Published in final edited form as:

Plast Reconstr Surg. 2020 February ; 145(2): 337e–347e. doi:10.1097/PRS.00000000000006483.

Bone Tissue Engineering in the Growing Calvaria Using Dipyridamole-Coated, Three-Dimensionally-Printed Bioceramic Scaffolds: Construct Optimization and Effects on Cranial Suture Patency

Samantha G. Maliha, B.A., Christopher D. Lopez, B.A., Paulo G. Coelho, D.D.S., Ph.D., Lukasz Witek, Ph.D., Madison Cox, B.A., Alan Meskin, B.A., Sejndi Rusi, B.A., Andrea Torroni, M.D., Ph.D., Bruce N. Cronstein, M.D., Roberto L. Flores, M.D.

Hansjörg Wyss Department of Plastic Surgery, New York University Langone Health; New York University School of Medicine; the Icahn School of Medicine at Mount Sinai; the Department of Biomaterials, New York University College of Dentistry; and the Division of Translational Medicine, New York University School of Medicine.

Abstract

Background: Three-dimensionally-printed bioceramic scaffolds composed of β -tricalcium phosphate delivering the osteogenic agent dipyridamole can heal critically sized calvarial defects in skeletally mature translational models. However, this construct has yet to be applied to growing craniofacial models. In this study, the authors implanted three-dimensionally-printed bioceramic/dipyridamole scaffolds in a growing calvaria animal model and evaluated bone growth as a function of geometric scaffold design and dipyridamole concentration. Potential adverse effects on the growing suture were also evaluated.

Methods: Bilateral calvarial defects (10 mm) were created in 5-week-old (approximately 1.1 kg) New Zealand White rabbits ($n = 16$ analyzed). Three-dimensionally-printed bioceramic scaffolds were constructed in quadrant form composed of varying pore dimensions (220, 330, and 500 μm). Each scaffold was coated with collagen and soaked in varying concentrations of dipyridamole (100, 1000, and 10,000 μM). Controls consisted of empty defects. Animals were killed 8 weeks postoperatively. Calvariae were analyzed using micro-computed tomography, three-dimensional reconstruction, and nondecalcified histologic sectioning.

Results: Scaffold-induced bone growth was statistically greater than bone growth in empty defects ($p = 0.02$). Large scaffold pores, 500 μm , coated in 1000 μM dipyridamole yielded the most bone growth and lowest degree of scaffold presence within the defect. Histology showed vascularized woven and lamellar bone along with initial formation of vascular canals within the scaffold lattice. Micro-computed tomographic and histologic analysis revealed patent calvarial sutures without evidence of ectopic bone formation across all dipyridamole concentrations.

Roberto L. Flores, M.D., Hansjörg Wyss Department of Plastic Surgery, New York University Langone Health, 307 East 33rd Street, New York, N.Y. 10016, roberto.flores2@nyumc.org.

Disclosure: Dr. Cronstein has patented the use of dipyridamole-coated three-dimensionally printed biodegradable scaffolds for the treatment of critical bone defects. Dr. Coelho has patented the three-dimensional printing device configuration for bone regeneration. The remaining authors have no financial interests to report.

Conclusion: The authors present an effective pediatric bone tissue-engineering scaffold design and dipyridamole concentration that is effective in augmentation of calvarial bone generation while preserving cranial suture patency.

Pediatric cranial reconstruction poses unique challenges, such as identifying replacements for bony defects that can remodel with the growing skeleton. Autologous bone graft from the skull, iliac crest, and rib¹ is limited by fixed volume and shape, donor-site morbidity, risk of infection, and resorption.^{2,3} In addition, after 2 years of age, endogenous calvarial healing is attenuated because of a decline in dura-mediated osteogenesis.⁴ This decline makes spontaneous healing unlikely, and the limitations of autogenous bone sources have been the impetus for the pursuit of tissue-engineering-based therapeutic alternatives that are also compatible with skeletal growth.

Biomaterials such as bioactive ceramics have demonstrated bone regenerative capacity for decades.^{5–7} Deployment of scaffolds made of these bioactive ceramics has been refined with the advent of modalities such as three-dimensional printing.^{8–11} Such manufacturing methods have allowed for stepwise geometric design of scaffold porosity known to facilitate bone healing.¹² These bioactive ceramic scaffold design principles have been successfully applied in skeletally mature, critically sized defects of the rabbit calvaria and mandible.^{13,14} Despite promising results, comparison of differing bioceramic scaffold pore sizes on osteogenesis is pending. Previous work has shown that hydroxyapatite scaffold interstices measured at $500 \times 500 \mu\text{m}$ presented higher bone healing capacity when compared to six other pore dimensions.¹³ Hydroxyapatite, however, has lower resorption/degradation kinetics compared with other calcium-phosphates such as β -tricalcium phosphate.¹⁵ β -Tricalcium phosphate three-dimensional lattice bone tissue-engineering constructs have shown favorable resorption and osteoconduction when designed with a lattice porosity of $330 \times 330 \mu\text{m}$.¹⁶ In addition, studies have demonstrated that bone tissue-engineering constructs may carry osteogenic agents to further enhance bone formation.^{17–25} However, the deployment of these agents has never been investigated along with scaffold pore size dimensions. Given the well-established safety profile and regenerative capacity of these constructs, there is impetus to compare scaffold pores together with osteogenic agents to further enhance bone regeneration.

The most studied osteogenic agent for calvarial regeneration is recombinant human bone morphogenetic protein-2,^{26–33} for which robust bone regeneration of calvarial defects in large-animal studies have been reported for decades.^{26–30} However, the clinical deployment of recombinant human bone morphogenetic protein-2 in pediatric patients remains contraindicated because of adverse side effects that have been reported.³⁴ Studies have reported its risks to premature suture fusion in the growing skeleton, osteolysis, malignant degeneration, and ectopic bone formation, calling into question the safety of recombinant human bone morphogenetic protein-2 in the growing craniofacial skeleton.^{4,35–37} These adverse sequelae have driven the investigation of alternative pediatric osteogenic agents, such as the recently described adenosine receptor pathway.

Dipyridamole is an indirect adenosine A2A receptor agonist with recently described osteogenic potential^{38–40} through increase of extracellular adenosine concentrations by

binding to the Ent1 transporter, thereby inhibiting purine entry into the cell.^{41,42} It is clinically known under the commercial name Persantine (Boehringer Ingelheim International GmbH, Ingelheim, Germany) as an antithrombotic agent and vasodilator with a well-established safety profile in both adult and pediatric patients.^{43–45} A2A ligation has been reported to induce bone growth–promoting activities such as increased osteoblast differentiation, decreased osteoclast activity, and reduction of regional inflammatory response.^{41,46,47} Dipyridamole has been previously used in a murine model to demonstrate bone regeneration within cranial defects consistent with levels previously described by recombinant human bone morphogenetic protein-2 but without evidence of bone overgrowth.³¹ These data are consistent with reports of dipyridamole’s safe and effective bone regeneration at larger, more translational calvarial models as well,⁴⁸ prompting its application in skeletally immature translational models.

Despite successful implementation of this construct in the craniofacial skeleton in adult models, the ideal pediatric bone tissue engineering construct capable of safely regenerating functional bone has yet to be realized. The purpose of this study is to explore the effect of varying three-dimensionally–printed bioceramic scaffold lattice dimensions and dipyridamole concentrations on calvarial bone formation and suture patency in a skeletally immature translational animal model.

MATERIALS AND METHODS

Scaffold Design

A custom-built, direct-write, three-dimensional printer (3D Inks LLC, Tulsa, Okla.) was used to fabricate a quadrant three-dimensionally–printed bioactive ceramic scaffold composed of 100 percent β -tricalcium phosphate (Fig. 1).^{13,15,48} Detailed information regarding ink production and extrusion methods is reported elsewhere.^{16,49,50} The scaffold cap was first created by extruding two concentric circles of progressively decreasing diameter. Subsequently, single layers of scaffold were created by extruding struts of β -tricalcium phosphate material parallel to one another in a raster pattern of preprogrammed distances. The struts of each consecutive layer were perpendicular to the previous. Pore sizes within each quadrant were categorized as small (strut thickness, $200 \pm 8 \mu\text{m}$; scaffold fraction, 0.682; pore fraction, 0.318; pore size, $220 \mu\text{m}$), medium (strut thickness, $200 \pm 8 \mu\text{m}$; scaffold fraction, 0.565; pore fraction, 0.435; pore size, $330 \mu\text{m}$), or large (strut thickness, $200 \pm 8 \mu\text{m}$; scaffold fraction, 0.441; pore fraction, 0.559; pore size, $500 \mu\text{m}$). Each scaffold core region contained one quadrant composed of small pores, one quadrant composed of large pores, and two directly opposing quadrants composed of medium pores (Fig. 1). The porous core component of the scaffold alone measured 11 mm in diameter and 1.5 mm in height, whereas the porous region plus solid cap overlain atop the core measured 13 mm in diameter. Caps, used to prevent downward growth of the soft tissue from the scalp and promote horizontal growth of bone through the defect, were $500 \mu\text{m}$ thick. The lateral and inferior aspect of the scaffold are porous and are exposed to the cellular environment of the calvaria and dura, respectively.

Before implantation, scaffolds were sterilized and immersed in 2% type I bovine collagen solution (Corning, Inc., Corning, N.Y.), incubated at 37°C for 20 minutes, and subsequently

cross-linked by irradiation ($120 \mu\text{W cm}^2$ for 15 minutes). Scaffolds (experimental group) were coated in varying concentrations of dipyridamole ($n = 9$ in $100 \mu\text{M}$, $n = 9$ in $1000 \mu\text{M}$, and $n = 9$ in $10,000 \mu\text{M}$). These solutions were created by a 1:100, 1:1000, and 1:10,000 dilution, respectively, of 5.04 mg of dipyridamole in 1 ml of dimethylsulfoxide with $1\times$ phosphate-buffered saline, a choice guided by our preliminary work demonstrating the ability of this immersion concentration to enhance bone growth in an in vivo setting.^{16,31} Five scaffolds were coated only in the previously described collagen solution (positive control group). Four defects were left unfilled, serving as negative controls.

Surgical Intervention

After approval of the institutional animal care and use committee, 18 skeletally immature 5-week-old New Zealand White rabbits were anesthetized with Telazol (Zoetis, Parsippany, N.J.) and isoflurane (1.5 to 3%) in oxygen/nitrous oxide (50/50). Following aseptic preparation of the surgical site over the calvaria, dissection was performed down to the periosteum (Fig. 2). A trephine burr (10-mm inner diameter, 11-mm outer diameter) (Hu-Friedy Manufacturing Co., Chicago, Ill.) was used to create full-thickness bilateral calvarial defects approximately 2 mm posterior and lateral to the coronal suture and sagittal sutures, respectively (Fig. 2). Scaffolds were deposited into these defects such that caps were flush with the calvaria and the porous core presenting the distinct lattice geometries was in contact with the dura. The scalp was closed with running 3-0 silk suture. Food and water were offered to the animals in the postoperative period ad libitum. All animals were killed humanely by means of anesthesia overdose 8 weeks postoperatively. Two animals died before sacrifice because of infection and intraoperative airway collapse.

Micro-Computed Tomographic Analysis

After calvarial harvest, samples were cleanly cut down by a band saw. They were then stored in vials containing 70% ethanol and subjected to micro-computed tomography ($\mu\text{CT } 40$; SCANCO Medical, Bassersdorf, Switzerland). Scans were converted to Digital Imaging and Communications in Medicine files for three-dimensional reconstruction. Samples were quantitatively analyzed by means of three-dimensional analysis using Amira software (Thermo Fisher Scientific, Waltham, Mass.). Each scaffold was reconstructed and cut into quadrants according to pore size, leaving four quadrants for analysis: a small pore section, a large pore section, and two medium pore sections. Bone volume generation and scaffold volume were measured using Amira (Fig. 3).

Histologic Preparation

After completion of micro-computed tomography, the samples were dehydrated in increasing concentrations of ethanol (70, 90, and 100%) in preparation for histologic analysis. Samples were then kept in methyl salicylate for 48 hours before immersion in methylmethacrylate I for 24 hours and methylmethacrylate II for 24 hours. Samples were finally embedded in methylmethacrylate and kept in ultraviolet light for polymerization. Nondecalfied histologic sections were prepared for each defect according to standardized methodology.^{13,51} A Buehler IsoMet saw (Buehler, Lake Bluff, Ill.) was used to obtain approximately 250- μm -thick histologic sections. Ten cuts were made through the defect extensions per sample. These were then polished to a 1200-grit finish. A 1- μm polishing

compound was used to remove residual scratches and to obtain a final thickness of approximately 100 μm . A Stevenel blue and van Gieson picrofuchsin differential tissue staining protocol was used for staining slides. On using Stevenel blue and van Gieson picrofuchsin stains, soft tissue is stained blue, mineralized tissue is stained red, and scaffold stains black. The stained sections were examined using a histology slide scanning system (Aperio Technologies, Vista, Calif.). All samples were qualitatively analyzed for bone growth pattern and for suture morphology.

Statistical Analysis

The number of rabbits ($n = 18$) was determined through statistical power analysis yielding greater than 80 percent power to detect an increase of bone regeneration of 25 percent (relative to control) with an SD of 15 percent at an alpha of 0.05. All micro-computed tomographic data (bone, scaffold, soft tissue) were assessed for normality using the Shapiro-Wilk test ($p > 0.05$) before further analysis. A generalized linear mixed model (IBM SPSS Version 23; IBM Corp., Armonk, N.Y.) was used for quantitative analysis with fixed factor variables of pore size and dipyrindamole concentration. The generalized linear mixed model takes into consideration random effects and fixed effects on experimental subjects. It was used in an effort to explore treatment effects of experimental groups such as pore size and dipyrindamole concentration on dependent variables such as amount of bone, scaffold, and soft tissue remaining. All data are presented as a mean value with corresponding 95 percent confidence interval.

RESULTS

Micro-Computed Tomography

Amira software reconstruction was used to quantify and visualize new bone formation and scaffold presence (Fig. 3). Negative controls remained patent, with bone growth forming only marginally (Fig. 3, *below*). Mean bone growth within empty defects was 12.1 ± 9.7 percent (estimated mean $\pm$ 95 percent CI). When analyzed as a function of dipyrindamole-treated scaffolds, the amount of growth within these empty defects was statistically less than that induced by scaffolds coated in 100 μM dipyrindamole (26.5 ± 3.7 percent; $p = 0.008$), 1000 μM dipyrindamole (27.9 ± 4.1 percent; $p = 0.004$), and 10,000 μM dipyrindamole (24.1 ± 3.5 percent; $p = 0.025$), resulting in approximately 120, 130, and 100 percent increase in bone growth when compared to negative controls, respectively.

When comparing scaffold-induced bone growth (defined as space within the defect occupied by bone) as a function of dipyrindamole dose irrespective of pore size (Fig. 4, *above, left*), there was significantly higher mean bone occupancy in scaffolds coated in 1000 μM dipyrindamole compared to collagen-coated scaffolds ($p = 0.023$). Borderline statistical significance was observed between the 100 μM relative to collagen-coated scaffolds ($p = 0.054$). No significant differences were observed for scaffold volume presence, and significantly higher amounts of empty space/soft tissue were observed for collagen-coated and 1000 μM dipyrindamole relative to the other groups.

When comparing bone growth within various pore sizes irrespective of dipyrindamole concentration, the highest mean bone regeneration was found among small pores when compared to medium and large pores (not significant at $p > 0.10$) (Fig. 4, *above, right*). Large-pore quadrants coated in 1000 μM dipyrindamole were shown to have the lowest mean volume occupied by scaffold before implantation and at the time of sacrifice at 8 weeks after implantation when compared to medium pore, which presented significantly smaller values relative to small pore quadrants (all $p < 0.01$) (Fig. 4, *above right*). A significantly higher degree of empty space/soft-tissue volume was observed for the medium and large pore sizes relative to the small pore size ($p < 0.001$) (Fig. 4, *above right*).

Analysis of dipyrindamole immersion concentration and pore size taken in conjunction revealed that large pores coated in 1000 μM dipyrindamole yielded the most overall mean bone growth when compared to collagen groups. Statistical summary data for all groups' bone formation are presented in Figure 4, *below*. All radiographic images across all scaffold porosity designs and dipyrindamole concentrations demonstrated patent calvarial sutures without evidence for ectopic bone formation (Fig. 3).

Histology

Nondecalcified histologic sections revealed no signs of inflammation or ectopic bone formation in any group (Fig. 5). Independent of scaffold treatment, intramembranous-like healing was observed, with highly cellular and vascularized regions of immature woven bone and mature lamellar reorganization (Fig. 5). Haversian-like sites were seen throughout new bone formations, and osteoconduction occurred throughout all scaffold porous dimensions from defect margins toward the center of critical-sized defects. All sutures were patent and undisturbed across all dipyrindamole-treated and control groups at the time of sacrifice (Fig. 6).

DISCUSSION

Although autogenous bone grafting is a common first-line treatment of growing cranial defects, limitations such as increased operative time, donor-site morbidity, graft resorption, and limited bone stock persist.⁵² These limitations are magnified in children who require radiation treatment after oncologic resection.⁵³ Biomaterials such as titanium, methylmethacrylate, polyetheretherketone, and porous polyethylene also have associated risks including exposure, infection, and potential need for replacement as the calvaria grows.⁵⁴ Although advances have been made in regenerative medicine, the ideal pediatric bone tissue-engineering construct has yet to be realized.

Three-dimensionally-printed bioceramic scaffolds have been investigated for bone regenerative capacity in translational models, but this is the first investigation of three-dimensionally-printed bioceramic scaffolds in a growing calvarial model that also assesses the effect of scaffold porous dimensions and local dipyrindamole effect. After 8 weeks, the greatest degree of bone formation and the lowest scaffold volumetric presence were recorded in three-dimensionally-printed bioceramic scaffolds of large porosity treated with 1000 μM of dipyrindamole. Cranial sutures remained patent at therapeutic (100 μM /1000 μM) and supratherapeutic (10,000 μM) doses of dipyrindamole even with the scaffold adjacent

(approximately 2 mm) to the growing cranial suture. These three findings are novel to the field of pediatric bone tissue engineering: significantly greater scaffold-induced osteogenesis compared to negative control; identification of an optimized dipyridamole dose (1000 μ M) for pediatric bone induction; and safety of dipyridamole to the growing sutures even at 10 times greater than the effective dose. The authors note that it is possible that suture fusion may be observed at later time periods of osteogenesis, and this is a subject of future study.

These data are consistent with a growing body of literature demonstrating that dipyridamole does not result in excessive bone formation and premature fusion of sutures commonly associated with osteogenic agents such as recombinant human bone morphogenetic protein-2.⁵⁵ Use of recombinant human bone morphogenetic protein-2 in the pediatric patient has been regarded with caution by a number of clinicians. Known to be a natural mediator in neural development and fracture healing,^{56,57} multiple studies attest to the utility of bone morphogenetic protein-2 in spinal and long bone fusion and alveolar reconstruction in adult patients.^{58–60} However, premature fusion of the cranial sutures associated with the use of recombinant human bone morphogenetic protein-2 has been reported in children³⁵ and pediatric rabbit studies on calvarial healing.⁵ The mechanism for this premature fusion of the cranial sutures is likely because of loss of the suture stem cell population and obliteration of Shh-responsive cells in the head of the mesoderm.⁶¹ Furthermore, recombinant human bone morphogenetic protein-2 has been associated with malignant degeneration and the development of vascular malformations.^{62,63} Therefore, despite studies describing its use in other areas of the growing craniofacial skeleton,^{62–64} bone morphogenetic protein-2 may not be the optimal osteogenic agent for use in the pediatric patient.

Irrespective of dipyridamole dosage, the choice of bioactive ceramic used and its varying porosities was critical for generating large quantities of vascularized bone. Many scaffold materials that have been investigated include products made from natural polymers, inorganic materials, synthetic materials, and composites.⁶¹ Consistent with our histologic observations, a previous investigation of scaffolds composed of hydroxyapatite demonstrated that mineralization patterns of newly formed bone at a cellular level arise from osteoblasts interacting with the scaffold as they would with preexisting bone matrix. Subsequent studies that explored the effects of scaffold porosity on osteogenesis found a trend toward increased bone growth in the smaller pore (220 μ m) dimensions.¹³ However, when assessing for scaffold resorption and degradation, β -tricalcium phosphate scaffolds demonstrated far more rapid resorption/degradation and also demonstrated robust osteoconduction.⁶⁵

Previous studies have demonstrated successful and enhanced bone generation using porous scaffolds composed of β -tricalcium phosphate in combination with the osteogenic agent dipyridamole in several adult models.^{16,49,65,66} However, the ideal pore size for osteogenesis in the growing craniofacial skeleton has yet to be defined. When assessing how a scaffold composed of varying pore sizes (small, medium, and large) and varying dipyridamole concentration affects osteogenesis in a growing animal model, we observed no detrimental effects at any pore size paired with any dipyridamole dosage. The lack of statistically significant differences between pore sizes suggests that all geometric dimensions facilitate osteoconduction to such a robust degree that dimensional considerations are not as critical in

skeletally immature models prone to large degrees of innate healing up to 8 weeks in vivo. Therefore, although a large-pore-size scaffold combined with 1000 μ M dipyridamole was found to be the optimal construct design, the pore size may have limited effects on osteogenesis.

At the 8-week time point, computed tomographic and histologic results showed full defect bridging throughout the scaffold, where bone represented an average of 28 percent volume, and scaffold fractions were approximately 32, 26, and 21 percent (small, medium, and large pores, respectively) of the total defect volume. Thus, the level of defect filling by a composite of bone and scaffold is on average close to 50 percent, uniformly distributed throughout the defect. Alternatively, controls presented bone formation limited to the defect periphery, an unfavorable clinical scenario. Full defect bridging through our tissue-engineering approach provides clinically desirable outcomes when the objective is to rapidly close a large cranial defect with a composite of newly formed bone and a degradable scaffold that has recently shown the potential for continuous degradation and bone formation over the course of 24 weeks.⁶⁷ Given that the present study investigated a relatively early time point, we are currently working on long-term groups to determine the dynamics of the bone/scaffold interaction.

Although this study demonstrates that bone conduction was greater in defects containing a scaffold when compared to empty defects, the use of only one time point limited the present study's ability to distinguish the temporal bone conduction properties of each quadrant. Finally, because of multiple variables being tested at once (dipyridamole concentration and pore size), there is a possibility for confounding effects on our data. In addition, the degree of bone formation at the 8-week time point is not sufficient to completely restore the cranial defect. Results may be optimized in the future by including longer term analysis of the described bone tissue-engineering construct, fewer experimental variables, and a comparison of bony healing to autogenous bone graft.

CONCLUSIONS

We present a three-dimensionally printed bioactive ceramic scaffold design and an optimized dipyridamole concentration that effectively generates bone in a growing calvarial model and preserves cranial suture patency without ectopic bone formation.

ACKNOWLEDGMENT

The investigation was completed in conjunction with an ongoing institutional animal care and use committee-approved grant (NIH R21/R33 HD090664-01), "Use of 3D Printing for Creation of Implantable Pediatric Devices."

REFERENCES

1. Elsalanty ME, Genecov DG. Bone grafts in craniofacial surgery. *Craniofacial Trauma Reconstr.* 2009;2:125–134. [PubMed: 22110806]
2. Bowers CA, Riva-Cambrin J, Hertzler DA II, Walker ML. Risk factors and rates of bone flap resorption in pediatric patients after decompressive craniectomy for traumatic brain injury. *J Neurosurg Pediatr.* 2013;11:526–532. [PubMed: 23473303]

3. Yadla S, Campbell PG, Chitale R, Maltenfort MG, Jabbour P, Sharan AD. Effect of early surgery, material, and method of flap preservation on cranioplasty infections: A systematic review. *Neurosurgery* 2011;68:1124–1129; discussion 1130. [PubMed: 21242830]
4. Kinsella CR Jr, Cray JJ, Durham EL, et al. Recombinant human bone morphogenetic protein-2-induced craniosynostosis and growth restriction in the immature skeleton. *Plast Reconstr Surg*. 2011;127:1173–1181. [PubMed: 21364420]
5. Gao C, Deng Y, Feng P, et al. Current progress in bioactive ceramic scaffolds for bone repair and regeneration. *Int J Mol Sci*. 2014;15:4714–4732. [PubMed: 24646912]
6. Bairo F, Novajra G, Vitale-Brovarone C. Bioceramics and scaffolds: A winning combination for tissue engineering. *Front Bioeng Biotechnol*. 2015;3:202. [PubMed: 26734605]
7. Lange TA, Zerwekh JE, Peek RD, Mooney V, Harrison BH. Granular tricalcium phosphate in large cancellous defects. *Ann Clin Lab Sci*. 1986;16:467–472. [PubMed: 3541772]
8. Warnke PH, Seitz H, Warnke F, et al. Ceramic scaffolds produced by computer-assisted 3D printing and sintering: Characterization and biocompatibility investigations. *J Biomed Mater Res B Appl Biomater*. 2010;93:212–217. [PubMed: 20091914]
9. Rath SN, Strobel LA, Arkudas A, et al. Osteoinduction and survival of osteoblasts and bone-marrow stromal cells in 3D biphasic calcium phosphate scaffolds under static and dynamic culture conditions. *J Cell Mol Med*. 2012;16:2350–2361. [PubMed: 22304383]
10. Inzana JA, Olvera D, Fuller SM, et al. 3D printing of composite calcium phosphate and collagen scaffolds for bone regeneration. *Biomaterials* 2014;35:4026–4034. [PubMed: 24529628]
11. Kim HD, Amirthalangam S, Kim SL, Lee SS, Rangasamy J, Hwang NS. Biomimetic materials and fabrication approaches for bone tissue engineering. *Adv Healthc Mater*. 2017;6:1700612.
12. Coelho PG, Jimbo R. Osseointegration of metallic devices: Current trends based on implant hardware design. *Arch Biochem Biophys*. 2014;561:99–108. [PubMed: 25010447]
13. Simon JL, Michna S, Lewis JA, et al. In vivo bone response to 3D periodic hydroxyapatite scaffolds assembled by direct ink writing. *J Biomed Mater Res A* 2007;83:747–758. [PubMed: 17559109]
14. Lopez CD, Diaz-Siso JR, Witek L, et al. Three dimensionally printed bioactive ceramic scaffold osseointegration across critical-sized mandibular defects. *J Surg Res*. 2018;223:115–122. [PubMed: 29433862]
15. Witek L, Smay J, Silva NR, Guda T, Ong JL, Coelho PG. Sintering effects on chemical and physical properties of bioactive ceramics. *J Adv Ceram*. 2013;2:274–284.
16. Bekisz JM, Flores RL, Witek L, et al. Dipyridamole enhances osteogenesis of three-dimensionally printed bioactive ceramic scaffolds in calvarial defects. *J Craniomaxillofac Surg*. 2018;46:237–244. [PubMed: 29292126]
17. Chen X, Zhao Y, Geng S, Miron RJ. In vivo experimental study on bone regeneration in critical bone defects using PIB nanogels/boron-containing mesoporous bioactive glass composite scaffold. *Int J Nanomedicine* 2015;10:839–846. [PubMed: 25653525]
18. Zhang J, Zhao S, Zhu Y, Huang Y, Zhu M, Tao C. Three-dimensional printing of strontium-containing mesoporous bioactive glass scaffolds for bone regeneration. *Acta Biomater*. 2014;10:2269–2281. [PubMed: 24412143]
19. van der Stok J, Wang H, Amin Yavari S, et al. Enhanced bone regeneration of cortical segmental bone defects using porous titanium scaffolds incorporated with colloidal gelatin gels for time- and dose-controlled delivery of dual growth factors. *Tissue Eng Part A* 2013;19:2605–2614. [PubMed: 23822814]
20. Ribeiro FO, Gómez-Benito MJ, Folgado J, Fernandes PR, García-Aznar JM. In silico mechano-chemical model of bone healing for the regeneration of critical defects: The effect of BMP-2. *PLoS One* 2015;10:e0127722. [PubMed: 26043112]
21. Wehrhan F, Amann K, Molenberg A. PEG matrix enables cell-mediated local BMP-2 gene delivery and increased bone formation in a porcine critical size defect model of craniofacial bone regeneration. *Clin Oral Implants Res*. 2012;23:805–813. [PubMed: 22151397]
22. Laurencin CT, Attawia MA, Lu LQ, et al. Poly(lactide-coglycolide)/hydroxyapatite delivery of BMP-2-producing cells: A regional gene therapy approach to bone regeneration. *Biomaterials* 2001;22:1271–1277. [PubMed: 11336299]

23. Fu YC, Nie H, Ho ML, Wang CK. Optimized bone regeneration based on sustained release from three-dimensional fibrous PLGA/HAp composite scaffolds loaded with BMP-2. *Biotechnol Bioeng.* 2008;99:996–1006. [PubMed: 17879301]
24. Lee SS, Huang BJ, Kaltz SR, et al. Bone regeneration with low dose BMP-2 amplified by biomimetic supramolecular nanofibers within collagen scaffolds. *Biomaterials* 2013;34:452–459. [PubMed: 23099062]
25. Lee JW, Kang KS, Lee SH, Kim JY, Lee BK, Cho DW. Bone regeneration using a microstereolithography-produced customized poly (propylene fumarate)/diethyl fumarate photopolymer 3D scaffold incorporating BMP-2. *Biomaterials* 2011;32:744–752. [PubMed: 20933279]
26. Hong L, Tabata Y, Yamamoto M, et al. Comparison of bone regeneration in a rabbit skull defect by recombinant human BMP-2 incorporated in biodegradable hydrogel and in solution. *J Biomater Sci Polym Ed.* 1998;9:1001–1014. [PubMed: 9747991]
27. Mao T, Wang C, Zhang S, et al. An experimental study on rhBMP-2 composite bone substitute for repairing craniomaxillary bone defects. *Chin J Dent Res.* 1998;1:21–25. [PubMed: 10557168]
28. Takahashi Y, Yamamoto M, Yamada K, Kawakami O, Tabata Y. Skull bone regeneration in nonhuman primates by controlled release of bone morphogenetic protein-2 from a biodegradable hydrogel. *Tissue Eng.* 2007;13:293–300. [PubMed: 17504062]
29. Cray J Jr, Henderson SE, Smith DM, et al. BMP-2-regenerated calvarial bone: A biomechanical appraisal in a large animal model. *Ann Plast Surg.* 2014;73:591–597. [PubMed: 23657046]
30. Zhang W, Zhu C, Wu Y, et al. VEGF and BMP-2 promote bone regeneration by facilitating bone marrow stem cell homing and differentiation. *Eur Cell Mater.* 2014;27:1–11; discussion 11–12.
31. Ishack S, Mediero A, Wilder T, Ricci JL, Cronstein BN. Bone regeneration in critical bone defects using three-dimensionally printed β -tricalcium phosphate/hydroxyapatite scaffolds is enhanced by coating scaffolds with either dipyridamole or BMP-2. *J Biomed Mater Res B Appl Biomater.* 2017;105:366–375. [PubMed: 26513656]
32. Ren LF, Shi GS, Tong YQ, Jiang SY, Zhang F. Effects of rhBMP-2/7 heterodimer and RADA16 hydrogel scaffold on bone formation during rabbit mandibular distraction. *J Oral Maxillofac Surg.* 2018;76:1092.e1–1092.e10. [PubMed: 29425753]
33. Kaito T, Morimoto T, Mori Y, et al. BMP-2/7 heterodimer strongly induces bone regeneration in the absence of increased soft tissue inflammation. *Spine J.* 2018;18:139–146. [PubMed: 28735764]
34. U.S. Food and Drug Administration. Bone graft substitutes containing recombinant proteins or synthetic peptides in patients under age 18: FDA Safety Communication. Reports of serious injuries. Available at: <https://ryortho.com/breaking/fda-issue-safety-communication-over-certain-bone-graft-substitutes/>. Accessed January 28, 2018.
35. Beidas OE, Deschamps-Braly JC, Morgan AM, et al. Safety and efficacy of recombinant human bone morphogenetic protein 2 on cranial defect closure in the pediatric population. *J Craniofac Surg.* 2013;24:917–922. [PubMed: 23714911]
36. Spiro AS, Beil FT, Baranowsky A, et al. BMP-7-induced ectopic bone formation and fracture healing is impaired by systemic NSAID application in C57BL/6-mice. *J Orthop Res.* 2010;28:785–791. [PubMed: 20063306]
37. Steib JP, Jean-Paul S, Bouchaïb J, et al. Could an osteoinductor result in degeneration of a neurofibroma in NF1? *Eur Spine J.* 2010;19(Suppl 2):S220–S225. [PubMed: 20449613]
38. Costa MA, Barbosa A, Neto E, et al. On the role of subtype selective adenosine receptor agonists during proliferation and osteogenic differentiation of human primary bone marrow stromal cells. *J Cell Physiol.* 2011;226:1353–1366. [PubMed: 20945394]
39. Mediero A, Wilder T, Perez-Aso M, Cronstein BN. Direct or indirect stimulation of adenosine A2A receptors enhances bone regeneration as well as bone morphogenetic protein-2. *FASEB J.* 2015;29:1577–1590. [PubMed: 25573752]
40. Mediero A, Wilder T, Reddy VS, et al. Ticagrelor regulates osteoblast and osteoclast function and promotes bone formation in vivo via an adenosine-dependent mechanism. *FASEB J.* 2016;30:3887–3900. [PubMed: 27511945]

41. Mediero A, Kara FM, Wilder T, Cronstein BN. Adenosine A(2A) receptor ligation inhibits osteoclast formation. *Am J Pathol.* 2012;180:775–786. [PubMed: 22138579]
42. Perez-Aso M, Mediero A, Cronstein BN. Adenosine A2A receptor (A2AR) is a fine-tune regulator of the collagen1:collagen3 balance. *Purinergic Signal.* 2013;9:573–583. [PubMed: 23749290]
43. McGrath LB, Gonzalez-Lavin L, Eldredge WJ, Colombi M, Restrepo D. Thromboembolic and other events following valve replacement in a pediatric population treated with antiplatelet agents. *Ann Thorac Surg.* 1987;43:285–287. [PubMed: 3827372]
44. Ueda N, Kawaguchi S, Niinomi Y, et al. Effect of dipyridamole treatment on proteinuria in pediatric renal disease. *Nephron* 1986;44:174–179. [PubMed: 3785481]
45. Dengler R, Diener HC, Schwartz A, et al.; EARLY Investigators. Early treatment with aspirin plus extended-release dipyridamole for transient ischaemic attack or ischaemic stroke within 24 h of symptom onset (EARLY trial): A randomised, open-label, blinded-endpoint trial. *Lancet Neurol.* 2010;9:159–166. [PubMed: 20060783]
46. Mediero A, Frenkel SR, Wilder T, He W, Mazumder A, Cronstein BN. Adenosine A2A receptor activation prevents wear particle-induced osteolysis. *Sci Transl Med.* 2012;4:135ra65.
47. Costa MA, Barbosa A, Neto E, et al. On the role of subtype selective adenosine receptor agonists during proliferation and osteogenic differentiation of human primary bone marrow stromal cells. *J Cell Physiol.* 2011;226:1353–1366. [PubMed: 20945394]
48. Bekisz JM, Flores RL, Witek L, et al. Dipyridamole enhances osteogenesis of three-dimensionally printed bioactive ceramic scaffolds in calvarial defects. *J Craniomaxillofac Surg.* 2018;46:237–244. [PubMed: 29292126]
49. Lopez CD, Diaz-Siso JR, Witek L, et al. Three dimensionally printed bioactive ceramic scaffold osseointegration across critical-sized mandibular defects. *J Surg Res.* 2018;223:115–122. [PubMed: 29433862]
50. Beutel BG, Danna NR, Granato R, et al. Implant design and its effects on osseointegration over time within cortical and trabecular bone. *J Biomed Mater Res B Appl Biomater.* 2016;104:1091–1097. [PubMed: 26034012]
51. Campos FE, Gomes JB, Marin C, et al. Effect of drilling dimension on implant placement torque and early osseointegration stages: An experimental study in dogs. *J Oral Maxillofac Surg.* 2012;70:e43–50. [PubMed: 22182660]
52. Rogers GF, Greene AK. Autogenous bone graft: Basic science and clinical implications. *J Craniofac Surg.* 2012;23:323–327. [PubMed: 22337435]
53. Oppenheimer AJ, Mesa J, Buchman SR. Current and emerging basic science concepts in bone biology: Implications in craniofacial surgery. *J Craniofac Surg.* 2012;23:30–36. [PubMed: 22337370]
54. Sahoo N, Roy ID, Desai AP, Gupta V. Comparative evaluation of autogenous calvarial bone graft and alloplastic materials for secondary reconstruction of cranial defects. *J Craniofac Surg.* 2010;21:79–82. [PubMed: 20061970]
55. Klimt CR, Knatterud GL, Stamler J, Meier P. Persantine-aspirin reinfarction study: Part II. Secondary coronary prevention with Persantine and aspirin. *J Am Coll Cardiol.* 1986;7:251–269. [PubMed: 2868029]
56. Ebendal T, Bengtsson H, Söderström S. Bone morphogenetic proteins and their receptors: Potential functions in the brain. *J Neurosci Res.* 1998;51:139–146. [PubMed: 9469567]
57. Chen D, Zhao M, Mundy GR. Bone morphogenetic proteins. *Growth Factors* 2004;22:233–241. [PubMed: 15621726]
58. Baskin DS, Ryan P, Sonntag V, Westmark R, Widmayer MA. A prospective, randomized, controlled cervical fusion study using recombinant human bone morphogenetic protein-2 with the CORNERSTONE-SR allograft ring and the ATLANTIS anterior cervical plate. *Spine (Phila Pa 1976)* 2003;28:1219–1224; discussion 1225. [PubMed: 12811263]
59. Jones AL, Bucholz RW, Bosse MJ, et al.; BMP-2 Evaluation in Surgery for Tibial Trauma-Allgraft (BESTT-ALL) Study Group. Recombinant human BMP-2 and allograft compared with autogenous bone graft for reconstruction of diaphyseal tibial fractures with cortical defects: A randomized, controlled trial. *J Bone Joint Surg Am.* 2006;88:1431–1441. [PubMed: 16818967]

60. Liang F, Yen SL, Imahiyerobo T, et al. Three-dimensional cone beam computed tomography volumetric outcomes of rhBMP-2/demineralized bone matrix versus iliac crest bone graft for alveolar cleft reconstruction. *Plast Reconstr Surg*. 2017;140:767–774. [PubMed: 28953728]
61. Haidar ZS, Hamdy RC, Tabrizian M. Delivery of recombinant bone morphogenetic proteins for bone regeneration and repair: Part B. Delivery systems for BMPs in orthopaedic and craniofacial tissue engineering. *Biotechnol Lett*. 2009;31:1825–1835. [PubMed: 19690811]
62. Herford AS, Boyne PJ. Reconstruction of mandibular continuity defects with bone morphogenetic protein-2 (rhBMP-2). *J Oral Maxillofac Surg*. 2008;66:616–624. [PubMed: 18355584]
63. Boyne PJ, Lilly LC, Marx RE, et al. De novo bone induction by recombinant human bone morphogenetic protein-2 (rhBMP-2) in maxillary sinus floor augmentation. *J Oral Maxillofac Surg*. 2005;63:1693–1707. [PubMed: 16297689]
64. Fallucco MA, Carstens MH. Primary reconstruction of alveolar clefts using recombinant human bone morphogenetic protein-2: Clinical and radiographic outcomes. *J Craniofac Surg*. 2009;20(Suppl 2):1759–1764. [PubMed: 19816345]
65. Yang K, Zhang J, Ma X, et al. β -Tricalcium phosphate/ poly(glycerol sebacate) scaffolds with robust mechanical property for bone tissue engineering. *Mater Sci Eng C Mater Biol Appl*. 2015;56:37–47. [PubMed: 26249563]
66. Sohier J, Daculsi G, Sourice S, de Groot K, Layrolle P. Porous beta tricalcium phosphate scaffolds used as a BMP-2 delivery system for bone tissue engineering. *J Biomed Mater Res A*. 2010;92:1105–1114. [PubMed: 19301273]
67. Tovar N, Witek L, Neiva R, et al. In vivo evaluation of resorbable supercritical CO₂-treated collagen membranes for class III furcation-guided tissue regeneration. *J Biomed Mater Res B Appl Biomater*. 2019;107:1320–1328. [PubMed: 30199603]

Fig. 1.

Schematic depiction of computer-aided design/computer-aided manufacturing software that facilitates three-dimensionally-printed scaffold design. (*Above*) Scaffold base with first layer of struts depicted in black for visualization in cross-sectional view. (*Center*) Top view depicting quadrant porous differences. (*Below*) Completed scaffold view.

Fig. 2. Intraoperative photographs before scaffold placement. (*Left*) Calvarial defect (11 mm in diameter) after trephination with intact dura mater. (*Right*) Defect replaced by three-dimensionally-printed scaffold (solid cap surface visualized).

Fig. 3.

Micro-computed tomographic slices of new bone formation within scaffold at 8 weeks. (*Above*) Three-dimensionally-printed scaffolds with smaller porous geometric design (*left, yellow arrow*) and larger porous geometric design (*right, green arrow*). (*Second row*) Identical slice as *above* with scaffold in *blue* and bone in *orange/yellow* to demonstrate osseointegration of new bone throughout scaffold porosity irrespective of geometric differences. (*Center*) At 8 weeks, scaffolds coated with dipyridamole induced bone regeneration in skeletally immature calvaria while maintaining suture potency (*red arrows*). (*Fourth row*) Identical slice as *center* with scaffold in *blue* and bone in *orange/yellow* to demonstrate suture potency is accompanied by osseointegration of new bone throughout scaffold. (*Below*) At 8 weeks, negative controls remained patent (*blue arrow*).

Fig. 4.

(Above, left) Bone, scaffold, and soft-tissue volume quantified as a function of dipyrindamole dose. (Above, right) Scaffold and soft-tissue volume quantified as a function of pore size. (Below) Bone regeneration as a function of both dipyrindamole dose and pore size. Letters indicate statistically homogenous groups. All *vertical bars* represent 95 percent confidence interval.

Fig. 5.

(*Above*) Quadrant scaffold demonstrates bone regeneration through scaffold porosity, at both larger (*red arrows*) and smaller (*white arrows*) pore dimensions. (*Below, left*) Highly cellular and vascularized bone formation is seen within scaffold interstices. Intramembranous-like healing is observed with regions of mature, lamellar-like bone formation (*blue arrows*).

(*Below, right*) Bone formation is guided by highly osseoconductive scaffold dimensions as new bone formation is directed from scaffold pore-to-pore (*green arrows*) while interacting with scaffold struts (*yellow arrows*)

Fig. 6.

Cranial sutures remained patent at 8 weeks. (*Above*) Scaffold demonstrates a robust degree of bone regeneration through its interstices (*green arrows*) while maintaining a patent sagittal suture (*yellow arrows*). (*Below, left*) The patent sagittal suture demonstrates a wide suture with collagen infiltrate (*yellow arrows*). (*Below, right*) High magnification of the suture infiltrate depicts cellular soft tissue (*yellow arrows*).