Precise Control of Osteogenesis for Craniofacial Defect Repair

The Role of Direct Osteoprogenitor Contact in BMP-2-Based Bioprinting

Darren M. Smith, MD, James J. Cray, Jr, PhD, Lee E. Weiss, PhD, Elmer K. Dai Fei, BS, Sameer Shakir, BS, S. Alex Rottgers, MD, Joseph E. Losee, MD, Philip G. Campbell, PhD, and Gregory M. Cooper, PhD

Background: Success with bone morphogenetic protein-2 (BMP-2) has been widely reported in the osseous reconstruction of large calvarial defects. These efforts have required enormous doses of BMP-2 and are not sufficiently refined to facilitate the detail-oriented repair required for intricate craniofacial structures. We have previously shown that inkjet-based bioprinting technologies allow for precisely customized low-dose protein patterns to induce spatially regulated osteogenesis. Here, we investigate the importance of direct contact between bioprinted BMP-2 and the dura mater (a source of osteoprogenitors) in mediating calvarial healing.

Methods: Five-millimeter osseous defects were trephinated in mouse parietal bones (N = 8). Circular acellular dermal matrix (ADM) implants were prepared such that 1 semicircle of 1 face per implant was printed with BMP-2 bio-ink. These implants were then placed ink-toward (N = 3) or ink-away (N = 5) from the underlying dura mater. After 4 weeks, osteogenesis was assessed in each of the 4 possible positions (BMP-2-printed area toward dura, BMP-2-printed area away from dura, unprinted area toward dura, and unprinted area away from dura) by faxitron.

Results: The BMP-2-printed portion of the ADM generated bone covering an average of 66.5% of its surface area when it was face-down (printed surface directly abutting dura mater). By comparison, the BMP-2-printed portion of the ADM generated bone covering an average of only 21.3% of its surface area when it was face-up (printed surface away from dura). Similarly, the unprinted portion of the ADM generated an average of only 18.6% osseous coverage when face-down and 18.4% when face-up.

Conclusions: We have previously shown that inkjet-based bioprinting has the potential to significantly enhance the role of regenerative therapies in craniofacial surgery. This technology affords the precise control of osteogenesis necessary to reconstruct this region's intricate anatomical architecture. In the present study, we demonstrate that direct apposition of BMP-2-printed ADM to a source of osteoprogenitor cells (in this case dura mater) is necessary for bio-ink-directed osteogenesis to occur. These results have important implications for the design of more complex bioprinted osseous structures.

Key Words: craniofacial healing, BMP-2, bioprinting

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Inadequate native bone and the morbidity profile of alloplastic implants have propelled osseous reconstruction of the craniofacial skeleton to become a heavily studied subject of tissue engineering efforts in plastic surgery laboratories. The question has long been "how much bone can we make?" With the popularization of bone morphogenetic protein-2 (BMP-2)-based strategies, it is becoming increasingly practical to generate large quantities of bone with an off-the-shelf implant. BMP-2, however, is a potent morphogen and is not without its own drawbacks. The large doses of BMP-2 required for clinically useful bone generation are especially concerning. Regardless, massive doses of BMP-2 are being used to generate large amounts of bone. The relevant question in bone tissue engineering is becoming "can we make the *right amount* of bone *in the right place*."[1] Toward this end, our laboratory has previously introduced a novel protocol that allows for precise spatial patterns of BMP-2 to be deposited, and for osteogenesis to occur in tight register to these biopatterns.[2] Moreover, this protocol may facilitate ossification with significantly lower doses of BMP-2 than conventional modalities. The next step in the evolution of this technology is to stack 2-dimensional constructs to generate 3-dimensional bone shapes. This report addresses one of the questions most fundamental to the 3-dimensionalization of this technology. Specifically, the study discussed here examines the importance of direct contact between osteoprogenitors and biopatterned BMP-2 in inducing ossification. The outcomes of these trials will inform the design of 3-dimensional bioprinted constructs as this technology matures.

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Reprints: Darren M. Smith, MD, University of Pittsburgh Medical Center, Pittsburgh, PA. E-mail: smithdm3@upmc.edu.

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METHODS

Implant Preparation

Bio-ink was printed on 5-mm-diameter acellular dermal matrix (ADM) discs (DermaMatrix; Synthes, West Chester, PA) with our custom 2-dimensional bioprinting system as previously described,[2,3] based on a piezoelectric inkjet printhead (30-mm diameter orifice) from MicroFab Technologies (Plano, TX). One semicircle of the dermal surface of each ADM disc was printed with BMP-2 bio-ink (Fig. 1). Each semicircle printed with BMP-2 (Medtronic; Memphis, TN) received 50 overprints (passes of the printhead) delivering a cumulative total of 155.4 ng of BMP-2. Excess (unbound) BMP-2 was rinsed from the discs by a sterile PBS bath for 24 hours. Notches were cut in the discs opposite the BMP-2-printed area to maintain orientation on implantation.

Printed Disc Implantation

Eight male C57-BL6 mice (Jackson Labs; Bar Harbor, ME), 7 to 8 weeks of age, were used in this study. The mice were anesthetized and the scalps were shaved and sterilized before surgery. A midline scalp incision was used to expose the periosteum. The periosteum overlying the planned craniectomy defect was excised. Under an operating microscope, a 5-mm craniectomy defect was trephinated in the right parietal bone. Meticulous care was taken to ensure that underlying dura was not disturbed. Each craniectomy defect was filled with a BMP-2-printed disc prepared as described previously, with orientation guided by the aforementioned notches. The first group of animals (N = 3) had the BMP-2-printed surface placed face-down against the dura. The second group of animals (N = 5) had the BMP-2-printed surface placed face-up away from the dura. In this manner, 4 semicircular treatment conditions were created:

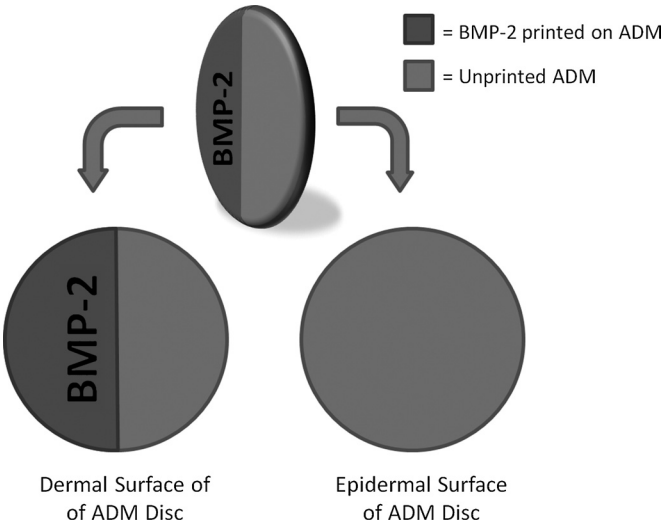

FIGURE 1. Schematic demonstrating configuration of BMP-2 bio-ink on ADM discs.

BMP-printed dermal surface toward dura, BMP-printed dermal surface away from dura, unprinted dermal surface toward dura, and unprinted dermal surface away from dura (Fig. 2). The skin was closed with a 4–0 nylon suture, and the mice received appropriate postoperative analgesia and antibiotics.

Analysis of Osteogenesis

The mice were euthanized 4 weeks after surgery, at which point the surgical sites were explanted and subjected to radiographic and histologic evaluation. Radiographic analyses were based on faxitron images imported into ImageJ (NIH) and Photoshop (Adobe, San Jose, CA). For histological analysis, tissues from the defect region of the calvaria were fixed, decalcified, and embedded in paraffin. The specimens were sectioned in the coronal plane at a thickness of 5 μm. Conventional hematoxylin and eosin staining for bone morphology was performed. Mann-Whitney *U* analysis was performed in SPSS (IBM, Armonk, NY).

RESULTS

Radiographic Evaluation

Percent ossification on faxitron was compared between each of the 4 semicircular treatment conditions (Fig. 3): BMP-printed dermal surface toward dura (66.45%), BMP-printed dermal surface away from dura (21.33%), unprinted dermal surface toward dura (18.58%), and unprinted dermal surface away from dura (18.36%). Although these results were not statistically significant by Mann-Whitney *U* analysis ($P > 0.05$), the BMP-printed dermal surface toward dura treatment condition had substantially greater ossification (66.45%) than any of the other groups. Qualitatively, there was more robust and consistent ossification on the semicircles with the BMP-printed dermal surface toward dura than in any of the other treatment conditions (Fig. 4). Moreover, the boundary between printed and unprinted semicircles was much more distinct on printed-side-toward dura than on printed-side-away from dura ADM discs (Fig. 4).

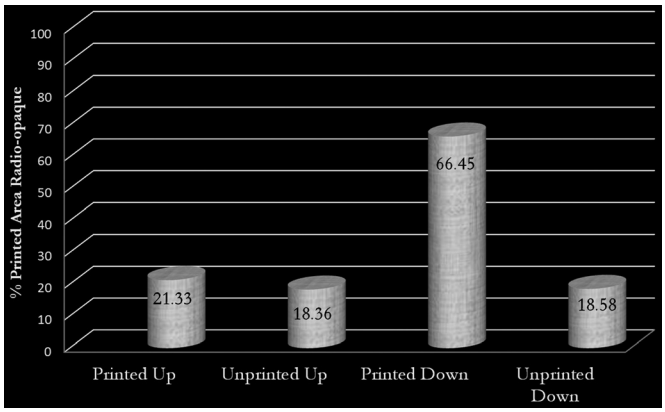

FIGURE 3. Graph comparing the average percent of each treatment condition that was ossified.

Histology

Histologic analysis revealed that ossification occurred primarily on the side of the ADM construct facing the dura (Fig. 5). This is to say that even when the BMP-2-printed surface of the ADM was facing away from the dura, it was the surface in direct contact with dural osteoprogenitors that underwent ossification. The bone generated by the bioprinted BMP-2 seemed histologically normal; it was fairly compact and demonstrated lacunae with viable osteoblasts (Fig. 5). The presence of cartilage in the ossifying constructs indicated that endochondral ossification was underway, which is characteristic of

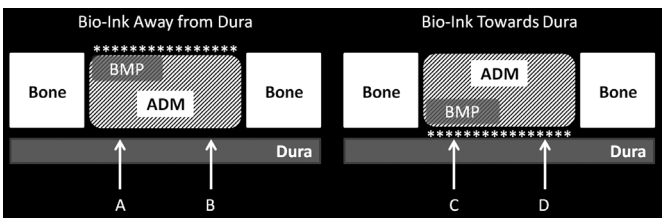

FIGURE 2. Two schematic coronal sections of cranial defects. ADM discs are depicted (hashed) filling osseous defects with white bone edges. The defect on the left has ADM with the dermal surface (****) facing away from the dura, and the defect on the right has ADM with the dermal surface facing towards the dura. The four treatment conditions are labeled A (BMP-printed dermal surface away from dura), B (unprinted dermal surface away from dura), C (BMP-printed dermal surface towards dura), and D (unprinted dermal surface towards dura).

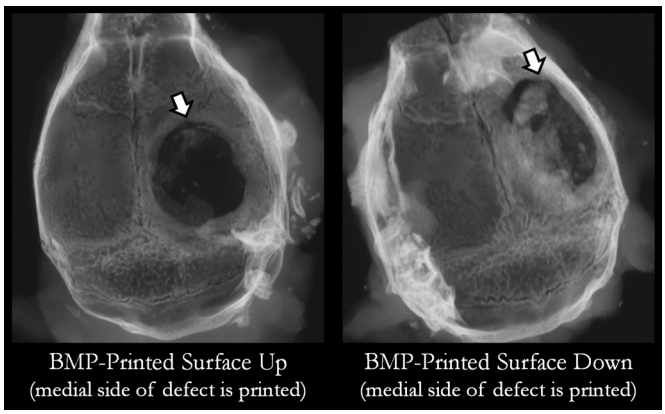

FIGURE 4. Faxitrons comparing ossification between discs with BMP-printed surface up (away from dura) and down (toward) dura. The arrows indicate the border between BMP-printed and unprinted semicircles. The printed semicircle is medial in both faxitrons.

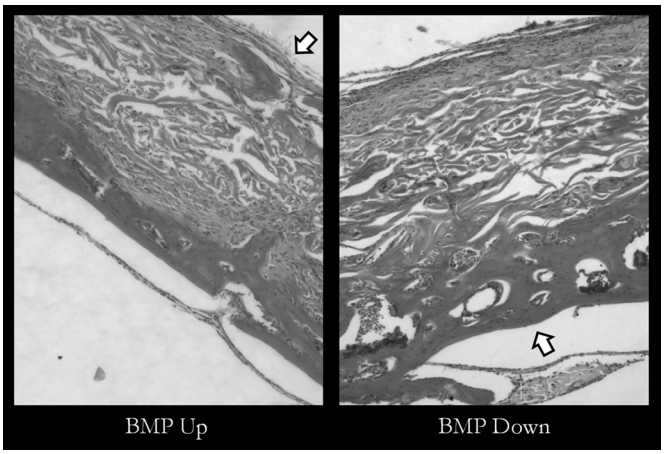

FIGURE 5. Histologic coronal sections of defects repaired with printed ADM printed surface up (away from dura) and down (toward) dura. The arrows indicate the printed surface.

BMP-2-driven osteogenesis in the calvarium, which natively undergoes intramembranous ossification.

DISCUSSION

The use of BMP-2 for reconstruction of the pediatric craniofacial skeleton is an appealing option in the context of limited autogenous donor bone and the difficulties inherent to alloplastic implants ranging from growth restriction to infection and extrusion. Many animal studies have been published by our group and others that demonstrate the potential of BMP-2 in craniofacial applications.[4–14] BMP-2 has recently seen early clinical use in craniofacial surgery. A BMP-2-based system was used for mandibular reconstruction in a #7 facial cleft and in a 12-cm hemimandibulectomy defect.[15,16] BMP-2 has also been applied successfully to alveolar cleft repair.[17] Early reports of in situ cranial vault reconstruction with BMP-2 are promising.[18,19] In these clinical instances, however, BMP-2 has been used in extremely high doses (on the order of milligrams) and without regard to fine morphologic control: these early efforts were oriented solely toward making bone, not toward achieving fine control of its deposition. Although this approach may be adequate for large defects with simple geometry, it does not allow for the surgical precision necessary in reconstructing 3-dimensionally complex craniofacial defects; additionally, the large quantities of BMP-2 used is cost-prohibitive for widespread clinical use.

The substantial osteoinductive potential of BMP-2, capable of fostering osteogenesis in even the most hostile environments,[20] is also one of the greatest stumbling blocks to its widespread clinical application in the pediatric population. We have previously demonstrated that this protein, in fact, is capable of inducing a craniosynostosis-like phenotype in a juvenile New Zealand White rabbit model.[21] That study also clearly demonstrated the lack of control inherent to BMP-2 as it is presently available (collagen sponge soaked with BMP-2 solution): although the implanted BMP-2-soaked sponges were only 5 mm in diameter, sutural fusion was observed well beyond areas directly abutting the constructs.

Two dominant strategies have emerged to reduce the amount of BMP-2 required to produce an osteogenic response: supplementing BMP-2 delivery systems with osteogenic progenitor cells and increasing the host's sensitivity to exogenously introduced BMP-2. Each of these strategies has significant limitations. Briefly, any attempt to introduce additional cells to a bioengineered construct exponentially increases the complexity of the endeavor and removes it further from the realm of immediate translational utility.[1] First, the osteogenic potential of the cell used must be carefully verified.[22,23] Second, if a suitable cell type is identified, a practical source must be determined. If harvested in advance from donors to allow off-the-shelf utility, the immunologic hurdles of any tissue transplant must be cleared. The second strategy involves augmenting the host's response to a given dose of BMP-2. One recently explored application of this approach uses AMD3100, a bone marrow progenitor cell mobilizing agent. AMD3100 was found to significantly enhance calvarial defect bone regeneration in a murine model.[24] AMD3100 also lead to improved bone generation in the context of a murine distraction study by the same group.[25] Although these results are encouraging, the prospect of systemically mobilizing bone marrow progenitors must be carefully vetted before it is used in skeletally immature individuals.

It is in this context that our group has introduced a novel approach to growth factor delivery for craniofacial reconstruction.[2] In this methodology, bio-inks (proteins) are printed onto a substrate (ADM) in much the same manner as an inkjet prints colored ink onto paper. This solid-phase technology offers 2 principal advantages over classic liquid-phase BMP-2 delivery systems. First, the morphology of tissue-engineered ossification can be carefully regulated. User-defined patterns of BMP-2 can be printed. These patterns persist on the ADM constructs and direct site-specific ossification.[2] Second, substantially lower doses of BMP-2 are required to induce ossification. The precise control afforded with bioprinting applies not only to 2-dimensional patterns but to 3-dimensional patterns as well.

In this study, the dura mater was the source of osteoprogenitors. As expected, the most robust osteogenesis occurred when the BMP-2-printed face of the ADM was in direct contact with the dura mater (Fig. 4). The amount of ossification present in ADM whose BMP-2-printed face was away from the dura was not appreciably different from the amount of ossification seen in ADM that was not printed at all (Fig. 3). The significance of this finding is that the BMP-2 bio-ink did not exert an osteogenic effect across the ADM. The implication of these results is that osteoprogenitors must be in direct contact with a BMP-2 printed surface to ossify. Although this requirement is cumbersome in that 3-dimensional constructs will require osteoprogenitors to be in close proximity, it is another assurance of spatial specificity: bone will not form in areas that do not meet the strict criteria for ossification under this scheme (direct apposition of BMP-2 and osteoprogenitors).

Direct apposition of bio-ink to osteoprogenitors resulted not only in more robust ossification but also in a more precise distinction between the degree of ossification observed between the printed and unprinted sides of the ADM discs (Fig. 4). Although this finding is likely simply a reflection of the fact that there is no significant difference between the amount of ossification occurring on the printed and unprinted sides of an ADM disc whose printed surface is facing away from the dura, it is still must be accounted for when planning to stack bioprinted discs.

Histologic analysis revealed that ossification occurred on the face of the ADM directly abutting the dura, regardless of which face was printed (Fig. 5). It is expected that ossification would occur on the dura-abutting face of an ADM construct when this was the face printed with BMP-2. More surprising is the finding that when ossification did occur on ADM with the printed surface away from the dura, it was the dura-abutting face that ossified. There was no evidence of BMP-2 inducing ossification of an ADM surface not in direct contact with the dura. Therefore, the BMP-2 bio-ink was not able to recruit and differentiate osteoprogenitors from distant tissues, nor able to induce migration of osteoprogenitors from the unprinted face of ADM abutting dura to the printed face not abutting dura (Fig. 5). This finding again underscores the spatial specificity to be expected of a 3-dimensional ADM stacking protocol.

CONCLUSIONS

Bioprinting may represent a viable strategy to precisely engineer intricately detailed 3-dimensional bone constructs. Although this technology has proven promising in 2-dimensional, it must be applied in 3-dimensional to be practically useful. From the results observed here, it seems clear that direct contact between osteoprogenitors and printed proteins is required for efficient osteogenesis. Therefore, when 2-dimensional constructs are stacked to construct 3-dimensional shapes, an adequate interface must be provided between osteoprogenitors and printed surfaces. Although the extremely localized effects of bioprinting represents a challenge in the form of an increased osteoprogenitor requirement, this same quality also ensures a high degree of precision in resultant osteogenesis.

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