene, and embedded in paraffin. Histology and immunohistochemistry were performed on 3, 7 μ m sections in the coronal plane at least 40 μ m apart per sample for each analysis. Harris' hematoxylin (VWR, Radnor, PA, 10143–606) and Eosin (VWR, 95057–848) (H&E) staining proceeded by standard protocol. Images of the H&E stained defects were obtained using a Motic Inverted Microscope with attached camera (Motic, British Columbia, Canada) and analyzed using NIH ImageJ software to count nuclei. A mask was created for each image to count purple stained nuclei in the identified region of interest: center of the defect overlying the removed sagittal suture; intermediate not suture or margin related area; margin of the defect containing approximately 50% native bone (Figure 1b).

For immunohistochemistry, slides were deparaffinized in xylene and rehydrated in graded ethanol (100–0%). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and then washed. Sections were blocked in 1% goat serum with 1% bovine serum albumin. Sections were incubated with primary antibody (Gli1, NBP1–78259, 1:500, Novus Biologicals Littleton, CO; CD44, Ab157107, 1:1000, AbCam; PECAM, Ab28364, 1:50, AbCam) overnight at 4°C followed by washing and incubation with HRP conjugated secondary antibody (ab6721, AbCam) for one hour at room temperature. Diaminobenzidine (DAB; Vector Laboratories, Burlingame, CA) chromagen was used to identify immunoreactive structures. Sections were analyzed using Image J Software and the IHC Profiler Open Source Plugin for automated scoring of percent positivity within region of interest (Figure 1b) (27).

Statistics

Standard two-way ANOVA with post-hoc Bonferroni analyses were conducted where appropriate. Transformations of the data were performed where needed to correct for violations of homogeneity of variance or normality. Differences were considered significant if $p < 0.05$. Data are presented as means $\pm$ SE.

Results:

A regional assessment of healing in the form of bone volume/tissue volume (BV/TV) within a standardized volume indicated that BMP2 drove greater healing at both 4 and 8 weeks ($p < 0.001$, Figure 2). Analysis of healing within the defect via μ CT indicates greater healing over time. Additionally, the center of the defect (overlying the extirpated sagittal suture) precipitated less healing than the anterior and posterior regions of the defect associated with the remnants of the coronal and lambdoid suture mesenchyme, respectively, at both 4 and 8 weeks ($p < 0.01$). At 4 weeks, the margin not associated with existing suture presented with less healing as compared to the area associated with the coronal suture at the anterior of the defect ($p < 0.05$). By 8 weeks, the margin precipitated more healing than the center of the defect ($p < 0.001$).

Histologically, it appears that at 4 weeks, both groups present with disorganized regenerate throughout the defect site. Assessing the histological cell count at 4 weeks post-surgery, the

BMP treated defects contained more cells than the control defects ($p<0.01$). By 8 weeks this relationship was reversed (Figure 3). The regional assessment of cell density did not reveal any differences by region.

As there were no differences in the number of cells present in each region of the defect we investigated each region for stem cells to elucidate if the type of cell in each region drove the observed healing pattern. An investigation of Gli1, a marker of suture derived stem cells, revealed no statistically significant differences in proportion of stem cells within regions associated with the surgical margin, the extirpated sagittal suture, or an intermediate region unassociated with suture or surgical margin at either time point. Additionally, an assessment of CD44 positive cells also revealed no statistically significant differences by region, time, or treatment (Figure 4).

As differences in stem cell activity by location in this healing model were not observed, we assessed if the increased healing observed at the surgical margins could be correlated with increased vasculature. Staining for PECAM, a marker associated with blood vessels and angiogenesis, indicated increased PECAM staining with BMP2 treatment ($p<0.001$) and with time ($p<0.001$). However, our regional assessment of this staining did not reveal statistically significant differences by location (Figure 5).

Discussion:

Results confirmed the hypothesis that healing in the defect would be greater along the osteogenic front associated with undisturbed suture mesenchyme than in the center of the defect. However, our conjecture that this increased healing was due to an increased presence of stem cells from the suture mesenchyme was not supported. This result lends support to the hypothesis that availability of vasculature and/or other necessary signaling cascade (e.g., matrix proteins) may be responsible for this healing within a calvarial defect (6). The assessment of PECAM staining within the defect also supports this hypothesis with increased PECAM presence at the margin of the defect associated with native diploe and at the center associated with the undisturbed underlying sagittal sinus. When considering the margins of the defect, we found that BMP2 treatment could greatly augment healing, particularly in this region, reinforcing the principle that BMP2 intervention is a primary factor robustly augmenting healing along the osteogenic front (28).

Investigation of isolated regions within the defect site revealed that healing of the central portion of the defect is likely only influenced by the underlying dura mater and lacks the contribution of the osteogenic fronts until margins reapproximate over time allowing for more direct healing (8). Interestingly, our data indicate that there may be an increased presence of stem cells (Gli1) in the center of the defect overlying the extirpated sagittal suture. The presence of this marker within the entire defect area may be evidence of the role of Gli1 cells in healing in general (9, 29). Further, though our assessment did not reveal statistically significant differences in stem cell location within the healing defect, the specific distribution that we observed could be biologically relevant to the healing process (30, 31). Our results show that BMP2 can drive healing at the center of the defect, potentiating “ectopic bone” that can later be incorporated and remodeled. However, without

augmentation by this powerful peptide, little healing occurs, suggesting the prior presence (potentially imprinting of signals within the underlying dura) of the sagittal suture contributes very little to healing in this model (11, 12).

Use of an osteoinductive peptide (BMP2) along with an osteoconductive matrix (ACS) precipitated the greatest amount of bone growth in this experiment, reinforcing previous studies (22–24, 28, 32). Further, this treatment modality demonstrated the greatest amount of cellular infiltration at the 4 week time point. Investigating the cell counts along with the corresponding μ CT analyses, it appears that a greater amount of cellular infiltration at 4 weeks post-op correlates, not unexpectedly, to more healing. The 8 week histological sections, particularly those from the BMP group are thinner and more organized than the 4 week sections indicating both a degradation of the osteoconductive scaffold and a remodeling of naïve bone. These data suggest that the ability to recruit as many regenerative cells as possible to the defect site is crucial in the process of bone healing. Additionally, it may be that in compromised healing there is a deficiency in available cells for healing. Interestingly, we found that there were comparatively fewer CD44 stem cells recruited to the defect site at 4 weeks for the BMP treated group. This may be an indication of a change in the stepwise timing of the bone healing process indicating that stem cells may contribute earlier in the healing process resulting in the abundance of differentiated cells observed at 4 weeks (33–35). Our hypothesis that stem cells are contributing to this healing process was not validated at these time points.

Overall, employing osteoconductive matrices in conjunction with osteoinductive peptides results in better healing of large bony defects of the calvarium. This healing appears to emanate from the surgical margin where there is an abundant supply of vasculature and both hematopoietic and mesenchymal progenitor cells.

Acknowledgments

Source of funding:

This work was supported by the AO Foundation [S-16–108C (JC)]; NIH/NIDCR [5T32DE017551]; [F31DE026684 to ELD]; NIH/NIGM [P30GM103331]; and research support toward this study was received from Medtronic Sofamor Danek USA. F31DE026684 to ELD. This study utilized the facilities and resources of the MUSC Center for Oral Health Research (COHR).

References:

1. Mountziaris PM, Mikos AG. Modulation of the inflammatory response for enhanced bone tissue regeneration. *Tissue Eng Part B Rev* 2008;14(2):179–86. [PubMed: 18544015]
2. Bauer TW, Muschler GF. Bone graft materials. An overview of the basic science. *Clinical orthopaedics and related research* 2000(371):10–27.
3. Silber JS, Anderson DG, Daffner SD, Brislin BT, Leland JM, Hilibrand AS, et al. Donor site morbidity after anterior iliac crest bone harvest for single-level anterior cervical discectomy and fusion. *Spine (Phila Pa 1976)* 2003;28(2):134–9. [PubMed: 12544929]
4. Sen MK, Miclau T. Autologous iliac crest bone graft: should it still be the gold standard for treating nonunions? *Injury* 2007;38 Suppl 1:S75–80. [PubMed: 17383488]
5. Grabowski G, Cornett CA. Bone graft and bone graft substitutes in spine surgery: current concepts and controversies. *The Journal of the American Academy of Orthopaedic Surgeons* 2013;21(1):51–60. [PubMed: 23281471]

6. Shenag Deana SD. Characterization of Reversibly Immortalized Calvarial Mesenchymal Progenitor Cells. *Journal of Craniofacial Surgery*;26(4):1207–13.
7. Maruyama T, Jeong J, Sheu TJ, Hsu W. Stem cells of the suture mesenchyme in craniofacial bone development, repair and regeneration. *Nat Commun* 2016;7:10526. [PubMed: 26830436]
8. Savaridas T, Wallace RJ, Salter DM, Simpson AHRW. Do bisphosphonates inhibit direct fracture healing? *The Bone & Joint Journal* 2013;95(B):1263–8.
9. Park Shery S Sutures Possess Strong Regenerative Capacity for Calvarial Bone Injury. *Stem Cells and Development* 2016;25(23):1801–7. [PubMed: 27762665]
10. Zhao Hu H The suture provides a niche for mesenchymal stem cells of craniofacial bones. *Nature Cell Biology*;17(4):386–96.
11. Cooper Gregory MG. Tissue interactions between craniosynostotic dura mater and bone. *Journal of Craniofacial Surgery*;23(3):919–24.
12. Greenwald JAJ. Immature versus mature dura mater: II. Differential expression of genes important to calvarial reossification. *Plastic and Reconstructive Surgery*;106(3):630–8. [PubMed: 10987470]
13. Stetzer Kraig K Effects of fixation type and guided tissue regeneration on maxillary osteotomy healing in rabbits. *Journal of Oral and Maxillofacial Surgery*;60(4):427–36.
14. Mundell RDR. Osseous guided tissue regeneration using a collagen barrier membrane. *Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons*;51(9):1004–12.
15. Verschuere DSD. The effects of guided tissue regeneration (GTR) on modified Le Fort I osteotomy healing in rabbits. *International Journal of Oral and Maxillofacial Surgery*;34(6):650–5. [PubMed: 16053889]
16. Geiger MM. Collagen sponges for bone regeneration with rhBMP-2. *Advanced Drug Delivery Reviews* 2003;55(12):1613–29. [PubMed: 14623404]
17. Cray James J BMP-2-regenerated calvarial bone: a biomechanical appraisal in a large animal model. *Annals of Plastic Surgery*;73(5):591–7. [PubMed: 23657046]
18. MacIsaac Zoe MZ. Novel animal model of calvarial defect: part IV. Reconstruction of a calvarial wound complicated by durotomy. *Plastic and Reconstructive Surgery*;131(4).
19. Herberg Samuel S Mesenchymal stem cell expression of SDF-1 α synergizes with BMP-2 to augment cell-mediated healing of critical-sized mouse calvarial defects. *Journal of tissue engineering and regenerative medicine*;11(6):1806–19.
20. Schmitz JPI. The critical size defect as an experimental model for craniomandibulofacial nonunions. *Clinical orthopaedics and related research* (205):299–308.
21. Mooney M, Siegel M. Animal models for bone tissue engineering of critical-sized defects (CSDs), bone pathologies, and orthopedic disease states. *Fundamentals of Bone Tissue Engineering* 2005:217–44.
22. Herberg S, Kondrikova G, Periyasamy-Thandavan S, Howie RN, Elsalanty ME, Weiss L, et al. Inkjet-based biopatterning of SDF-1 β augments BMP-2-induced repair of critical size calvarial bone defects in mice. *Bone* 2014;67:95–103. [PubMed: 25016095]
23. Herberg S, Susin C, Pelaez M, Howie RN, Moreno de Freitas R, Lee J, et al. Low-dose bone morphogenetic protein-2/stromal cell-derived factor-1 β cotherapy induces bone regeneration in critical-size rat calvarial defects. *Tissue engineering Part A* 2014;20(9–10):1444–53. [PubMed: 24341891]
24. Cray J, Jr., Henderson SE, Smith DM, Kinsella CR, Jr., Bykowski M, Cooper GM, et al. BMP-2-regenerated calvarial bone: a biomechanical appraisal in a large animal model. *Ann Plast Surg* 2014;73(5):591–7. [PubMed: 23657046]
25. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol* 2010;8(6):e1000412. [PubMed: 20613859]
26. Bouxsein ML, Boyd SK, Christiansen BA, Guldberg RE, Jepsen KJ, Muller R. Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. *J Bone Miner Res* 2010;25(7):1468–86. [PubMed: 20533309]

27. Varghese F, Bukhari AB, Malhotra R, De A. IHC Profiler: an open source plugin for the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples. *PloS one* 2014;9(5):e96801. [PubMed: 24802416]
28. Lissenberg-Thunnissen SN, de Gorter DJ, Sier CF, Schipper IB. Use and efficacy of bone morphogenetic proteins in fracture healing. *Int Orthop* 2011;35(9):1271–80. [PubMed: 21698428]
29. Zhao H, Feng J, Ho T-V, Grimes W, Urata M, Chai Y. The suture provides a niche for mesenchymal stem cells of craniofacial bones. *Nature Cell Biology* 2015;17(4):386–96. [PubMed: 25799059]
30. Wu Y, Chen L, Scott PG, Tredget EE. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. *Stem Cells* 2007;25(10):2648–59. [PubMed: 17615264]
31. Branski LK, Gauglitz GG, Herndon DN, Jeschke MG. A review of gene and stem cell therapy in cutaneous wound healing. *Burns* 2009;35(2):171–80. [PubMed: 18603379]
32. James AW, LaChaud G, Shen J, Asatrian G, Nguyen V, Zhang X, et al. A Review of the Clinical Side Effects of Bone Morphogenetic Protein-2. *Tissue Eng Part B Rev* 2016;22(4):284–97. [PubMed: 26857241]
33. Kalfas IHI. Principles of bone healing. *Neurosurgical Focus* 2001;10(4).
34. Costantino Peter DP. Bone healing and bone substitutes. *Facial Plastic Surgery* 2002;18(1):13–26. [PubMed: 11823929]
35. Mackie EJ, Ahmed YA, Tatarczuch L, Chen KS, Mirams M. Endochondral ossification: how cartilage is converted into bone in the developing skeleton. *Int J Biochem Cell Biol* 2008;40(1):46–62. [PubMed: 17659995]

Figure 1: Methodological Schematic of Areas Assessed for Healing and Cell Presence and Type. A. Representative μ CT reconstruction with regions of interest identified (small circles) at the anterior abutting the coronal suture, posterior abutting the lambdoid suture, margin and center of the 5 mm calvarial defect (large circle). B. Representative histological section of a defect with areas of interest (boxes) indicated in the center of the defect overlying the removed sagittal suture, in an intermediate not suture or margin related area, and at the margin of the defect containing approximately 50% native bone.

Figure 2: Regional Assessment of Bone Healing.

A. Representative μ CT reconstruction of 4 and 8 week 5 mm critical sized calvarial defect healing with the original defect margin noted (circle). Note overall greater healing with BMP2 (right). **B–C.** Regional assessment of healing at 4 (b) and 8 (c) weeks post-surgery confirms greater bone volume (bone volume/tissue volume; BV/TV) in BMP2 treated defects in all areas at both time points. Assessment of healing by area indicates greater healing along the margins of the defect particularly in areas associated with suture mesenchyme (anterior and posterior). * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$ $n=9$ (4 weeks) or 10 (8 weeks)

Figure 3: Regional Assessment of Cell Presence in a Healing Defect.

A. Representative H&E stained healing defects with high magnification images of the center (left) and margin (right) indicate that remodeling of the defect area occurs over time. Arrows indicate highly cellular areas and boxed areas on the low magnification images indicate areas used for higher magnification. **B–C.** Regional assessment of cell infiltration within the healing defect at 4 (b) and 8 (c) weeks post-surgery indicates that BMP2 drives more initial cell infiltration throughout the defect which is then resolved by 8 weeks. ** $p < 0.01$ $n=4$ individuals per group per time $\times$ 3 sections at least 40 μ m apart per individual.

Figure 5: Regional Assessment of Vascularity in a Healing Defect.

A. Representative immunohistological staining for PECAM in healing defects with high magnification images of the center (left) and margin (right) regions indicate that remodeling of the defect area occurs over time. Arrows indicate positive (brown) staining and boxed areas on the low magnification images indicate areas used for higher magnification. **B–C.** Regional assessment of PECAM positive staining within the healing defect at 4 (b) and 8 (c) weeks post-surgery indicates no statistically significant difference in the distribution of vascularity to correlate with the noted regional differences in healing. $n=4$ individuals per group per time $\times$ 3 sections at least $30\mu\text{m}$ apart per individual.