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14. ABSTRACT 1. The number and severity of battlefield injuries to the craniofacial region increased significantly with the operations in Iraq and Afghanistan as has the survival of personnel with hemifacial injuries after ballistic trauma. Non-battlefield craniofacial injuries, including blunt trauma from motor vehicle accidents and falls, also provide a significant challenge in military operations and are relevant to the general population. Because of the complex nature of these injuries and the location in the craniofacial region, multiple tissue types suffer damage. These defects often involve a loss of maxillary and periorbital architecture resulting in poor malar projection, orbital dystopia, and visual sequelae. These craniofacial injuries often require numerous sequential complex surgeries that often do not achieve adequate aesthetic restoration or functional recovery. Therefore, there is a critical need for new solutions and improved surgical methods to treat these injuries. 2. This work will deliver GMP grade scaffolds comprised of human bone extracellular matrix (ECM) blended with polycaprolactone (PCL) into hybrid ECM-PCL scaffolds that can be 3D-printed into precise anatomical structures and effectively integrated with the patients' stromal vascular fraction cells for the treatment of geometrically complex bone defects in craniofacial trauma, focusing on the periorbital regions. This single distinct technology developed during Grant W81XWH-11-2-0022 will be integrated for this complex reconstruction of hard (bone) tissues in periorbital defects.					
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1. INTRODUCTION:

Background. The number and severity of battlefield injuries to the craniofacial region increased significantly with the operations in Iraq and Afghanistan as has the survival of personnel with hemifacial injuries after ballistic trauma. Non-battlefield craniofacial injuries, including blunt trauma from motor vehicle accidents and falls, also provide a significant challenge in military operations and are relevant to the general population. Because of the complex nature of these injuries and the location in the craniofacial region, multiple tissue types suffer damage. These defects often involve a loss of maxillary and periorbital architecture resulting in poor malar projection, orbital dystopia, and visual sequelae. These craniofacial injuries often require numerous sequential complex surgeries that often do not achieve adequate aesthetic restoration or functional recovery. Therefore, there is a critical need for new solutions and improved surgical methods to treat these injuries.

Objective/Hypothesis. This work will deliver GMP grade scaffolds comprised of human bone extracellular matrix (**ECM**) blended with polycaprolactone (**PCL**) into **hybrid ECM-PCL scaffolds** that can be 3D-printed into precise anatomical structures and effectively integrated with the patients' stromal vascular fraction cells for the treatment of geometrically complex bone defects in craniofacial trauma, focusing on the periorbital regions. This single distinct technology developed during Grant W81XWH-11-2-0022 will be integrated for this complex reconstruction of hard (bone) tissues in periorbital defects.

Specific Aims. Based on the critical military need for surgical management of complex craniofacial injuries including the mid-face orbital region, and the emphasis on translational approaches the following specific aims will be addressed:

Specific Aim 1. Develop ECM-PCL composite biodegradable scaffolds for reconstructing periorbital, bony defects. We will develop protocols for printing scaffolds with the appropriate mechanical and degradation properties.

Specific Aim 2. Integrate 3D-printed ECM-PCL scaffolds for regenerating vascularized bone in cranial (mouse) and periorbital (pig) bone defects. The ECM-PCL scaffolds will be integrated with human SVF for mouse studies. For pig studies, we will 3D-print anatomically-shaped ECM-PCL scaffolds for the treatment of zygomatic bone segmental defects in Yorkshire pigs with and without porcine SVF.

Specific Aim 3. Transition steps for GMP production of clinical-grade ECM-PCL scaffolds will be performed.

2. KEYWORDS:

Bone defects, periorbital, stem cells, reconstruction, trauma, porcine, craniofacial, scaffold

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1	Timeline	Progress
Major Task 1: Develop defined bioactive, biodegradable ECM-PCL composite bone implants.	Months	
Task 1A: Define the formulation (wt/wt ratios) of ECM-PCL scaffolds.		
a. Evaluate quality of 3D prints as a function of ECM concentrations.	1-12	100%
b. Assess mechanics and degradation rates of ECM-PCL scaffolds with respect to ISO compliance.	1-12	100%
Task 1B: Assess biological characteristics of 3D-printed ECM-PCL scaffolds.		
a. Perform proteomic analysis of bovine ECM pre- and post-printing.	1-24	100%
b. Cultivate human SVF in ECM-PCL scaffolds in vitro to assess osteoinductivity.	1-24	100%
Milestone(s) Achieved: Task 1 will result in the optimized formulation of the ECM-PCL scaffolds based on bovine bone ECM to be 3D printed for use in the large animal periorbital defect.		
Specific Aim 2		
Major Task 2: Evaluation of scaffold technology for orbital bone reconstruction.		
Task 2A: Calvarial bone defect reconstruction in murine model.		
a. Combine bioscaffolds with human SVF to regenerate vascularized bone in 4-mm calvarial defect in immunocompromised mice.	13-24	100%
Task 2B: Periorbital bone defect reconstruction in porcine model.		
a. Short-term (6 weeks) and long-term (12 months) term assessments of zygomatic reconstruction in pig model	10-36	100%
Milestone(s) Achieved: Task 2 will demonstrate the utility of the scaffolds with and without autologous cells and with/without growth factor to regenerate vascularized bone in the periorbital regions. We will obtain valuable insight on materials design leading to more standardized, reproducible implants that can be manufactured for large, anatomical reconstructions with no problems in donor tissue acquisition.		
Specific Aim 3		
Major Task 3: Translation of technology for orbital bone reconstruction.		
Task 3A: Transition to clinical grade manufacturing.		
a. Define SOPs for manufacturing scaffold.	13-24	100%
b. GMP production of anatomically shaped, ECM-PCL composite scaffolds.	13-36	75%
Milestone(s) Achieved: Task 3 will establish the ground work for translation to a pre-clinical model and manufacture following GMP. Efficacy for composite tissue repair will be established along with translation.		

What was accomplished under these goals?

Accomplishments:

1. Development and characterization of PCL-DCB scaffolds

a) Fabrication of custom PCL-DCB filaments for 3D printing scaffolds

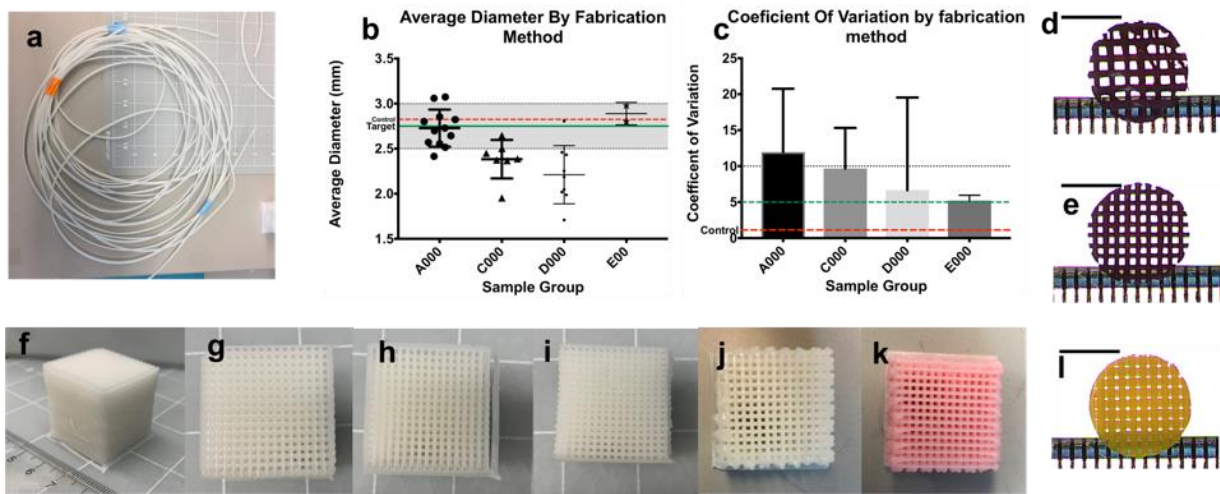


Figure 1: Filament Manufacture, Standardization, and 3D-Printing. A. Example of manufactured filament. B. Filaments have been standardized to be produced with diameters inside the target zone. C. Filaments do not vary in diameter. D., E., I., Alizarin Red staining of scaffolds with varying concentrations of DCB (more bone = more red color.) F – K, different prints of the manufactured filament show the replicable high quality of the print.

The pneumatic printer setup we formerly used for preparing our scaffolds had poor temperature distribution and control. It was not possible to closely constrain the thermal aspect of the printing process. Thermal processing can directly impact polymer material properties such as molecular weight, crystallinity and ultrastructure. To improve the quality of our results, we upgraded to a filament-based printing system using a different 3D-printer in our lab. This new printer featured closely constrained thermal parameters among other features. It was necessary to purchase a polymer extruder so that we could create filaments containing our customized dopant.

Our manufacturing process was adapted to use a Laboratory Mixing Extruder (LME) extruder from Dynisco to prepare polymer filaments, which in turn would be fed into the 3D-Printer in order to produce an implant in the exact shape of the facial bone. This is a new manufacturing would allow us to fabricate clinically relevant implants with dimensions >0.5cm in height.

The LME was successfully delivered and installed at the Grayson Lab at JHU. It was able to prepare consistent polymer filaments of pure polycaprolactone and of 70% polycaprolactone and 30% decellularized bone matrix. These filaments had high tolerance of their diameter, appropriate to feed into

the LulzBot 3D-Printer. These filaments were used on the 3D-Printed to make scaffolds of regular porous shapes to assess print quality and complex anatomic shapes for use in porcine studies.

b) Evaluation of degradation property of PCL-DCB composite

We used an accelerated hydrolysis degradation protocol and compared mass loss in scaffolds made of pure PCL to PCL scaffolds containing dopant. This was compared under two different hydrolytic degradation conditions. We found that adding dopant to our scaffold material increases the mass loss of the scaffold at a given time point. This effect is independent of the rate of hydrolysis.

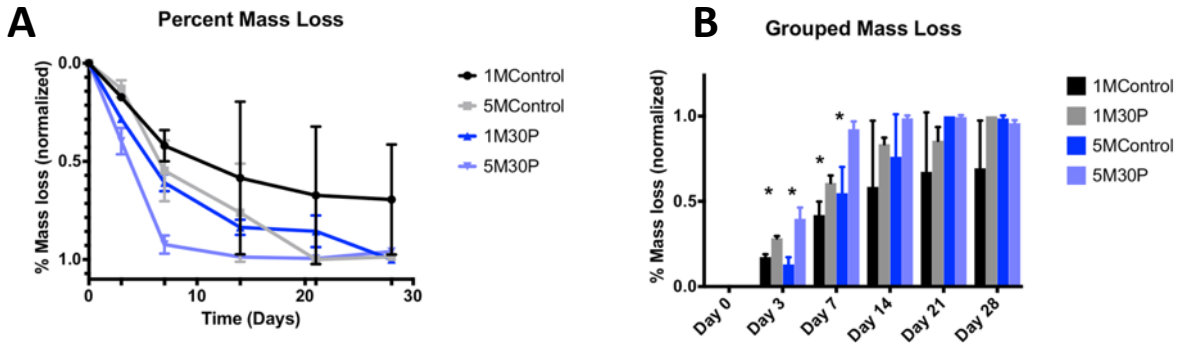


Figure 2: Bone Dopant increases the mass loss of the scaffold under hydrolytic degradation. Degradation conditions are 1M NaOH and 5M NaOH concentrations. Percent mass loss when compared between PCL control and 30 percent dopant scaffolds (by weight) is greater at each timepoint.

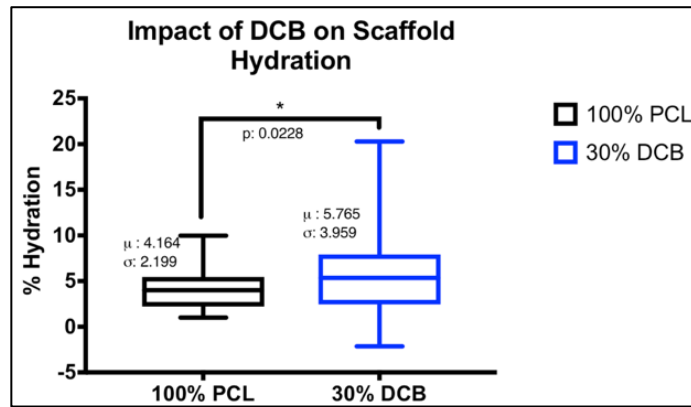


Figure 3: Bone Dopant increases the hydration capacity of the scaffold. 30% dopant scaffolds showed a statistically significant increase in percent hydration (measured by change in weight after hydration) over pure PCL scaffolds.

We also investigated the ultra-structure of the scaffold and how this structure changes due to the addition of dopants. Using a fundamental hydration protocol where the scaffold sample is incubated overnight in PBS, we compared the hydration capacity (evaluated through change in weight before and after hydration) of pure PCL scaffold to that of the scaffold containing dopant. The scaffold containing dopant was shown to have a greater hydration capacity than the pure PCL scaffold. This increase in hydration was statistically significant (Figure 3).

Finally, we have shown that the molecular weight of the polymer in our scaffold (an important consideration for degradation) is not significantly affected by our printing process or by the addition of dopants. This was done by preparing Gel Permeation Chromatography (GPC) samples of the pure PCL powder used in our scaffolds (before printing), a pure PCL scaffold and a PCL-dopant scaffold. (Figure 4)

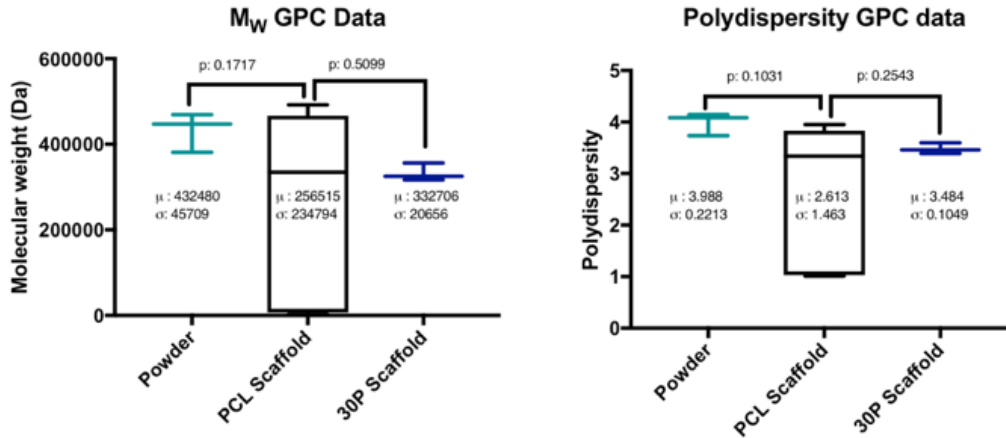


Figure 4: Neither printing process or addition of bone dopant significantly change polymer molecular weight (MW) or polydispersity: GPC results of pure PCL polymer powder, PCL scaffold and 30% bone dopant PCL scaffold. These samples have not been treated prior to preparation of GPC.

Using these methods we have been able to confirm our expected results that the presence of dopant has no statistically significant effect on the molecular weight of our scaffold material but does impact the thermal properties of the material.

c) Design and fabrication of large volume anatomical shaped scaffolds from PCL-DCB composite

Pre-operative CT scans of pigs were used as a template for designing custom surgical guides and zygomatic implants for each of the pigs using MIMICS software. Once the implants were designed, they were exported out of MIMICS in the form of slices (.bmp files) which could then be imported by a MATLAB script and generate a 3D volume in space of the implant i.e. a 3D matrix. Each elementary unit of this 3D matrix i.e., a voxel contains the greyscale values from the CT scan (spatially distributed within the 3D array) which is proportional to local mineral density of the bone. This 3D matrix is then discretized with each of its elements (or voxels) assigned/tagged a value for e.g. 0, 0.7 or 1 where 0, 0.7 and 1 imply empty space, body of the implant and fixation tab respectively. This discretized matrix is then passed into an inhouse developed Slicer script which slices the implant volume incorporating desired porosity at right locations and creates the print path instructions for the 3D printer and these instructions are stored in the form of g-code (.gcode file). The g-code can be read using a 3D printer slicing software like CURA (opensource) and relay the print path instruction to the 3D printer. The nozzle of the 3D printer draws in either ABS or custom Corbion-BioOss filaments and prints the implant. Using this technique, anatomically precise PCL-DCB scaffolds with 500 μm strut width and 800 μm pore width were fabricated. The above-mentioned workflow can be better visualized in Figure 5.

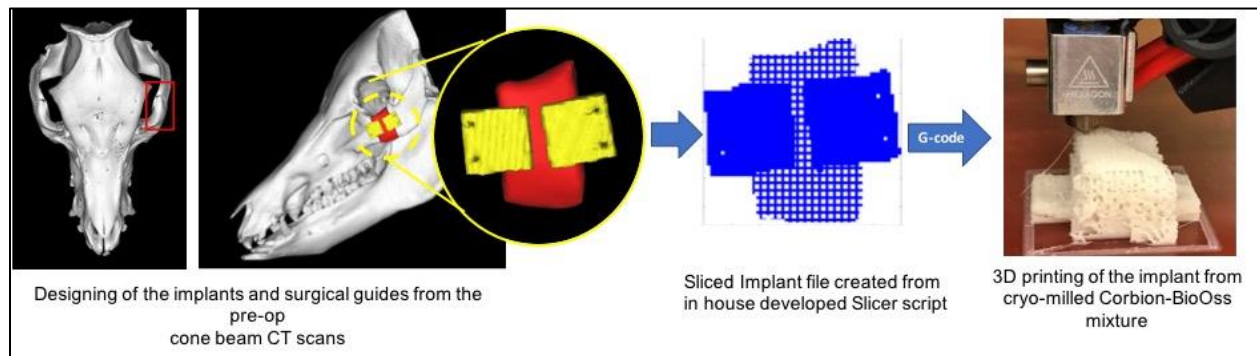


Figure 5. Design and 3D Printing of Implants: Workflow depicting the process of designing a custom implant from pre-op CT scans using MIMICS and 3D printing the designed implant.

d) Developing a methodology to quantitatively assess print quality of PCL-DCB scaffolds

Post fabrication, cone beam computed tomography (CBCT) scans of the 3D printed PCL-DCB scaffolds were acquired and using an inhouse MATLAB script the scaffold 3D volumes were skeletonized i.e., the original scaffold volumes were converted into single connected voxel thread providing a strut skeleton. Inversing the strut skeleton (grayscale) would generate the pore skeleton. A 6mm width cube whose center was concentric with the center of mass of the scaffold volume was identified as a representative volume for the whole scaffold which would then be evaluated for print quality. Average strut width and average pore width were chosen as the metrics for print quality assessment wherein average strut width would be defined as the Euclidean distance between each voxel in the strut skeleton and the nearest voxel of the pore volume (assuming these distances were half of the strut width). Similarly, the Euclidean distance between each voxel of the pore skeleton and nearest voxel in the strut volume could be measured to calculate average pore width. This method was applied to 3D printed PCL-DCB scaffolds and our analysis showed that the print quality parameters of the fabricated scaffolds were in close agreement with the intended design parameters i.e., strut width of 500 μm and pore width of 800 μm .

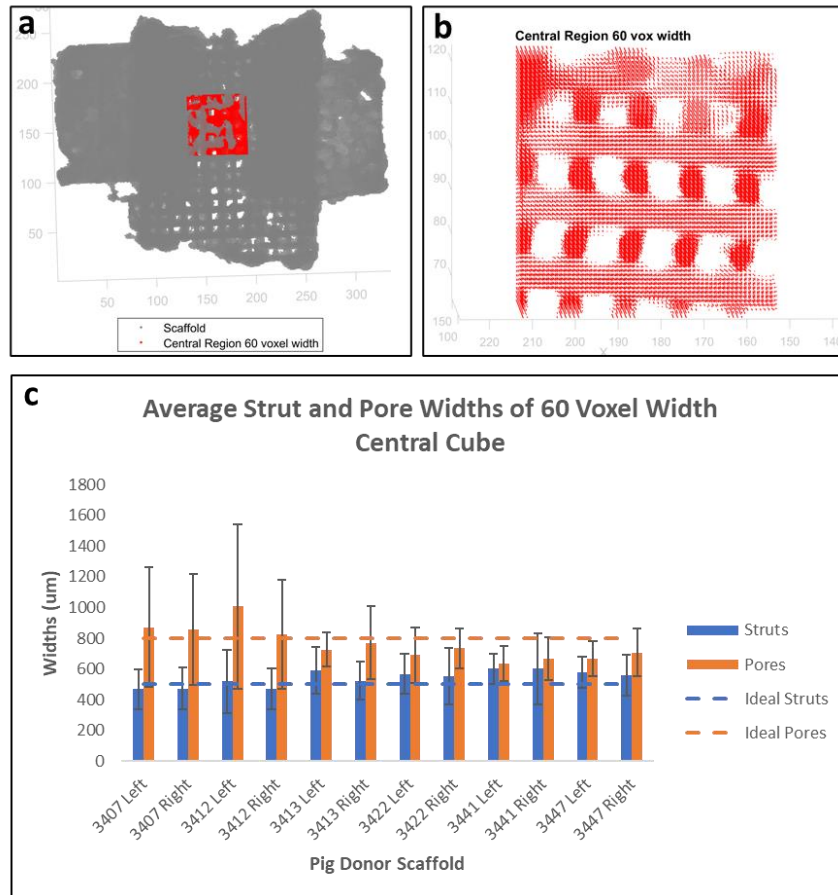


Figure 6. Print Quality assessment of 3D printed scaffolds (a) Cone beam computed tomography (CBCT) scan of 3D printed PCL-DCB scaffolds with a 6mm x 6mm x 6mm representative volume (red) (b) magnified image of the representative volume highlighting the open and regular pores within the scaffold (c) histogram comparing scaffold dimensions (strut width and pore width) calculated from the CBCT of 3D printed scaffolds with that of the intended design value i.e., strut width of 500 and pore μm width of 800 μm .

2. Isolation of Stromal Vascular Fraction (SVF) from human lipoaspirate and cell characterization

Critical-sized craniofacial bone defects are challenging to treat due to their geometric complexity. Our lab has previously reported on novel 3D-printed scaffold technologies used in conjunction with adipose-derived stem cells (ASCs) for regenerating bone in murine calvarial defects. However, these strategies are limited by the need for extensive in vitro manipulation prior to implantation. To address this, we explored the bone forming potential of the stromal vascular fraction (SVF) obtained directly from human lipoaspirate with the potential to be used intraoperatively.

Briefly, human lipoaspirate was collected from 6 donors undergoing cosmetic liposuction (with written informed consent and IRB approval). The fat was washed with PBS, digested with collagenase I at 1mg/mL for 1hr, RBC lysed, and filtered at 100 μm in standard fashion. SVF was used directly after filtering, while P2 ASCs were cultured for two passages on tissue culture plastic in standard media (high glucose DMEM + Fetal bovine serum (FBS) + Pencillin/Streptomycin(P/S)) before use. Flow cytometry was performed on both the SVF and P2 ASC population to gain insight into the cellular heterogeneity and donor-to-donor variability.

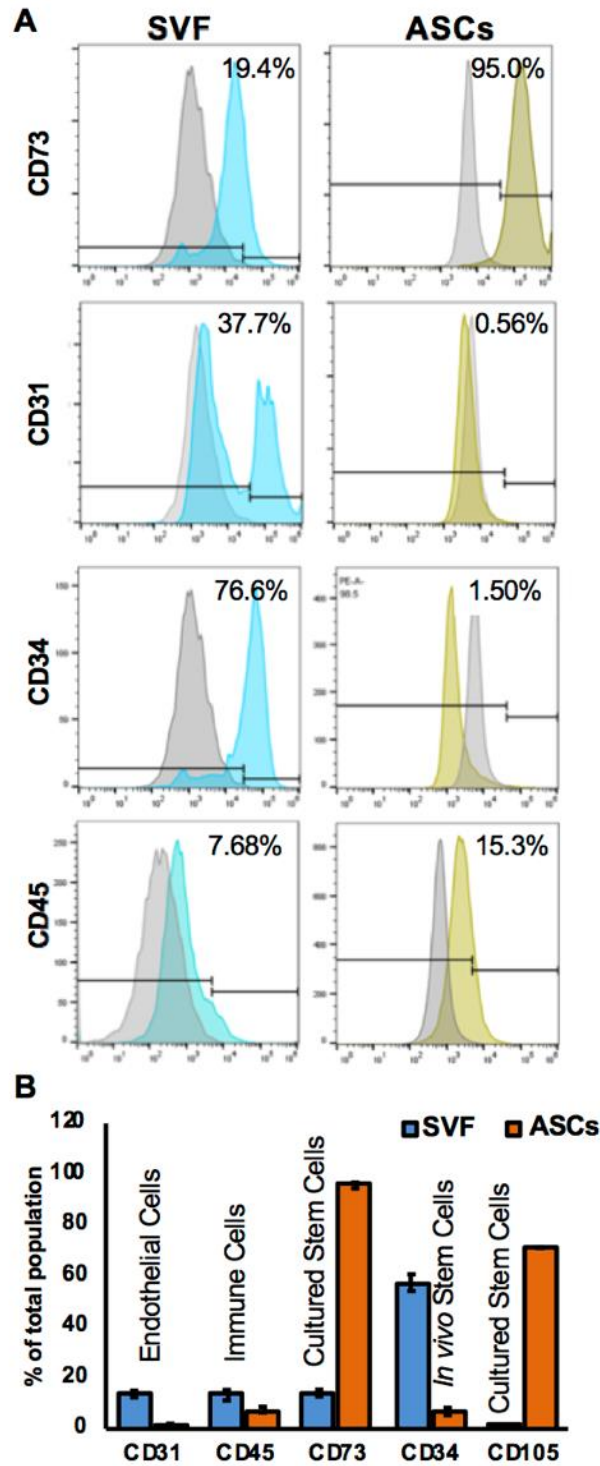


Figure 7. Flow Cytometry. (A) Representative flow cytometry histograms for endothelial (CD31), hematopoietic (CD45), and stem cell (CD34, CD73) markers before and after culture. (B) Summary of marker profiles for 6 donors.

Table 1: Donor profiles and cellular yield

Donor	Age	Race	Sex	BMI	Location(s)	Yield (cells / mL of clean fat)
1	54	Caucasian	F	25	Flank / Scapular	63,750
2	37	Caucasian	F	23.9	Flank / Inner Thigh	120,000
3	54	Caucasian	F	28.6	Flank / Scapular	700,000
4	43	Caucasian	F	20.8	Flank / Abdomen	145,000
5	32	African Am	F	25.8	Flank	676,000
6	22	African Am	F	25.7	Flank / Abdomen	278,000

Results: The flow cytometry characterization highlights a greater presence of endothelial cells (CD31+) in the SVF population. A decrease of CD34 (marker of hematopoietic stem cell and increase in CD73 was observed in SVF as compared to P2 ASC population which might be a result of culturing on tissue culture plastic which leads to enrichment of adherent cell population. Moreover, the relative expression of cell surface markers seem to be consistent among different donors.

3. Demonstration of osteogenic potential of PCL-DCB/SVF combination using in vitro assays and in vivo murine critical-sized calvarial defect model

In order to evaluate the in vitro osteogenic potential of PCL-DCB/SVF combination, SVF and passage 2 ASCs were suspended in a fibrin hydrogel at 20,000 cells/ μ L and delivered into porous 3D-printed PCL-DCB (osteo-inductive) scaffold. Cell seeded scaffolds were cultured for three weeks in control media (low glucose DMEM + 10% FBS) or osteo-induction media (control media + osteo-inductive supplements), harvested and analyzed biochemically for calcium and DNA content.

Animal Studies: All animal work was approved by JHU IACUC. Male nude immunocompromised mice (fox1n1, Charles River) were used at 8 weeks of age.

Cranial Defect Model: A 4mm circular saw was used to create bilateral defects in the parietal bone. Scaffolds were press-fit into the defects, and post-mortem μ CT was conducted 6 weeks after surgery.

Intramuscular Defect and microfil assay: Blunt dissection was used to create pockets in the hamstring portion of each leg. Scaffolds were placed into the pockets and the tissue was sutured closed. After 10 days or 6 weeks, mice were sacrificed and microfil was perfused through the vasculature trans-cardially. μ CT was performed to determine the extent of scaffold vascularization.

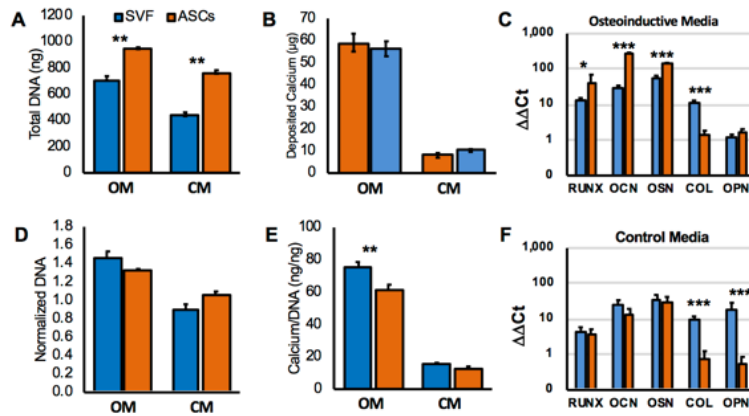


Figure 8. In Vitro Mineralization Potential Over 21 Days. (A & D) Total DNA and DNA normalized to day 0 amounts. (B & E) Deposited calcium and deposited calcium normalized to total DNA. Osteoinductive (OM) and Control (CM) media, in PCL-DCB scaffolds. n = 6 donors. (C & F) Bone gene expression of one donor at 21 days, on PCL-DCB scaffolds.

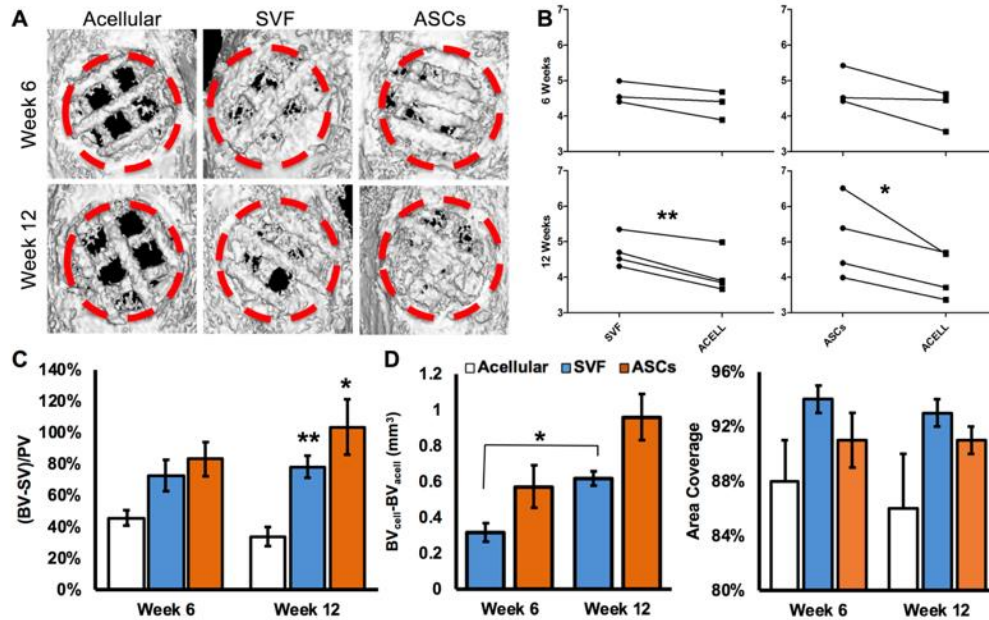


Figure 9. In Vivo Bone Forming Potential. (A) microCT reconstructions of 4mm diameter cranial defects (red outline) (B) Pair-wise comparison of bone volume per animal. Each connected pair indicates a single animal containing two defects (cellular and acellular). (C) Percentage of porous space filled with bone. Bone volume reduced by the initial scaffold volume and normalized to the porous volume: (Bone Volume-Scaffold Volume) / Pore Volume. (D) Bone volume within defect reduced by contralateral acellular defect bone volume. (E) Area coverage of defect area. Measured in ImageJ as (number of non-black pixels) / (total number of pixels) in the 4mm-diameter circle.

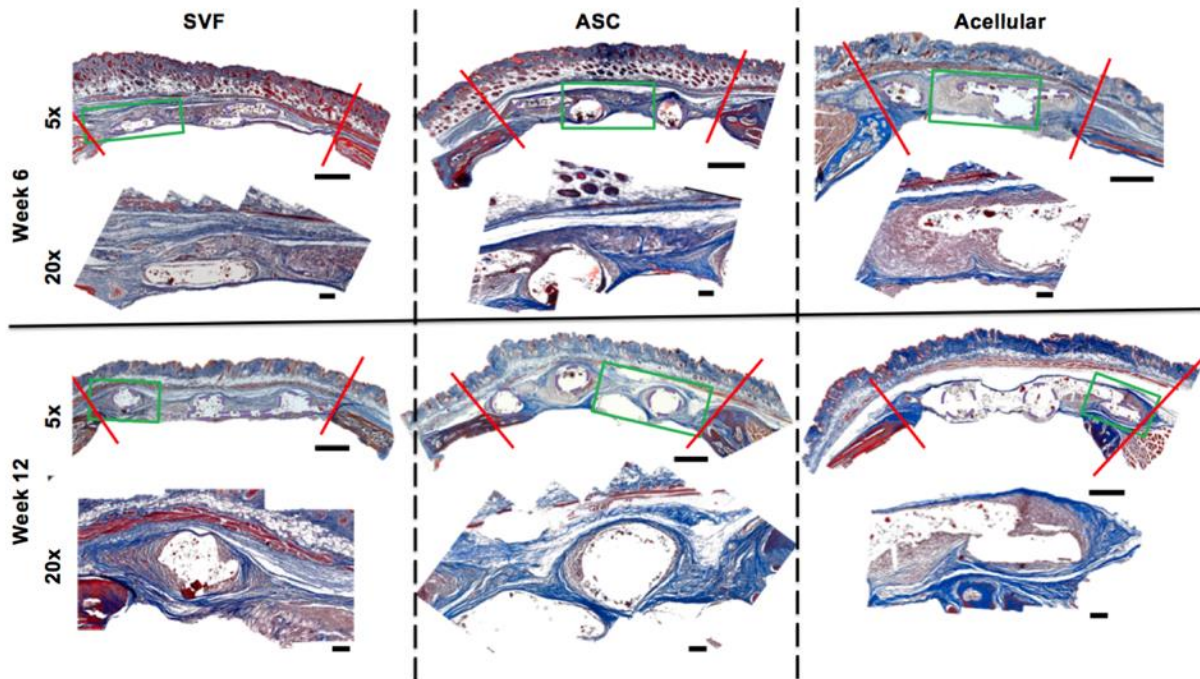


Figure 11. Histology Staining of Murine Cranial Defects. Mason's Trichrome Staining on cross sections of samples at 6 (top row) and 12 weeks (bottom row). Scaffold struts indicated by empty white space, dense collagen as blue. Red lines indicate boundaries of the defect, and green boxes are the zoomed in section. Scale bars 5x = 500 μ m, 20x = 100 μ m.

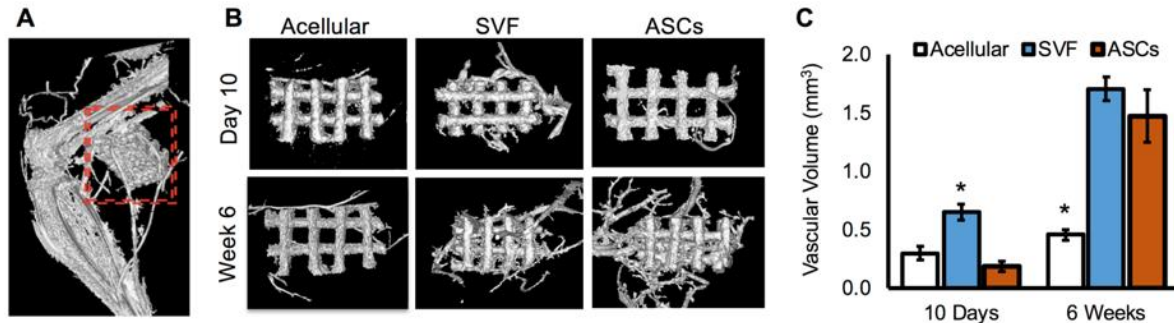


Figure 12. Intramuscular Implantation and Microfil Perfusion (A) Example CT showing scaffold implant (red box) in relation to leg. (B) microCT reconstructions of scaffolds implanted intramuscularly, with the vasculature filled with radiopaque microFil. (C) Total vascular volume within scaffold.

Results:

In Vitro osteogenesis: We hypothesized that SVF responds to the osteoinductive PCL-DCB scaffolds similarly to P2 ASCs. (1) In vitro culture in osteogenic conditions reveals that both SVF and P2 ASCs deposit mineral in scaffolds. (2) SVF slightly out-performs passaged ASCs when depositing mineral. (3) PCL-DCB scaffolds enhanced the mineralization of both SVF and ASCs. Figure 2.

Animal Implantation: (1) SVF and P2 ASCs improve the healing outcomes of critical-sized cranial defects with increased bone volume and area coverage. (2) Intramuscularly, P2 ASCs increase the vascular infiltration into the scaffold, particularly at 6 weeks.

The 12-week data shows the same healing trend as the 6-week data. ASCs slightly outperform the SVF, but either cell type is able to fill the scaffold with bone. Scaffolds and cells enabled the bone regeneration. We see the intervention is useful filling the defect with bone volume, in adding new bone volume to that of the scaffold, and in covering the area of the defect with bone. Thus, we are encouraged about the use of DCB-PCL scaffolds and SVF in the upcoming porcine study. In conclusion, we observed that both populations successfully mineralized the scaffold demonstrating robust bone formation properties of SVF and a potential autologous stem cell source.

4. Reconstructing critical-sized peri-orbital defect in a pre-clinical porcine model

a) Developing a zygomatic porcine critical-sized defect model

The porcine surgical model was tested using a cadaveric pig head and evaluated using a prototype scaffold (figure 13). Simple ABS scaffolds and cutting guides were designed to facilitate the practice surgery. The surgical guides are used as a template to create the osteotomies in the porcine zygomas. 3D printed ABS scaffolds which exactly fit the dimensions of the defect were inserted into the defect site. The boundaries of the defect were aligned with the contours of the surrounding native bone.

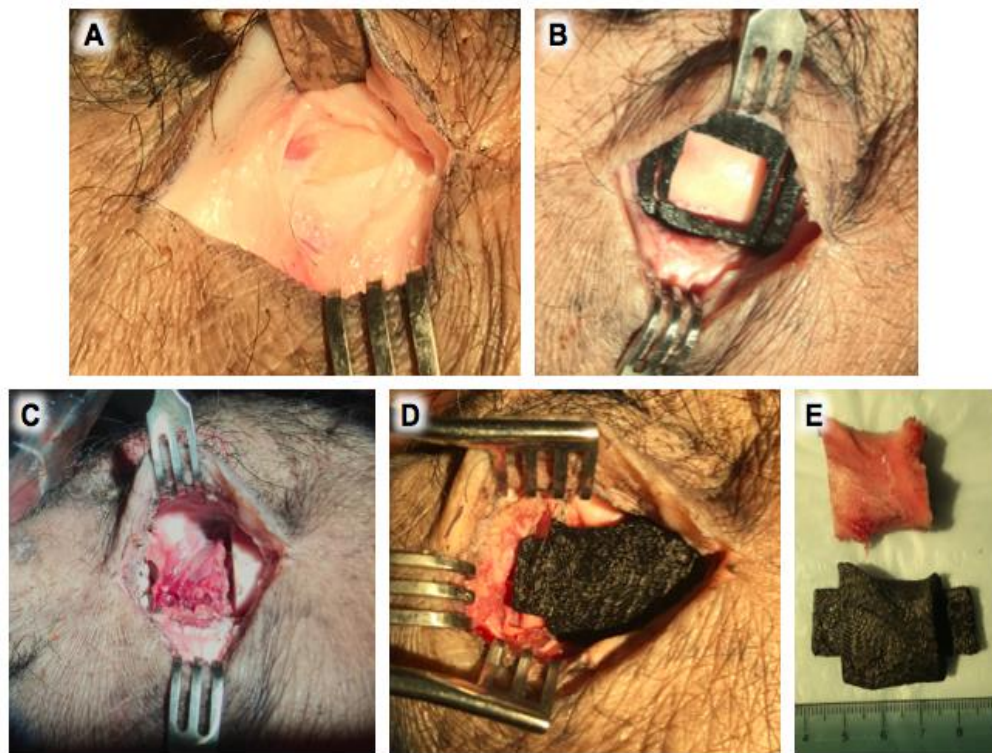


Figure 13. Dissection of cadaveric porcine head. A. Transcutaneous approach to the zygomatic arch and dissection of the periosteum. B. Placement of the cutting guide on the zygoma. C. Zygoma with the osteotomy. D. Prototype scaffold placed in the osteotomy. E. Comparison of scaffold and excised bone.

Developing an isolation technique for swine SVF within narrow surgical window

The inclusion of stromal vascular fraction (SVF) within the implant is critical to the regeneration of bone. Our previous studies using a murine model have shown that acellular scaffolds have significantly less new bone growth than those with SVF. As we progress towards a pre-clinical swine study, we took into consideration the substantial differences in approach to isolate SVF from human vs swine. After consultation with other experts who had experience isolating SVF from swine, we prepared adapted versions of our protocols and tested them with pig fat obtained from a local butcher and other freshly sacrificed research swine.

The swine fat was different from any of our previous experience with human or rodent fat. At room temperature, it was a solid block instead of loose and elastic. Additionally, it was substantially more fibrous than human fat and required more intense mechanical processing before the introduction of digestive enzymes to separate the stromal vascular fraction from the adipocytes. Despite these differences, we were able to successfully obtain SVF cells from porcine adipose tissue.

Our protocol for isolation of SVF from swine initially required more time than is available during the swine operation (the animal can only be kept on anesthesia for about 2h total, while the cell isolation required 3 hours). We have developed adaptations to the protocol to reduce the time to 50min by increasing the collagenase enzyme concentration, halving the enzyme digestion time period, and reducing the number and time of spins (Figure 9).

In 5 practice isolations, we were able to successfully obtain viable cells from swine fat of the same breed, location, and amount of fat as we intend to use in the swine zygomatic arch study. These cells grow in monolayer culture as expected. Furthermore, they were successfully seeded into 3D-printed DCB-PCL scaffolds and proliferated and mineralized the scaffold.

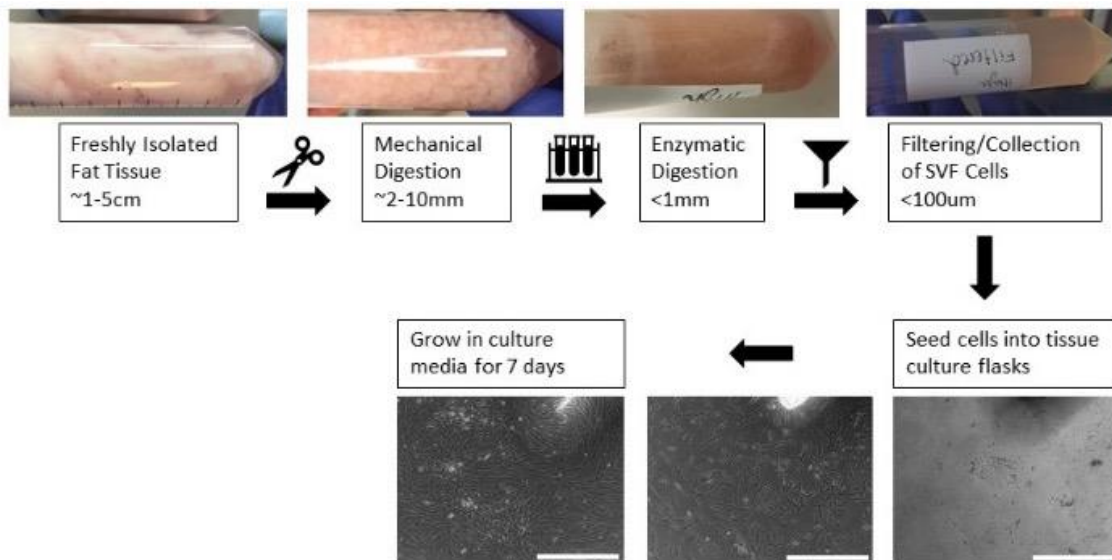


Figure 14. Porcine SVF Isolation. Freshly isolated fat tissue is minced with scissors. Then the collagen is digested enzymatically, before the stem cells are separated from the fat and tissue via centrifugation separation and size filtration. Cells are plated on tissue culture plastic and grown for 5 days. Scale Bars = 500µm.

Reconstruction of critical sized porcine zygomatic defect within a surgical suite

Table 2: Summary of all the experimental groups highlighting the evaluations that will be carried out along with the CT time points. Yellow shading indicates the animals which underwent surgery in December 2018 and the ones in green indicate that of June 2019.

Experimental Groups	Animals/group (n)	CT Time Points	End of Study	Evaluation
Empty	2	Pre-Op, Post-Op, 6wk, 12 wk, 24wk, 52wk	52 weeks	Bone Formation (CT) Histology Mechanical strength Blood Work
Acellular	2	Pre-Op, Post-Op, 6wk, 12 wk, 24wk, 52wk	52 weeks	
Cellular	2	Pre-Op, Post-Op, 6wk	6 weeks	
	4	Pre-Op, Post-Op, 6wk, 12 wk, 24wk, 52wk	52 weeks	

To validate the therapeutic efficacy of anatomically precise PCL-DCB scaffolds in combination with SVF, surgeries were performed on a set of 10 pigs at LSU in 2 rounds: the first set comprising of 4 pigs (Pig ID: 3077, 3097, 3105, 3134) in December 2018 and the second set comprising the rest of 6 pigs (ID: 3407, 3422, 3412, 3413, 3441, 3447, 3412) in June 2019. Briefly, a 2cm full thickness, segmental defect was created bilaterally in the zygomatic bone of each of the pigs. The pigs were divided into three groups namely empty, cellular and acellular. The acellular and cellular groups received the 3D printed custom implants with or without stromal vascular fraction (SVF) harvested from the corresponding animals respectively. The empty group was left untreated and received no implants. All the groups and the evaluations are summarized in Table 2.

In Dec 2018, the first round of surgeries was performed essentially as a pilot study to assess the feasibility of the method and to identify potential sources of error/complications which may include anything from hygiene to logistics. Osteotomy was performed on 2 pigs (ID: 3077,3097) and received no implants. These pigs would serve as the control (Empty) group for the study. 2 more pigs (ID: 3105, 3134), part of the cellular group underwent osteotomy and received implants with autologous SVF in (Tisseel) hydrogel.

The next round of surgeries was performed in June 2019 which saw 6 pigs undergoing osteotomy. For the acellular group (Pig ID: 3407, 3441) the implants were placed in the defect and injected with 5ml of Tisseel (hydrogel) only whereas for the cellular groups (Pig ID: 3412, 3413, 3422, 3447) the implants were injected with 5 ml of Tisseel (hydrogel) containing autologous SVF from the corresponding pigs. Post surgery, the pigs resumed normal activities and were regularly observed. Evaluations like CT scans, blood work were carried out at the allotted time points and evaluations like mechanical strength and histology were performed once the implants are harvested when the end point is reached.

5. Post-operative evaluations

Post-surgery, CT scans were performed on the pigs of different groups at the designated time points as shown in Table 1. The scans were analyzed using Materialise Mimics software. Briefly, the raw CT Dicom files were imported into mimics and thresholding was done at 226 HU (Hounsfield Units). The VOI (volume of interest), in this case the implant, was isolated using the same wedge-shaped solid object (.stl file) which was used to create the segmental zygomatic defect initially as shown in figure 1. Once the VOI was isolated, a mask was created from it and the volume of the mask (i.e. the volume of the voxels contained in the respective mask) was recorded as bone volume (BV) for the respective time point. The total volume of the defect was calculated by performing a “wrap” function on the isolated VOI from the pre-op CT of the animals (performed a few weeks before the surgery) and was recorded as total volume (TV) for the respective animal (constant for all time points). 3D rendered images were created from the CT scans of the defect site to monitor scaffold aided bone regeneration over 52 weeks. Empty defects did not bridge validating the

critical nature of the defect. (Figure 15 (top)). CT evaluations demonstrated that at all time points (6, 12, 24 and 52 weeks) both the acellular and SVF groups had superior bone growth at the defect site as compared to the control group. However, we did not observe any statistically significant difference in the amount of bone formation between the acellular and SVF group (figure 15 (bottom)).

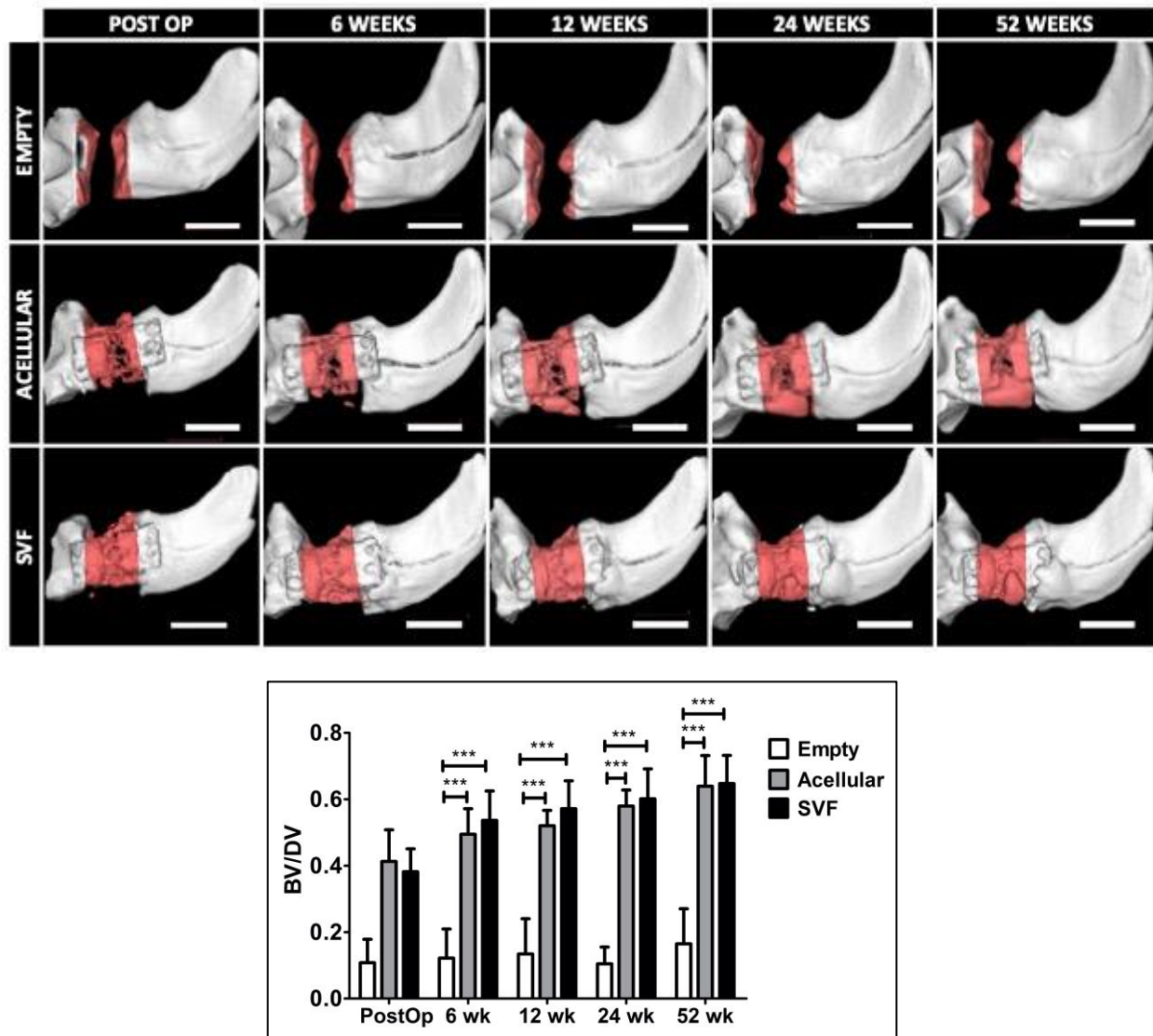


Figure 15: (Top): 3D rendered images of CT scans of the zygomatic defect site belonging to all experimental groups namely Empty, Acellular and SVF, demonstrating scaffold aided bone regeneration over a period of 52 weeks. Scale bar = 2cm. **(Bottom):** Quantitative evaluation comparing new bone regeneration at the defect site within the three experimental over a period of 52 weeks.

Upon reaching the 52 weeks endpoint, all animals were euthanized and the zygomas were harvested. The zygomatic specimens were subjected to mechanical testing and histological evaluations.

a) Mechanical testing of harvested zygomas

Harvested zygomas (left side) were potted in polymethyl methacrylate (PMMA) and subjected to cantilever beam bending test in order to assess osseointegration. A load beam was made to indent at the center of the scaffold on the medial side to initiate fracture at the scaffold-native bone (posterior) interface. Fracture torque was calculated and compared between both the groups. It was demonstrated that SVF group required a higher fracture torque as compared to acellular group suggesting higher degree of osseointegration.

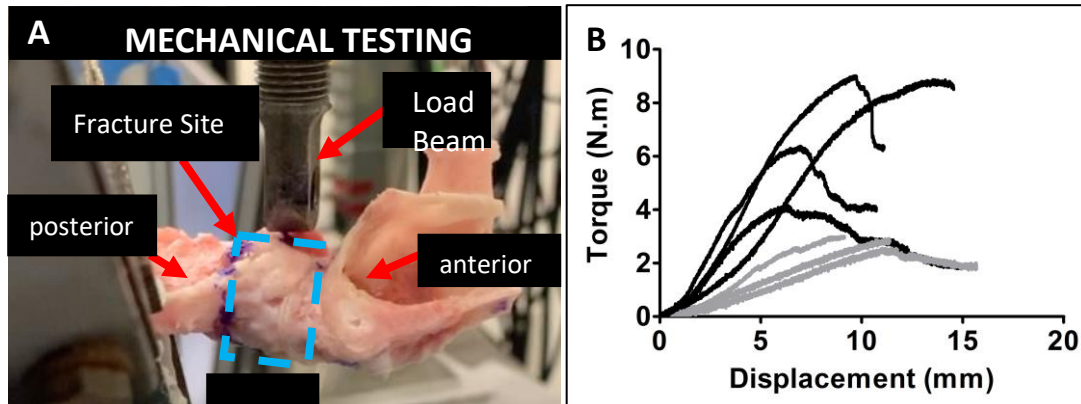


Fig. 16. (A) Cantilever beam bending test of harvested zygoma specimens (B) Plot of fracture torque for specimens belonging to acellular (gray) and SVF (black) group for comparison of osseointegration.

b) MicroCT of the harvested zygomas

Micro-CT scans of the harvested zygomas (right side) were acquired to gain insight into the microstructure of new bone growing within the defect site. Bone nodules could be seen nucleating within the pores of the scaffolds which were found to be in close association with the PCL-DCB (polycaprolactone – Decellularized bone) struts. At 52 weeks, the bone nodules demonstrated radiodensity similar to that of the native bone. It is noteworthy that most of these new bone nodules were found to the lateral aspect of the scaffold where the fixation tab is located.

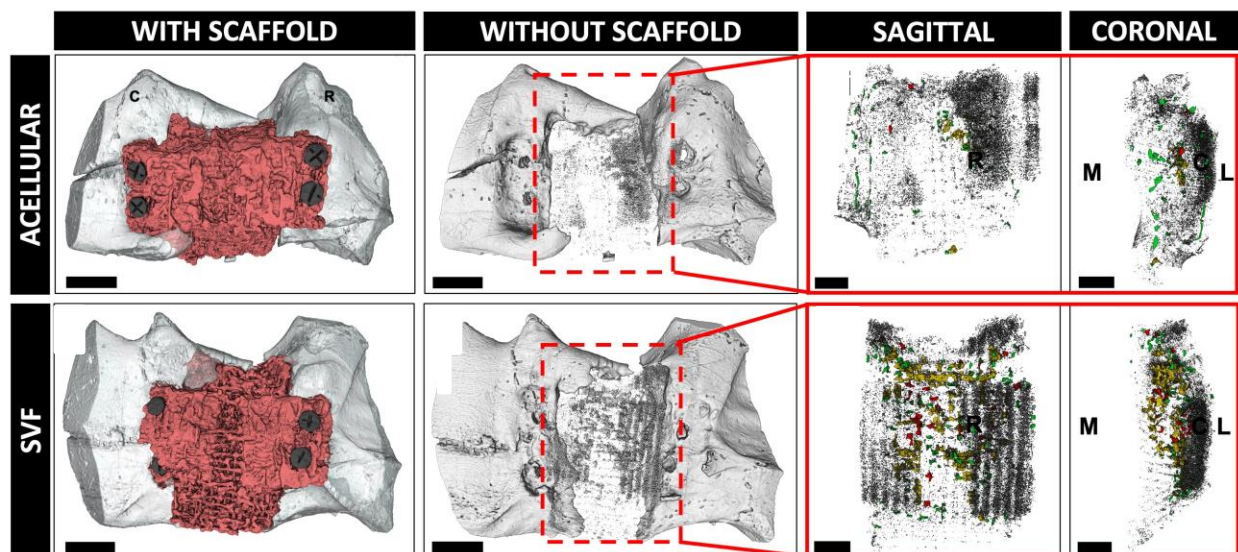


Fig. 17. 3D rendered images of MicroCT scans of the representative harvested zygoma from acellular and SVF group demonstrating new bone nodules growing within the scaffold at 52 weeks. Scale bar: 1 cm (with and without scaffold); 0.5 cm (sagittal and coronal)

c) Hard Tissue Histology of the harvested zygomas

After acquiring the microCT scans, the harvested zygomas were embedded in methyl methacrylate and hard tissue sections were created. Thereafter, the sections were stained with Stevenel's blue stain to identify new bone from the existing bone. Osteoid matrix was detected within the pore. In addition, osteoid matrix in close association with the PCL-DCB struts were seen mineralizing. Bone nodules nucleating within the pores were found to be woven bone whereas new bone growing on the periphery were lamellar bone.

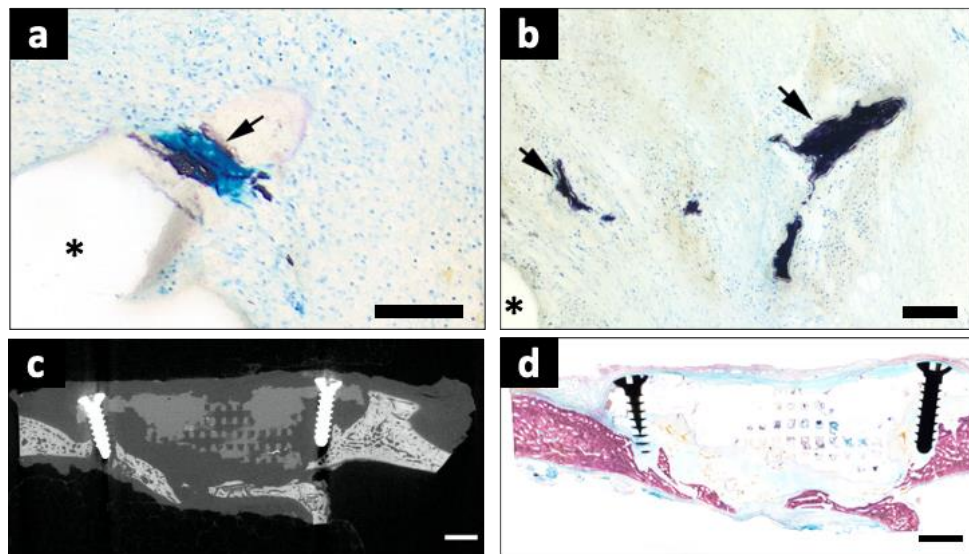


Fig. 18. Hard Tissue Histology of harvested zygomas. (a) Osteoid matrix mineralizing and (b) woven bone detected within the pore spaces of the scaffold. (c) MicroCT scan and (d) Stevenel's blue staining of the harvested zygomas show lamellar bone growing on the periphery of the scaffolds. Scale bar = 4 cm.

Our post-operative evaluations suggest that PCL-DCB scaffold in combination with SVF was able to promote robust bone regeneration and osseointegration in critical sized peri-orbital bone defect.

Transition to clinical grade manufacturing.

We recruited The LaunchPort, Inc., a team of subject matter experts in the fields of manufacturing engineering, FDA regulatory navigation, operations management, design engineering, supply chain strategic sourcing, and business management to review the manufacturing process. While on site, the team performed critical analysis of the currently established process flow for building the additively manufactured PCL-DCB lattice structure. The team identified several high priority control mechanisms necessary to reduce manufacturing risk in a production environment. These include both capital investments and operational procedure controls. The process flow consists of four primary phases: Cryomilling, Filament Extrusion, Additive Manufacturing (3D Printing), and Sterilization & Packaging. The following observations and recommendations of each of these phases have been established as the output of this initial failure mode & effects analysis.

Cryomilling: This first phase, consists of mixing two materials, polycaprolactone (PCL) and commercially sourced decellularized bone matrix, **BioOss®**, into a homogenous powder with a targeted equivalent particle size. This particle size has been deemed critical to subsequent manufacturing processes. Several details within the process are continuing to be established including the particle size verification, its

associated tolerance limits, and the impact of these variables in the later manufacturing process phases. Raw material is then stored in a temperature-controlled environment to ensure the integrity of the mix until use. We will further investigate the impacts of temperature-controlled handling.

Filament Extrusion: This phase of the process has the greatest opportunity area for GMP (Good Manufacturing Processes) interventions. The cryomilled PCL-BioOss® powder is loaded into a heated vessel and extruded into a fine filament. Equipment control mechanisms, such as temperature regulation and filament extruder size, were not utilized in the early development phases of this process to allow for research flexibility. As the process moves through maturity, the pertinent areas of GMP focus will include vessel temperature monitoring, extrusion speed detection, filament output metrology monitoring, and the associated feedback loop control mechanisms of the extruder itself.

3D Printing: The 3D-printed implantable scaffold results in a highly customizable and personalized product. The nature of these unique products present challenges in maintaining GMP. Printing error anomalies identified during the validation scan process requires a manual operation to bring the product into compliance. The scanning fidelity and error identification also has an ambiguity regarding the measured-to-modeled precision requirement. A quantitative threshold for dimensional – internal and external – tolerances, density, as well as any other key variables, will be critical in aiding in the rework operation quality control.

Packaging and Sterilization: This is a critical final step towards regulatory compliance. As previously mentioned, batch size becomes a critical parameter, but in addition sterilization is typically validated on standard volume and configurations. In this case of personalized configurations, the challenges with sterilization validation needs further investigation. It will also be key to understand the unique packaging requirements for each customized lattice structure to assure structural support and integrity during transportation.

Future steps: The fundamental principle of FMEA documentation is risk prioritization. There are various FMEA techniques that can be applied to evaluating future capital expenditures and the associated production process controls. A detailed FMEA, focused on each of the four process phases, should assess the clinical outcomes that result from the variations noted above. Traditionally, the FMEA documentation that supports a commercially approved quality management system would include failure mode, impact, severity, causes, occurrence, detection mode, ease of detection, & risk prioritization to determine where process controls need to be implemented and monitored.

Discussion of stated goals not met These goals have been partially met due to delays in obtaining the IACUC and the subsequent ACURO approvals as well as due to delays in IRB approvals for the SVF isolation. However, we have since secured approvals for all of these elements and expect to achieve those goals over the next several months.

Nothing to report.

What opportunities for training and professional development has the project provided?

Courses: Makeda Stephenson took the course, Advanced Orthopaedic Tissue Engineering (AOTE), taught by the PI, Dr. Warren Grayson. Srujan Singh took the courses Human Anatomy, AOTE and Genes to Society- Immunology which helped him with the animal study.

One-on-one work with mentor: The PI has 2 one-on-one meetings each year with Ethan Nyberg, Makeda Stephenson, and Aine O'Sullivan to discuss project related elements as well as all other factors related to professional development.

Weekly or bi-weekly meetings: Ethan Nyberg, Makeda Stephenson, and Aine O'Sullivan meet weekly in a group meeting with the PI, Dr. Warren Grayson where progress on the project is discussed. Srujan Singh engaged with Dr. Warren Grayson to discuss and design post operative studies which included mechanical testing and histological evaluation .

Meetings with clinicians: Ethan Nyberg and Makeda Stephenson have had meetings with Dr. Michael Grant to plan for the pig surgery and have performed an osteotomy on cadaveric pig head. Ethan Nyberg has also had meetings with a veterinarian/scientist (DVM/PhD) to discuss plans for the pig studies. Srujan Singh engaged with the clinicians frequently to make clinically correct assessments and draw right inferences from the post operative evaluations.

Conferences: Ethan Nyberg attended and presented posters at the Tissue Engineering and Regenerative Medicine International Society and the Mid-Atlantic Biomanufacturing Conference. Aine O'Sullivan attended and presented at Northeast Bioengineering Conference, and the Institute for NanoBiotechnology Fall Conference. Srujan Singh presented at the 11th World Biomaterials Congress and is scheduled to present at the 6th TERMIS World Congress.

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

We have observed very distinct pattern of bone formation in response to PCL-DCB/SVF combination therapy in between murine and porcine critical sized bone defects. For instance:

1. Bone growth seemed to be uniform throughout the defect volume within a critical sized calvarial defect in murine studies when augmented with PCL-DCB/SVF combination therapy.
2. Two distinct patterns of new bone formation was observed at the zygomatic defect site in porcine studies. Majority of the new bone were found to be growing along the periphery of the scaffolds from the neighboring native bone due to osteo-conduction. They were found to be lamellar bone histologically. In addition, dense new bone nodules were found out to be nucleating within the pores of the scaffold in close association with the PCL-DCB scaffolds suggesting osteo-induction. The volume of these new bone nodules within the pores was much lower as compared to the new bone growing on the periphery. Histology demonstrated that these bones were woven bone which may hint to limited bone remodeling within the scaffold volume.

Our study provides insight into spatio-temporal growth patterns of new bone in a clinically relevant, critical sized periorbital defect in response to a point-of-care management strategy involving custom PCL-DCB scaffolds and autologous SVF. The findings from this study demonstrate that bone regeneration in small volume porous scaffolds can vary a lot from that of a large volume scaffold. This provides motivation and opportunity for conceiving new design strategies for fabricating large volume porous scaffolds to treat clinically relevant defects. Moreover, it gives a chance to understand the technical and logistical challenges associated with translating bench side research into a clinical setting.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals.

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS:

- **Publications, conference papers, and presentations**
Journal publications.

- Manuscript titled “**Point-of-Care Treatment of Geometrically Complex Midfacial Critical-Sized Bone Defects with 3D-Printed scaffolds and Autologous Stromal Vascular Fraction**” has been submitted to the journal “**Biomaterials**” and is currently under review.
- Manuscript titled “**A robust, autonomous, volumetric quality assurance method for 3D printed porous scaffolds**” has been submitted to the journal “**3D Printing in Medicine**” and is currently under review.
- Article titled: **Comparison of Stromal Vascular Fraction and Passaged Adipose-Derived Stromal/Stem Cells as Point-of-Care Agents for Bone Regeneration** published in the journal **Tissue Engineering Part A**
- Article titled: **scafSLICR: A MATLAB-based slicing algorithm to enable 3D-printing of tissue engineering scaffolds with heterogeneous porous microarchitecture** published in the journal **PLOS ONE**.

Books or other non-periodical, one-time publications.

Nothing to report

Other publications, conference papers, and presentations.

1. Poster presentation at TERMIS-Americas: **E. L. Nyberg**, A. Farris, W. Grayson. *Assessing the Osteogenic Potential of Adipose-derived Stromal Vascular Fraction Cells*. Poster at the Tissue Engineering and Regenerative Medicine International Society Annual Conference. Charlotte, North Carolina. (2017) **** Winner, Student and Young Investigator Section ****
2. Poster presentation at JHU Department of Medicine Annual Retreat **E. L. Nyberg**, A. Farris, W. Grayson. *Assessing the Osteogenic Potential of Adipose-derived Stromal Vascular Fraction Cells*. (2018) **** Finalist, School of Engineering Trainee ****
3. Invited presentation at World Congress of Biomechanics. “Design and Manufacture of 3D-Printed Scaffolds for Regeneration of Massive Craniofacial Bone Loss”, Ethan Nyberg, Aine O’Sullivan, Warren Grayson.
4. Oral presentation at the Northeast Bioengineering Conference "3D-Printing Heterogeneous Porous Patterns in Tissue Engineered Bone Scaffolds", Aine O'Sullivan, Ethan Nyberg, and Warren Grayson.
5. Poster presentation at 2019 Military Health System Research Symposium (MHSRS) with the poster title “**Point-of-Care cell based strategy for treating large craniofacial bone defects**”.
6. Oral presentation (virtual) at **11th World Biomaterials Congress, Glasgow 2020**. Abstract title: “Point-of-Care Cell-Based Strategy for Treating Large Craniofacial Bone Defects”.
7. Abstract titled “Point-of-Care Cell-Based Strategy for Treating Large Craniofacial Bone Defects” selected for oral presentation at the **6th TERMIS World Congress 2021, Maastricht, The Netherlands**.

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Nothing to report

- **Inventions, patent applications, and/or licenses**

Full patent application in review:
US Patent Application: 15/739946
Grayson WL, Hung BP, Elisseeff JH, 3D-Printed Extracellular Matrix Mixture and
ECM Scaffolds Made with Polycaprolactone and decellularized bone.

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name	Project Role	Researcher Identifier (e.g. ORCID ID):	Nearest person month worked	Contribution to project
Warren Grayson	Principal Investigator	0000-0001-6099-6469	15	Coordinated interaction with LSU facility for design of pig study; Performed work towards implant design. Worked on new IRB application.
Srujan Singh	Graduate Student		24	Analysis of data and post operative evaluations
Ethan Nyberg	Graduate Student		36	Design of 3D-printed implants; evaluated surgical model; design of pig study; mouse study. Swine cell isolation
Makeda Stephenson	Graduate Student Fellowship Support		12	Filament manufacture; development of degradation protocol, degradation studies
Aine O'Sullivan	Research Technologist		12	Swine cell isolation. Implant manufacturing.
Ashley Farris	Graduate Student		12	Swine cell isolation
Alexandra Rindone	Graduate Student		2	Swine cell isolation
Nicholas Zhang	Undergraduate student		12	Print Quality Assessment

Emma Whitehead	Undergraduate Student		12	Analysis of post operative CT data
Yuxiao Zhou	Post-doctoral candidate		12	Analysis of microCT data
Chukwuebuka C. Achebe	Undergraduate student		12	Mechanical Testing
Eszter Mihaly	Graduate Student		2	Flow cytometry characterization
Michael Grant	Orthopedic Surgeon		12	Reconstruction surgery and implant harvest
Joseph Lopez	Orthopedic surgeon		12	Reconstruction surgery and implant harvest
Mandi Lopez	Veterinarian		12	Reconstruction surgery and implant harvest
Catherine Takawira	Veterinarian		12	Reconstruction surgery and implant harvest
Takashi Taguchi	Veterinarian		12	Reconstruction surgery and implant harvest

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Active grants that have closed:

NIH/NIBIB

Role: PI

Period: 09/01/14-08/31/16 0.6 CM

Title: Tunable Oxygen Delivery to Cells Using Novel Microtank Technology

Program Officer: Rosemarie Hunziker, hunzikerr@mail.nih.gov

New active grants:

NSF CAREER Award - 1350554

Role: PI

Period: 05/15/14 – 05/14/19 0.6 CM

Program Officer: Steven Peretti, speretti@nsf.gov

Title: Modeling Stem Cell Decision Making During Vascularized Bone Development

Maryland Stem Cell Research Fund – Investigator-Initiated Research Proposal

Role: PI

Period: 07/01/16 – 06/30/19 1.2 CM

Program Officer: Dan Gincel, dgincel@tedco.md

Title: Engineering Contractile Muscle for Treatment of Volumetric Muscle Loss

Congressionally Directed Medical Research Programs

Role: Multi-PI (Contact PI)

Period: 9/30/17-9/29/20 1.2 CM

Scientific Officer: Nicole Enman nicole.m.enban.ctr@mail.mil

Title: Multi-Parametric Bioreactor for Functional Preservation of Vascularized Composite Allografts

Maryland Stem Cell Research Fund

Role: PI

Period: 07/01/18 – 12/31/19 0.6 CM

Program Officer: Dan Gincel, dgincel@tedco.md

Title: 3D-Printed, Oxygen-Delivering Scaffolds for Regenerating Vascularized Craniofacial Bone

What other organizations were involved as partners?

Geistlich Pharma North America Inc.
202 Carnegie Center
Princeton, NJ 08540

Provided in-kind support by contributing Bio-Oss, the clinical grade bone ingredient in the implants.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Nothing to Report.

QUAD CHARTS:

9. APPENDICES:

Nothing to Report.

ORIGINAL ARTICLE

Comparison of Stromal Vascular Fraction and Passaged Adipose-Derived Stromal/Stem Cells as Point-of-Care Agents for Bone Regeneration

Ethan Nyberg, BS,^{1,2} Ashley Farris, BS,^{1,2} Aine O'Sullivan, BS,^{1,2} Ricardo Rodriguez, MD,³ and Warren Grayson, PhD^{1,2,4,5}

Large craniofacial bone defects remain a clinical challenge due to their complex shapes and large volumes. Stem cell-based technologies that deliver osteogenic stem cells have shown remarkable regenerative potential but are hampered by the need for extensive *in vitro* manipulation before implantation. To address this, we explored the bone forming potential of the clinically relevant stromal vascular fraction (SVF) cells obtained from human lipoaspirate. SVF cells can be isolated for acute use in the operating room and contain a subpopulation of adipose-derived stromal/stem cells (ASCs) that can develop mineralized tissue. ASCs can be purified from the more heterogeneous population of SVF cells via secondary and tertiary culture on tissue culture plastic. In this study, the relative potential for using SVF cells or passaged ASCs to induce robust bone regeneration was compared. Isogenic SVF and ASCs were suspended in fibrin hydrogels and seeded in three-dimensional-printed osteoinductive scaffolds of decellularized bone matrix and polycaprolactone. *In vitro*, both cell populations successfully mineralized the scaffold, demonstrating the robust bone formation properties of SVF. In murine critical-sized cranial defects, ASC-loaded scaffolds had greater (but not statistically significant) bone volume and bone coverage area than SVF-loaded scaffolds. However, both cell-laden interventions resulted in significantly greater bone healing than contralateral acellular controls. In conclusion, we observed substantial *in vitro* mineralization and robust *in vivo* bone regeneration in tissue-engineered bone grafts using both SVF and passaged ASCs.

Keywords: bone tissue engineering, stromal vascular fraction, adipose-derived stromal/stem cells, 3D printing, osseointegration

Impact Statement

The inability to effectively regenerate bone within critical-sized craniofacial defects is a present clinical challenge and overcoming this limitation using tissue engineering strategies would significantly advance current treatment outcomes. The present study tests the feasibility of harvesting stem cells intraoperatively, combining them with three-dimensional (3D)-printed osteoinductive scaffolds and, without culturing *in vitro*, implanting them into the bone defect to stimulate regeneration. The data from this study demonstrated that SVF isolated from lipoaspirate and used *in vivo* with minimal processing could be combined with a 3D-printed bioactive material in a point-of-care approach to promote bone regeneration.

Introduction

ANNUALLY, THERE ARE OVER 200,000 bone replacements for reconstruction of craniomaxillofacial (CMF) bones in the United States.¹ Reconstructive surgeries are performed following trauma, congenital malformation, or cancerous resectioning. While bone is a self-healing organ, critical-sized

(nonhealing²) CMF bone defects are challenging to treat due to their geometric complexity and the volume of viable bone needed. Compared with other bony defects, massive loss of CMF bone structure is more likely to cause disfigurement and psychosocial pathologies.^{3,4} The current standard of care is the free fibular bone graft, which poorly replicates the mechanics and anatomy of defects, is resorbed at high rates,⁵

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⁵Institute for NanoBioTechnology, Johns Hopkins University, Baltimore, Maryland.

exhibits a slow rate of conduction from the surrounding bone (~ 1 cm/month),⁶ and may be negatively impacted by other comorbidities that impair bone healing, such as radiation osteonecrosis.⁷ Alternatively, stem cell-mediated bone regeneration⁸ using tissue engineering strategies has significant potential for regenerating massive CMF defects.

Adipose-derived stromal/stem cells (ASCs) have been considered a potent cellular agent for tissue engineering and regeneration; they are readily available in patients, their isolation causes limited morbidity, they possess multipotency for bone and cartilage, and they are potent agents of neovascularization.^{9,10} Compared with the bone marrow mesenchymal stem cells (MSCs), however, ASCs reportedly have a lower relative osteogenic potency.^{11,12} Consequently, additional processing steps are required to promote osteogenic differentiation and thus for them to function as skeletal progenitor stem cells.¹³ Accordingly, most reported strategies are limited by the need for extensive *in vitro* manipulation of the ASCs to generate an osteogenic phenotype before implantation.

ASCs are derived from the adherent subpopulation of the stromal vascular fraction (SVF) of cells from lipoaspirate. They are a less heterogeneous population of cells than SVF.^{14,15} In particular, the subpopulation of endothelial cells is reduced in ASC cultures. Prior studies have demonstrated that bone forming cells and endothelial cells together elicit greater bone formation responses than alone.^{16,17} Therefore, the inherent heterogeneity of SVF might be advantageous to bone regeneration.

SVF is a potential alternative cell source for bone regeneration to ASCs that may be used intraoperatively.^{18–20} Prior uses of SVF in small-animal models and in human clinical trials²¹ have yielded mixed results. Güven *et al.* compared SVF seeded for 5 days in a perfusion bioreactor before ectopic implantation in rats with isogenic ASCs and MSCs that had been similarly prepared. They found SVF-seeded constructs had faster tissue ingrowth, increased vasculature, and bone formation compared with ASCs and MSCs.²² Rhee *et al.* used syngeneic rat SVF in a cranial model, which increased regeneration from 13% in acellular controls to 58% in SVF groups.²³ Finally, Prins *et al.* used autologous SVF in maxillary elevation surgery in humans and found very slight improvements in bone volume and area when SVF was included, compared with acellular contralateral treatments.²⁴

There are several case reports of SVF/ASCs used to treat volumetric CMF defects in human. First, Lendeckel *et al.* reported on SVF in combination with milled iliac bone, resorbable microporous sheets, and fibrin glue with excellent results 3 months postoperatively.²⁵ Mesimäki *et al.* used ASCs, bone morphogenic protein-2, and beta-tricalcium phosphate to develop an ectopic bone flap to successfully create a neomaxilla with good results 1 year postoperatively.²⁶ Sándor *et al.* used autologous cultured ASCs as an adjuvant to implants/bone granules in a variety of craniofacial defects. They found acceptable hard tissue graft function in mandibular and midface locations. However, cranial defects showed unexpected resorption rates²⁷ and the 6-year follow-up was unsatisfactory.²⁸ SVF treatments have not demonstrated a robust amount of bone formation in humans, nor have they improved bone healing compared with standard-of-care or acellular treatments.

In this study, we explored the potential of human SVF to enable robust bone regeneration *in vitro* as well as within orthotopic *in vivo* murine calvarial defect models. The SVF was obtained from human donors and immediately combined with

three-dimensional (3D)-printed osteoinductive scaffolds,^{29,30} in the absence of growth factors, to mimic an intraoperative procedure. Furthermore, we sought to determine whether the bone regeneration potential of the SVF was comparable with that of isogenic ASC populations similarly seeded in 3D-printed scaffolds. We performed a similar study in scaffolds of larger volumes and implanted intramuscularly to evaluate the angiogenic characteristics of SVF and ASCs. To our knowledge, this is the first study to directly compare the osteogenic and angiogenic potential of intraoperative SVF with isogenic ASCs.

Materials and Methods

Study design

SVF was isolated from human lipoaspirate and cultured to obtain isogenic ASCs. Flow cytometry characterization was performed on all cell populations. The bone forming potential of the cells was then assessed in 3D-printed scaffolds *in vitro* and orthotopically *in vivo*. The vascular potential of the cells was also examined *in vivo* using an intramuscular model. *In vitro* results were the average of all six donors, while only one donor was used for *in vivo* studies. The study design is summarized in Figure 1.

Cell isolation and culture

Human subcutaneous adipose tissue was obtained in the form of lipoaspirate from six female Caucasian donors undergoing elective surgery, with written informed consent and with institutional review board approval (#846242-1). ASCs were isolated as previously described.^{31,32} Briefly, tissue was digested with collagenase type 1 (1 mg/mL; Worthington Biochemical Corp.) to isolate the SVF of cells. Cells were filtered through a 100 μ m filter and termed “SVF.” SVF was directly seeded into scaffolds or used for flow cytometry. Cells not used as SVF were plated onto tissue culture plastic and were termed “passage 0 ASCs.” Cells were passaged with trypsin when they reached 80–90% confluence, termed “passage 1 ASCs.” On reaching 80–90% confluence, these cells were lifted with trypsin and used as “passage 2 ASCs” for all experiments with ASC groups. Growth medium consisted of high-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco) with 10% fetal bovine serum (FBS; Atlanta Biologicals), 1% penicillin/streptomycin (Gibco), and 1 ng/mL basic fibroblast growth factor-2 (PeproTech).

Flow cytometry

Cell surface markers were analyzed using flow cytometry to determine cell phenotype: mesenchymal (CD73), endothelial (CD31), stem/progenitor (CD34), and hematopoietic (CD45). Briefly, SVF cells were resuspended and passaged ASCs were detached and resuspended in phosphate-buffered saline (PBS) containing 2% FBS. Cells were then incubated with monoclonal antibodies conjugated to fluorescein isothiocyanate, phycoerythrin, or PerCP-Cy5.5 for 30 min at 4°C and cell surface markers were analyzed on a Sony SH800 cell sorter. All markers were acquired from BD Biosciences (Supplementary Table S1).

Preparation of decellularized bovine bone extracellular matrix

All materials were obtained from Sigma unless otherwise stated. Calf knees were obtained from Green Village Packing

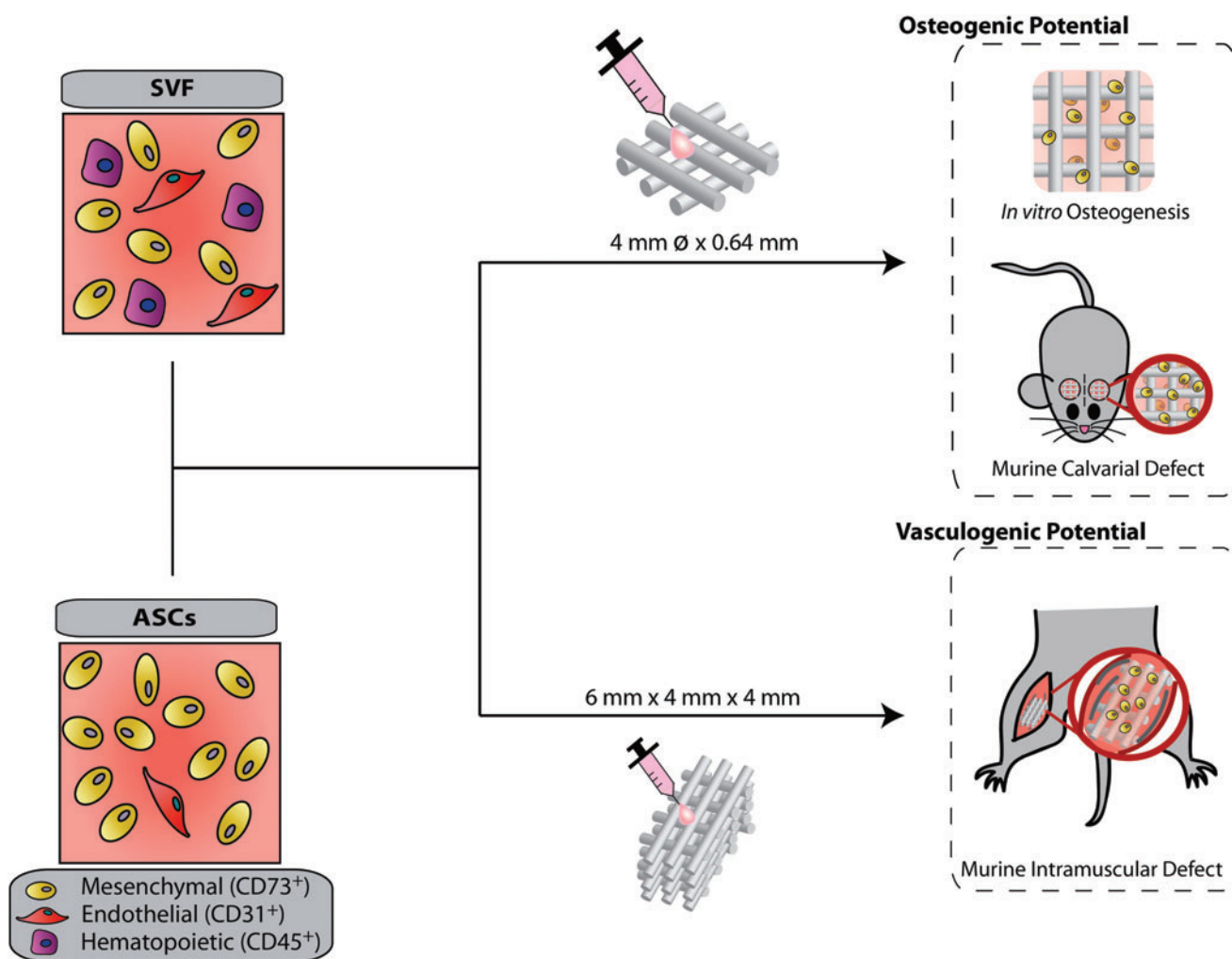


FIG. 1. Schematic of study outline. Cells were sourced from human donors undergoing elective liposuction. Freshly isolated cells (SVF) and isogenic cultured cells (ASCs) were used in all the experiments. Cells were evaluated with flow cytometry and used for *in vitro* differentiation and *in vivo* tissue formation assays. ASC, adipose-derived stromal/stem cell; SVF, stromal vascular fraction. Color images are available online.

Co. (Green Village, NJ). Decellularized bovine bone extracellular matrix (DCB) was obtained by isolating trabecular bone from calf knees. The bone was decellularized using a protocol as described previously.³³ Briefly, trabecular bone fragments were blasted with water to remove as much cellular debris as possible. The bone fragments were then placed in a series of four detergent washes of 0.1% EDTA for 1 h, 0.1% EDTA/10 mM Tris for 12 h, 0.5% sodium dodecyl sulfate (SDS)/10 mM Tris for 24 h, and 50 U/mL DNase, 1 U/mL RNase, and 0.1% EDTA/10 mM Tris for 5 h. Following the washes, the bone was rinsed with PBS and lyophilized. The decellularized bone fragments were pulverized using SPEX SamplePrep 6770 cryomill (SPEX SamplePrep, Metuchen, NJ) at a frequency of 10 cycles per second for 45 min to obtain powdered bone.

Three-dimensional printing of scaffolds

Cryomilled decellularized bone powder was mixed 30% w/w with powdered polycaprolactone (PCL; 43k–50k MW; Polysciences 25090) by sifting through a stainless steel 400 µm mesh three times. PCL-DCB scaffolds were manufactured using an in-house pneumatic fused deposition system mounted

to a CNC machine with a nozzle diameter of 460 µm.¹⁰ Briefly, a powdered mixture of PCL-DCB was loaded into the nozzle chamber and heated to 100°C to bring the polymer to a liquid phase. Pneumatic pressure was then applied to expel the liquefied mixture out of a 460 µm brass extrusion die at the bottom of the chamber. Scaffolds for cell studies^{29,30,33} were prepared using rectilinear patterns with 60% void volume and two layers in height (0.640 mm) and punched to 4 mm in diameter. Intramuscular PCL-DCB scaffolds were printed in the same pattern and cut to 6×4×4 mm (length×width×height). Intramuscular scaffolds are larger to provide 3D depth to assess vascular formation. Before seeding cells, scaffolds were treated with 1 M NaOH for 1 h to increase hydrophilicity, washed with PBS, soaked in 100% EtOH for 1 h to sterilize, and immersed in 100% FBS at 37°C for 1 h to facilitate protein adsorption to the surface of the scaffold.

Scaffold seeding

Cells were suspended in fibrinogen (8 mg/mL final; Sigma) and then mixed with thrombin (2 U/mL final; Sigma) for a final cell concentration of 2×10^4 cells/µL gel. Constructs

were seeded by pipetting 12 μ L of gel and cell solution into 4-mm-diameter scaffolds and incubating at 37°C for 30 min to allow complete gelation before the addition of medium. Twice the gel volume (24 μ L) was used for intramuscular scaffolds to account for the larger volume of the scaffold. For *in vitro* culture, control medium (CM) scaffolds were fed with 0.5 mL of low-glucose DMEM (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin. Osteogenic medium (OM) scaffolds were fed with 0.5 mL of control media supplemented with 10 mM β -glycerol phosphate and 50 μ M ascorbic acid-2-phosphate. Media were exchanged every other day. For *in vivo* studies, scaffolds were incubated for 10 h in 0.5 mL of low-glucose DMEM (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin before implantation.

Gene expression

After the culture periods, scaffolds were digested with TRIzol (Life Technologies) and isolated mRNA was used to produce cDNA. cDNA was subject to real-time polymerase chain reaction for osteogenic genes *COL1A1*, *RUNX2*, osteopontin (*OP*), osteocalcin (*OCN*), and osteonectin (*ON*) as previously described.³⁴ The primers used are presented in Supplementary Table S2. For analysis, the delta/delta Ct method was used, in which β -actin served as the housekeeping gene, and gene expression was normalized to that of SVF cells at the time of isolation.

Biochemical assays

Scaffolds were cultured for 3 weeks in control or osteogenic conditions ($n = 4$ per assay). Calcium and DNA were measured as previously described.³⁰ Briefly, cellular DNA quantities were assessed using the Quant-It PicoGreen dsDNA assay (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total DNA at 21 days and the fold change from day 0 total DNA are reported. Total calcium was measured by agitating scaffolds in 0.5 N hydrochloric acid for 24 h and then measuring the calcium in solution using a Stanbio LiquiColor calcium assay (Stanbio, Boerne, TX) to determine calcium content. Samples were mixed with the color reagent 1:100 and absorbance was read on a plate reader at 550 nm according to assay instructions. Samples were reduced by the absorbance from wells without sample and interpreted as a fraction of the absorbance of the standard (10 mg/dL). PCL-DCB scaffolds were also reduced by the amount of calcium retrieved from acellular scaffolds, resulting in "deposited calcium." Calcium content was normalized to the amount of cellular DNA. Mineralized calcium was detected using alizarin red S (Sigma A5533) staining and detected using brightfield microscopy.

Animal studies

In conducting research using animals, the investigator(s) adhered to the laws of the United States and regulations of the Department of Agriculture. All studies were approved by the Johns Hopkins University Institutional Animal Care and Use Committee (Protocol No. M016M468). Male nude immunocompromised mice (*fox1n1*; Charles River) were used at 8 weeks of age.

Assessing bone regeneration in cranial defects. To determine the effect of cells and scaffolds *in vivo*, the critically

sized murine calvarial defect model was used as previously described.^{34–36} Briefly, a 4-mm-circular knife (Medicon, Tuttingen, Germany) was used to excise a 4-mm disk of calvaria between the coronal and lambdoid sutures and 1 mm lateral to the sagittal suture. Constructs consisting of SVF or ASCs seeded in fibrin inside DCB-PCL scaffolds were press-fit into the resulting defect. A total of 14 mice were operated, 7 for SVF and 7 for ASCs, with $n = 3$ at 6 weeks and $n = 4$ at 12 weeks. The contralateral sides of each animal were also operated and treated with acellular control scaffolds. Postmortem imaging of defects with computed tomography (CT) used a nanoScan PET/CT (Mediso, Budapest). Imaging was performed at 35 kV peak voltage and 800 μ A current (0.48 mAs). Reconstruction was done with a voxel size of 40 μ m. Scans were conducted at 6 and 12 weeks postimplantation. Samples were fixed in 3.7% formalin overnight for histological analysis.

Assessing vascular potential in intramuscular implantation. Blunt dissection was used to create pockets in the hamstring portion of each leg.³⁷ Scaffolds were placed into the pockets and the tissue was sutured closed. Each animal received an SVF- or ASC-loaded scaffold and a contralateral acellular control. After 10 days ($n = 3$) or 6 weeks ($n = 4$), mice were sacrificed and MicroFil-MV[®] (Flow Tech, Inc., Carver MA),³⁸ a radiopaque polymer, was perfused through the vasculature. Microfil-MV was mixed at 20:8:1 (diluent:compound:curing agent) and perfused intracardially at 250 μ L/min following perfusion of heparinized saline (5 U/mL). Samples were dissected out and fixed in 3.7% formalin overnight. Finally, μ CT (Bruker SkyScan 1275, 10 μ m resolution, 65 keV with 1-mm aluminum filter, 0.3° rotation between images) was used to determine the extent of scaffold vascularization in implanted scaffolds. Analysis was done in Mimics (Materialise). First, the vasculature and scaffold were identified by thresholding. Second, the vasculature was manually segmented from the scaffold based on different morphologies of scaffold and vasculature. Finally, the segmented vasculature was cropped to the boundaries of the scaffold, and the volume of cropped vasculature within the scaffold was reported.

Histology

Samples were decalcified in 14% EDTA for 14 days and then trimmed to the cranial region. Samples were then embedded in 2% w/v agarose gel, dehydrated, and placed in chloroform overnight to remove the PCL phase. They were then washed three times with hot paraffin to remove residual chloroform and cast into paraffin wax blocks. Blocks were sectioned at 7 μ m using a microtome (Leica). Masson's trichrome staining was performed according to the manufacturer's specifications (Sigma HT15). Stained sections were imaged at 5 $\times$ and 20 $\times$ magnification on an upright microscope (Zeiss) and stitched³⁹ in ImageJ (Bethesda, MD).

Statistical analysis

Quantitative data are expressed as mean $\pm$ standard error. Statistical analyses were performed using GraphPad Prism 5 software. Statistical significance was determined by one-way ANOVA with Tukey's post-test and is denoted as * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

TABLE 1. DONOR PROFILES AND CELLULAR YIELD

Donor	Age	Race	Sex	BMI	Location(s)	Yield (cells/mL of clean fat)
1	54	Caucasian	F	25	Flank/Scapular	63,750
2	37	Caucasian	F	23.9	Flank/Inner thigh	120,000
3	54	Caucasian	F	28.6	Flank/Scapular	700,000
4	43	Caucasian	F	20.8	Flank/Abdomen	145,000
5	32	African American	F	25.8	Flank	676,000
6	22	African American	F	25.7	Flank/Abdomen	278,000

Results

Cell characterization

Cells were collected from six donors; the donor demographics and cellular yields are summarized in Table 1. Cell populations were analyzed using flow cytometry and markers for endothelial cells, hematopoietic cells, and stem cells (Fig. 2A). There was little donor-to-donor variability in the number of cells positive for each cell surface marker. The percentages are presented as the average $\pm$ standard deviation of the six donors. There was a greater presence of endothelial cells (CD31⁺) in the SVF population ($12.9\% \pm 0.82\%$) compared with the ASC population ($1.3\% \pm 0.13\%$). Likewise, we observed a decline in hematopoietic cells (CD45⁺) in the SVF ($15.6\% \pm 1.6\%$) compared with ASCs ($0.60\% \pm 0.065\%$). We observed a decrease in CD34 (SVF = $68.2\% \pm 2.54\%$; ASCs = $8.116\% \pm 0.63\%$) and increase in CD73 from SVF to ASCs (SVF = $17.6\% \pm 1.25\%$; ASCs = $95.7\% \pm 1.43\%$) (Fig. 2B).

In vitro mineral deposition

DNA and calcium contents as well as gene expression in the PCL-DCB scaffolds were measured after 21 days (Fig. 3). There were greater cell numbers in OM versus CM, and SVF scaffolds had less total DNA than those seeded with ASCs (Fig. 3A, OM: SVF = 704.2 ± 72.6 ng; ASCs = 944.7 ± 9.0 ng; $p < 0.005$, CM: SVF = 433.8 ± 18.6 ng; ASCs = 757.3 ± 15.2 ng; $p < 0.005$). SVF scaffolds had greater proliferation than ASCs in OM (Fig. 3B, SVF = 1.5 ± 0.15 -fold, ASCs = 1.3 ± 0.012 -fold). *In vitro* cell cultures were used to test whether SVF had the potential to mineralize PCL-DCB scaffolds to a similar extent as passaged ASCs. *In vitro* culture over 3 weeks revealed that both SVF and ASCs mineralized scaffolds. While SVF and ASCs grown on PCL-DCB scaffolds had similar total mineralization (Fig. 3C, OM: SVF = 59.0 ± 3.3 μ g Ca²⁺; ASCs = 56.2 ± 3.2 μ g Ca²⁺ n.s.), SVF had a greater mineralization per cell (Fig. 3D, DCB-OM: SVF = 75.6 ± 4.3 ng Ca²⁺/ng DNA; ASCs = 61.0 ± 3.8 ng Ca²⁺/ng DNA; $p < 0.005$).

To confirm that the DCB in the scaffolds was osteoinductive, we measured DNA and calcium in PCL-only scaffolds (Supplementary Fig. S1, PCL-OM: SVF = 51.2 ± 3.2 ng Ca²⁺/ng DNA; ASCs = 45.3 ± 3.6 ng Ca²⁺/ng DNA; SVF vs. ASCs $p < 0.005$, PCL vs. PCL-DCB $p > 0.05$). Both SVF and ASCs responded to osteoinductive cues in the PCL-DCB scaffolds. The cells also responded to the PCL-DCB scaffolds by upregulating key bone forming genes independent of media condition. While *RUNX2*, *OCN*, and *OPN* were significantly more upregulated in ASCs in OM, *COL1A1* was more significantly upregulated in SVF groups

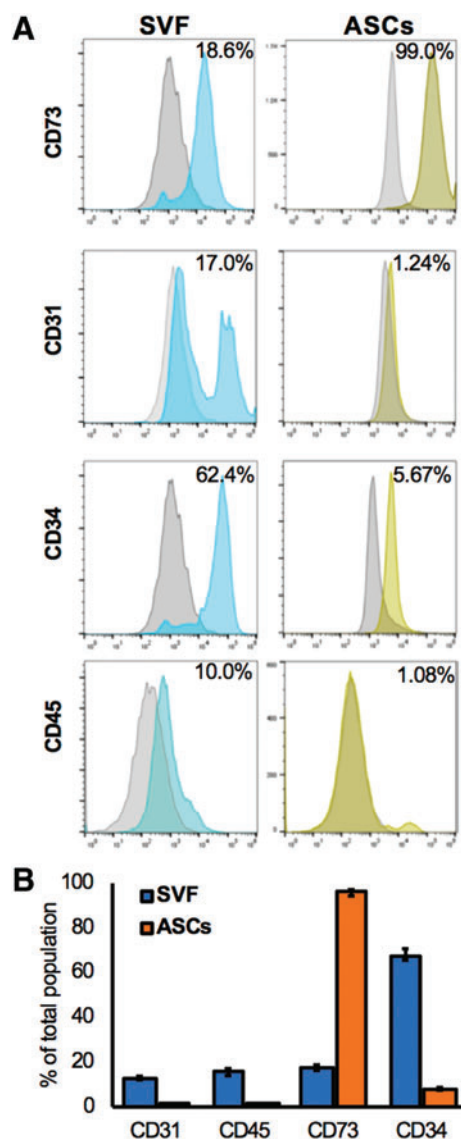
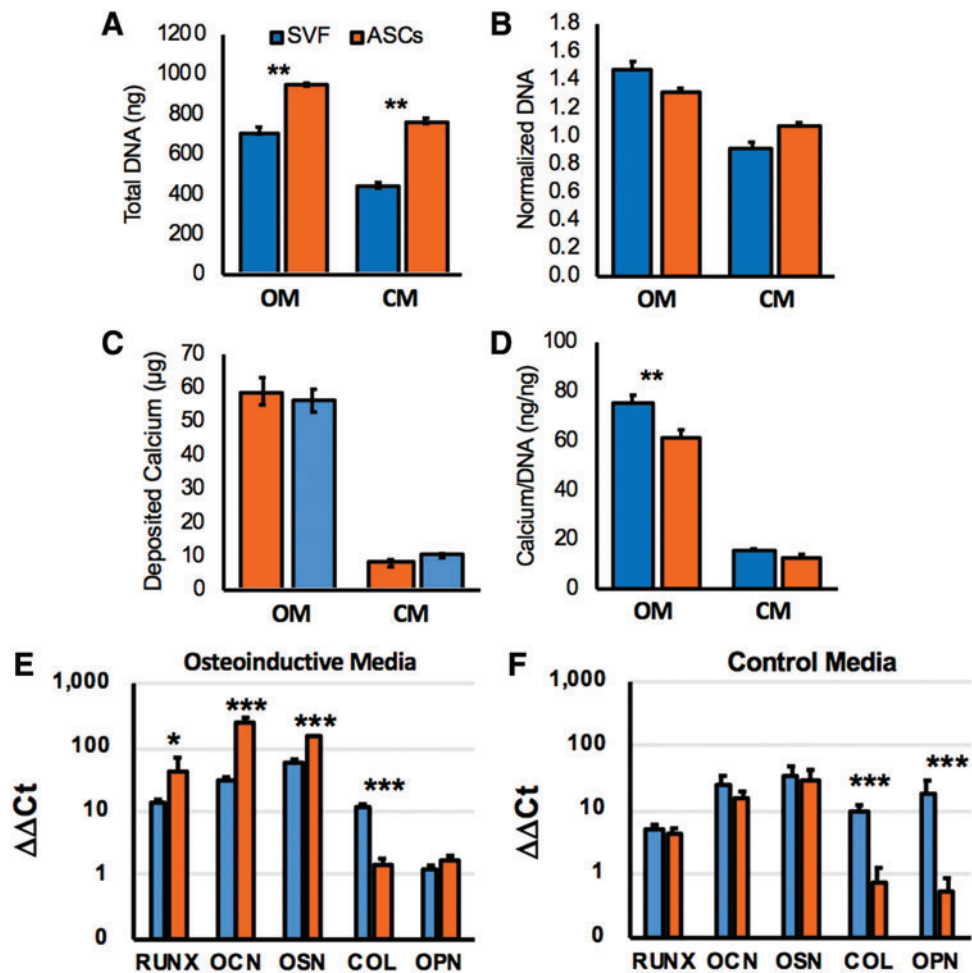


FIG. 2. Flow cytometric analysis of cell populations. (A) Representative flow cytometry histograms for endothelial (CD31), hematopoietic (CD45), and stem cell (CD34, CD73) markers before and after culture. (B) Surface marker profiles averaged for all six donors. Data shown as average $\pm$ standard deviation. Color images are available online.

FIG. 3. *In vitro* mineralization of SVF and ASCs in PCL-DCB scaffolds over 21 days. SVF and ASCs from six donors were cultured in OM and CM in PCL-DCB scaffolds. (A) Total DNA content. (B) DNA normalized to day 0 amounts. (C) Deposited calcium in the scaffolds. (D) Deposited calcium normalized to total DNA. (E, F) Bone gene expression of one donor at 21 days, on PCL-DCB scaffolds. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. CM, control media; DCB, decellularized bovine bone extracellular matrix; OM, osteoinductive media; PCL, polycaprolactone. Color images are available online.



regardless of media condition (Fig. 3E, F, OM: SVF = 11.3 ± 0.41 ; ASCs = 1.4 ± 0.41 ; $p < 0.005$, CM: SVF = 9.8 ± 0.53 ; ASCs = 2.6 ± 0.25 ; $p < 0.005$).

Bony healing

To test the ability of these cells to regenerate bone in an orthotopic environment, SVF and ASCs were loaded onto PCL-DCB scaffolds and implanted into critical-sized cranial defects in mice (Fig. 4A and Supplementary Fig. S2). The inclusion of cells led to an increased amount of bone volume when compared with contralateral acellular defects. At 12 weeks after surgery, SVF (Fig. 4B, paired SVF = $4.72 \pm 0.10 \text{ mm}^3$ and acellular = $4.10 \pm 0.12 \text{ mm}^3$; $p < 0.005$) and ASCs (Fig. 4C, paired ASCs = $5.07 \pm 0.24 \text{ mm}^3$ and acellular = $4.11 \pm 0.14 \text{ mm}^3$; $p < 0.05$) both had significantly more bone volume.

At 6 and 12 weeks, acellular scaffolds had open pore spaces with slight amounts of bone formation along the border of the defect. Acellular groups had less than 40% of the porous space filled (Fig. 4C, 6 weeks = $46\% \pm 5\%$; 12 weeks = $34\% \pm 10\%$). SVF groups had increased amounts of bone filling the pore spaces (Fig. 4C, 6 weeks = $73\% \pm 6\%$; 12 weeks = $78\% \pm 7\%$), and bone volume increased between weeks 6 and 12 (Fig. 4D, BV-PV at 6 weeks = $0.32 \pm 0.052 \text{ mm}^3$; 12 weeks = $0.62 \pm 0.04 \text{ mm}^3$, $p < 0.05$). ASCs filled the most pore volume with bone (Fig. 4C, 6 weeks = $83\% \pm 11\%$; 12 weeks = $100\% \pm 18\%$).

Histology

The osteogenic performance of implanted scaffolds at 6 and 12 weeks postsurgery was evaluated using Masson's trichrome stain. Cell-laden scaffolds (both SVF and ASC) showed greater amounts of dense collagen formation, as indicated by the dark blue coloring around scaffold struts, when compared with the acellular scaffolds, which showed more open red-colored fibrotic tissue around the struts (Fig. 5). Scaffolds with ASCs showed denser collagen formation, particularly between scaffold struts, at 6 and 12 weeks; although by 12 weeks, SVF scaffolds also had dense collagen indicative of bone matrix deposition between struts. In ASCs, the new bony matrix also appeared integrated into host bone on both sides of the defect. By 12 weeks, the defect was filled with new tissue in both SVF and ASC scaffolds, with a high concentration of collagen around scaffold struts and bridges of collagen in between them. By contrast, acellular scaffolds showed very little dense collagen present anywhere in the defect.

Vascular infiltration

To observe the ability of cells to promote an angiogenic response, larger constructs were implanted in the hind limbs of mice (Fig. 6A). Microfil perfusion was used to examine the extent of vascularization at 10 days and 6 weeks (Fig. 6B). At 10 days, there was minimal vascular infiltration

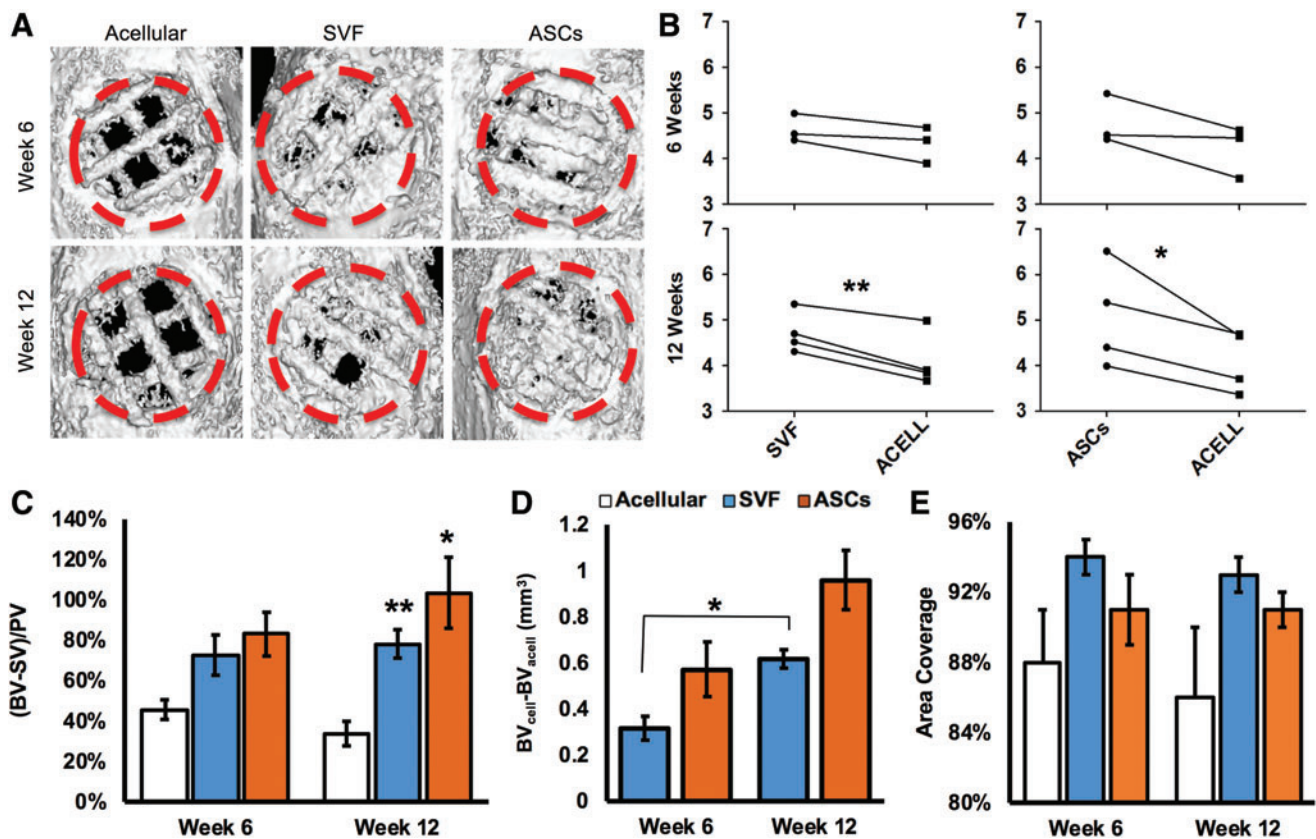


FIG. 4. *In vivo* bone forming potential. (A) MicroCT reconstructions of 4-mm-diameter cranial defects (red outline). (B) Pairwise comparison of bone volume per animal. Each connected pair indicates a single animal containing two defects (cellular and acellular). (C) Percentage of porous space filled with bone. Bone volume reduced by the initial scaffold volume and normalized to the porous volume: (Bone Volume – Scaffold Volume)/Pore Volume. (D) Bone volume within defect reduced by contralateral acellular defect bone volume. (E) Area coverage of defect area. Measured in ImageJ as (number of non-black pixels)/(total number of pixels) in the 4-mm-diameter circle. * $p < 0.05$, ** $p < 0.005$. CT, computed tomography. Color images are available online.

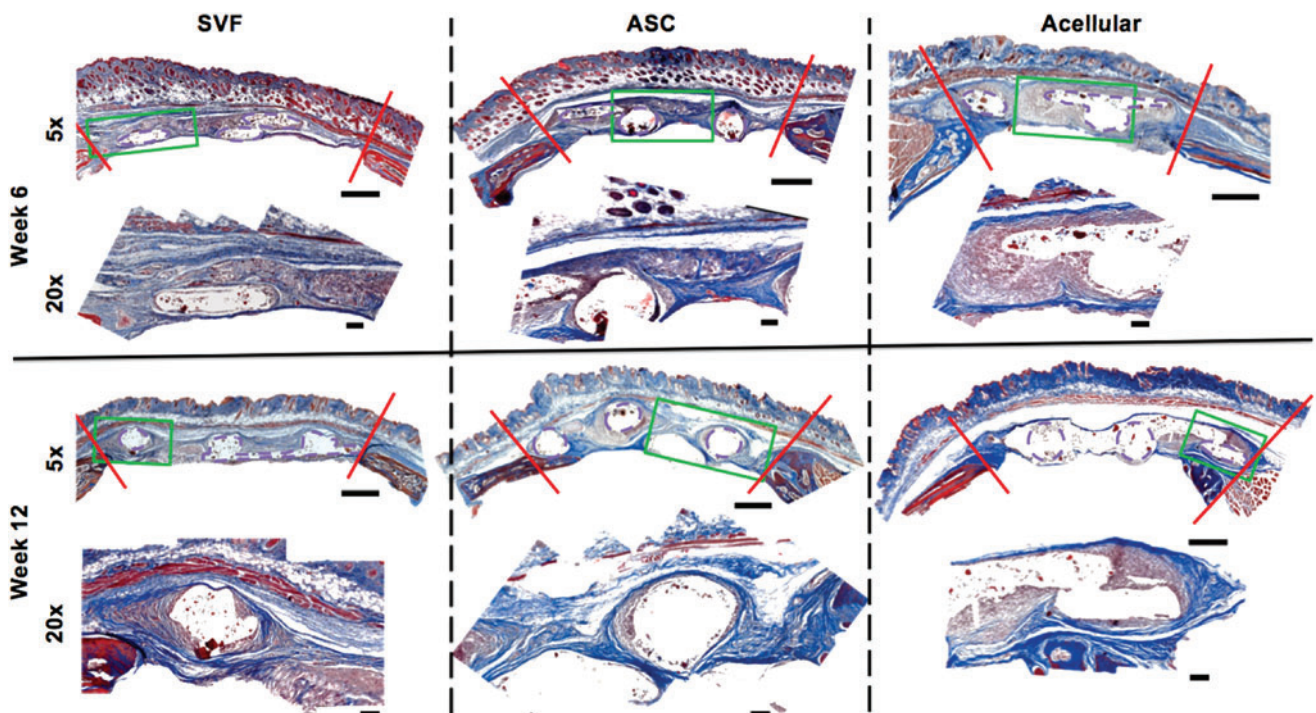


FIG. 5. Histology staining of murine cranial defects. Masson's trichrome staining on cross sections of samples at 6 (top row) and 12 weeks (bottom row). Scaffold struts indicated by empty white space, dense collagen as blue. Red lines indicate boundaries of the defect, and green boxes are the zoomed in section. Scale bars 5x = 500 μm; 20x = 100 μm. Color images are available online.

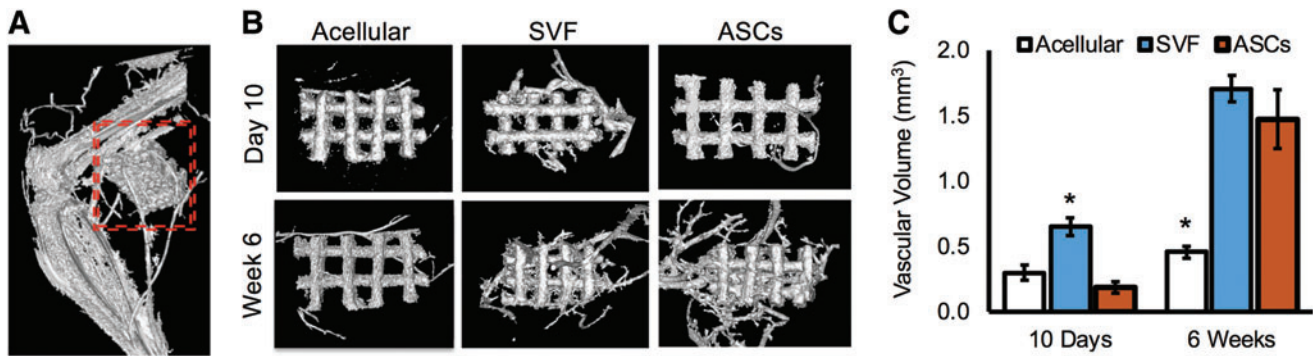


FIG. 6. Intramuscular implantation and Microfil perfusion (A) Example CT showing scaffold implant (red box) in relation to leg. (B) MicroCT reconstructions of scaffolds implanted intramuscularly, with the vasculature filled with radiopaque Microfil-MV. (C) Total vascular volume within scaffold. * $p < 0.05$. Color images are available online.

in the acellular and ASC groups (Fig. 6C, 0.30 ± 0.058 and $0.19 \pm 0.044 \text{ mm}^3$, respectively), and some infiltration into the SVF group (Fig. 6C, $0.65 \pm 0.068 \text{ mm}^3$, $p < 0.05$ vs. acellular and ASCs). At 6 weeks, cellular groups were both significantly greater than the acellular groups (Fig. 6C, ~3-fold, SVF = $1.71 \pm 0.10 \text{ mm}^3$, ASCs = $1.47 \pm 0.22 \text{ mm}^3$, acellular = $0.45 \pm 0.045 \text{ mm}^3$, $p < 0.05$) and the SVF group was similar to the ASC group.

Discussion

The inability to effectively regenerate bone within critical-sized craniofacial defects is a present clinical challenge and overcoming this limitation using tissue engineering strategies would significantly advance current treatment modalities. One approach would be to harvest SVF, combine it with 3D-printed osteoinductive scaffolds, and within the same surgical procedure, implant it into the bone defect to stimulate regeneration. This approach offers a number of advantages compared with traditional tissue engineering methods where cells (e.g., ASCs) are culture expanded and maintained in the scaffold *ex vivo* for a period of time before implanting into the defect site. Specifically, it would reduce the required number of operations for the patient, eliminate the cost and practical limitations associated with manipulating the cells *ex vivo*, and leverage the regulatory feasibility of an intraoperative versus multioperative procedure. This study addresses two potential limitations of this strategy, which is (i) whether minimally processed SVF delivered to a critical-sized bone defect within an osteoinductive scaffold in a manner that mimics a clinical intraoperative procedure could promote robust bone regeneration, and (ii) determining whether the bone formation by SVF is as effective as that obtained from culture-expanded ASCs.

To assess the feasibility of the SVF-based bone regeneration, we first characterized the isolated cells. While there was donor-to-donor variability in cell yield, the trends of osteoinduction and proliferation remained the same across donors, and we were able to isolate large quantities of cells that would be suitable for acute clinical use. Potential intervention for a defect of 4–10 cc would require on the order of 10^7 – 10^8 cells if seeded at concentrations ranging from 1 to 25×10^6 cells/mL. This corresponds to 250 mL of lipoaspirate. For this study, we obtained over 500 mL from each

donor of average BMI of 25. Cell marker distributions were also consistent across donors. The values in our study also correlated with those reported previously by other groups, which describe decreases in endothelial (CD31), hematopoietic (CD45), and CD34 subpopulations and a concomitant increase in mesenchymal (CD73) subpopulations.^{14,15,40}

As reported in a recent review, several groups are working to implement SVF or ASCs in clinical trials for bone regeneration.²¹ Yet, the ability of SVF or ASCs to directly differentiate into osteoblasts and produce bone *in vivo* remains contested. A recent study reported that despite the ability of ASCs to deposit mineral and turn on bone-forming genes *in vitro*, they were unable to spontaneously form ectopic bone¹² even though they promoted a robust angiogenic response and exhibited trophic function.⁴¹ Hence, demonstrating bone regeneration capacity in a critical-sized defect remains a crucial hurdle in clinical translation. While others have used autologous rodent SVF²³ in bone healing, we believe this study is the first reported case of nonpredifferentiated human SVF significantly contributing to new bone volume in small animals. As such, this work corroborates other studies, which have shown that SVF used in humans is safe and effective in promoting bone regeneration.²⁴

This study applied the SVF to the calvarial defect with minimal manipulation to simulate a clinically applicable intervention for bony regeneration that did not rely on *ex vivo* culture or “priming” of cells before implantation. While the ASCs were cultured for two passages, they were also implanted in the defect site without prior *in vitro* osteogenic differentiation, yet both cell types promoted mineral deposition that was significantly greater than the acellular controls. The amount of bone formation formed by the SVF was lower than that formed by ASCs although the difference was not statistically significant. The reason for this is not clear: While this result could potentially correlate with the relatively lower number of mesenchymal (CD73⁺; believed to be osteogenic progenitors) subpopulations present in SVF than ASCs, if this were true, the same trend should have been observed *in vitro*. Yet, both SVF and ASCs exhibited similar osteogenic potential *in vitro*.

In our prior studies, we have shown that human cells are present at the site of new bone formation³⁴ although we have not validated in either study that the cells responsible for new bone formation are human. Alternatively, it is possible that the cells act on endogenous cells to affect bone

formation. Recent work has shown that transplanted mesenchymal stem cells act on surrounding host cells with trophic, paracrine, and immunomodulatory functions.^{42–44} In future studies, we will use cells transduced with bone-forming gene reporter labels to probe the direct contribution of transplanted cells. The translational relevance of this study is limited by the use of xenogenic human cells in immunocompromised mice and also by the size differences between murine and human bones. However, these data are useful for informing subsequent studies using autologous cells in larger animals.

These experiments highlight the strength of a 3D-printed bioactive material-based approach to mediate bone regeneration. Three-dimensional printing technologies are powerful tools to facilitate patient-specific approaches.⁴⁵ Regenerative approaches that utilize 3D-printed scaffolds may avoid the stress shielding, loosening, and implant extrusion that cause permanent plastic, metal, and ceramic implants to fail.^{46,47} We have previously reported that 3D-printed PCL-DCB scaffolds enhanced the expression of bone-related genes (*RUNX1*, *COL1A1*, *OCN*) in ASCs³⁰ and regenerated bone in murine calvarial defects³³ in a dose-dependent manner, that is, the higher the concentration of DCB in the 3D-printed scaffolds, the greater the amount of mineral deposition *in vitro* and *in vivo* bone formation.³⁰ We have also shown that embedded DCB in 3D-printed PCL scaffolds was a more potent osteoinductive stimulus than other clinically used bone replacement materials such as tricalcium phosphate and hydroxyapatite.²⁹

In this study, SVF and culture-expanded ASCs were encapsulated in 3D-printed PCL-DCB scaffolds and implanted orthotopically *in vivo* without prior osteogenic differentiation and still gave rise to significant amounts of new bone formation. Several groups have demonstrated that predifferentiation of ASCs to osteoblasts results in substantial bone formation.^{11,22,48,49} Moving that differentiation process to the implanted scaffold environment eliminates *in vitro* preparation time and enables the osteoinductive signals to act, additionally, on endogenous cells. It should also be noted that the addition of exogenous growth factors induces robust bone regeneration,^{50–52} but the limitation is that they are provided at supraphysiological levels, which is not the case with the PCL-DCB 3D-printed scaffold system. Even the acellular scaffolds induced some bone healing, showing the robustness of the PCL-DCB-based scaffold approach.

The study also evaluated the relative angiogenic potential of SVF and ASCs. The formation of healthy regenerated bone in large, critical-sized defects in humans would rely on well-established vascular networks. To assess the vascular response induced by the cells, we used an ectopic model that enabled us to test scaffolds of larger volumes. Scaffolds were filled with fibrin hydrogel, which promoted vascular ingrowth even in acellular scaffolds, and we assessed specifically the perfused vascular networks by using Microfil-MV. Both SVF and ASCs exhibited roughly three times greater vascular responses than acellular scaffolds at 6 weeks. This later time point was chosen to investigate sustained vascular networks.

At the early time point (10 days), only SVF-seeded scaffolds showed increased vasculature compared with the acellular controls. This early vascular presence correlated with the greater presence of endothelial cells in the SVF,

suggesting those endothelial cells might assemble into vasculature and patently anastomose with the host. In this study, we did not investigate the contributions of blood vessels of human or mouse origin. It may also be due to a higher secretion of proangiogenic factors by SVF compared with ASCs.⁵³ This early vascular response in our system might enable more of the implanted cells to survive implantation conditions and contribute to subsequent mineralization. Thus, the robust vascular formation and bone formation observed in this study using SVF and ASCs may be correlated.

Conclusion

The data from this study demonstrated that SVF isolated from lipoaspirate and used *in vivo* with minimal processing could promote substantial bone healing. The SVF-seeded scaffolds filled 80% of the void volume of scaffolds in critical-sized murine calvarial defects. In an ectopic intramuscular model, the SVF also induced a robust angiogenic response that was sustained at 6 weeks postimplantation. We compared both the bone healing and angiogenic responses with that obtained from isogenic passage 2 ASCs. We found that the SVF and ASCs behaved comparably. The ASCs stimulated new bone formation that filled up to 100% of the pore volume of the scaffolds and exhibited similar levels of vascularization at 6 weeks. Altogether, the study supports the potential intraoperative use of SVF combined with bone scaffolds for the treatment of critical-sized bone defects.

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Authors' Contributions

E.N.: Conception and design, collection of data, data analysis and interpretation, article writing. A.F.: Conception and design, collection of data, data analysis and interpretation, article writing. A.O.: Collection and analysis of data. R.R.: Provision of study patients and final approval of article. W.G.: Conception and design, financial and administrative support, article writing, and final approval of article.

Disclosure Statement

No competing financial interests exist.

Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Table S1
Supplementary Table S2

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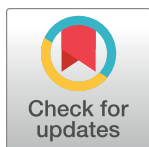
scafSLICR: A MATLAB-based slicing algorithm to enable 3D-printing of tissue engineering scaffolds with heterogeneous porous microarchitecture

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Abstract

3D-printing is a powerful manufacturing tool that can create precise microscale architectures across macroscale geometries. Within biomedical research, 3D-printing of various materials has been used to fabricate rigid scaffolds for cell and tissue engineering constructs with precise microarchitecture to direct cell behavior and macroscale geometry provides patient specificity. While 3D-printing hardware has become low-cost due to modeling and rapid prototyping applications, there is no common paradigm or platform for the controlled design and manufacture of 3D-printed constructs for tissue engineering. Specifically, controlling the tissue engineering features of pore size, porosity, and pore arrangement is difficult using currently available software. We have developed a MATLAB approach termed **scafSLICR** to design and manufacture tissue-engineered scaffolds with precise microarchitecture and with simple options to enable spatially patterned pore properties. Using scafSLICR, we designed, manufactured, and characterized porous scaffolds in acrylonitrile butadiene styrene with a variety of pore sizes, porosities, and gradients. We found that transitions between different porous regions maintained an open, connected porous network without compromising mechanical integrity. Further, we demonstrated the usefulness of scafSLICR in patterning different porous designs throughout large anatomic shapes and in preparing craniofacial tissue engineering bone scaffolds. Finally, scafSLICR is distributed as open-source MATLAB scripts and as a stand-alone graphical interface.

Introduction

3D-printing technologies have become widely available with a large number of commercially available low-cost hardware systems and printable materials used to fabricate scaffolds for tissue regeneration [1,2,11–13,3–10]. Thus far, much of the work using fused deposition

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modeling (FDM) to produce constructs for tissue engineering has focused on developing suitable biomaterials with a variety of mechanical and biological properties [14]. Scaffolds for tissue engineering necessarily contain highly-defined porous networks. The interplay between scaffold mechanics and porous volumes for uniform cell seeding, promoting *de novo* tissue growth, and the diffusion of nutrients throughout presents a challenge in determining the optimal microarchitectural design for a scaffold. While there have been design approaches for selective laser sintering 3D-printing using unit cell libraries [15], topology optimization [16,17], and mathematical design [18], the structures are not reasonably transformed to the fiber deposition paradigm of FDM [19].

An ongoing tissue engineering design goal is to create spatially controlled, heterogeneous patterns of pores throughout anatomic shapes to mimic differences in mechanical requirements throughout the tissue. In prior studies, this has most commonly been achieved by modifying the fiber-fiber spacing between *z*-levels, which results in different pore sizes at different heights in the print. For example, Sobral *et al.* designed a gradient of pore sizes by systemically increasing or decreasing fiber-fiber spacing in each print layer [20]. Additionally, Woodfield *et al.* 3D-printed cartilage constructs with a gradient of pore sizes in the *z*-direction [21]. These fiber-spacing approaches are constant across an *xy*-plane and limit designs to changes in spacing in the *z*-direction. While such an approach is applicable in small-scale prints, it does not easily transfer to human-scale complex anatomic shapes. Di Luca *et al.* demonstrated fiber-fiber spacing across the *xy*-plane, resulting in a step gradient across the plane [22–24]. Therefore, the fiber-fiber spacing could be controlled in *xy* and *z* directions simultaneously, enabling designs with different pore sizes across 3D-space. Thus far, this approach has only been shown on a small scale, cuboid scaffold with a three-pattern linear gradient. Implementation of such gradients in the *xy* plane across a variety of large, more complex geometries remains an unmet challenge for bone tissue engineering.

Additionally, specific control over pore architecture is desirable. Fiber height is often mismatched from desired pore sizes, and cross-hatching fiber patterns on alternating print layers result in pore diameters that are determined by the versatile fiber-fiber spacing in the *z*-direction but limited by fiber height in the *xy*-direction. Varying the fiber height can change the height of the pores in the *xy*-plane, but this approach must be implemented across the entire print plane which prevents in-plane patterning. Further, fiber height is limited by the range of nozzle hardware. However, repeating the same print pattern without changing the fiber location could result in stacked, taller fibers and therefore pores, as shown by Moroni *et al.* [25] and Xu *et al.* [26]. Struts designed from congruent, stacked fibers could be used to make pore microarchitectures isotropic and vary pore size in all three spatial dimensions.

The process of transforming a 3D-design into the *xy*-layers of hot element paths is termed *slicing*. The slicing process determines the fiber laydown pattern, and the resulting geometric and mechanical properties of the print [27]. Traditional slicing software systems create a solid wall or shell around the exterior surface of the shape with a single, infilling, truss pattern applied to the interior bulk of the shape. These resulting designs are not useful for tissue engineering constructs as they do not contain interconnected pore networks. Research groups have been limited by the set of functionalities in broadly-used software (such as Slic3r [28] or Cura [29]) or in the proprietary software delivered with the bioprinter—which has restricted the availability of useful tissue engineering designs.

To overcome the limitations of the available slicing software, several groups have prepared custom porous designs through ‘brute force’ design: i.e. they manually design each pore and strut in CAD programs and then pass the CAD file to a traditional slicing program, which best approximates fiber placement across the design [5,30]. This design process is labor and computationally intensive and disconnects the design process from the design space of the 3D-

printer, which can cause infidelities in the final product. Alternatively, custom slicing software can enable the creation of gradients or custom porous structures. For example, Kang *et al.* developed an integrated tissue-organ printer and custom software integrated into the system to design and manufacture of constructs. However, their published source code is unique to their hardware and does not appear to allow for the design of gradients [9]. Trachtenberg *et al.* developed a Python and Pronterface system to generate GCODE that can vary the fiber-fiber spacing on different print levels on a custom-built 3D-printer [31]. However, these programs are specialized to each design and manufacturing system and are not easily replicable or adaptable.

Therefore, currently available slicing programs do not allow precise control over porous patterns and microarchitectural features needed for tissue engineering scaffolds. Here, we present an approach to designing 3D-printed scaffolds with patterns of porous and mechanical properties. The goals of this approach are to (i) develop a software which can design and implement patterns of pore properties throughout 3D space which contain isotropic, fully connected pores relevant to tissue engineering, (ii) validate the printability and mechanical integrity of such designs, and (iii) provide this software as a tool that researchers can use when 3D-printing tissue engineering scaffolds. Additionally, we demonstrate that the resulting approach allows the independent patterning of pore size and porosity across a variety of anatomic shapes relevant to craniofacial bone regeneration.

Materials & methods

3D-printing on Lulzbot

The methods in this paper were developed for use on a Lulzbot Taz 5 3D-Printer (Aleph Objects, Loveland, CO), which is representative of the many low-cost desktop 3D-printers that are broadly in use. The printer uses gears to drive a solid polymer filament through a melt chamber and narrow extruder nozzle. The nozzle is moved in the *x* and *y* directions as it deposits material in a single *z* level before proceeding in a layer-by-layer fashion until the build is complete. The cooling and solidification rate of the extruded polymer is critical for determining print quality, and it is controlled by adjusting air fans and the heat of the print surface. This paper uses the following terms to describe the 3D-printed part:

- **Fiber**—the structure of extruded material from the extruder head along a toolpath on a single print layer. Fibers are assumed to be rectangular with the width of the extruder nozzle and the height of the print layer.
- **Strut**—the solid material resulting from a set of adjacent fibers, often composed of multiple fibers in width and height.
- **Pore**—the channel-like void spacing between struts, in horizontal and vertical directions. Pores have square projections with equal width and height when viewed from the top or side of the scaffold.

Most importantly, the Lulzbot uses the Marlin operating system to process the standard RepRap flavor of GCODE instructions to control the robotic behavior of the system. The machine responds to commands to deposit a fiber of the material (*extruder diameter*) at a given temperature (*extruder temperature*), at a given rate (*extrusion rate*), and move in *x-y* space (*tool paths*, *extruder movement speed*). Additionally, the print surface can be heated to prevent warping (*bed temperature*) and the print can be cooled by turning the fan on at various print heights (*fan speed*, *fan start height*).

Scaffolds in this paper were printed using acrylonitrile butadiene styrene (ABS) plastic filament (IC3D, Columbus, OH) with the following printer settings. A 0.5mm diameter extruder was used with the extruder temperature set to 240°C and the bed temperature set to 110°C. The layer height was set to 0.2mm and fans were turned on to 50% speed after the first layer was deposited. The feed rate was set to 1200 mm/min and a 1% over-extrusion factor was applied throughout the entire scaffold. scafSLICR could work with a range of materials in an FDM printer, optimized with printing parameters.

Scaffold design with MATLAB Script

The code was written to enable the feature-driven design of tissue engineering scaffolds. The key features of porosity and pore size are used to create a 3D-template, which is then applied to the desired areas of the scaffold shape (Fig 1). Pores are designed as isotropic square pores. Porosity is tuned by both the pore width and the strut width (design porosity, Eq 1). The strut width can be increased by placing multiple fibers directly adjacent to each other (fiber-fiber spacing = 0 mm) and pore width is controlled by the strut-strut distance. By repeating the same strut pattern on consecutive layers, the strut height can be increased to equal the pore width and result in square pores. The porosity of the repeating unit is calculated in Eq 1: a

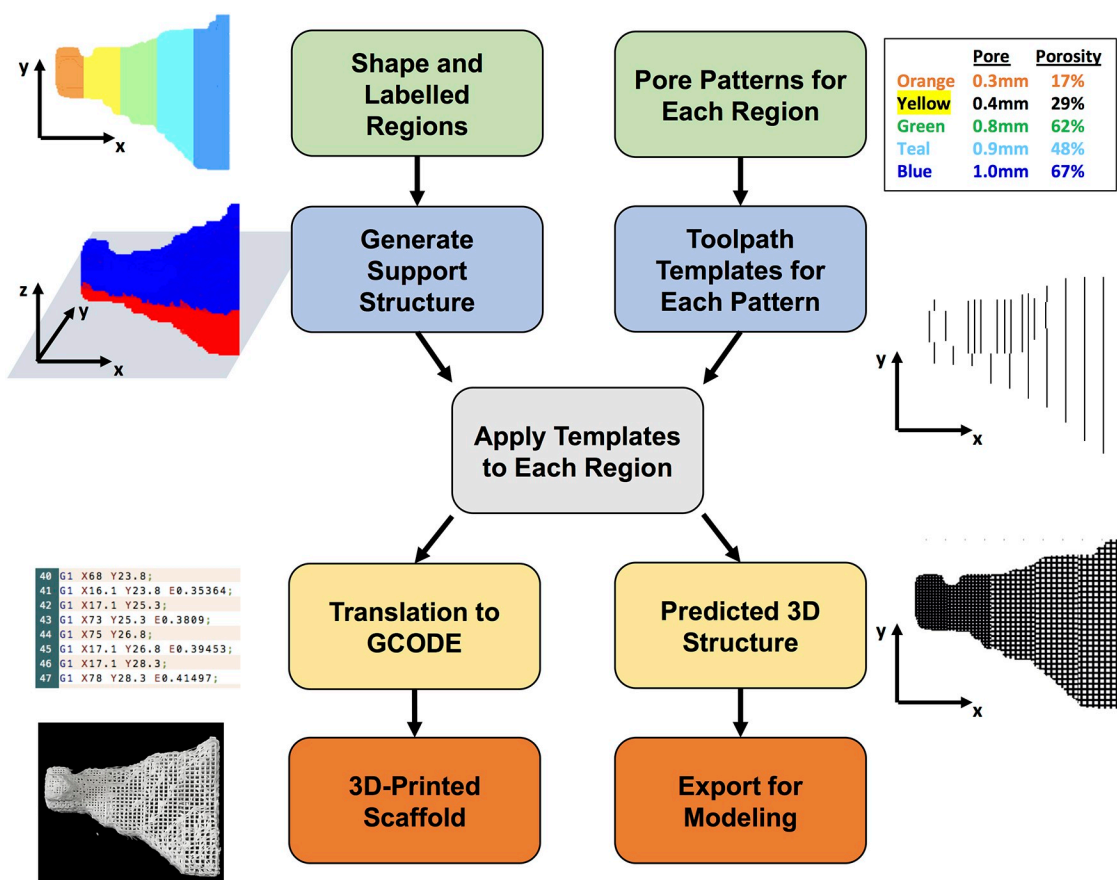


Fig 1. Overview of scafSLICR approach. User inputs a labeled 3D shaped and the pore properties for each label (green boxes). The program then generates a support structure between the shape and the print bed (blue/red shape) and tool path templates for each pore pattern (blue boxes). The slicing process convolves these tool path templates with each x-y level of the shape according to the label (gray box). The result of this convolution is then translated into a set of GCODE instructions or into a predicted porous model of the shape (yellow boxes). These outputs can be manufactured on a 3D-printer or used for *in silico* modeling (orange boxes).

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repeating unit has the width of a strut + pore, the length of a strut + pore, and the height of pore, while the void volume is given by pore width*pore width*pore height.

$$porosity = \frac{(pore\ width)^2}{(pore\ width + strut\ width)^2} \quad (1)$$

The inputs to the code are 3D-shape, pore sizes, and porosities. The possible combinations of pore sizes and associated porosities are dependent on the thickness of the struts and are available as design options. The shape can be easily adapted from CT scans, STL files, or other 3D-data. The pore size and porosity can be inferred from the initial biologic data (e.g. CT density), mathematically defined in a variety of gradients, or any desired pattern that can be applied to a 3D-matrix.

The scafSLICR function generates templates of toolpaths in both x and y directions based on these parameters and convolves them with the shape matrix. Additionally, it generates support material between the input shape and the print bed surface. The function includes the options to improve print quality by pausing, back-tracking, or retracting material at the end of each fiber to prevent dragging strands across pore spaces.

The program outputs include common GCODE instructions that are conserved across many common FDM (tested on the RepRap Marlin system [32]) printers and 3D rendering of expected design (as STL and volumetric data). For ease-of-use, the function was incorporated into a graphical user interface [S1 Appendix]. It uses 3D-plotting [33] and STL import [34] scripts from the Mathworks repository.

scafSLICR is freely distributed on the Mathworks repository at www.mathworks.com/matlabcentral/fileexchange/72856-scafslicr as well as in supplements to this article.

Scaffold manufacturing

Scaffolds were manufactured to assess the print quality of different porous patterns (homogenous scaffolds), the transition between different patterns (hybrid scaffolds), and gradients of patterns in three dimensions (gradient scaffolds). Homogenous and hybrid scaffolds were $20 \times 20 \times 10$ mm and gradient scaffolds were $30 \times 30 \times 30$ mm. The exact porous features of all scaffold groups are listed in Table 1. 3D-Design models were generated by scafSLICR by assuming fibers to be perfectly rectangular (nozzle width x print layer height). The design was assembled into a volumetric 3D-matrix which could be examined directly in MATLAB using matrix property analyses, MATLAB 3D-plotting functions, or exported as an STL to be viewed and analyzed in a range of software programs.

Table 1. Pore features of homogenous, biphasic, and gradient scaffolds.

Homogenous		Hybrid		Gradient	
Pore Size (mm)	Porosity (%)	Pore Size (mm)	Porosity (%)	Pore Size (mm)	Average Porosity (%)
0.2	28%	0.2 → 0.5	28% → 25%	0.2	28%
0.5	25%	0.5 → 0.8	25% → 28%	0.35	26%
0.8	28%	0.2 → 0.8	28% → 28%	0.5	50%
	45%	0.8 → 0.8	28% → 45%	0.65	56%
	62%	0.8 → 0.8	45% → 62%	0.8	62%
		0.8 → 0.8	28% → 62%		

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Print quality assessment

Design features were measured in manufactured scaffolds and evaluated for accuracy compared to the input values. scafSLICR was used to design and print scaffolds ($20 \times 20 \times 10$ mm) with a variety of combinations of pore size and porosity, along with a solid ABS cube. The porosity of printed porous scaffolds was determined by mass measurements compared to solid prints of the same dimensions (Eq 2), and they were compared to the porosity of the computer design by computing the void fraction the 3D-MATLAB matrix.

$$Porosity_{mass} = 1 - \frac{mass_{porous}}{mass_{solid}} \quad (2)$$

Alternative Eq 2:

$$Porosity_{mass} = 1 - \frac{mass_{porous} / volume_{porous}}{mass_{solid} / volume_{solid}}$$

Scaffolds were imaged on a stereoscope (Zeiss Z8). Images were taken of top and side views at 2X magnification. Pore size and strut width were measured separately for top and side views. Pore size was analyzed using the DiameterJ plug-in for FIJI [35,36] by measuring the area of each pore of the binarized image. The size of the pore was then reported as the square root of the pore area. All of the pores were measured in each scaffold and each scaffold design was printed in triplicate. Strut widths were measured by hand in FIJI using the original stereoscope image. Between 59 and 69 struts were measured over three scaffolds per group. For pore size and strut width, the ratio of the measured value to the predicted value reported +/- standard deviation.

Mechanical testing

Scaffolds were tested to assess the base mechanical properties of homogenous and hybrid scaffolds. Scaffolds measuring $20 \times 20 \times 10$ mm were loaded into an MTS Criterion Model 43 (Eden Prairie, MN) with a 5 kN load cell and subjected to unconfined uniaxial compression. The scaffolds were compressed perpendicular to the print axis at a rate of 1.27 mm/min. The compressive modulus was determined from the linear region of the stress-strain curve ($n = 3$).

Analysis of porous boundaries

In order to assess the connectivity of pores between regions of different porous microarchitectures, the area of the interface was analyzed in the digital model in MATLAB. The interface was isolated digitally at a $200\mu\text{m}$ thickness, and the area of each connecting pore was measured. The porous area fraction of the boundary surface was found by summing the individual pore areas and dividing by the area of the interface boundary between regions.

Anatomic shapes

Large portions of the craniofacial skeleton were selected to serve as anatomic test shapes. STLs or DICOMs of the shapes were exported from MIMICs (Materialise, Plymouth, MI) and imported into scafSLICR. The shapes were divided into regions arranged linearly along the length of the shape (zygoma), or according to shape thickness (orbital bones), or according to depth (hemimandible). Different porous patterns appropriate for tissue engineering were selected from the design space and applied to the different regions of the anatomic shapes.

Results

scafSLICR was used on a standard desktop computer to generate the designs in this study [S2 Appendix, Examples 1–9]. The largest shape (orbital bone) took six minutes to slice and generate the GCODE file, which is similar to the computing time when using Slic3r. Smaller volume shapes required proportionally less time to slice. 3D-printing time similarly scaled with volume, with large shapes requiring on the order of 10 hours and smaller shapes on the order of 20 min. Designed scaffolds were manufactured using the output GCODE without complications. Isotropic, regular, cubic pores were visible from top-down and side-on views of the scaffold (Fig 2A). The support material was automatically generated for anatomic shapes and removed from prints with minor artifacts.

Available design space

Based on the diameter of the printer nozzle in use, the strut width can be modulated by depositing adjacent fibers (Fig 2A), thus a variety of strut widths may be achieved that are integer multiples of the printer nozzle diameter. This allows the decoupling of pore diameter and overall porosity. By modulating the strut width, a multitude of porosities may be achieved for a given pore diameter (and *vice versa*) as shown in Fig 2B. Pore diameters ranging from 0.2 mm to 1.0 mm were successfully printed using the 0.5 mm nozzle on the Lulzbot Taz5 printer. Within this range of pore diameter, many different porosities may be achieved by varying the strut width. For example, for a pore diameter of 500 μm , eight different porosities may be achieved between 11% and 50% by increasing the strut width from 0.5 mm to 4 mm.

The maximum porosity is determined by the pore size and printer nozzle diameter. The maximum porosity for pore diameters ranging from 0.2 mm to 1.0 mm is summarized in Table 2. For a 0.5 mm nozzle diameter, porosities may be achieved between 29% and 67%, and the porosity may be further increased to 74% by using a printer nozzle with a diameter of 0.35 mm.

Similarly, a specific porosity may be achieved using multiple different pore diameters. A porosity of 28.57% can be achieved at 0.2, 0.4, 0.6, 0.8, and 1.0mm pores by modulating the strut width between 0.5mm and 2.5mm (Fig 2B).

Print quality/validation of predicted designs

This study evaluated five combinations of pore size and porosity that were considered to be highly relevant for bone tissue engineering applications (summarized in Table 2). These designs were printed and used to validate that the predicted designs from scafSLICR could be successfully manufactured with a high degree of fidelity. Printed scaffolds are shown with their respective design previews in Fig 3A. Manufactured scaffolds matched predicted designs to a high degree in both the top and side views. There was slight over-deposition of material, with pore sizes consistently below the predicted value irrespective of the actual pore diameter (Fig 3B and 3C). Pore diameter ranged from 76% to 93% of the expected value while the strut width varied from 3.5% under deposition to 13% over deposition.

The measured gravimetric porosity (Fig 3D) is strongly correlated to the specified porosity of input design. The deviation of measured porosity from input porosity is due to the dimensions of the printed scaffold not being exact multiples of the characteristic distances of the individual microarchitectures (pore width in z , pore width + strut width in x and y).

Homogenous scaffolds were compressed to find the effective compressive modulus (Fig 3E). Primarily, the effective compressive modulus decreased with increased porosity. Increasing the porosity from a solid cube to 28% porosity with 200 μm pores resulted in a 44% decrease in compressive modulus. Further increasing the porosity to 62% with 800 μm

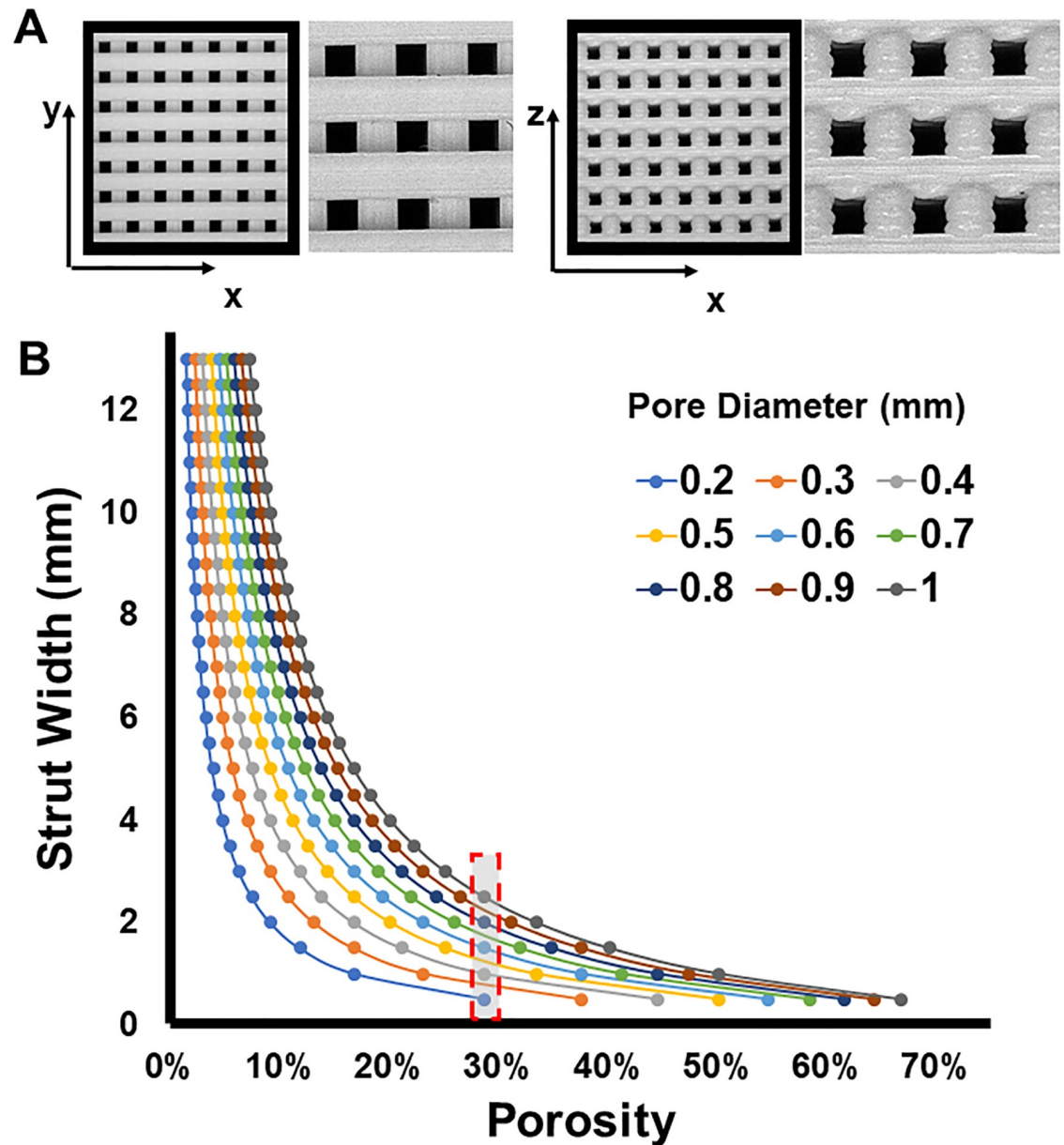


Fig 2. Available design space. (A) Stereoscope pictures (1X, 5X) of scaffolds produced with scafSLICR demonstrating isometric pores. (B) Relationship of Strut Width and Porosity: Modulating the width of struts can produce a range of discrete porosities that are manufacturable at a given pore diameter for 0.5 mm nozzle.

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pores resulted in an 84% decrease in compressive modulus relative to the solid cube. Despite the clear inverse relationship between porosity and compressive modulus, the three designs with near 25% porosity had different compressive moduli, demonstrating that the mechanics also vary with the specific microarchitecture (pore size and strut size). Increasing pore size also resulted in decreased modulus with scaffolds containing 200, 500, and 800 μm pores with near 28% porosity having a compressive modulus of 503, 486, 328 MPa, respectively.

Table 2. Maximum porosity for a range of pore diameters.

Pore Size	Upper Porosity Limit (0.5mm nozzle)	Upper Porosity Limit (0.35mm nozzle)
0.2mm	29%	36%
0.3mm	38%	46%
0.4mm	44%	53%
0.5mm	50%	59%
0.6mm	55%	63%
0.7mm	58%	67%
0.8mm	62%	70%
0.9mm	64%	72%
1.0mm	67%	74%

<https://doi.org/10.1371/journal.pone.0225007.t002>

Hybrid scaffolds

The porous interconnectivity between different microarchitectures in hybrid scaffolds (Fig 4A) was analyzed in predicted designs (Fig 4C). Because the nature of the interface depends on the position, extent, and curvature of the interface surface, pattern-to-pattern interconnectivity could not be assessed experimentally and was instead measured using *in silico* designs of the presented examples. All interface designs included connected pores. A portion of the connected pores was often reduced in individual area, but together represent a large area fraction of connected porous space (10–30% of boundary area) per interface. Hybrid scaffolds were also tested for mechanics in compression normal to the plane of transition between microarchitectures (Fig 4B and 4D). The modulus of the hybrid scaffold was compared to the modulus of the more porous (softer) design and less porous (stiffer) design. In all cases, the hybrid scaffolds had moduli between those of the two constitutive homogenous designs. This result indicates that the transition between microarchitectures did not weaken the mechanics of the scaffold.

3D printed scaffolds with heterogeneous porous patterns

Gradient patterns of different porous microarchitectures were applied to cubes (Fig 5). First, we demonstrate the ability of scaSLICR to prepare gradients. It readily applied gradients in the print (z) direction (Fig 5A) or across the print layer (xy plane) (Fig 5B). Further, a 3D gradient was applied which graded the porous microarchitectures from the exterior to the interior of the cube (Fig 5C). The cubes were larger than homogenous or hybrid scaffolds in order to accommodate the characteristic sizes (twice the sum of the pore and strut width) of the five patterns. The designs were 3D-printed without complication.

Portions of the craniofacial skeleton were used to test shape complexity, pattern complexity, and scale. The zygomatic bone (Fig 6A) was printed with a linear gradient in the pore structures from left to right, arranged so the less porous design was at the narrow portion of the bone and the more porous was at the wider portion of the bone.

The hemi-mandible (Fig 6B) was graded into shells based on depth from the surface of the shape. A more porous pattern was applied to the outer shell, versus a more solid pattern along the inner core. This shell design could allow for cell ingrowth into the scaffold along the surface with some added stability from the inner core. There is satisfactory porous connection between the outer two shells, however, the inner core was nearly solid and did not have many pores for connectivity.

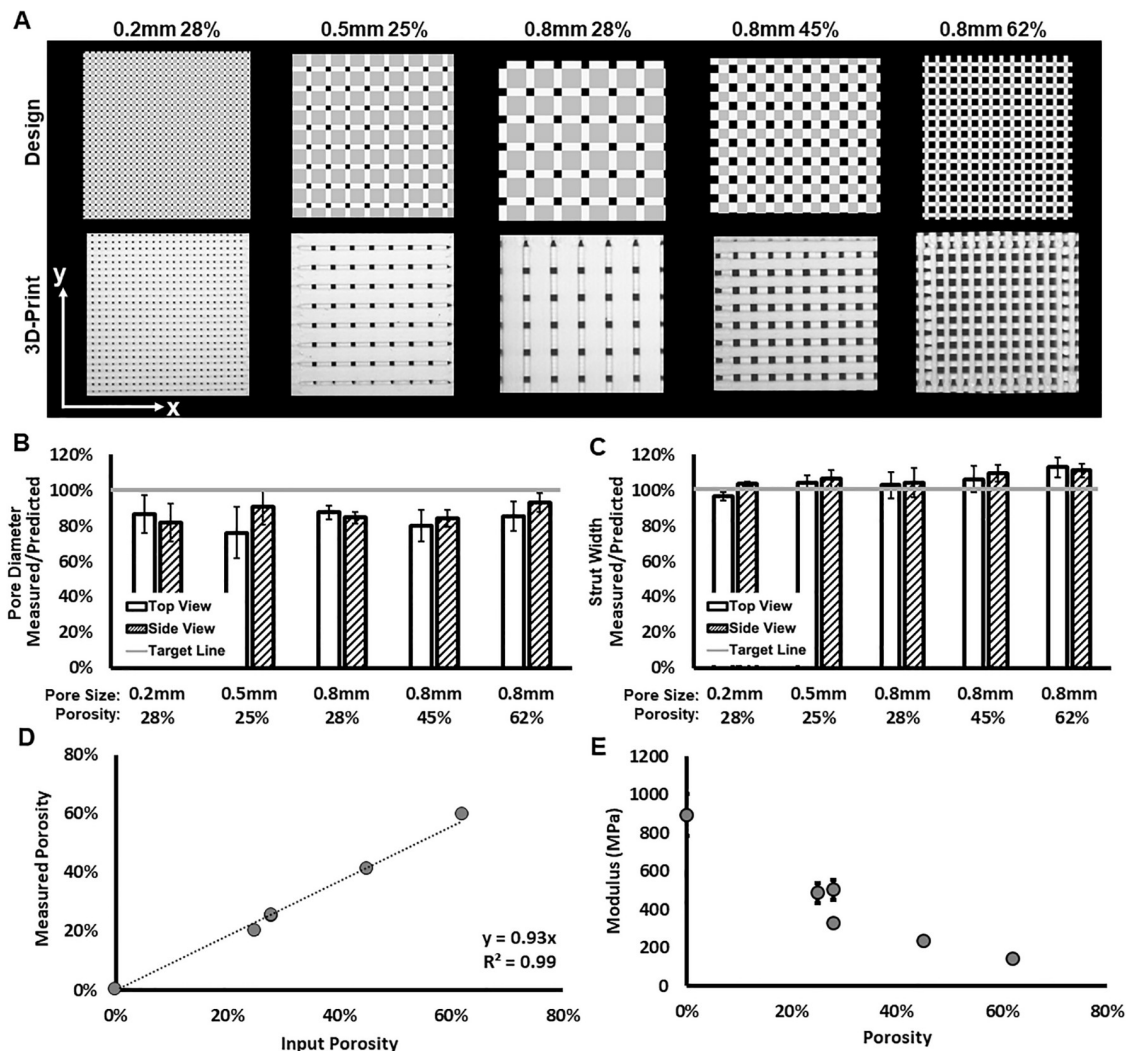


Fig 3. 3D-printed scaffolds with uniform isotropic pores. (A) Side-by-side comparison of scaffold previews (top row) and 3D-printed scaffolds (bottom row) for different patterns of pore size and porosity. (B, C) Assessments of print fidelity of pore diameter and strut width to design from top and side views. (D) Observed gravimetric porosity and expected design values. (E) Compressive modulus varies with porosity.

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3D-printing thin structures is difficult, more so when the structure is manufactured with a porous pattern. The orbital bone shape (Fig 6C) has characteristically thin bones across the orbital floor. To print these faithfully, the shape was divided into regions based on the average thickness, which allowed the thin regions to be assigned a less porous, more stable pattern. Thicker, more stable regions were assigned more porous patterns. The arrangement of the patterns resulted in curved and interwoven boundaries throughout the shape. These boundaries maintained 10% and 20% area pore-connectivity for the three most porous patterns while the less porous designs had much lower connectivity (1.4% and 4.1% area fraction) concurrent with their decreased porosity and pore size.

These large, curved shapes show step/staircase artifacts (particularly in the zygoma example) because there are multiple print levels for a single level of input voxels (input voxel edge = 0.600 mm, slicing voxel edge = 0.100 mm, printing layer height = 0.200 mm). This staircase artifact could be resolved by smoothing the surface of the slicing design 3D-matrix.

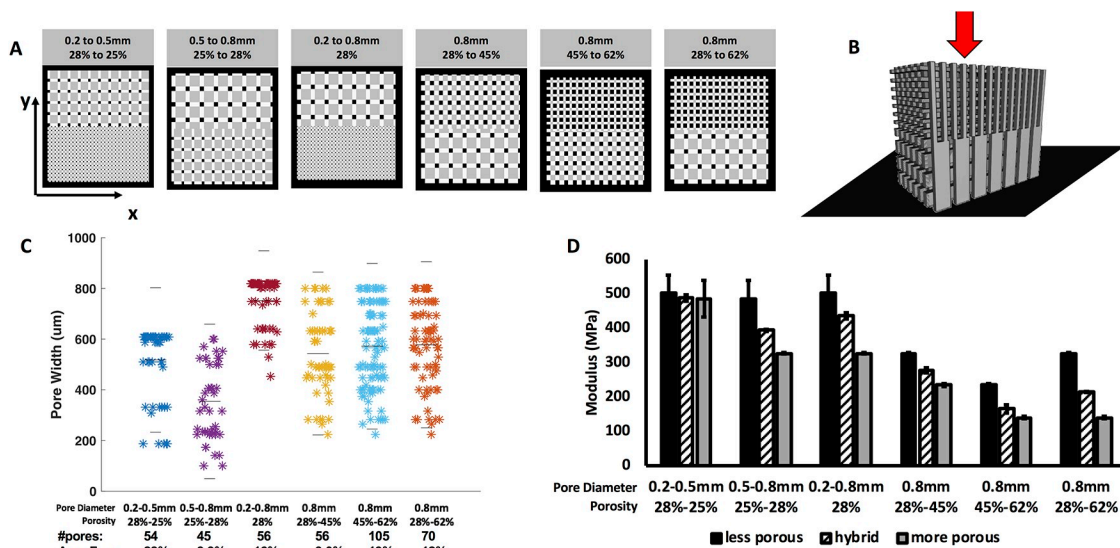


Fig 4. 3D-printed scaffolds with hybrid pore structures. (A) 3D previews of scaffold designs featuring a more porous region and less porous region which meet at a center boundary. View is top-down onto the xy surface of the scaffold (B) Schematic showing application of force (red arrow) and alignment of scaffold on the platen (black plane) (C) Pore connectivity of transition plane: measured pore areas, number of pores, and area fraction of boundary plane that is connected pore space. Gray lines indicate median and upper and lower quartiles. (D) The compressive modulus of each transition scaffold compared to homogenous scaffolds composed of one of the pore diameter-porosity combinations found in the transition scaffold.

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Discussion

This work develops and implements an approach for the design and manufacture of 3D-printed scaffolds for tissue engineering applications. scafSLICR provides the ability to easily leverage the available design and manufacturing space available in additive manufacturing. In

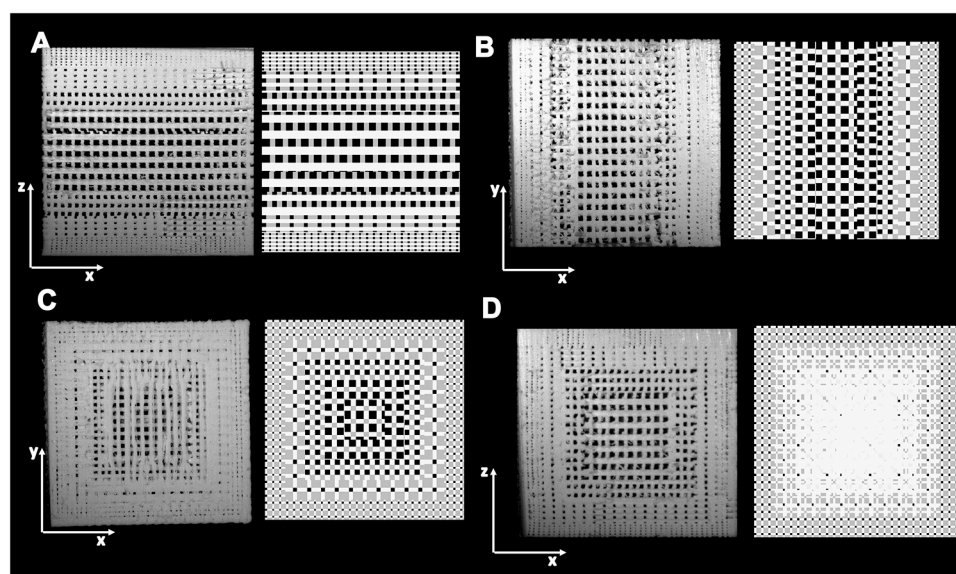


Fig 5. 3D-printed scaffolds with heterogeneous pore structures. Pictures of cross-sections of $2 \times 2 \times 2 \text{ cm}^3$ ABS scaffolds (left) and design (right). (A) Graded in z. (B) Graded in xy. (C) Graded in xyz.

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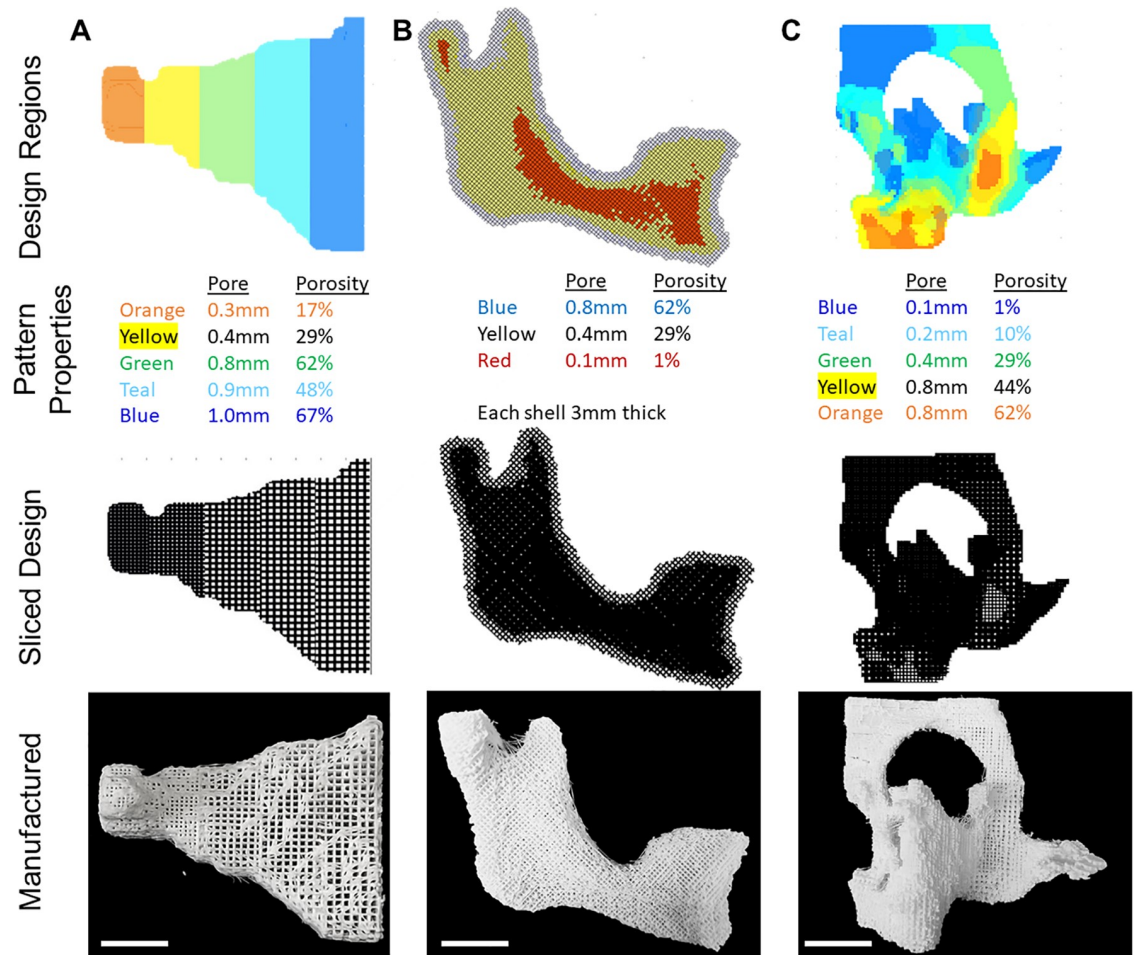


Fig 6. 3D-printed anatomically shaped scaffolds with heterogeneous pore structures. Anatomic shapes from the craniofacial skeleton were labeled with different design regions, sliced with scafSLICR, and 3D-printed. (A) Zygomatic arch patterned linearly left-to-right. (B) Hemi-mandible patterned with shells from exterior to interior. (C) Orbital midface complex patterned according to average shape thickness. Scale bar = 1cm.

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particular, the broad subset of porous microarchitectures can be dependably mixed together in patterns with mechanical integrity and porous interconnectivity.

The cubic cross-hatch pore pattern used in scafSLICR has been used broadly throughout 3D-printing applications to create tissue-engineered scaffolds. We provide complexity to this structure by changing both fiber-fiber spacing and the width and height of struts *via* adjacent and stacked fibers. scafSLICR operates across the design space of the hardware (nozzle diameter) to create porous micropatterns according to desired features (pore size, porosity). This design approach permits multiple porosities with the same pore size and multiple pore sizes with the same porosity. The availability of this breadth in the design space is important because porosity is most directly attuned with print mechanics and pore size with biologic function. Thus, by decoupling the pore size and overall porosity, we have increased the versatility of the application to control the porous microarchitecture.

Different strut patterns beyond the classic cross-hatched rectangular patterns are possible. By off-setting the print direction to different angles or curves, the base pattern can be drastically alternated by z-layer and *xy*-location to create more complex patterns. Changing the base

design from regular cubic cross-hatched struts to another with different offset angles or arching fibers would increase the design space further, and perhaps influence mechanics and porosity in beneficial ways. For example, Moroni *et al.* manufactured scaffolds with 0-45-90 degree patterns of strut offset in order to closely match scaffold mechanics to the cartilage microenvironment [25]. Additionally, Szojka *et al.* 3D-printed scaffolds with alternating layers of parallel fibers with layers of radial ring fiber pattern [37]. Such changes could be implemented into scafSLICR by changing the template creation sub-routine. Unit cell libraries [15,38] of pore and strut shapes for 3D-printed tissue-engineered scaffolds are common for AM techniques such as SLS and powder binding, but the complex strut architecture is infeasible in fused deposition manufacturing methods.

The design limits of the microarchitectures of this study are well suited to bone tissue engineering. There are well-established constraints for bone tissue engineering scaffolds with regards to porosity, pore size, and mechanics. Porosity should be greater than 50% to provide space for tissue growth and regeneration [39–41]. Pores should range between 100µm and 1mm [39,42,43]. Mechanical moduli needed to mimic bone vary from 14 MPa of trabecular bone to 2 GPa of cortical bone [11,39,44,45].

Scaling the manufacture of unique porous architectures to large shapes has been a challenge using FDM, limiting the applications of FDM-based 3D-printing of anatomically shaped scaffolds. Many studies establish their techniques at scales less than 2 cm in regular cubes and cylinders, which facilitates insights into material properties and enables a greater understanding of the cell-material interactions. In this study, we consider challenges associated with scale-up of 3D-printed scaffolds, such as load-bearing bones, which need to be addressed to facilitate long-term clinical applications. The complex geometric nature (curves, gaps, peaks, and small walls and divots) of anatomic shapes challenges the 3D-printing processes developed for cubes and cylinders. Moreover, when developing a slicing system for tissue engineering scaffolds, it is essential that the system can readily adapt to a variety of complex anatomic shapes. scafSLICR easily scaled to large prints, with regional heterogeneity that did not compromise porous or mechanical interconnectivity.

One of the major weaknesses of this study is the choice of material. While it is bioinert, ABS was used because of manufacturing simplicity, speed, and cost. The mechanical assessments were used to validate that porosity influenced mechanics and they were not intended to demonstrate appropriateness for bone scaffold implantation. Towards that end, our research group has used scafSLICR to design and manufacture scaffolds in polycaprolactone and bioactive variations thereof (data not shown). While the examples demonstrate the applicability of 3D-printing for bone tissue engineering, many other tissue engineering applications require porous scaffolds with known pore structures and mechanics [46]. Additionally, food 3D-printing primarily uses extrusion-based printing methods, and scafSLICR could be adapted to these printing systems, enabling different porous and mechanical regions in the food structure [47,48]. Drug 3D-printing has become popular [49], but the application scales are too small for scafSLICR to be useful.

The outputs of scafSLICR enable design validation *in silico* before proceeding to manufacture or implantation. The manufactured scaffolds in this study precisely matched the porous designs. These digital porous models of the scaffolds could be used to assess properties such as mechanics, diffusion, or degradation. Such properties are difficult to directly measure, particularly in complex anatomic shapes [50–52]. The ability to validate such critical attributes are within desired ranges before manufacturing or implantation provides a low-cost means to assure implant functionality [53]. Despite the validation of the print quality and print accuracy, scafSLICR is not validated at the level needed for medical software. It would need additional dimensional and resolution tests to demonstrate reliability with many complex shapes, design

transitions, and materials. Importantly, the software validation can be compromised by the resolution and calibration of the specific 3D-printer and its ability to properly execute the GCODE. At a base-level, scafSLICR operates on a volumetric 3D-matrix. This matrix approach can be memory intensive (design matrix variables sometimes reach 5GB) but allows for the inclusion of more spatial specific information across the 3D-shape [54]. This volumetric matrix could include additional functional information (explicitly and implicitly [55])—for example, fixation attachments, or bone-implant interfaces—to improve computer-aided implant design and slicing process. Additionally, the 3D-matrix has a direct correlation to the DICOM format used to obtain patient-specific anatomic shapes and allows for minimal manipulation of that data along the design and manufacturing process. In contrast, many slicing software systems operate on the common STL format, which only includes information on the surface topography and therefore slice based on 2D contours of the design. Breaking away from the STL format to more of a computer aided design format echoing many of the thoughts from <https://www.fabbaloo.com/blog/2019/2/25/at-last-the-end-of-stl-is-in-sight>.

Conclusion

This work developed an approach to designing and manufacturing 3D-printing scaffolds for tissue engineering, with direct control over scaffold features. It was successfully implemented in MATLAB (or the open-source OCTAVE) and is available at Mathworks Repository as a modifiable source code and as a user-friendly graphical user interface. Scaffolds manufactured with the approach were validated with sliced designs. Complex designs of graded pore patterns were demonstrated in regular cubes and complex anatomic shapes at scale. scafSLICR provides both an approach to designing tissue engineering scaffolds with controlled, heterogeneous complexity and scale as well as a readily available tool for tissue engineers to use in designing and manufacturing scaffolds across a variety of 3D-printing systems.

Supporting information

S1 Appendix. scafSLICR user guide.

(DOCX)

S2 Appendix. Methods to design the scaffolds used in the manuscript.

(DOCX)

Author Contributions

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Formal analysis: Ethan Nyberg, Aine O’Sullivan.

Funding acquisition: Warren Grayson.

Methodology: Ethan Nyberg, Aine O’Sullivan.

Writing – original draft: Ethan Nyberg, Aine O’Sullivan.

Writing – review & editing: Warren Grayson.

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Point-of-Care Treatment of Geometrically Complex Midfacial Critical-Sized Bone Defects with 3D-Printed scaffolds and Autologous Stromal Vascular Fraction

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Abstract: Critical-sized midfacial bone defects present a unique clinical challenge due to their complex three-dimensional shapes and intimate associations with sensory organs. To address this challenge, a point-of-care treatment strategy for functional, long-term regeneration of 2 cm full-thickness segmental defects in the zygomatic arches of Yucatan minipigs is evaluated. A digital workflow is used to 3D-print anatomically precise, porous, biodegradable scaffolds from clinical-grade poly- ϵ -caprolactone and decellularized bone composites. The autologous stromal vascular fraction of cells (SVF) is isolated from adipose tissue extracts and infused into the scaffolds that are implanted into the zygomatic osteotomies. Bone regeneration is assessed up to 52 weeks post-operatively in acellular (AC) and SVF groups ($BV/DV = 0.64 \pm 0.10$ and 0.65 ± 0.10 respectively). In both treated groups, bone grows from the adjacent tissues and restores the native anatomy. Significantly higher torque is required to fracture the bone-scaffold interface in the SVF (7.11 ± 2.31 N.m) compared to AC groups (2.83 ± 0.23 N.m). Three-dimensional microcomputed tomography analysis reveals two distinct regenerative patterns: osteoconduction along the periphery of scaffolds to form dense lamellar bone and small islands of woven bone deposits growing along the struts in the scaffold interior. Overall, this study validates the efficacy of using 3D-printed bioactive scaffolds with autologous SVF to restore geometrically complex midfacial bone defects of clinically relevant sizes.

Keywords: 3D printing, Yucatan pigs, decellularized bone, stromal vascular fraction, critical-sized bone defects.

1. Introduction

Midfacial fractures are among the most commonly occurring bone fractures in the facial region^[1]. Critical-sized defects (CSDs)^[2] occurring in midfacial bones are particularly challenging as they may cause many significant complications including psychological distress; disruption of vision, olfaction, mastication, and speech; and they may also be life threatening.^[3,4] Reconstructing midfacial bones involves unique challenges due to their complex geometry, their mechanical requirements to support masticatory forces, their close spatial association with the eye, important nerves and vasculature, and the potential risk of infection due to the presence of the sinuses.^[5] Autologous bone grafts remain the gold standard for treating midfacial CSDs but common drawbacks include donor-site morbidity, limited tissue availability, and the inability to replicate the geometric complexity.

Anatomically precise tissue engineered bone grafts offer a compelling alternative to autologous bone grafts.^[6] In particular, customized defect-specific bone scaffolds used in conjunction with autologous adipose-derived stem/stromal cells (ASCs) significantly enhance regeneration of craniofacial CSDs.^[7,8] These promising bioreactor-based strategies were used to engineer mature bone grafts *in vitro* that were then implanted to integrate with native bone. ‘*In vivo bioreactors*’, where synthetic graft were implanted in a chamber against the rib periosteum, have also been used to generate anatomically shaped, vascularized bone prior to reconstruction.^[9] Other tissue-engineered approaches sacrificed the geometric customization provided by the bioreactor-based strategies in order to employ a single surgical ‘point-of-care’ (POC) event by directly combining the stromal vascular fraction of cells from lipoaspirate tissue (SVF) with mineralized granules to treat alveolar bone in humans and in proof of principle studies.^[10,11] Recently, 3D-printed bioactive scaffolds have been used together with human SVF to regenerate calvarial CSDs in immunocompromised mice confirming the *in vivo* osteogenicity of SVF cells and demonstrating the feasibility of a cell-based point-of-care approach for treating CSDs.^[12]

Few tissue engineering studies to evaluate the treatment of craniofacial CSDs have focused on regenerating midfacial skeletal defects. Porcine facial bones are similar to those in humans in terms of cross-section, mineral density, lamellar bone structure, and growth rates.^[13,14] Consequently, porcine models of unilateral full-thickness, segmental defects in the zygomatic bones have been established.^[15,16] However, neither of these studies provided scaffold-based or

cell-based treatments to assess the potential for bone regeneration though their findings demonstrated that untreated 2 cm defects in the porcine zygoma would not spontaneously heal during the period of the study and therefore fit the criteria of being CSDs. We hypothesized that treating similar defects with 3D-printed poly- ϵ -caprolactone-decellularized bone (PCL-DCB) scaffolds augmented with autologous porcine SVF would promote robust bone regeneration and facilitate a clinically-translatable intraoperative reconstruction strategy.

To test this hypothesis and assess the feasibility for clinical use, we designed and tested a POC therapeutic strategy for the treatment of 2 cm full-thickness, segmental, bilateral defects in the zygomatic arches of skeletally mature Yucatan minipigs as a surrogate for critical-sized midfacial bone defects in human patients (Figure 1). We created the defects with animal-specific cutting guides generated from pre-treatment computed tomography (CT) scans and then treated the defects with PCL-DCB scaffolds. The defect-specific PCL-DCB scaffolds with integrated fixation tabs were fabricated from clinical-grade materials and 3D-printed via a fused deposition modeling (FDM) process with isotropic, interconnected pores^[17] to promote ingrowth of vasculature and de novo bone tissue. In SVF groups, dorsal fat was harvested immediately before creating the bony defects. We employed a modified protocol for the enzymatic digestion of subcutaneous adipose tissue from the dorsal lumbar region to successfully isolate SVF within a narrow surgical window.^[18] The SVF was suspended in a fibrinogen solution and loaded into a double-barreled hydrogel applicator containing thrombin in the second barrel. We infused autologous SVF into the defect site during the same operative procedure. We assessed regeneration out to 1 year post-operatively via gross morphology, computed tomography, mechanical testing, and histology. Our findings demonstrate that the POC strategy facilitates anatomic restoration of midfacial bone and integration of the scaffolds with the native bone.

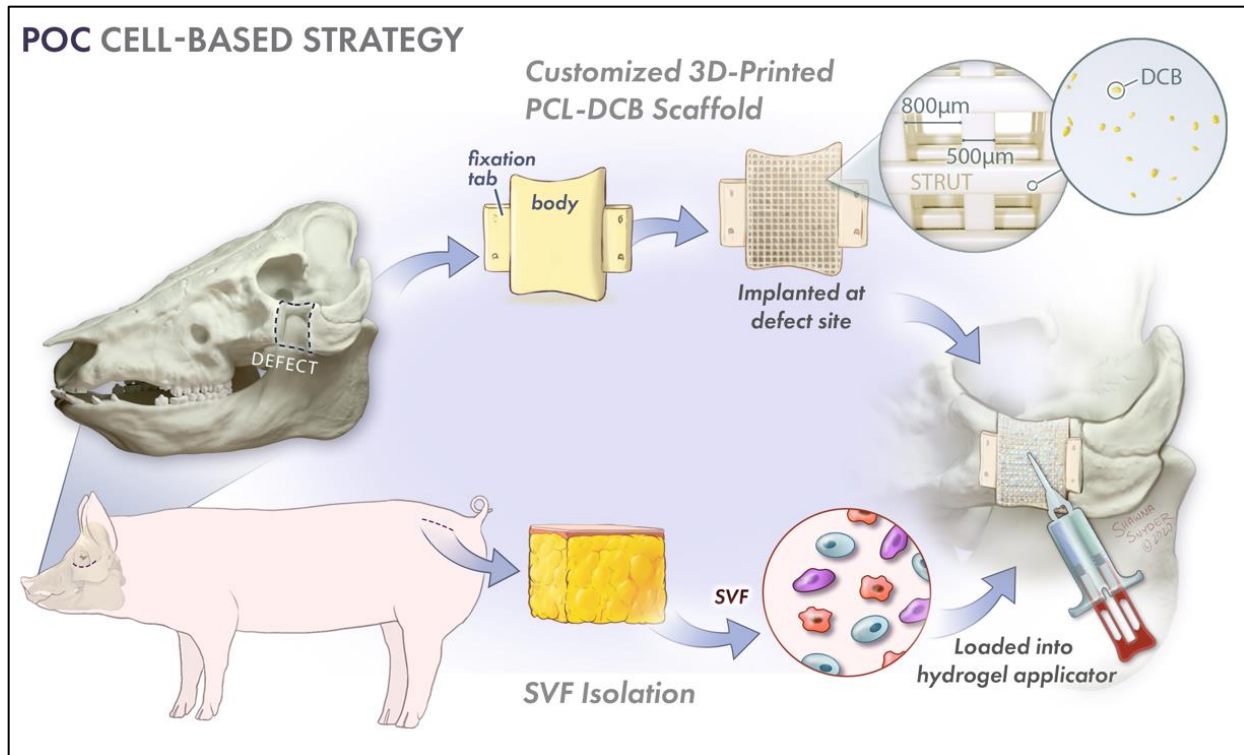


Figure 1. Point of care (POC) cell-based strategy for treating critical-sized midfacial bone defects. CT images of each skull were used to custom-design porous PCL-DCB scaffolds which were 3D-printed with solid fixation tabs. At the time of surgery, custom-fit cutting guides were used to create 2 cm bilateral full-thickness defects within the zygomatic bone of Yucatan minipigs. Concurrently, subcutaneous adipose tissue was harvested from the dorsal lumbar region and the stromal vascular fraction of cells (SVF) was isolated and resuspended in fibrinogen. The cells were injected into the pore-spaces of the implanted scaffold without any pre-cultivation.

2. Midfacial CSD were successfully managed with 3D-printed PCL-DCB and autologous SVF POC strategy

In this study, we tested three experimental groups. The untreated controls were left empty (E; $n=2$ pigs). PCL-DCB scaffolds were implanted in all other defects and filled with fibrin only for AC ($n=2$ pigs) or SVF suspended in fibrin ($n=4$ pigs) (Table S1). In AC and SVF groups, print quality assessments were established and performed on each scaffold to validate the isotropic, interconnected pore structure (Figure 2; Movie S1). The minipigs were under anesthesia for a maximum of 3 hours to harvest fat and extract the SVF (within approximately ninety minutes

(Table S2)), create the defects, implant the scaffolds, and infuse the scaffolds with autologous SVF. In some cases, minor trimming of the scaffolds was performed during surgery to facilitate the press-fit into the defect regions. Flow cytometry analysis of passage one porcine adipose derived stem cells (P1 pASCs) showed that large percentages of the P1 pASC population expressed mesenchymal stem cell surface markers CD73 (94.87 ± 4.89) and CD90 (97.87 ± 1.55), although CD105 (17.55 ± 8.42) was not highly expressed. Hematopoietic stem cell markers CD34 (5.00 ± 0.95) and CD45 (12.55 ± 7.17) were expressed by a small percentage of cells. The osteogenic potential of P1 pASCs was confirmed following monolayer osteogenic differentiation in vitro by Alizarin Red staining (Figure S1).

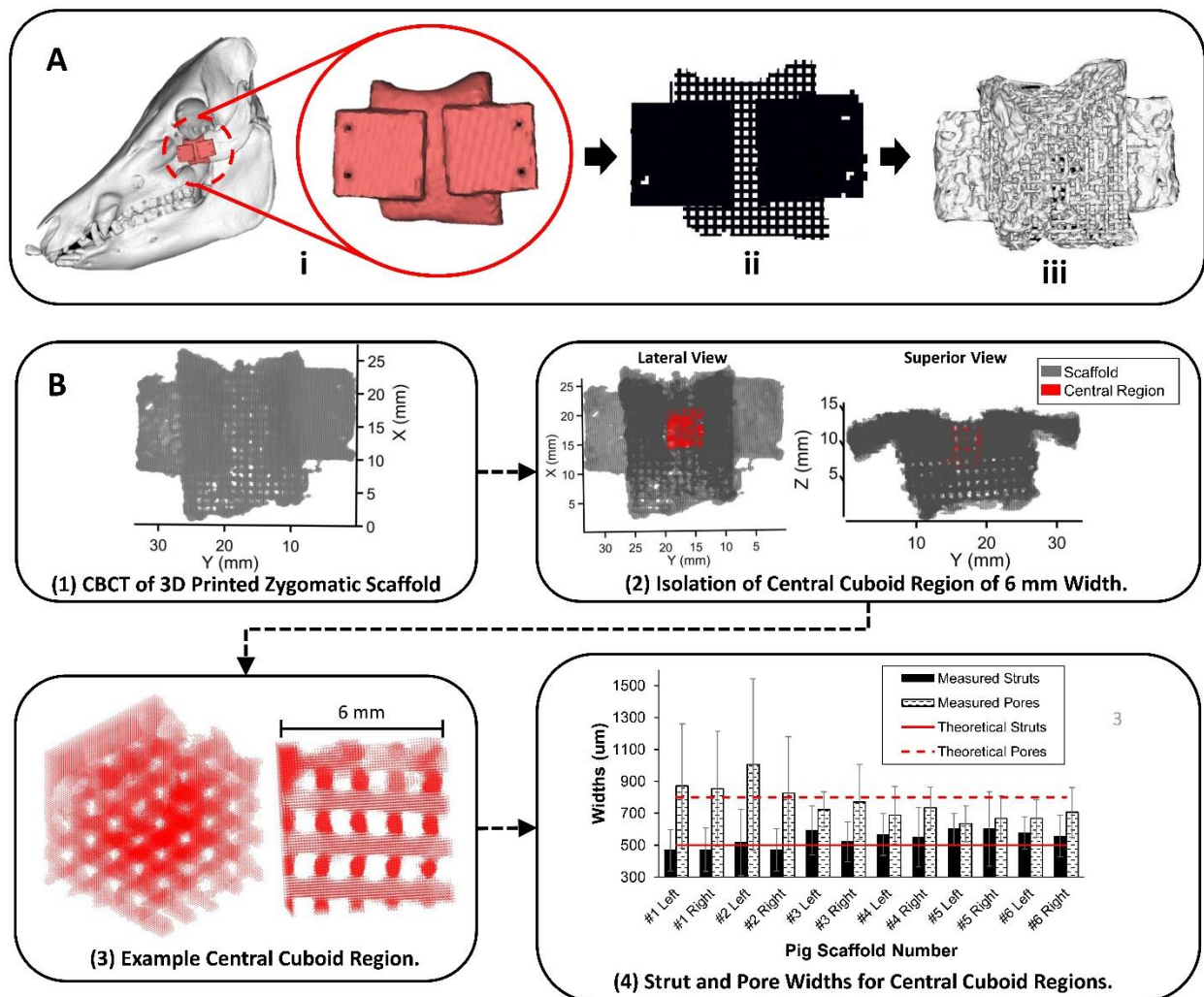


Figure 2. Design and Fabrication of custom PCL-DCB scaffolds. (A) Pre-operative CT scans of the porcine skull were acquired and used as a template to design the custom PCL-DCB scaffolds.

(i) Fixation tabs were added computationally to the scaffold body to complete the scaffold design. (ii) An in-house slicer program (17) was used to incorporate 800 μm isotropic pores into the scaffold and generate the g-code files for (iii) 3D-printing the PCL-DCB scaffolds. **(B)** Cone beam CT scans of each fabricated PCL-DCB scaffold were acquired. The central region of the scaffold (6 mm x 6mm x 6mm) was considered as representative volume and compared with the 3D theoretical design of the porous structure using a custom MATLAB® program to quantitatively evaluate that percent agreement between the design and the printed parameters.

3. PCL-DCB scaffolds restored native zygoma anatomy

Comparison of post-operative CT scans at different time points indicated that the porcine skulls grew throughout the period of study (Figure S2). We quantified the lengths and widths of the cranial vault for all animals at 52 weeks and compared them with that of the post-operative skull measurements. The changes in skull width were identical among the three groups, but the changes in length were greater in the AC and SVF groups compared to the empty group (Figure S3). This difference is likely attributable to differences in the age of animals at the time of the implantation of the scaffolds. This observation highlights that scaffolds demonstrated long term survivability without mechanical failure in an active growth zone. All scaffolds remained secure within the defect sites with fracturing observed in only 1 out of the 12 treated defects. This occurred before the 6-week time-point and was possibly due to external impact. However, this scaffold remained intact throughout the study possibly due to the contemporaneous growth of new bone at the dorso-rostral boundary of the scaffold.

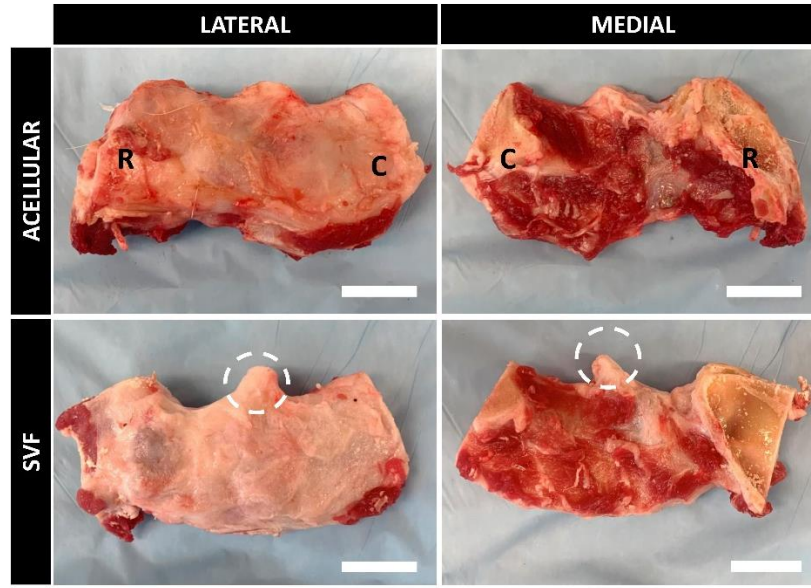


Figure 3. PCL-DCB scaffolds restored the native anatomy following critical-sized bone loss. Representative images of zygomas harvested after 52 weeks of implantation at the zygomatic defect site exhibited restoration of native anatomy in both the AC and SVF experimental groups. In some cases, there appeared to be some exuberant bone growth at the dorso-caudal region of the implant (white circle). There was also significant soft-tissue coverage of the grafts. R: Rostral, C: Caudal; Scale bar = 2 cm

Gross examination of the harvested zygomas indicated restoration of the presurgical anatomy. The medial and lateral surfaces of the scaffolds were flush with those of the flanking native bone. Some harvested zygomas exhibited exuberant new bone growth from the native tissue inwards into the defect region in a caudo-rostral fashion resulting in bony prominences at the dorso-caudal margin of the defect (Figure 3). The defect region was completely covered with a fibrous soft tissue capsule enveloping the scaffold. The capsule was contiguous with the periosteum of the adjacent bone and connected to the fascia of the masseter muscle. Palpation of all the harvested zygomas indicated robust osseointegration at the caudal aspect of the scaffolds while in some cases limited mobility was observed at the rostral aspect (adjacent to the maxillary sinus).

4. PCL-DCB scaffolds promoted robust bone regeneration and osseointegration

We examined bone regeneration at the zygomatic defect sites via post-operative CT scans of the porcine skulls acquired at the pre-determined time points i.e., 0 (post-operative), 6, 12, 24 and 52 weeks (Figure 4A). 3D rendering of CT scans demonstrated that one of the animals from the empty (control) group had undergone only a partial osteotomy. Only the animal from the control group that successfully received full thickness segmental defects was included in subsequent quantitative analyses (Figure S4A, S4B). No bridging was observed at the defect site at 52-weeks, which validated the critical-sized nature of the defect model (Figure S4B). Bone Volume/Defect Volume (BV/DV) was calculated and used as a metric to compare bone regeneration across all experimental groups. Implantation of PCL-DCB scaffolds in the AC and SVF groups promoted significant new bone regeneration at the defect site compared to untreated controls at 52 weeks ($BV/DV = 0.17 \pm 0.11$, 0.64 ± 0.10 , 0.65 ± 0.10 for Empty, AC and SVF groups, respectively; Figure 4B). The increase in new bone volume was greatest within the first six weeks post-surgery and thereafter a reduced rate of volumetric growth was observed throughout the period of study. Interestingly, no significant difference in bone regeneration (BV/DV) was observed between the AC and SVF groups.

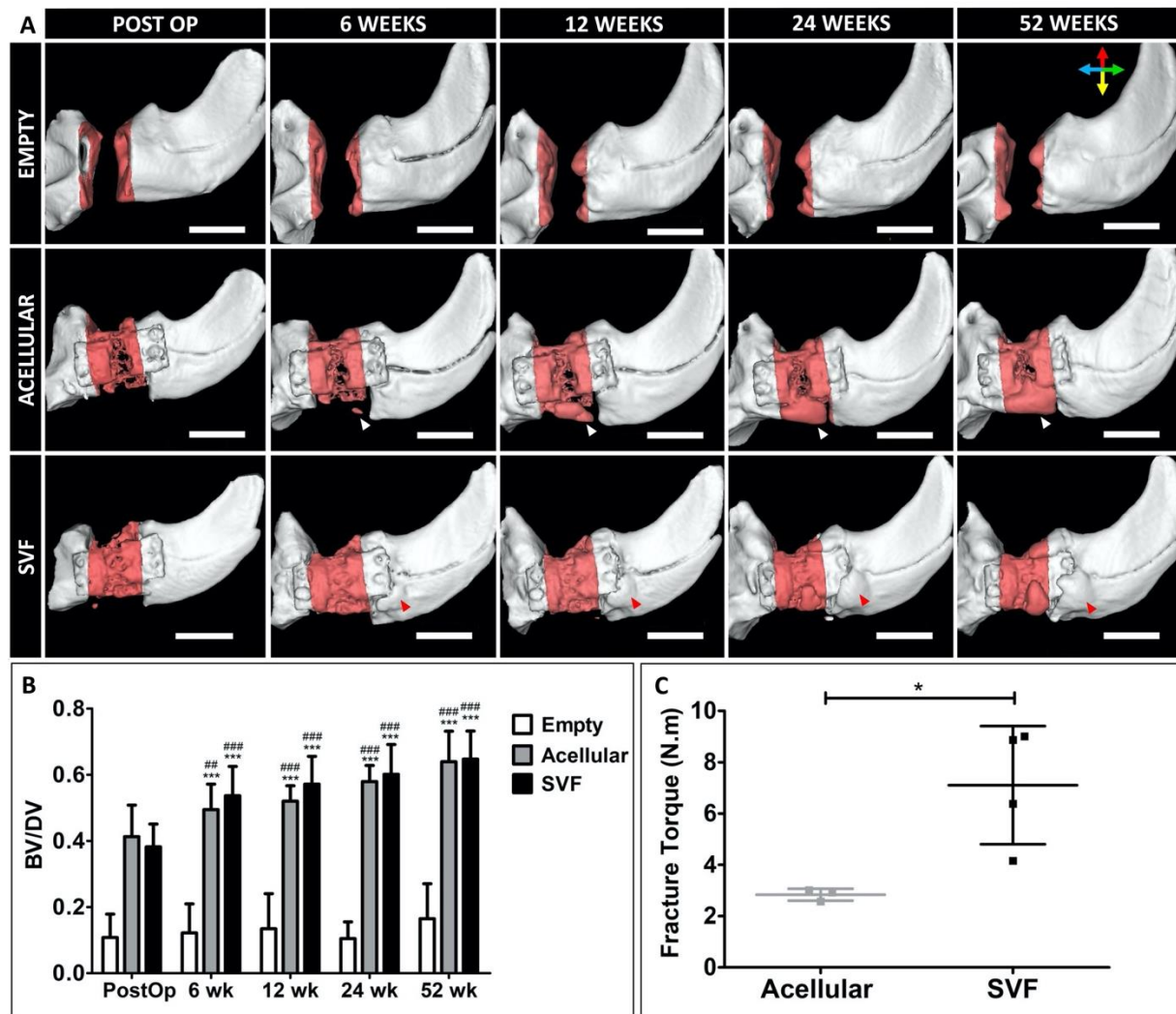


Figure 4. Bone regeneration and functional osseointegration with the native bone. (A) CT scans of the zygomatic defect sites depicting the region of interest (ROI, red), representative of the Empty, AC, and SVF groups at various post-operative timepoints. Note: Bony islands growing along the ventral aspect of the defect fill up the defect volume and restore native anatomy (white arrow heads). Growth of bony extension from the caudal aspect of zygoma in a caudo-rostral fashion (red arrow heads). Scale bar = 2 cm. Orientation is conveyed by colored arrows: dorsal (red), ventral (yellow), rostral (blue) and caudal (green). (B) Quantitative evaluation of bone volume/defect volume (BV/DV) within the ROI ($n= 2$ (E), 4 (AC) and 8 (SVF) defects). Note: Less bone was removed in the Empty group than originally planned, hence BV/DV is greater than zero even at the post-op time point. * indicates comparison with Empty group at the same time point whereas # indicates comparison with corresponding groups at PostOp. (C) Mechanical

testing was performed to quantitatively assess osseointegration ($n= 3$ (AC) and 4 (SVF) samples) of the scaffolds with the native bone (* indicates $p<0.05$).

From the post-operative CT scans, we identified spatially distinct and morphologically unique patterns of new bone growth in relation to the PCL-DCB scaffolds. For example, at the inferior (ventral) aspect of the implanted scaffold and towards the caudal end of defect boundary, we observed the emergence of bony islands at the inferior defect margin as early as 6 weeks (2 out of 12 defects). These bony deposits eventually filled up almost the entire defect volume restoring native anatomy at 52 weeks (Figure S5, S6). In some defects belonging to the SVF group, new bone grew in a caudal to rostral fashion into the defect region to integrate with the dorso-caudal edge of the scaffold (Figure S6, S7). In one AC defect, (Figure S5) the incipient bone was able to bridge the entire defect along the superior margins of the scaffold. In both the AC and SVF group (5 out of 12 defects), lateral bone growth emerged from the ventro-caudal aspects of the zygoma (zygomatic flange) which was prominent around 6 weeks. These features were more pronounced towards later time points (24, 52 weeks) where the new bone grew towards the defect site in a caudal to rostral fashion as well, brushing past the inferior edges of fixation tabs and ultimately growing over the lateral surfaces of the scaffolds (Figure S5, S6, S7). In one of the defects from the SVF group, this new bone encapsulated most of the fixation tab (Figure 4A, S6). Interestingly, most of the new bone grew in a caudal to rostral direction with respect to the defect. However, in some defects the new bone grew in the rostral to caudal direction (Figure S6, S7). Marked bone resorption was observed in some defects from both AC and SVF groups particularly at the ventro-caudal boundary of the defect (Figure S5, S7). New bone growth was observed at the medial aspect in 1 out of the 12 treated defects at 24 weeks at both the caudal and rostral margins of the defect (Figure S8)

Mechanical testing (cantilever beam bending) of the harvested zygomas was performed to quantitatively evaluate osseointegration of the scaffolds with native bone (Figure S9, Movie S2). Fracture torque was calculated and compared between the AC and SVF groups (Figure 4C). The SVF group required significantly ($p<0.05$) higher torque (7.11 ± 2.31 N.m (corresponding to ~ 30 – 40 kilograms of force)) to fracture the interface suggesting superior osseointegration compared to the AC specimens (2.83 ± 0.23 N.m).

5. PCL-DCB scaffolds provided a template for deposition of mineralized bone nodules

To understand why the SVF groups had higher fracture torques as well as to learn more about the growth pattern and spatial distribution of incipient bone within the scaffold body and its surrounding tissue, microCT (μ CT) scans of the harvested zygomas were acquired. The μ CT data illustrated two distinct patterns of new bone growth (Figure 5). Firstly, bone originating from the native bone in apposition with the scaffold boundaries could be seen growing around the scaffolds in both AC and SVF groups (Figure S10). At 52 weeks these bony structures exhibited radiodensity very similar to that of native bone. We did not identify any trend unique to either of the two groups as these features were noticed at dorso-caudal, dorso-rostral, ventro-caudal, and ventro-rostral sites in both groups (Figure S11, S12). Secondly, dense mineralized bone nodules could be seen nucleating within the pores of the scaffold body. Scaffolds were segmented and removed to analyze the distribution of these nodules within the defect space. Although high radiodensity indicated good mineral content of these nodules, the volume percentages of these features within the defect space were relatively small ($1.05 \pm 0.85\%$ (AC; $n=2$ samples) and $1.02 \pm 0.41\%$ (SVF; $n=4$) compared to that of the bony extensions that were observed at the periphery of the scaffold ($9.09 \pm 5.26\%$ (AC; $n=2$) and $9.24 \pm 3.82\%$ (SVF; $n=4$). We categorized the bone nodules into four different groups based on their volumes: fine (grey), small (red), medium (green) and large (yellow) (Figure 5). The majority of the bone nodules belonged to the “fine” category (99.74% (AC; $n=2$) vs. 99.70% (SVF; $n=4$)) and the frequency of larger nodules (i.e., small, medium and large) was low in comparison. The “fine” bone nodules were preferentially distributed towards the lateral surface of the scaffolds and were concentrated around the fixation tabs (Figure 5A (iii), (iv); S11, S12). In addition, the fine bone nodules were also found in higher concentrations proximal to both the defect boundaries (rostral and caudal) and lower concentrations at the center of the scaffold. We did not observe any obvious distribution trend in the dorso-ventral direction. “Small” mineralized nodules displayed higher incidence at the center of the scaffolds for both groups (Figure 5B, 5C (Insets)). For the larger bone nodules (medium and large) we did not find any preferential distribution pattern within the scaffold. Closer examination of the defect site demonstrated that these nodules formed in close association with the PCL-DCB struts; essentially using the scaffold as a template for depositing new bone (Figure 6).

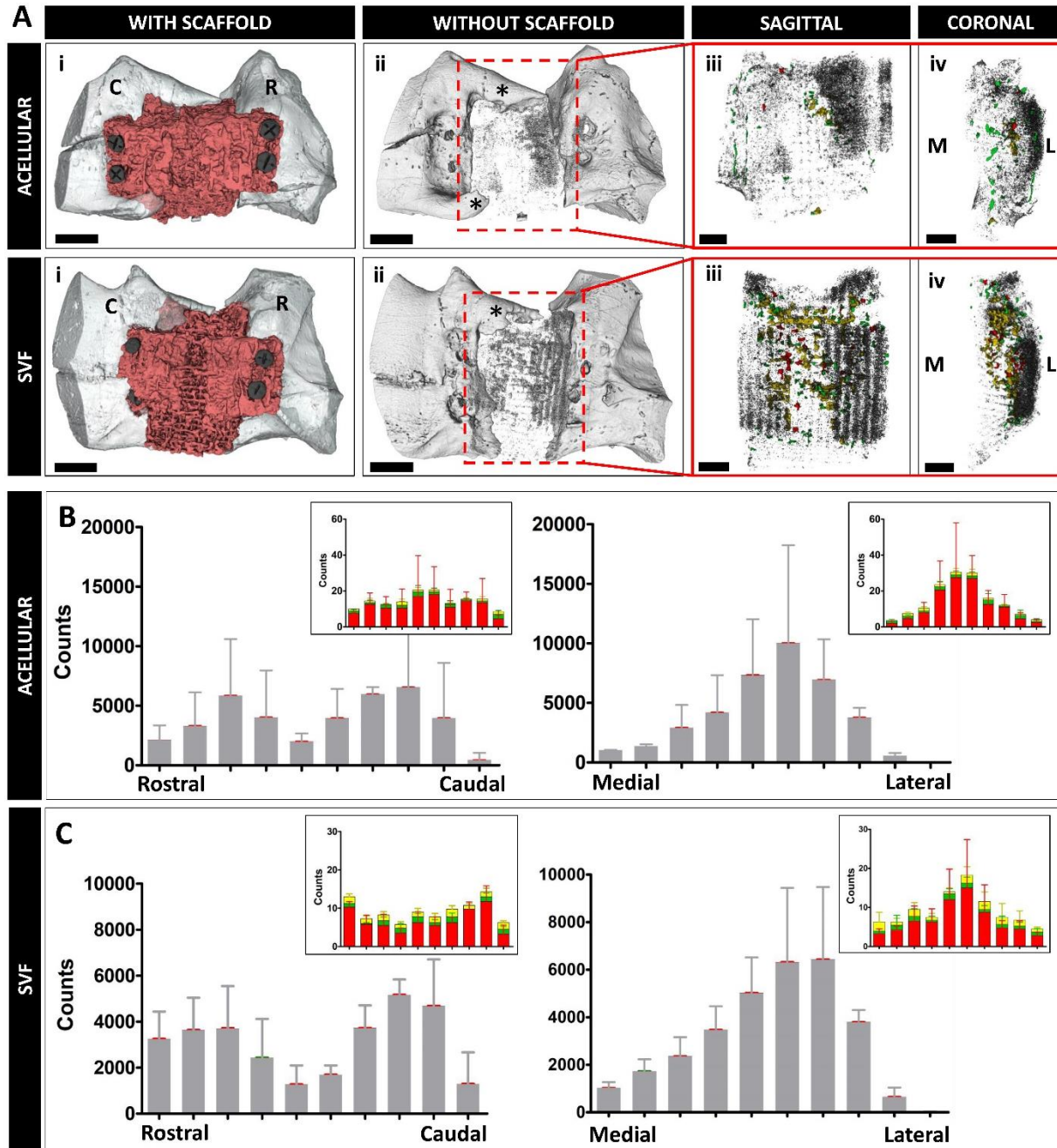


Figure 5. Analyzing the distribution of mineralized nodules within scaffolds. (A) (i) 3D reconstructions of μ CT scans of representative AC (defect volume = 4191.73 mm³) and SVF (defect volume = 4895.68 mm³) zygomas at 52 weeks with the scaffolds (red) segmented out and removed. (ii) Bone nodules formed within the pore spaces of the scaffold and bony extensions (asterisk) grew along the periphery. (iii, iv) With the bony extensions segmented out and removed, the sagittal and coronal views captured the spatial as well as volumetric distribution of new bone nodules within the scaffold pore spaces. The bone nodules were color coded based on their sizes,

namely fine ($< 0.0216 \text{ mm}^3$, grey), small ($0.0216 - 0.108 \text{ mm}^3$, red), medium ($0.108 - 0.216 \text{ mm}^3$, green), and large ($> 0.216 \text{ mm}^3$, yellow). (B, C) Histograms quantitatively depicting the spatial bone distribution of all bone nodules within the scaffold in the AC and SVF groups ($n=2$ and 4 for Acellular (AC) and SVF group respectively). Insets depict the spatial distribution of bone nodules, especially with volume larger than 0.0216 mm^3 , as their frequency is much lower as compared to those with volume less than 0.0216 mm^3 (grey). C: Caudal, R; Rostral, M: Medial, L: Lateral. Scale bar = 1 cm (A i,ii); 0.5 cm (A iii,iv)

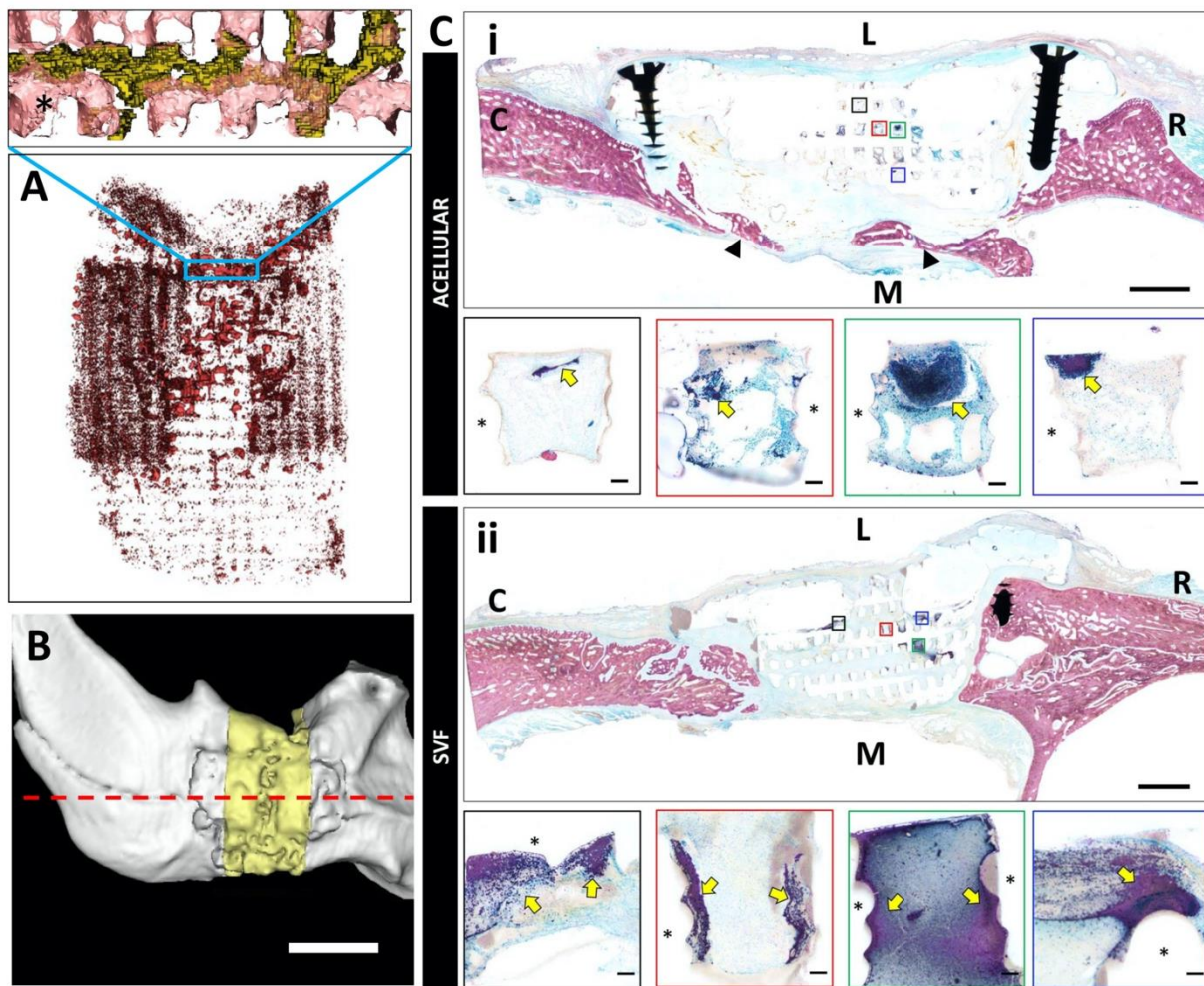


Figure 6. Bone nodules closely associated with PCL-DCB struts. (A) 3D rendered images of microCT scans of the defect site with the scaffold removed digitally (bottom panel) reveal that the bone nodules (yellow, top panel) nucleating within the pores of the scaffold are closely associated

with the PCL-DCB struts (pink; asterisk). (B) Red dashed line indicates the level of histological section for Stevenel's Blue (SB) staining. Scale bar = 2 cm (C) SB staining of representative samples (i,ii) elucidates presence of lamellar bone (black arrowheads) on the periphery of the scaffolds and woven bone (yellow arrows) within the scaffold pores. Scale bar = 4 mm (i,ii top panel); 100 μ m (i,ii bottom panel) C: Caudal, R: Rostral, L: Lateral, M: Medial.

6. PCL-DCB scaffolds elicited a mild local inflammatory response

Hard tissue histology of the explanted zygomas was performed to study the inflammatory and local tissue response to implantation of PCL-DCB scaffolds at the defect sites as well as to better understand bone remodeling around and within the scaffold. Histological studies indicated the presence of diffuse fibrovascular stroma within the porous spaces and collagen around the exterior surfaces of the scaffold (Figure 7A). The detection of hemosiderin pigment within the scaffold body suggested episodes of hemorrhage. Post hemorrhage, the pigments resided in the cytoplasm of the macrophages that were found in close association with the struts. Within the scaffold volume, osteoid deposition undergoing mineralization could be seen close to the struts and was identified as woven bone (Figure S13A, S13B). We did not find strong evidence of lamellar bone within the scaffold body, however the de novo growth into the defect region from the adjacent tissue appeared as lamellar bone at 52 weeks (Figure 7A). There was very limited remodeling observed histologically at the native bone-scaffold interface and limited bone infiltration into the scaffold body. We injected tetracycline (0 & 12 weeks) and calcein (6 & 24 weeks) into the AC and SVF animals to evaluate mineral deposition at the defect site (Figure S14, S15). Consistent with the Stevenel's Blue stains, fluorochrome stains did not indicate strong mineral deposition within the scaffolds of AC and SVF groups. However, the fluorochrome signal was pronounced in the neighboring native bones reflective of the skull growth. The residual implant area calculated from histological sections (n=18 i.e., AC=6, SVF=12) was found out to be $34.8 \pm 8.7\%$ (as a percentage of region of interest) which is similar to the theoretical scaffold material volume of 38% (or 62% porosity).

H&E staining of specimens revealed the presence of diffused sheets of lymphocytes within connective tissue and multinucleated giant cells (MNGCs) in very close association with the PCL-DCB struts (Figure 7B). The presence of macrophages, lymphocytes and MNGCs suggested local

inflammation at the defect sites within both the AC and SVF groups, but no inflammation was detected histologically within the empty (control) defect sites. However, hematological parameters like white blood cells, lymphocytes and monocytes were within normal physiological levels in the systemic circulation in all groups (Figure S16). Moreover, the animals did not exhibit any abscess formation or extrusion at the defect site throughout the duration of the study. Semi-quantitative evaluation of the H&E-stained samples compared local tissue as well as inflammatory responses among the empty, AC, and SVF defects (Figure 7C, 7D). From these evaluations, the PCL-DCB scaffolds appeared to induce a mild foreign body response.

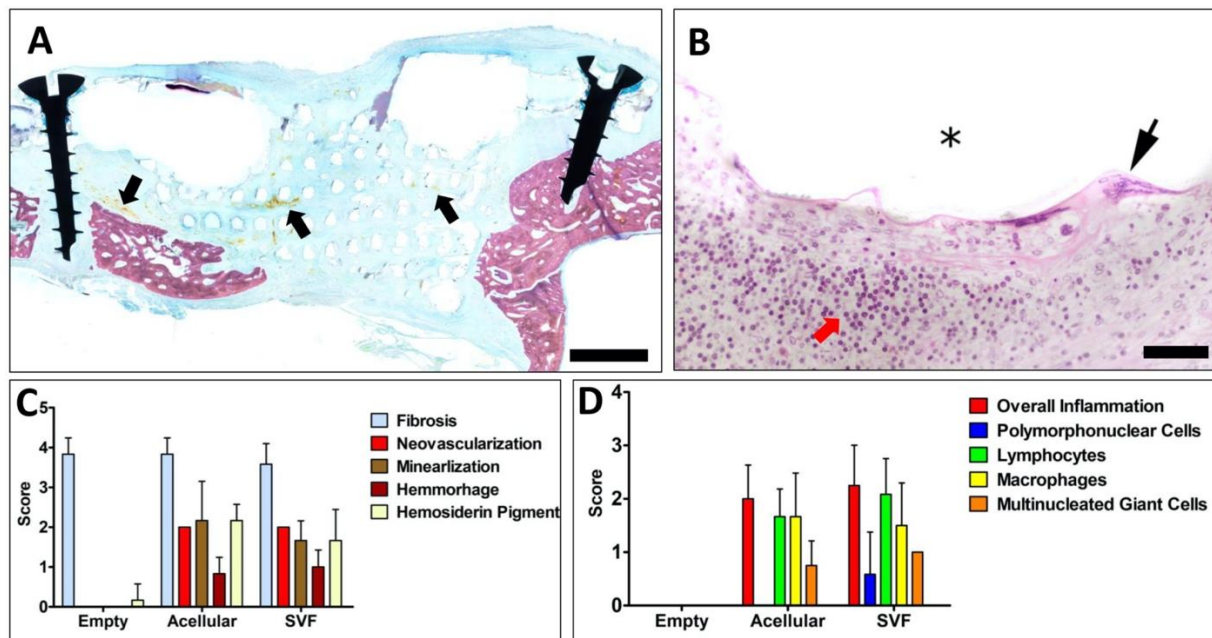


Figure 7. Local inflammation within the PCL-DCB scaffolds. (A) Representative Stevenel's Blue staining of undecalcified samples (hard tissue histology) highlights the presence of diffuse fibrovascular stroma within the porous spaces and collagen around the exterior surfaces of the scaffold. Hemosiderin pigment was also detected within the scaffolds (black arrows). Scale bar = 4 mm (B) H&E staining of the harvested zygomas demonstrate the presence of lymphocytes (red arrow) within the PCL-DCB scaffold pore spaces and MNGCs (black arrow) in close association with the PCL-DCB struts (asterisk). Scale bar = 50 μ m. (C) Semi-quantitative scoring of Stevenel's Blue stained sections for assessing fibrosis, neovascularization, mineralization, hemorrhage and hemosiderin within the scaffolds. Scores indicate 0 = not present, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe. (D) Semi-quantitative scoring of H&E stained sections for assessing overall inflammation, polymorphonuclear cells, lymphocytes, macrophages, and multinucleated giant cells. Scores indicate 0 = not present, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe.

moderate and 4 = marked/severe. (D) H&E-stained sections were used to assess overall inflammation, and presence of polymorphonuclear cells, lymphocytes, macrophages and MNGCs within the scaffolds. Scores indicate: 0 = absent, 1 = rare, 2 = mild infiltrate, 3 = moderate/heavy infiltrate, 4 = packed infiltrate/sheets for MNGCs.

H&E staining and immunostaining for CD31 and α -smooth muscle actin were performed on sections obtained from the center of the decalcified scaffolds (soft tissue histology). We detected the presence of vasculature in both the AC and SVF groups (Figure 8). Close association of CD31⁺ cells with the PCL-DCB struts was also observed which could be immune cells like macrophages or multinucleated giant cells as indicated by the semi-quantitative evaluations (Figure 7D).

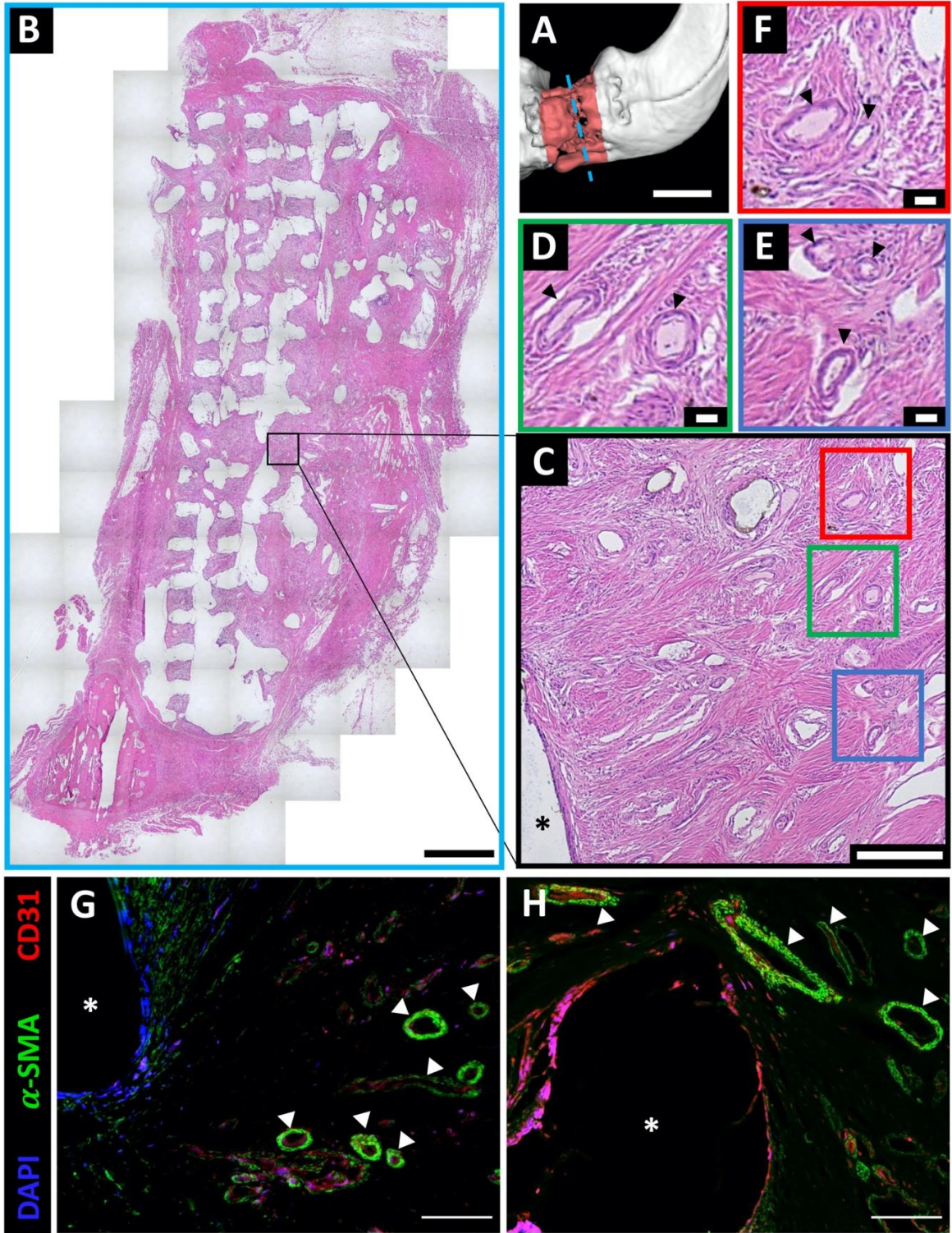


Figure 8. Presence of vasculature within the center of scaffold. (A) Blue dashed line indicates the site of histological sectioning from representative decalcified specimens to perform H&E and antibody staining. Scale bar = 2cm (B) H&E staining of the whole cross section of the specimen. Scale bar = 2 mm. (C-F) Zoomed in view of H&E stained specimen (B) to highlight presence of vasculature within the center of the scaffold. Scale bar = 200 μm (C); 20 μm (D-F). (G,H) Immunostaining of decalcified specimens with DAPI, CD31 and α -smooth muscle actin (SMA) antibody highlights the presence of vasculature (white arrowheads) which is consistent with the observations from H&E stained specimens. * indicates PCL-DCB scaffold struts. Scale bar = 100 μm .

7. Conclusions

The ultimate clinical solution for treating segmental craniofacial bone defects is a customizable biomaterial implant with the appropriate biochemical and biophysical properties to induce complete healing. This study provides a fundamental milestone en route to realizing this ideal and also demonstrates some limitations of the approach. The application of these 3D-printed PCL-DCB scaffolds with autologous SVF to regenerate complex midfacial bones has not been attempted in human patients and their long-term (1 year) outcomes in large animal models has not been previously reported. This study therefore provides critical insights on the advantages of using this biomaterial-based POC strategy clinically while also highlighting ongoing challenges that will need to be addressed.

We employed the scaffSLICR method established in our lab to 3D-print custom-designed, porous, biodegradable PCL-DCB scaffolds with large, isotropic pores and solid fixation tabs and demonstrated that the PCL-DCB scaffolds can restore the three dimensional structure of porcine midfacial bone CSDs with adequate soft-tissue coverage negating the need for osteosynthesis plates and their associated limitations.^[20,21] Intraoperatively sourced autologous SVF was incorporated using clinical grade fibrin hydrogel to facilitate cellular retention within the pores of the scaffolds avoiding leakage out of the defect site. We surmised that use of fibrin hydrogel would promote angiogenesis into the center of the scaffold. The 3D-printed PCL-DCB scaffolds have previously been shown to stimulate robust bone regeneration when used in combination with uncultured human SVF^[12] and without the need for exogenous growth factors such as bone

morphogenetic proteins which may elicit deleterious side effects.^[19] In addition, 3D-printed PCL-DCB scaffolds retained their structural integrity during the entire period of study in an environment where the bone was actively growing. Future studies will examine whether this adaptability facilitates their use in load-bearing defects and in skeletally immature animal models. Throughout the implantation period, the scaffolds mechanically integrated with the adjacent native bone and facilitated bone regeneration with or without SVF.

SVF has been used clinically for the treatment of alveolar bone and its use as an autologous cell source confers key regulatory and practical advantages. However, the osteogenic potential of SVF is widely considered to be lower than that of cultured ASCs due to the cellular heterogeneity and the potential for inhibitory signaling.^[22,23] PCL-DCB scaffolds with human SVF generated significantly more bone than acellular groups in mice^[12]. Therefore, we surmised that in this study we would induce significantly higher bone formation in the porcine SVF group. While the similarity in bone formation between SVF and AC groups in this study confirms that the scaffolds have strong osteoinductive and osteoconductive properties, the lack of additional bone volume in the SVF group might be attributed to the low seeding density or cellular trauma arising from the high concentration of collagenase and vigorous mechanical digestion steps used for expediting the digestion of porcine adipose tissue that negatively impacted their osteogenic capacity. (Notably, in the human scenario, the SVF would come from lipoaspirate, which can be digested more easily with reduced trauma). It may also point to a need to purify the cell population intraoperatively prior to injecting into the scaffolds as certain sub-populations of SVF are considered to be more osteogenic.^[24,25] The concentration of osteoprogenitors in porcine SVF was not ascertained in this study though it is assumed to be low based on the extended time needed to culture p1 pASCs. While we could not evaluate the cell composition or osteogenicity of porcine SVF, the P1 pASCs exhibited very similar surface marker expression profiles to human P1 ASCs^[26] as well as in vitro osteogenic capacity. Future studies may further explore dosing studies and assess the composition of porcine SVF to provide mechanistic insights.

The incorporation of SVF into the scaffolds did enhance the integration strength greater than two-fold. Direct comparisons to native bones were not possible in our study. Additionally, it should be noted that they may be of limited value given that the cantilever test would have required us to initiate a crack prior to applying load in order to control the fracture site. Nevertheless, the forces required to induce fracture (300 – 400 kN) suggest that the integration would be sufficiently

robust to support normal physiologic functions. To further explain the differences between the two experimental groups, we assessed μ CT data to determine whether the differences in integration strength between AC and SVF scaffolds could be attributed to distinct bone formation patterns. The patterns of bone growth in and around the scaffolds demonstrated by advanced image analysis of μ CT data and hard tissue histology were insightful even though they did not reveal an explanation for the differences between the mechanics of the AC and SVF groups. We designed scaffolds with isotropic 800 μ m interconnected pores to promote enhanced cell-biomaterial interaction^[27] and facilitate vascular and bony ingrowth to the scaffolds.^[28] Yet, most of the new bone in the defect regions appeared to be well-organized lamellar bone that grew from the adjacent tissue along the periphery of the scaffolds with limited infiltration into the pores. Future studies might examine whether the scaffold may be impeding the ingrowth of this dense lamellar bone while simultaneously inducing the deposition of mineralized nodules. The mineralized woven bone nodules and osteoid in the scaffold interior were closely associated with the struts of the PCL-DCB scaffold strongly suggesting that the scaffolds directly signaled to resident cells to undergo osteogenesis. However, the presence of woven bone nodules (confirmed by histological evaluation) within the pores of the scaffolds one year after transplantation reflected limited remodeling possibly due to a lack of transmission of compressive mechanical cues to the cells within the pores. Interestingly, in almost all cases, we observed near-perfect geometric reconstruction even when there was initial misalignment of the scaffold and the contours of the adjacent bone (which arose through scaffold trimming during surgery to facilitate their implantation in this intricate surgical model). For instance, when there was post reduction misalignment in the dorso-ventral direction it did not affect native anatomical restoration. This observation raises questions about how precisely the scaffold needs to replicate the geometry of the missing bone for clinical application. There are at least three key differences between this porcine model and putative clinical situations: (i) we were treating a fresh defect rather than chronic injury, (ii) the treated bone in this study was healthy and did not include disease-induced bone loss, and (iii) we preserved the periosteum around the implanted scaffold. Future studies might investigate an even more stringent model to better address this question of gross anatomic precision.

The model did introduce the complexity of treating bone containing a sinus, which can be variable in size and shape. The presence of a sinus at the rostral end reduced the end-to-end contact

between the scaffold boundary and the native bone which could lead to sub-optimal osseointegration. Conversely, at the caudal aspect of the implant where the interfacing native bone was compact and facilitated good bone-scaffold apposition and scaffold fixation, we observed rigid osseointegration in both groups. Despite abutting the maxillary sinuses, there were no episodes of mechanical failure of the scaffolds, wound dehiscence, or abscess formation that might suggest a loss of fixation. Hematological indicators indicated no systemic immune responses throughout the duration of the study. However, histological findings suggested some local inflammation within the scaffolds even after 52 weeks post-surgery. The fibrovascular tissue, macrophages, and multinucleated giant cells that were found to be in close association with the struts are hallmarks of a mild foreign body response (FBR)^[29]. It is unclear whether this impacted the bone formation efficacy in the scaffold interior, though future studies might incorporate the use of clodronate^[29] or other agents to reduce FBR^[30].

Overall, this study demonstrated the significant potential for in situ tissue regeneration of a CSD in non-load bearing midfacial bone using autologous cells in a large animal model with long-term follow up. We demonstrated a digital workflow, with 3D printing of defect-specific, mechanically strong, porous, bioactive scaffolds. Long-term follow-up over 52 weeks provided significant insights into long term implant survivability, unique patterns of new bone formation, mechanical integration of the scaffolds with native bone, and angiogenesis within the scaffolds while exposing areas that still need to be addressed like minimal biodegradation of the PCL-DCB scaffold and local inflammation. Envisioning clinical translation of our POC strategy, we have developed and utilized an image-based algorithm to quantitatively evaluate the 3D print quality of the scaffolds (pore and strut widths) which can be used for future quality control assessments.^[31] The acellular scaffolds significantly enhance bone regeneration. Additionally, infusing the scaffolds with autologous SVF obtained intraoperatively validates the feasibility of regenerating bone using a POC strategy^[32] and increases the likelihood of clinical translation.

8. Experimental Section/Methods

Experimental Design:

All experiments were conducted with skeletally mature, castrated, male Yucatán pigs at Louisiana State University under an approved Institutional Animal Care and Use Committee protocol. We aimed to have a sample size of at least $n = 4$ for statistical comparisons. With bilateral defects, this

corresponded to a minimum of 2 pigs per group. We created 2 cm full-thickness, segmental, bilateral defects in the zygomatic arches. We tested three experimental groups: (1) The untreated controls with empty defects (E; $n=2$ pigs); (2) acellular PCL-DCB scaffolds filled with fibrin only (AC; $n=2$ pigs); and (3) PCL-DCB scaffolds infused with autologous SVF suspended in fibrin ($n=4$ pigs) (**Table S1**). Following surgeries, the pigs were immediately returned to their housing and placed on their regular diet. Bone regeneration outcomes were evaluated by using CT and μ CT imaging, histology of decalcified and calcified samples, immunohistochemistry, immunostaining, and biomechanical testing. Isolated cells were culture expanded for one passage and characterized using flow cytometry and osteogenic assays. Surgery on the two animals in the empty (E) group (22 months old; 68 ± 3.7 kg) was performed together with two pilot animals to establish the surgical protocols. The six animals for AC and SVF (18 months old; 53.3 ± 5.4 kg) were obtained six month later and randomized. The discrepancy in ages between the AC/SVF and E animals was due to animal availability. The implantations were not blinded as we only performed fat harvest for the SVF group. CT and μ CT quantification, mechanical testing, and hard-tissue histology were all blinded to eliminate any bias.

Sedation of animals for blood collection and acquisition of pre- or post-operative CT scans:

All animals were sedated with a combination of tiletamine 2 mg kg^{-1} , ketamine 3 mg kg^{-1} and xylazine 3 mg kg^{-1} (intramuscular administration). Anesthesia was induced and maintained with isoflurane on oxygen via endotracheal tube. 15 – 20 ml of blood was collected from the cranial vena cava for complete blood counts (Siemens Bayer Avia 120 Hematology System) and serum chemistries (Beckman Coulter AV680). Blood was collected into ethyldiamine tetraacetic acid (EDTA) tubes (Becton Dickinson Bioscience, Franklin Lakes, NJ) for complete blood count and serum tubes ((PST™ Gel and lithium heparin, BD Bioscience)) for serum chemistry. Computed tomography images were acquired with the animals under general anesthesia and in ventral recumbency (GE light speed 16, GE, USA; 120kVp, 625 μm slices).

Fabrication of custom osteo-inductive PCL-DCB filaments:

Clinical grade poly- ϵ -caprolactone (PCL; PURASORB® PC 12, Corbion, The Netherlands) and decellularized bone (DCB; Bio-Oss®, Geistlich Pharma) were mixed in small batches in the ratio 70:30 (w/w), respectively and were stored at -80°C for at least 12 hours to render them suitable

for cryo-milling. Thereafter, cryo-milling of the mixture was performed in liquid nitrogen to attain a homogenous mixture of near uniformly sized granular particles (<0.5 mm). The cryo-milled mixture was then fed into the filament extruder (Dynisco Polymer Test Laboratory Machine Extruder, LME-120) and extruded at $75 - 90^{\circ}\text{C}$ to form filament with an average diameter of 2.85 mm. The custom PCL-DCB filament was further used for 3D printing custom scaffolds.

Design and fabrication of custom scaffolds:

Pre-operative CT scans were used as a template for designing the custom scaffolds for each animal (**Figure S1**). The raw DICOM files of CT scans were imported into the 3D medical image processing software, Mimics (Materialise Mimics 14.0®, Leuven, Belgium), and thresholding was performed at 226 Hounsfield units (HU) to create the masks. 2 cm wide wedge shaped solid objects (.stl) were inserted into the zygomas bilaterally to define the defect boundaries. Using the “Boolean” tool, the overlapping bone structure was segmented out creating a virtual osteotomy. The sinuses of the segmented zygomatic bone were filled in using the “Wrap” tool, and fixation tabs were added digitally to complete the design of the custom implant. The solid scaffold volumes were converted into masks. Thereafter, the lateral aspect of each mask was segmented out to obtain the masks of the corresponding surgical guides. Finally, the implant masks were exported out of Mimics in the form of bitmap (.bmp) slices.

These bitmap slices were in turn imported into a MATLAB®-based, in-house scafSLICR program^[17] which allowed for incorporation of isotropic porosity into the otherwise solid scaffold volume and subsequent generation of the print-path or the g-code file. The volume was sliced in a fashion such that the scaffold body contained isotropic pores of width $800\text{ }\mu\text{m}$ and struts of width $500\text{ }\mu\text{m}$ (which translates to a theoretical porosity of 62%)^[17], whereas the fixation tabs had no pores. The g-codes for each custom scaffold were relayed into a Lulzbot TAZ5 3D printer (Aleph Objects) provided with the osteoinductive PCL-DCB filaments and 3D printed at an extruding temperature of 110°C and a feedrate of 300 mm min^{-1} . Prior to implantation in the animals, all the scaffolds and cutting guides were subjected to surface treatment and sterilization. Briefly, the 3D printed scaffolds were treated with 3M sodium hydroxide solution for 20 minutes to increase the hydrophilicity of the scaffold surface. Thereafter, both the scaffolds and cutting guides were washed 3 times for 20 minutes with sterile phosphate buffer saline (PBS) . After the wash, they

were subjected to incubation in an ethanol ladder (70%, 80%, 90%, 95% and 100% ethanol with each step lasting for 20 minutes). All the treatment steps were carried out at room temperature on a rocker. KimWipes were used to wick dry all the treated scaffolds and cutting guides, following which they were all packed in sterilization pouches and treated with ethylene oxide gas for 12 hours to sterilize them. Once sterilized, the aseptic pouches containing the scaffolds and cutting guides were stored and transported along with silica gel packets to protect from moisture.

Assessment of print quality of the 3D printed PCL-DCB scaffolds

Post fabrication print quality of the PCL-DCB scaffolds was assessed to ensure that the print dimensions, especially pore width and strut width, closely matched with that of the desired design parameters. Cone beam computed tomography (CBCT) scans of the 3D printed scaffolds were acquired using prototype CMOS detector-based high-resolution extremity CBCT based on Carestream OnSight3D equipped with a Teledyne Dalsa Xineos 3030 CMOS detector and 0.3 mm focal spot x-ray tube (IMD RTM 37). Scans were performed at 99 μm detector pixel size, 420 projections over the short scan range of $\sim 220^\circ$, 10 mA tube current/x-ray pulse, 10 mAs pulse length, 90 kVp tube potential. Thereafter the scans were reconstructed at 100 μm resolution using bone “sharp” reconstruction protocol. The reconstructed volume was imported into Mimics and thresholding was performed at 800 HU and the resulting masks were exported into MATLAB® workspace. Using a custom MATLAB® script, scaffold volumes were skeletonized, thus reducing the original volume (strut volume) to a single, connected voxel thread (strut skeleton). Similarly, the initial strut volumes could be inversed (grayscale) to obtain the pore volume which could then be skeletonized to obtain the pore skeleton.

To calculate strut widths throughout the volume, the Euclidean distance between each voxel in the strut skeleton and the nearest voxel in the pore volume was calculated. These distances were assumed to be half of the actual strut width i.e., distance from strut boundary to strut center. Similarly, to calculate pore widths, the Euclidean distance between each voxel in the pore skeleton and strut volume was calculated. As earlier, these distances were assumed to be half of the actual pore width i.e., distance from pore boundary to pore center. A 6 mm width cube whose center was concentric with that of the center of mass of the scaffold volume was identified and qualified as a representative volume for the whole scaffold volume. Average strut width and pore width for the

representative cubes of all the scaffolds were quantified using the script and compared with that of the desired strut width and pore width of 500 μm and 800 μm respectively.

Post-Operative Bone Volume Quantification from CT:

Post-operative CT scans were analyzed using Mimics. The region of interest (ROI) was segmented out by using the boundaries of the wedge-shaped cutting guide that was previously used to design the scaffolds. Volume of voxels above a 226 HU threshold within the segmented ROI was measured at different time points and identified as the respective bone volume (BV). The wrapped volume (sinus of the zygomatic bone was closed using the “Wrap” tool of Mimics to obtain a compact volume) of the segmented native bone (used to design the scaffolds from pre-operative CT scans) was defined as the defect volume (DV). BV/DV was calculated for different time points and was used for comparison of new bone volume across all groups.

Isolation of SVF from subcutaneous porcine fat:

Under anesthesia subcutaneous adipose tissue was excised from the lumbar paravertebral area. Briefly, using a #20 blade a small incision was made over the area superficial to L3 - L6 following aseptic preparation and subcutaneous adipose tissue was sharply excised. Thereafter, the tissue was washed multiple times with phosphate buffered saline to remove blood, minced for five minutes into <2 – 3 mm pieces, and washed in RBC (red blood cell) lysis buffer (0.155 M ammonium chloride, 0.01 M potassium bicarbonate, 0.1 mM EDTA, dissolved in sterile deionized water) for 1 min to remove blood clots. After centrifugation to remove the RBC lysis buffer, the tissue was digested with collagenase solution (4 mg ml⁻¹ collagenase type 1, Worthington Biochemical; 25 mg ml⁻¹ bovine serum albumin, Sigma Aldrich; dissolved in 1 g l⁻¹ glucose Dulbecco’s modified eagle medium) under agitation for 30 minutes at 37°C. Following digestion, cells were centrifuged at 300 g for 5 minutes at 37°C to collect the stromal vascular fraction. Collagenase, oil, and residual undigested fat layers were removed, and the remaining cell pellet was resuspended in RBC lysis buffer. The cell suspension was filtered through an 800 μm mesh and a 100 μm cell strainer and then centrifuged.

Cells were resuspended in PBS and split into three groups: 45% of the volume for each defect and 10% for cell characterization. After centrifugation, pellets for each defect were resuspended in the fibrinogen component of a TISSEELTM packet (diluted 1:5 in phosphate buffered saline) and drawn into 5 ml double-barreled syringes along with the thrombin component

contained in the other side for *in situ* injection of SVF into the created defect. The remaining 10% of cells were plated in expansion media for further culture and characterization.

Zygomatic Defect Creation:

Once the animal was sedated, subcutaneous fat was harvested. Subsequently, a transcutaneous 5 cm sub tarsal incision was made with a #15 blade over the zygoma of Yucatan miniature pig. This was followed by dissection with a cautery down to the inferior orbital rim and then down to the zygomatic bone. The dissection proceeded in a subperiosteal fashion over the anterior, lateral portions of the zygomatic bone with a periosteal elevator. Careful attention was placed to the dissection of the masseter off the zygoma on its inferior margin to facilitate complete release of the zygomatic defect. A 3D-printed, animal-specific 2 cm osteotomy cutting guide was then placed onto the anterior wall of the zygoma/orbital rim and was held in place while the proximal and distal osteotomy sites were marked with a pencil. A 27 mm reciprocating saw tip (Johnson & Johnson – De Puy Synthes) was then used to make the proximal and distal osteotomies followed by the use of a 4 mm straight osteotome to complete the cuts. The cut bone was removed to create the defect and a thorough exploration of the defect and zygoma was performed. Any residual bone or sharp bone edges were removed. The animal-specific scaffold construct was then inserted into the zygoma defect and held in place with two molt elevators. Two 8 (or 10) × 1.8 mm screws were placed bilaterally on the built-in fixation tabs to fixate the implant onto the defect. The construct was copiously irrigated and injected with SVF or control solution when appropriate. Finally, the incision was closed in layers starting at the periosteal level, followed by a deep subcutaneous layer, and lastly in a subcuticular fashion using absorbable stitches. Finally, dermal skin glue was placed over the incision to achieve immediate closure of the wound. No surgical site dressing was utilized.

Scaffold Explantation:

Upon reaching the endpoint (52 weeks) all animals were euthanized and the zygomas (zygomatic (arch+flange) + squamosal bone) containing the scaffolds were harvested. Briefly, a transcutaneous 8 cm incision with 1 cm vertical extensions proximally and distally were made over the malar region of the swine with a 10 blade. This was followed by sharp dissection down to the anterior and posterior borders of the zygoma. Dissection proceeded in a subperiosteal fashion until the entire zygoma was exposed, including the former zygomatic defect region. At a distance of 2 cm proximal and distal to the defect margin, the zygomas were dissected free and

osteotomized using a reciprocating saw (Johnson & Johnson – DePuy Synthes) followed by a 4 mm osteotome. The entire zygomatic complex including the implanted construct was then removed *en bloc*.

MicroCT Scans:

Selected harvested zygomas were visualized using the Bruker SkyScan 1173 system. During the scan, each sample was rotated 360 degrees and projections were captured using the following scan settings, source voltage: 130kV, source current: 61 μ A, exposure time: 1200 ms, rotation step: 0.25 degrees, 0.25mm brass filter. Once all projections were obtained, Nrecon software was used to reconstruct the projection images and to generate computed axial cross-section slice images with 30 μ m pixel resolution. These axial sliced images were rendered with Bruker's CTVOx software whereas segmentation of the images was performed using Avizo (FEI Visualization Sciences Group, Burlington, MA). The zygomatic bones, scaffolds, the bone formation on the periphery and within the scaffold, and the metal pins were labeled and analyzed using the *watershed* tool, *volume edit* function, and *label analysis* functions in Avizo.

Mechanical Testing:

All the harvested zygoma specimens were scraped to remove excess tissue with care taken to not damage the soft tissue coverage around the PCL-DCB scaffolds. Rectangular cross-sectional plastic tubes (McMaster-Carr) were cut into small sections of approximately 1 inch depth to serve as pots for the zygomas. The zygomas were held vertically upright within the pot while polymethylmethacrylate (PMMA, Keystone Bosworth Fastray) was poured to uniformly fill the entire volume around the zygoma. The slurry was allowed to cure for approximately 20 minutes. The defect boundaries and the midpoint of scaffold were identified and marked for reference. The potted zygomas were clamped into a multiaxial vise on a servo hydraulic testing machine (Model 858, MTS, Eden Prairie, MN) such that the medial aspect of the zygoma faced the load applicator of the testing machine. The load was applied at the midpoint of the scaffold. The load applicator was displaced at a rate of 1mm s⁻¹ until failure occurred at the interface between the scaffold and the posterior (caudal) aspect of the native bone. The distance between the load application site and the fixed end of the specimen was measured and used to calculate the applied bending moment as follows:

$$\text{Applied bending moment} = \text{Load} \times \text{distance}$$

Hard Tissue Histology:

A special bone stain, Stevenel's Blue (SB)^[33] with Van Gieson's picrofuchsin counterstain, was employed to primarily distinguish new bone from pre-existing bone and identify sites of osteoid deposition or mineralization within the extra-cellular matrix whereas H&E stain was used to gain insight about the cellular entities present at the defect site. All zygoma specimens were radiographed (Bruker Digital Radiograph System, Bruker, Billerica, MA, USA) and the resulting radiographs were used to aid in trimming each specimen to include the implant area with adjacent pre-existing bone. Tissues were then dehydrated through a graded series of ethanol and infiltrated and embedded with methyl methacrylate (MMA). The resultant MMA blocks from each zygoma were then sectioned at three levels and each level ground/polished to approximately 100 µm thickness (Exakt® Systems, Norderstedt, Germany). For each level, three sections were obtained: one section was stained with H&E, one section was stained with SB for bone and one section left unstained for dynamic morphometry. All slides were scanned using an Olympus VS120 scanner (Olympus Corporation of America, Center Valley, PA, USA). SB-stained samples were scored semi-quantitatively by a pathologist for fibrosis, neo-vascularization, mineralization, hemorrhage and hemosiderin pigment where scores indicate 0 = not present, 1 = minimal, 2 = mild, 3 = moderate and 4 = marked/severe. H&E-stained slides were scored semi-quantitatively for overall inflammation, polymorphonuclear cells, lymphocytes, macrophages and multinucleated giant cells (MNGC) where scores indicate 0 = absent, 1 = rare, 2 = mild infiltrate, 3 = moderate/heavy infiltrate, 4 = packed infiltrate/sheets for MNGC.

Soft tissue histology for examining vasculature within the scaffolds:

Once mechanical testing was performed on the zygomas, one zygoma each belonging to the AC and SVF groups was chosen for immunostaining. The zygoma specimens were incubated in decalcifying medium (Thermo Scientific™ Richard-Allan Scientific™ Cal-Rite™ Decalcifying/Fixation Solution) for 10 – 12 days. Three different blocks (two close to the defect boundary and one in the center) of width ~3 mm from the decalcified scaffold region were cut out and embedded in paraffin. Thin tissue sections of the paraffinized specimens were obtained on glass slides for microscopy. The specimens were de-paraffinized by subjecting the sections to subsequent washes in xylene, 100%, 95%, 80% and 70% ethanol. Antigen retrieval was performed by microwaving the de-paraffinized sections in 1X Tris-EDTA Antigen Retrieval buffer

(Invitrogen 00-4956-58) for a minute. Thereafter the sections were incubated in a blocking solution of PBS containing 0.2% (v/v) Triton X-100 and 10% (v/v) donkey serum for 30 minutes. Once the blocking solution was discarded, the sections were incubated with CD31 (Abcam; ab28364 rabbit polyclonal) and α -smooth muscle actin primary antibodies (ThermoFisher Scientific; MA1-06110 Mouse monoclonal) diluted at 1:200 and 1:500 (v/v) ratio respectively in a solution made up of 10% (v/v) donkey serum and 2% (v/v) Tween-20 at 4°C overnight. For conjugating the secondary antibody, the primary antibody solution was discarded, and the sections are washed with PBS. Thereafter, they were treated with the secondary antibodies (Invitrogen A32794 Donkey anti-Rabbit Alexa Fluor Plus 555 and Invitrogen A32766 Donkey anti-Mouse Alexa Fluor 488), each diluted at 1:200 (v/v) and DAPI (4',6-diamidino-2-phenylindole) diluted at 1:2000 (v/v) in a solution made up of 10% (v/v) donkey serum and 2% (v/v) Tween-20 in PBS for an hour at room temperature. Finally, the sections were washed with 1x PBS and mounted with 1.5 μ m thick coverslips with the application of 50% (v/v) glycerol in 1x PBS. The immuno-stained slides were imaged using a Zeiss AxioObserver.Z2 (with apotome) inverted microscope.

Statistical Analysis:

Quantitative data were expressed as the mean $\pm$ standard deviation. Statistical analyses were performed using GraphPad Prism 5 software. Statistical analysis for bone volume was carried out by two-way ANOVA followed by a Bonferroni *post-hoc* test. Comparison of mechanical testing data was done using an unpaired, one-tailed t-test. For complete blood work data (dot plot), each dot represents an individual animal. Statistical significance has been denoted as $^*(p < 0.05)$, $^{**}(p < 0.01)$ and $^{***}(p < 0.001)$ or $\#(p < 0.05)$, $\##(p < 0.01)$ and $\###(p < 0.001)$.

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Conflict of interest : The authors declare no conflict of interest.

Credit Author Statement

SS: Designed research, performed research, analyzed data, wrote the paper. ELN: Designed research, performed research, analyzed data. ANO: Designed research, performed research, analyzed data. ALF: Designed research, performed research, analyzed data. ANR: Performed research. NZ: Performed research, analyzed data. ECW: Analyzed data. YZ: Analyzed data. EM: Performed research. CCW: Performed research. WZ: Provided resources. WG: Performed research. DG: Performed research, Analyzed data. NDJ: Analyzed data. TT: Performed Research. CT: Performed research. JL: Performed research. MJL: Performed research, analyzed data. MPG: Designed research, performed research, analyzed data, WLG: Designed research, analyzed data, wrote the paper.

Data Availability Statement

The raw/processed data required to reproduce these findings cannot be shared at this time due to technical or time limitations.

Supplementary Materials & Methods:

Flow cytometry:

In preparation for flow cytometry, 3 lots of cryopreserved P0 pASCs, derived from isolated porcine SVF (from 3 different animals), were thawed via immersion in a water bath and plated separately at a density of roughly 6,000 cells/cm² in T175 flasks (1 million cells per flask). The media used was composed of 10% fetal bovine serum (Sigma-Aldrich, F4135), 1% penicillin/streptomycin (Corning, 30-002-CI) in High Glucose DMEM (ThermoFisher, 11965118), supplemented with 20 µL of fibroblast growth factor-2 (PeproTech, 100-18B). The cells were allowed to expand for 3 days. Once sufficiently confluent, they were detached from the flasks via a brief incubation in warm accutase (Sigma-Aldrich, A6964-100ml) and resuspended in PBS. These cells were referred to as P1 pASCs. The cells were incubated in a Violet Live/Dead staining solution (Thermofisher, L34963) for 30 minutes at 4 °C, then centrifuged, washed with PBS, and resuspended in blocking buffer (5% Normal Donkey Serum, Sigma-Aldrich D9663-10ml in PBS). From this point on, the cells were protected from light. After a 10-minute incubation at 4 °C, the cells were again resuspended in a solution of 2% normal donkey serum (NDS) in PBS. At this stage, each sample was distributed into the wells of a 96-well V bottom plate (Millipore Sigma, M9686). The appropriate volumes of antibody (CD73 (Novus AF-4488-SP), CD90 (BD 555595), CD105 (Abcam ab53318), CD34 (Abcam ab81289), CD45 (Bio-Rad MCA1222F)) were added to each well of the plate, and the plates were incubated at 4 °C for 30 minutes. For the non-conjugated antibodies used, this step was followed by 3 PBS washes, and then a subsequent incubation with the appropriate secondary antibody, followed again by 3 PBS washes. Finally, the samples were fixed via incubation in ice-cold 4% PFA at room temperature for 5 minutes, followed by a 5-minute centrifuge cycle. A final PBS wash followed, before the samples were each resuspended in 100 µL PBS, with 200,000 cells in each sample. The samples were run on CytoFLEX S Flow Cytometer (Beckman Coulter Life Sciences) with the lasers set to 60 mW, 500 mW, and 30 mW for the FITC, APC, and PB channels, respectively. FlowJo Software was used for gating of the flow cytometry samples.

In vitro osteogenic culture

Prior to cell culture, well plates were coated with collagen I (Sigma Aldrich C8919) to facilitate cellular adhesion and to prolong the duration of the culture, as ASC begin to peel off once they start mineralizing. Cryopreserved P0 pASCs were thawed and plated on the collagen coated wells at a seeding density of 2500 cells/cm² to get P1 pASCs. The P1 pASCs were cultured in expansion media for 24 hours following which the media was switched to control or osteogenic media (day 0). The expansion media was comprised of 10% fetal bovine serum, 1% penicillin/streptomycin, 1 ng/ml fibroblast growth factor (FGF-2) in 4.5 g/L glucose Dulbecco's modified eagle medium. The control media was comprised of 10% fetal bovine serum, 1% penicillin/streptomycin dissolved in 1 g/ml glucose Dulbecco's modified eagle medium whereas the osteogenic media was prepared by supplementing the control media with beta glycerophosphate and ascorbic acid to have a final concentration of 10 mM and 50 µM, respectively. The control or osteogenic media was replenished 3 times per week. After 22 days of culture, bright field images of the pASC monolayer were acquired using a Zeiss Axio Observer.A1 microscope. Thereafter, cells were washed once in PBS and fixed with 3.7% paraformaldehyde solution for 30 minutes at room temperature. The fixed monolayers were stained with Alizarin Red (Sigma Aldrich A5533) and imaged using a Zeiss Discovery.V8 stereoscope to assess mineralization.

Supplemental Figures:

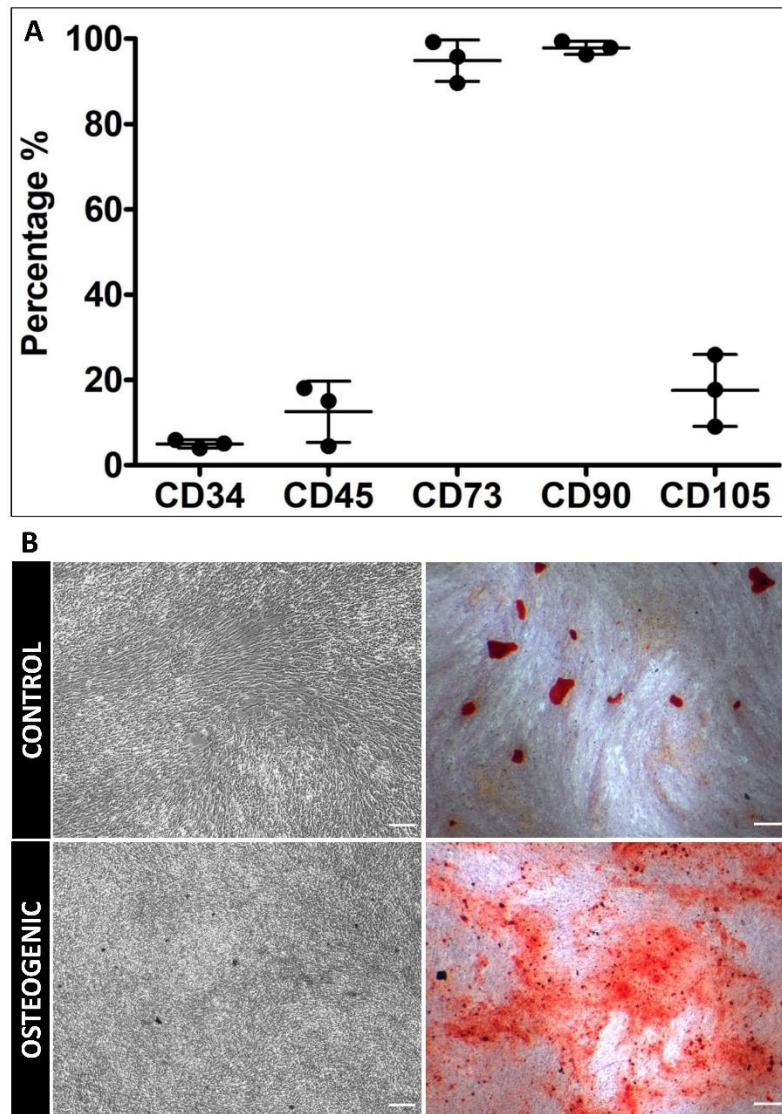


Fig. S1. Characterization of porcine adipose derived cells. (A) Isolated SVF was cultured for 1 passage in tissue culture flasks. The resulting P1 pASC were assessed with flow cytometry to determine the presence of CD34 (5.00 ± 0.95), CD45 (12.55 ± 7.17), CD73 (94.87 ± 4.89), CD90 (97.87 ± 1.55) and CD105 (17.55 ± 8.42) (B) The osteogenic capacity of P1 ASC was confirmed by culturing in media with osteo-inductive factors and observing mineral deposits in brightfield microscopy (Scale bar = 200 μm) and using Alizarin-Red staining (Scale bar = 500 μm).

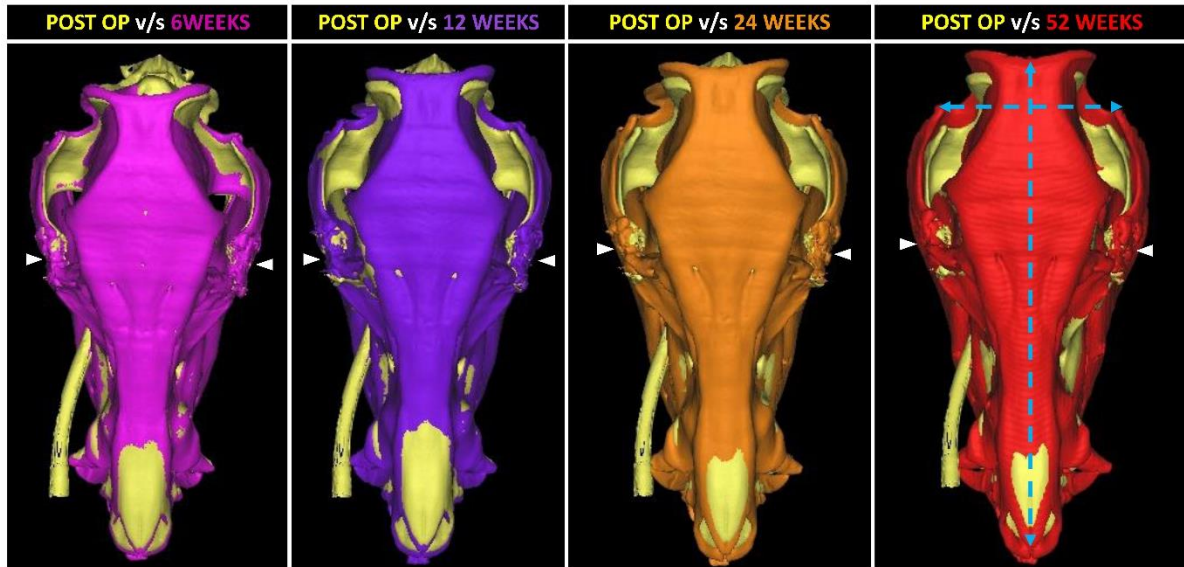


Figure S2. PCL-DCB scaffolds maintained their integrity in a skeletally evolving environment. Image registration was used to demonstrate significant post-operative skull growth. All 12 PCL-DCB scaffolds (AC and SVF group) in the study remained intact and attached to the adjacent native bone throughout the 52-weeks implantation period. Dashed lines indicate the axes of measurement for length and width of the skull. White arrowheads identify the zygomatic defect sites.

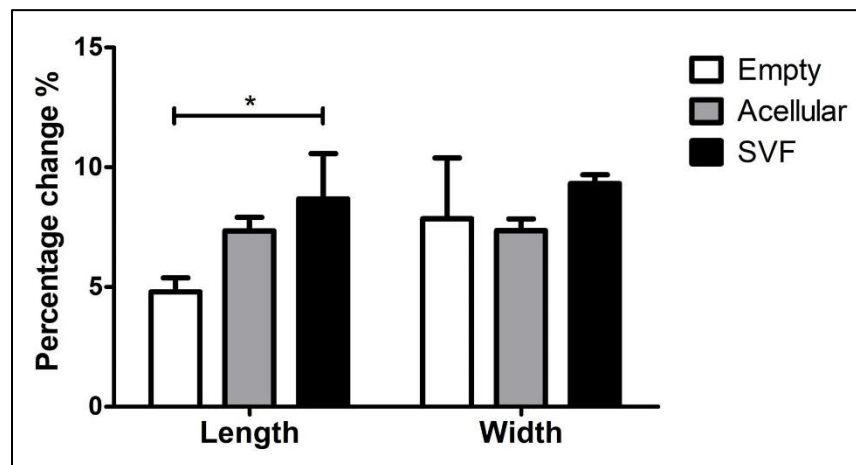


Fig. S3. Varying degree of skull growth in animals. Histograms depicting difference in magnitude of skull growth of animals belonging to different test groups (E, n=2 animals; AC, n=2; SVF, n=4) which might be arising due to difference in age of the animals (see Table S2).

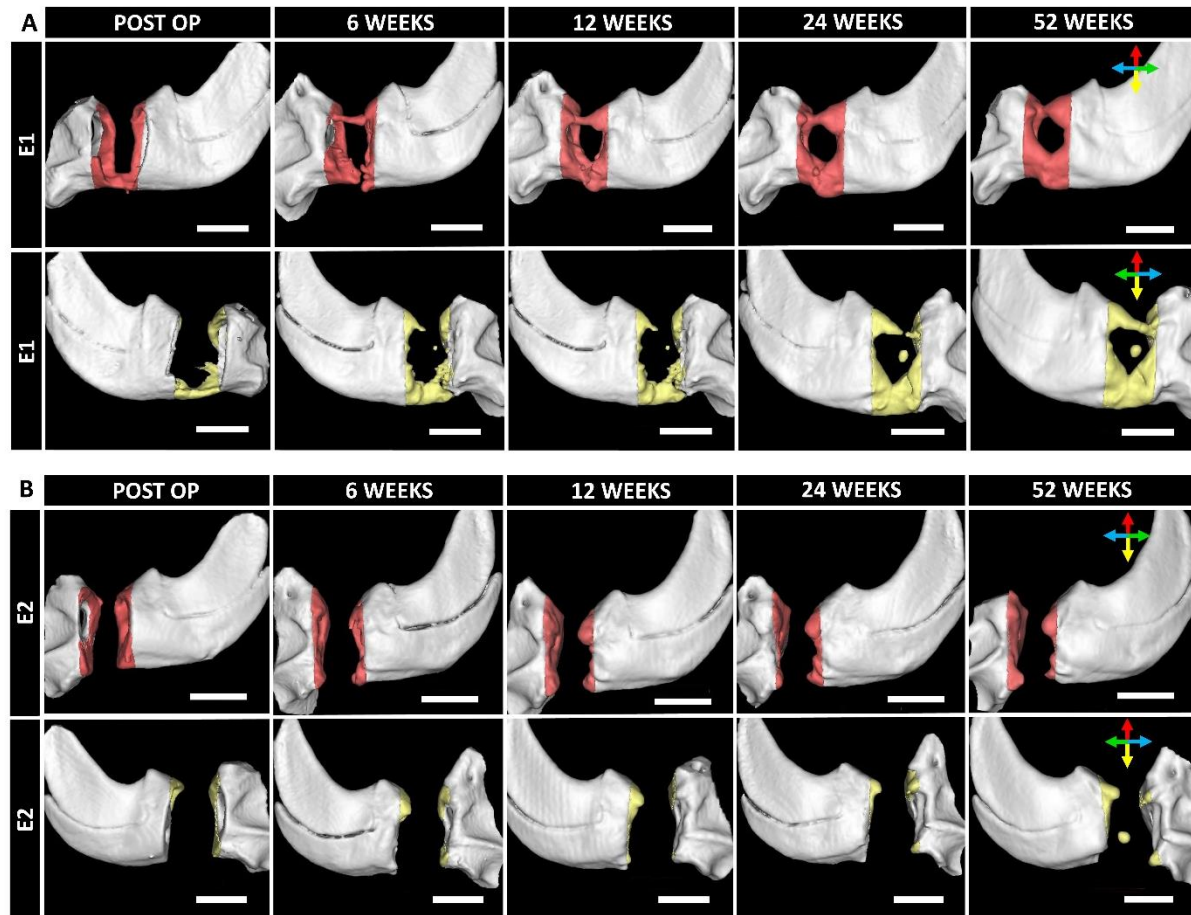


Fig. S4. CT scans of empty (control) defect at different timepoints. CT Scan suggesting (A) partial defect creation in both the zygomatic defects of one of the control animals which leads to significant bridging of the defect at 52 weeks timepoint. (B) creation of 2 cm full thickness segmental defect in porcine zygoma which demonstrates non-union at 52 weeks. Sense of orientation is conveyed by colored arrows: dorsal (red), ventral (yellow), rostral (blue) and caudal (green). Scale bar = 2 cm.

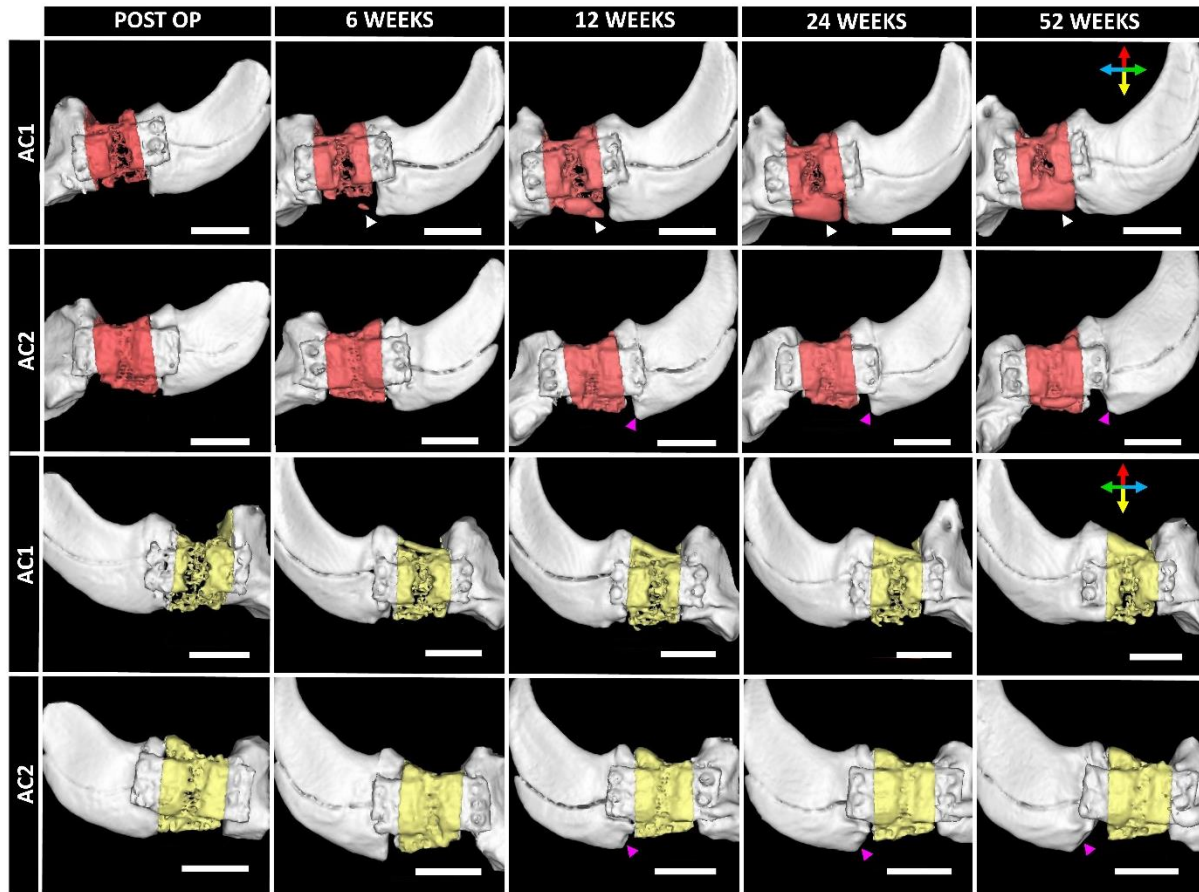


Fig. S5. CT Scans of AC group defects at different time point. Red and yellow color indicate region of interest belonging to the left and right zygomas respectively. Different bone growth patterns have been highlighted: formation of bony islands on the ventral aspect of the defect which fills in the deficit volume post reduction (white arrow heads), resorption at the ventro-caudal aspect of the defect (magenta arrow heads). Sense of orientation is conveyed by colored arrows: dorsal (red), ventral (yellow), rostral (blue) and caudal (green). Scale bar = 2 cm.

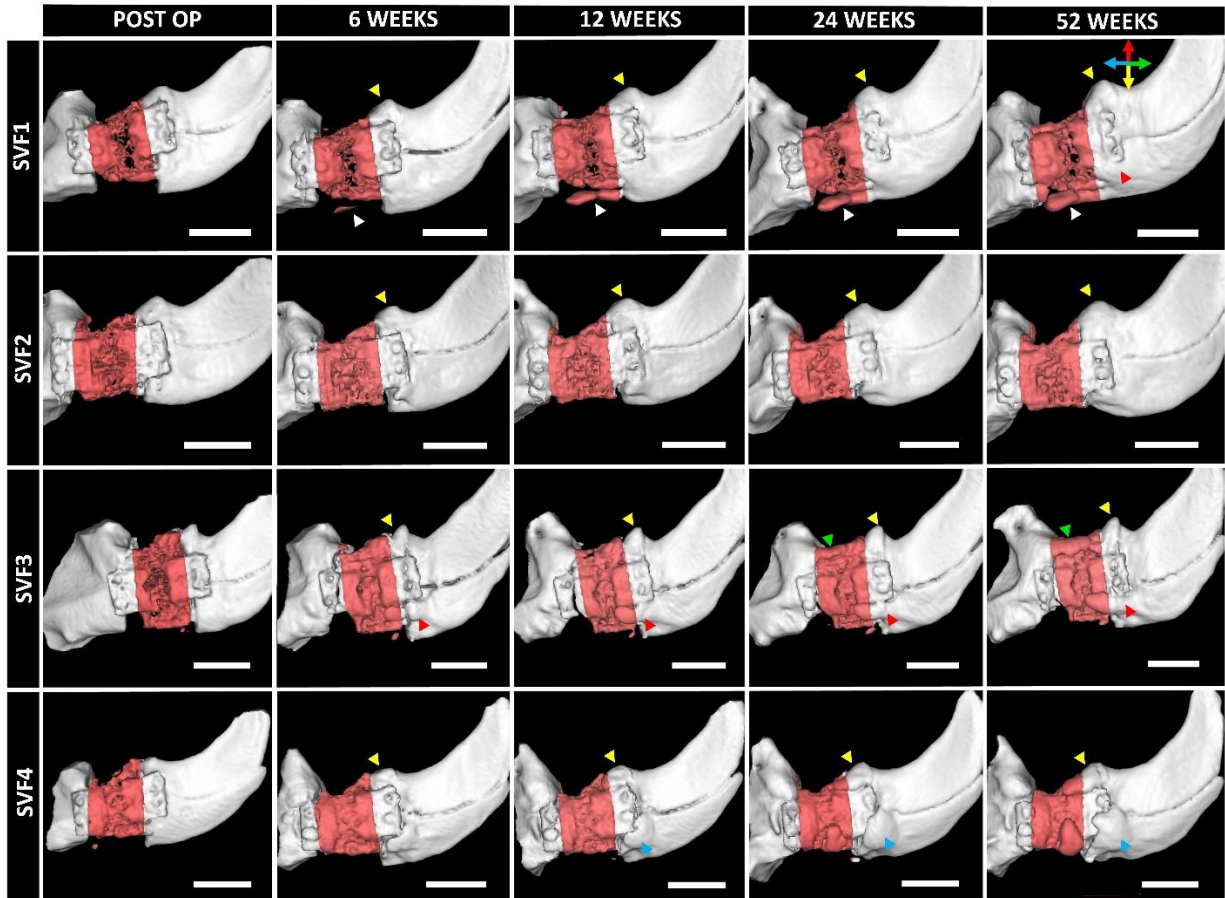


Fig. S6. CT Scans of SVF group (left) defects at different time point. Red color indicates region of interest. Different bone growth patterns have been highlighted: formation of bony islands on the ventral aspect of the defect which fills in the deficit volume post reduction (white arrow heads), bone growing into the defect site in a caudal to rostral fashion (yellow arrow heads), lateral bone growth from the ventro-caudal aspect of the zygoma which brushes past the inferior edge of the fixation tab (red arrow heads) or encapsulates the fixation tab (blue arrow heads), bone growing in a rostral to caudal fashion along the dorsal aspect of the defect (green arrow heads). Sense of orientation is conveyed by colored arrows: dorsal (red), ventral (yellow), rostral (blue) and caudal (green). Sale bar = 2 cm.

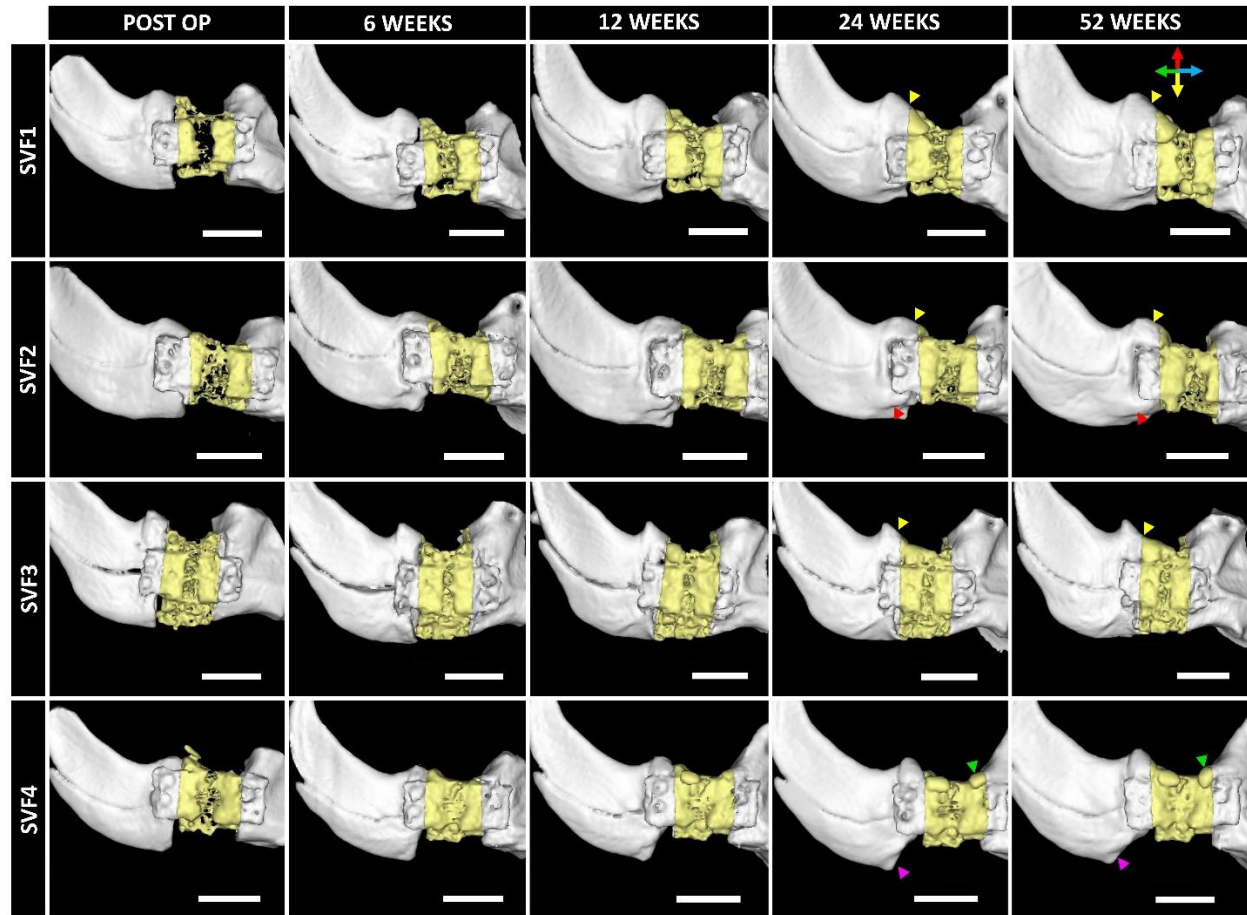


Fig. S7. CT Scans of SVF group (right) defects at different time point. Yellow color indicates region of interest. Different bone growth patterns have been highlighted: bone growing into the defect site in a caudal to rostral fashion (yellow arrow heads), lateral bone growth from the ventro-caudal aspect of the zygoma which brushes past the inferior edge of the fixation tab (red arrow heads), bone growing in a rostral to caudal fashion along the dorsal aspect of the defect (green arrow heads), bone resorption at the ventro-caudal aspect of the defect (magenta arrow heads). Sense of orientation is conveyed by colored arrows: dorsal (red), ventral (yellow), rostral (blue) and caudal (green). Scale bar = 2 cm.

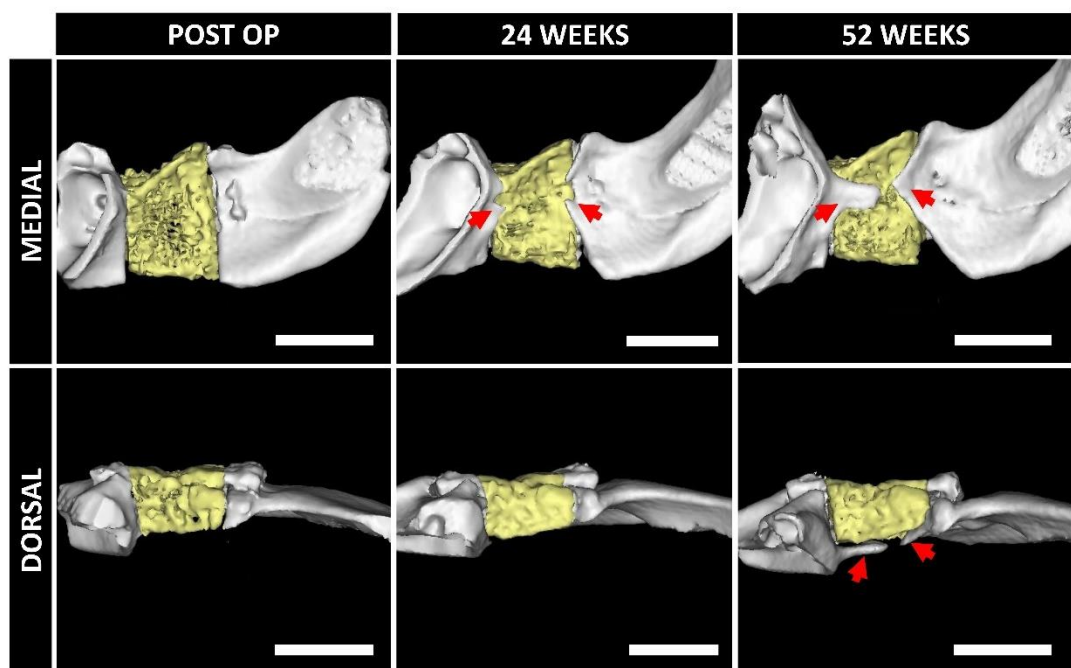


Fig. S8. Growth of bony extensions on the medial aspect of the zygomatic defect. CT scans showing growth of bony extensions (red arrow) on the medial aspect of the zygomatic defect at 24 weeks. These structures started growing from both the rostral and caudal aspect of the defect and became much more prominent at 52 weeks. Scale bar = 2 cm.

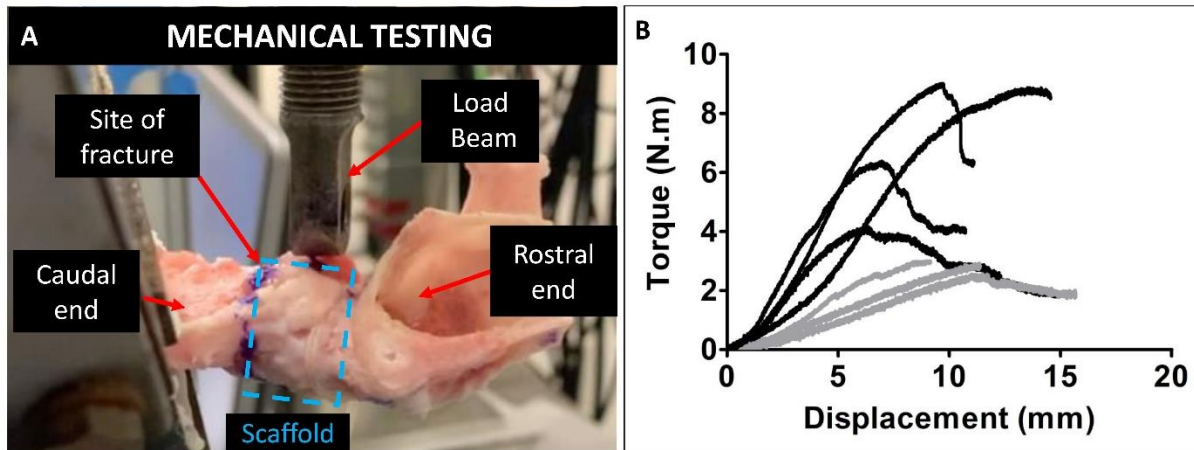


Fig. S9. Mechanical testing of harvested zygomas. (A) Experimental set up for mechanical testing of harvested zygomas identifying the location of scaffold and site of fracture initiation at scaffold-bone (caudal) interface (B) Torque v/s Displacement plots were generated for comparison of fracture torque between AC (grey) and SVF (black) groups.

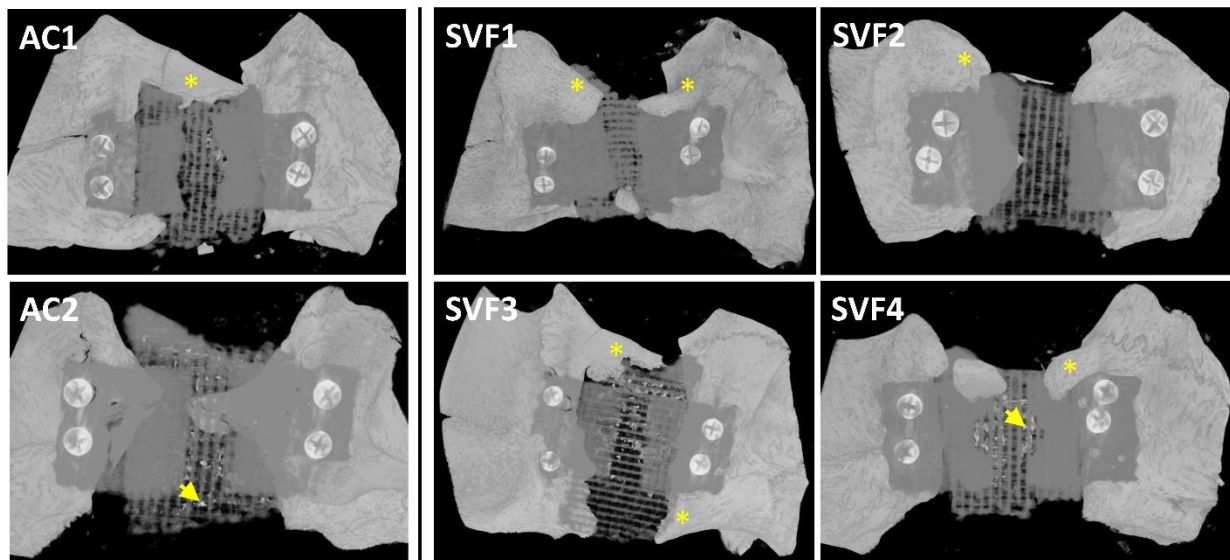


Fig. S10. 3D rendering of micro-CT scans of the harvested zygomas. The differences in radio-density between the scaffolds (dark grey) and the native bone (light grey) make them clearly distinguishable from each other. The growth of dense lamellar bone (yellow asterisk) from the

adjacent bone around the edges of the scaffold can be clearly observed. Dense mineralized nodules formed in the central regions of the scaffold (yellow arrowheads).

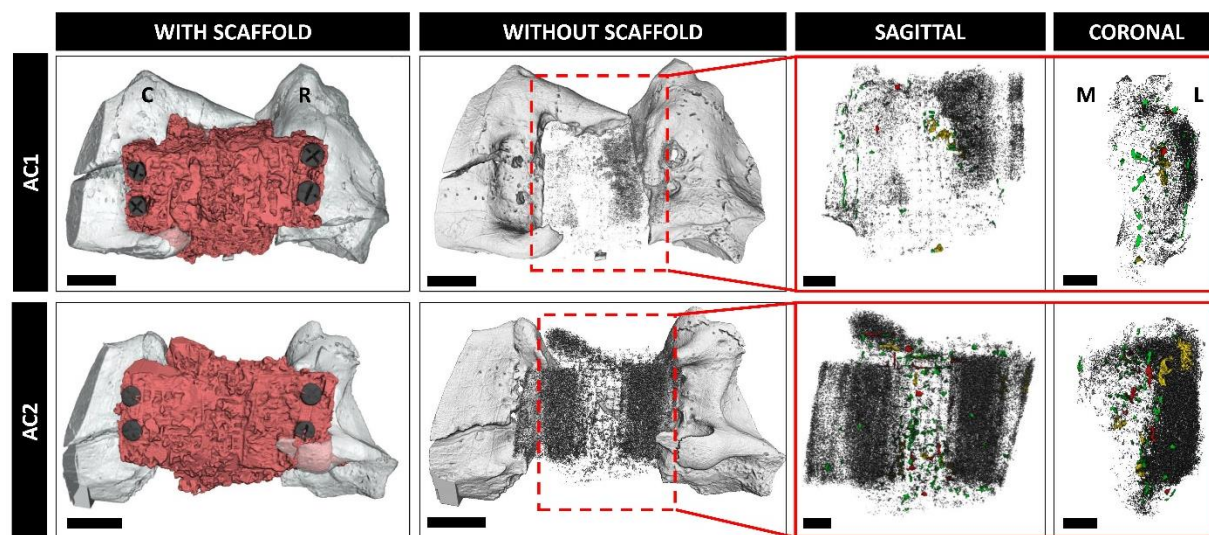


Fig. S11. 3D Rendering of microCT scans of AC group zygomatic specimens. 3D rendering of the microCT scans of the AC zygomas with or without the scaffolds. Spatial and volumetric bone nodules can be visualized within the defect sites. All the bone nodules have been color coded depending on their volume namely - fine ($< 0.0216 \text{ mm}^3$, grey), small ($0.0216 - 0.108 \text{ mm}^3$, red), medium ($0.108 - 0.216 \text{ mm}^3$, green), and large ($> 0.216 \text{ mm}^3$, large). C: Caudal; R:Rostral; M:Medial; L: Lateral. Scale bar = 1 cm (with/without scaffold); 0.5 cm (sagittal, coronal).

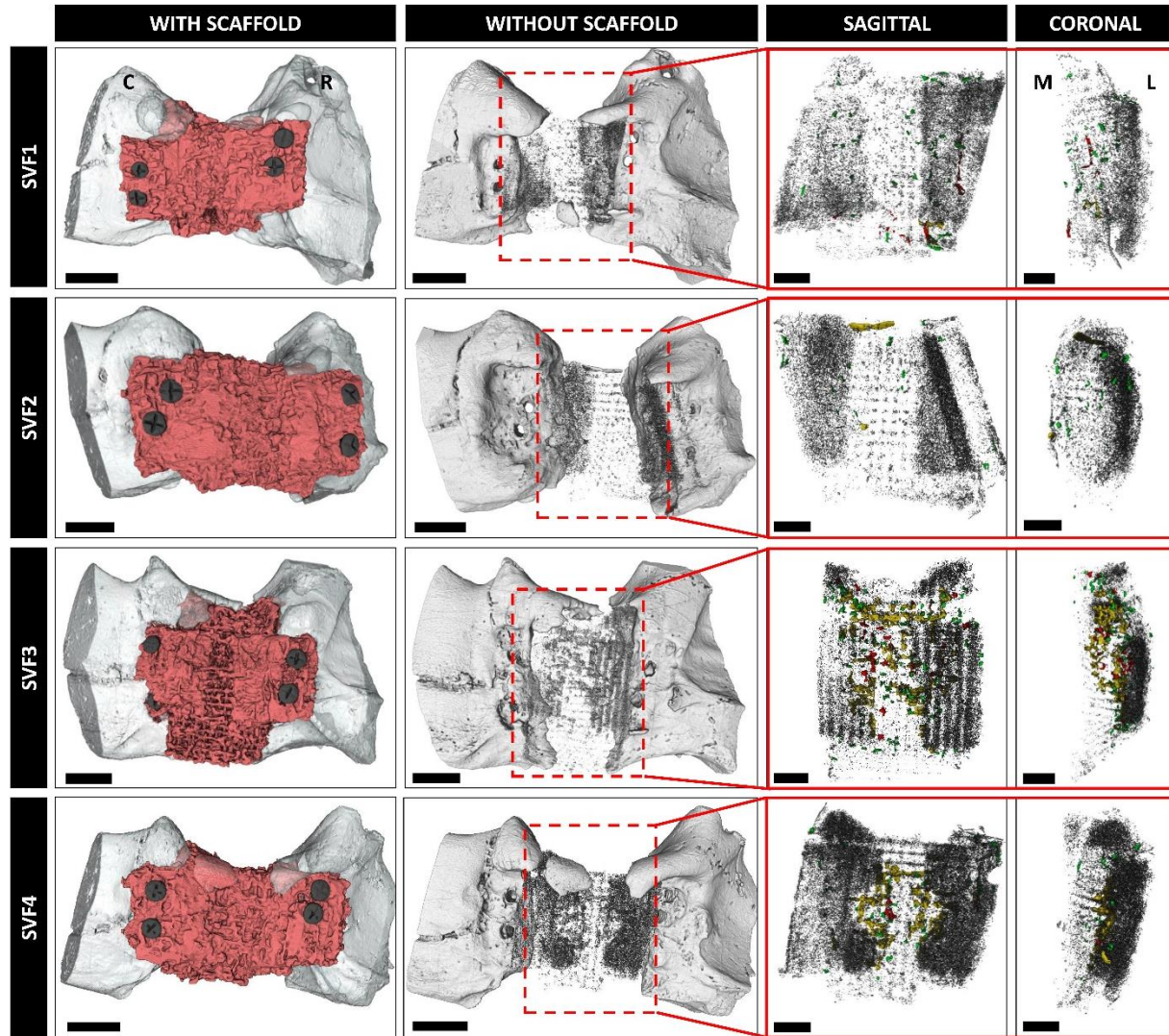


Fig. S12. 3D Rendering of microCT scans of SVF group zygomatic specimens. 3D rendering of the microCT scans of the SVF zygomas with or without the scaffolds. Spatial and volumetric bone nodules can be visualized within the defect sites. All the bone nodules have been color coded depending on their volume namely - small ($< 0.0216 \text{ mm}^3$, grey), small ($0.0216 - 0.108 \text{ mm}^3$, red), medium ($0.108 - 0.216 \text{ mm}^3$, green), and large ($> 0.216 \text{ mm}^3$, yellow). C: Caudal; R:Rostral; M:Medial; L: Lateral. Scale bar = 1 cm (with/without scaffold); 0.5 cm (sagittal, coronal).

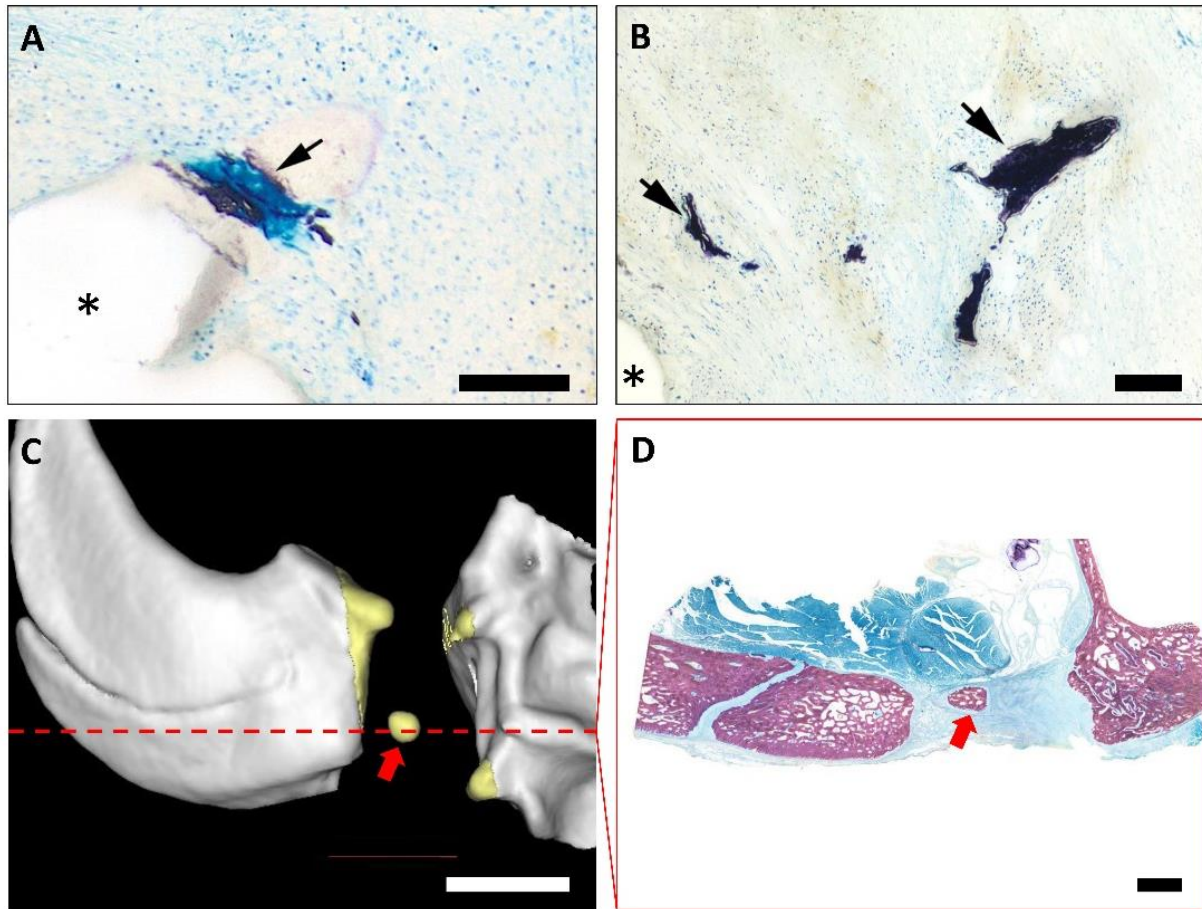


Fig. S13. Woven bone and lamellar bone within the defect site. (A) Osteoid matrix (black arrow) was detected within the pores of the scaffold which was mineralizing at the surface of PCL-DCB strut (asterisk) (B) Woven bone (black arrow) was observed within pores of the scaffold, present at a distance from the PCL-DCB strut (asterisk) within the connective tissue (C) CT rendered image of empty defect (AC2 right) at 52 weeks demonstrating formation of bone (red arrow) within the defect site in the absence of PCL-DCB scaffold (D) Stevenel's Blue (with van Gieson's picrofuchsin counterstain) histological staining confirming that the bone that was formed within the defect site is lamellar bone (red arrow). Scale bar = 100 μ m (A,B); 2 cm (C); 4 mm (D).

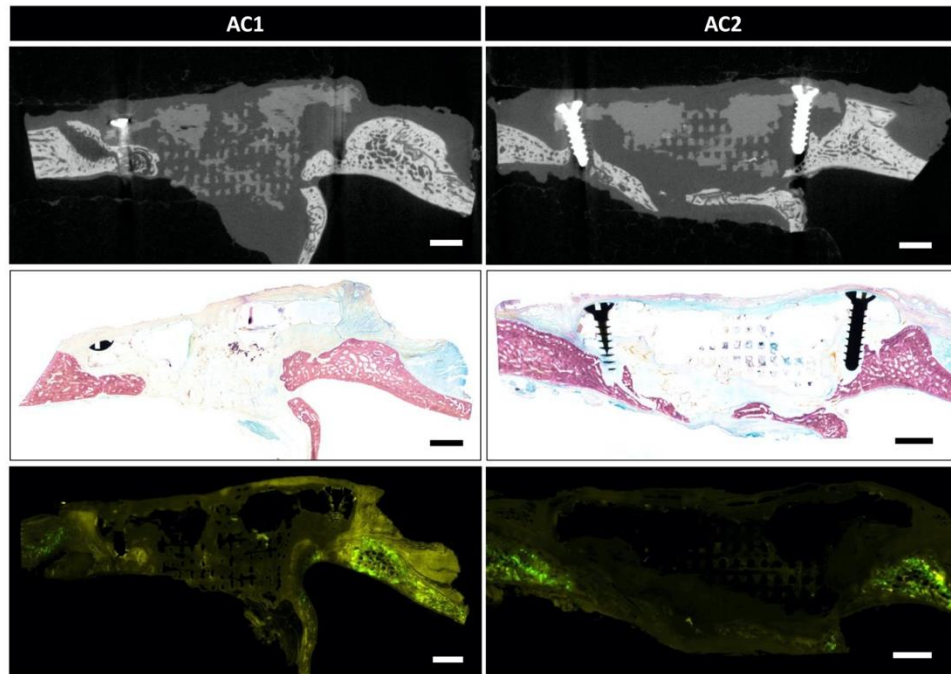


Fig. S14. Radiograph (top), Stevenel's Blue staining (middle), and fluorochrome (bottom) imaging of the harvested zygomatic specimens from the AC group. Scale bar = 4 mm.

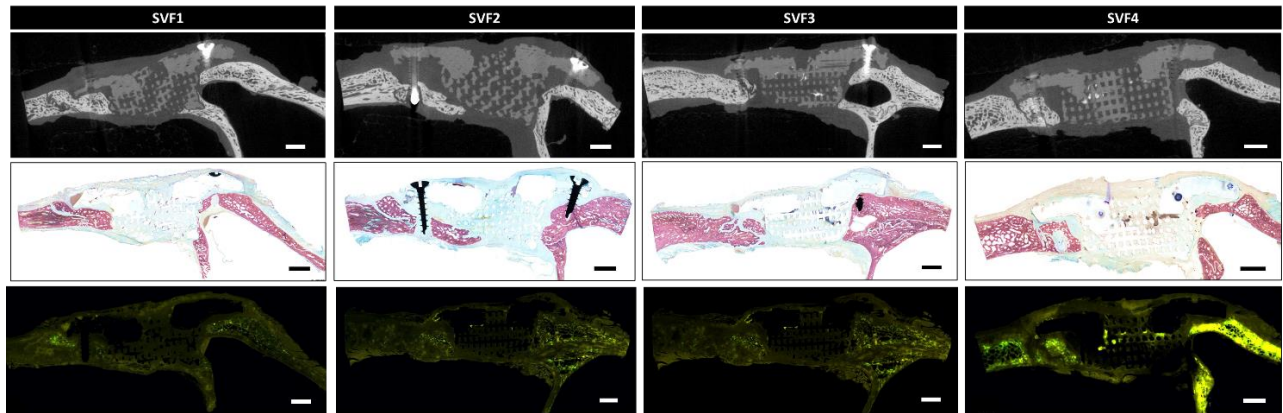


Fig. S15. Radiograph (top), Stevenel's Blue staining (middle), and fluorochrome (bottom) imaging of the harvested zygomatic specimens from the SVF group. Scale bar = 4mm

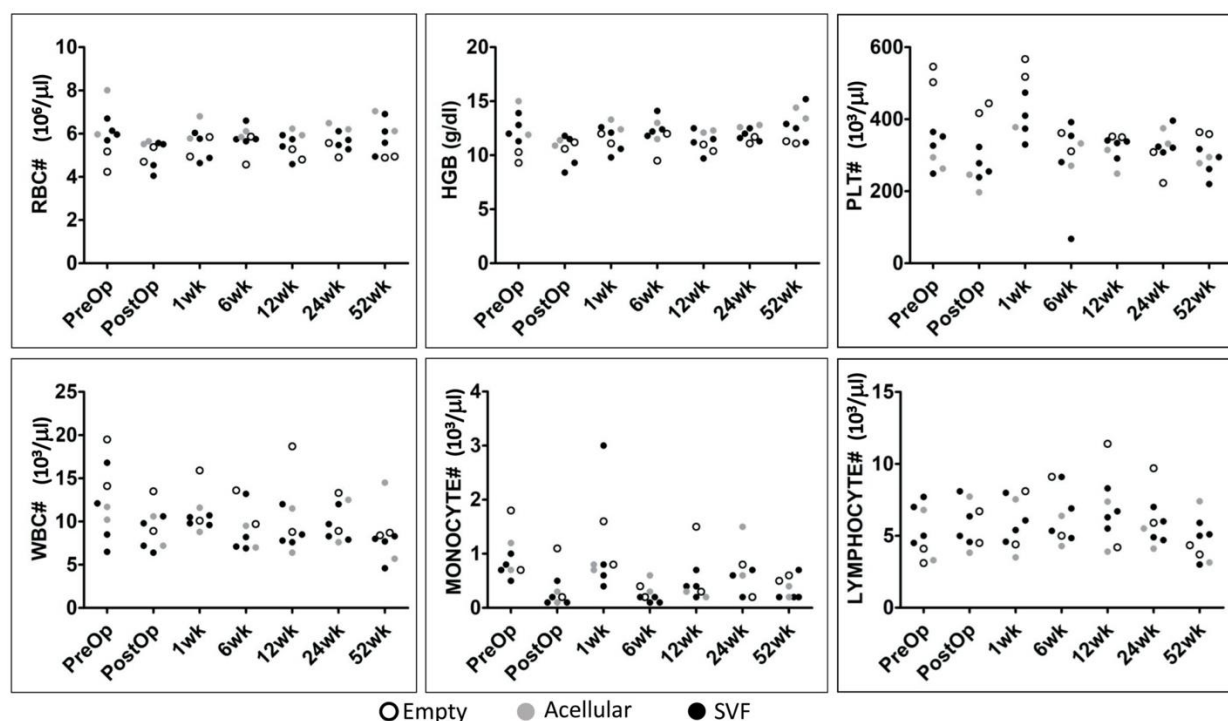


Fig. S16. Hematological indicators suggest absence of systemic immune responses in all groups throughout the post-implantation period. Except for a couple of time points for red blood cells (RBC), hemoglobin (HGB), platelets (PLT), there were no statistical changes in the measures of hematological indicators especially white blood cells (WBC), monocytes or lymphocytes in any of the test groups at any time point compared to the pre-op values. The measured values were also consistent with normal values for mini-pigs (34) at all time points.

Table S1. Experimental Summary. Nomenclature (E = Empty; AC = acellular; SVF = Stromal Vascular Fraction). Note: The left and right defects in E1 were both incomplete (i.e. a bone bridge remained at the inferior border). Hence E1 was omitted from end-point assessments.

Experimental Groups	Post-Operative Evaluations (Time-point in weeks)
• Empty (n=2 pigs) (No scaffold, hydrogel, or cells) • Acellular (n=2pigs) (PCL-DCB scaffold + hydrogel only) • SVF (n=4 pigs) (PCL-DCB scaffold + hydrogel +SVF)	• Quantitative CT (0, 6, 12, 24, 52): All pigs • Complete blood count (0, 6, 12, 24, 52): All pigs • Tetracycline injection (0, 12): Acellular and SVF pigs • Calcein injection (6, 24): Acellular and SVF pigs
	End-Point Assessments • Mechanical Testing AC1 (left), AC2 (left & right), SVF1-4 (left) • MicroCT AC1 & AC2 (right), SVF1-4 (right) • Hard Tissue Histology (Stevenel’s Blue, H&E, Fluorochrome (Tetracycline/Calcein) imaging) E2 (left & right), AC1 & AC2 (right), SVF1-4 (right) • Soft tissue Histology (H&E, CD31) AC1 & AC2 (left), SVF1 & SVF3 (left)

Table S2. SVF Isolation from porcine adipose tissue. Table summarizing amount of adipose tissue harvested, SVF extracted, cell seeding density* of scaffolds and time taken for SVF isolation. (*Note: It was challenging to obtain reliable cell counts on the hemocytometer. The small cell sizes made it difficult to distinguish them from debris).

Group	Age at the time of surgery (Days)	Weight (kg)	Total Fat Mass (g)	Total cells obtained (x10⁶)	Millions cells/gram fat	Seeding density of implants (x10⁶/ml)	Time taken for SVF isolation (mins)
Empty	671	65.8	-				
	668	71.1	-				
AC	571	47	-				
	546	47.4	-				
SVF	572	60.6	32.6	57.63	1.77	5.19	90
	571	56.2	37.6	39.85	1.06	3.52	90
	569	52.6	33.5	62.94	1.88	5.66	105
	546	56	29.9	51.35	1.72	4.81	90

Movie S1. Interconnected isotropic pores of the PCL-DCB scaffold. All PCL-DCB scaffolds were fabricated with interconnected, isotropic orthogonal pores of width 800 μm which can be visualized using cone beam CT scans of the 3D printed scaffolds.

Movie S2. Mechanical testing of harvested zygoma specimens. Cantilever beam bending test was performed on the harvested zygomas to assess osseointegration at the caudal end of the scaffold.

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