

Vascularized Bone-like Organoids: A Model System and Regenerative Strategy

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INTRODUCTION: Autologous bone grafts are the clinical gold standard to effectively heal osseous injuries due to their dense mineralization, osteoinductive and angiogenic cues, and vascularity. For critically-sized defects, vascularity becomes a driving factor in the success of healing - a lack of adequate neovascularization leads to necrosis at the core. Yet vascularized autologous grafts require secondary surgical sites and are limited based on size, shape, and sufficient vascular supply. Currently, no clinical alternatives exist to replace vascularized autologous bone grafts. However, recent advances in tissue engineering of bone can now replicate the highly mineralized collagen and cellularity of native bone in-vitro¹. This process occurs without supplemental growth factors and has been shown to be modular, in that both bone marrow stromal cells (BMSCs) and endothelial cells, can be encapsulated in the mineralized matrix separately or together¹. Therefore, we developed a vascularized bone-like organoid to serve both as an autograft alternative and a model system of bone development and vascularity in-vitro. We hypothesized that inclusion of vascular fragments into a mineralized bone-like organoid would enhance mineralization and mature bone and vascular cell phenotypes, similar to native bone. To test this, we utilized robotic bioprinting to create large numbers of small (< 0.5 mm diameter) bone-like organoids with and without microvascular fragments. Bone-like organoids were then characterized for mineral content, quality, and distribution as well as histology to glean morphological information for the resulting organoids. Our findings outline a novel approach to the scalable production of mineralized, vascularized, bone-like organoids utilizing automated robotic technology towards high-throughput fabrication of a vascularized autologous bone graft substitute for complex osseous defects and as an in-vitro microphysiological model system.

METHODS: *Organoid Fabrication:* Cell-laden mineralized collagen was prepared as described¹, briefly, rat tail type I collagen (3 mg/mL, Gibco) and human BMSCs (Lonza) were mixed to a final concentration of 1.5 mg/mL collagen containing either BMSCs (3.9x10⁵ cells/mL) or BMSCs plus rat adipose derived microvascular fragments² (MVFs) (20,000 fragments/mL). The hydrogels were cold (4°C) printed in 2 µL using the BioAssembly Bot@ 400 robotic bioprinter, then assembled into large volume flasks and agitated over the period of mineralization using described methods¹, followed by standard culture conditions up to 12 days. *Histology:* The resulting cellular organoids were decalcified then processed for hematoxylin and eosin staining. *Mineral Characterization:* Standard and ultra-high resolution microcomputed tomography (microCT) as well as Fourier transform infrared spectroscopy (FTIR) were performed on mineralized and non-mineralized samples for each group of BMSCs only or BMSCs+MVFs (n=3-5 organoids/assay/group)(Unpaired, two tailed t-tests were used when comparing two groups, otherwise a one-way analysis of variance (ANOVA) was applied). Significance defined as a p-value < 0.05.

RESULTS SECTION: The mineral to matrix ratio was 34% higher in the MVF+MSC bone-like organoids (3.56±0.32) as compared to MSC alone (2.33±0.30) (p=0.002) (Fig. 1A). The hydroxyapatite carbonation was higher in the MVF+MSC bone-like organoids (0.127±0.003) as compared to MSC alone (0.118±0.005) (p=0.05) (Fig. 1B), but no change in crystallinity (p=0.7). Microcomputed tomography (microCT) showed no difference in the bulk, average mineral density with or without MVFs. The mean densities were comparable with 283±109 mgHA/cm³ for MSC and 290±117 mgHA/cm³ for MSC+MVF. The higher density perimeter reached 430±64 mgHA/cm³ and 436±69 mgHA/cm³ for MSC and MSC+MVF, respectively. Histological sections revealed complex networks of pores which subsequently filled with either osteocyte-like cells (with mineral) or vasculature (without mineral) as the MVFs developed (Fig. 1C). Qualitatively, high resolution microCT showed increased mineral around encapsulated cells with MVFs as compared to BMSCs alone (data not shown).

DISCUSSION: This study is the first to utilize high-throughput robotic bio-printing assisted fabrication of cellularized, mineralized, and vascularized bone-like organoids. These bone-like organoids are approximately half of the native bone mineral density level for human trabecular bone. Inclusion of microvascular fragments increased the FTIR detectible mineral/matrix ratios, however, the mineral density as measured by microCT was no different between groups. The distribution of the mineral was qualitatively different with inclusion of MVFs, where the encased cells showed regions of higher density than the remaining matrix. Interestingly, the non-mineralized organoids showed a qualitative increase in pores, whereas the mineralized groups showed more osteocyte-like cells contained within the pores. This was enhanced with MVF inclusion. Ongoing work will characterize the cellular phenotype (Nanostring) and pore network density. While the current observations of biological mechanisms are limited, *the potential to both study bone differentiation processes and mineralization in under 12 days in-vitro is a fundamental shift from standard osteoblast/osteocyte differentiation in-vitro studies.* This is consistent with our previous data that our mineralization process induces differentiation of BMSCs to osteoblast and osteocyte phenotypes between 14-21 days¹. Additionally, our new method includes vascular fragments and reaches a higher mineral density than our previous formulation¹, which may accelerate differentiation. With further development, these bone-like organoids could both serve as a model to study osteogenesis as well as a vascularized autograft alternative.

SIGNIFICANCE/CLINICAL RELEVANCE: This work describes novel robotic bio-printing assisted fabrication of cellularized, vascularized, and mineralized bone-like organoids which may be used as a bone autograft alternative and an in-vitro microphysiological model of bone. With further study, we aim to create patient-specific bone organoids and autograft alternatives to provide a new precision medicine testing platform as well as to reduce the need for donor bone as a surgical therapy.

REFERENCES: 1. *Nature Communications*, 10, 3520 (2019), 2. *In Vitro Cell & Dev Bio.*, 32, 409-419 (1996).

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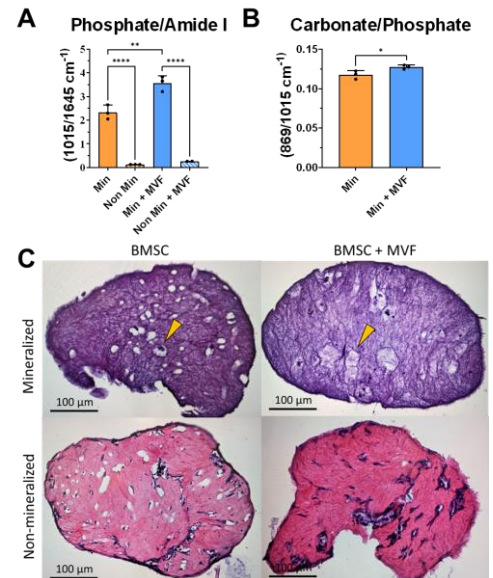


Figure 1. Mineral and cellular quality of bone-like organoids. (A) Quantification of the phosphate to amide I (mineral to collagen matrix ratio) increased with MVF inclusion in the bone-like organoids and (B) carbonate to phosphate ratio also increased, indicated a more mature mineral. (C) Histologically, there was an increased pore density with BMSCs alone, which filled with osteocyte and vessel like structures in the mineral and non-mineral organoids, respectively. Yellow arrow designates cell filled pore. (n=3-5/group). Scale 100 µm.