ORIGINAL ARTICLE

Dynamic Bioreactor Culture of High Volume Engineered Bone Tissue

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Within the field of tissue engineering and regenerative medicine, the fabrication of tissue grafts of any significant size—much less a whole organ or tissue—remains a major challenge. Currently, tissue-engineered constructs cultured *in vitro* have been restrained in size primarily due to the diffusion limit of oxygen and nutrients to the center of these grafts. Previously, we developed a novel tubular perfusion system (TPS) bioreactor, which allows the dynamic culture of bead-encapsulated cells and increases the supply of nutrients to the entire cell population. More interestingly, the versatility of TPS bioreactor allows a large range of engineered tissue volumes to be cultured, including large bone grafts. In this study, we utilized alginate-encapsulated human mesenchymal stem cells for the culture of a tissue-engineered bone construct in the size and shape of the superior half of an adult human femur ($\sim 200\text{ cm}^3$), a 20-fold increase over previously reported volumes of *in vitro* engineered bone grafts. Dynamic culture in TPS bioreactor not only resulted in high cell viability throughout the femur graft, but also showed early signs of stem cell differentiation through increased expression of osteogenic genes and proteins, consistent with our previous models of smaller bone constructs. This first foray into full-scale bone engineering provides the foundation for future clinical applications of bioengineered bone grafts.

Introduction

CRITICALLY SIZED BONE defects affect an average of 1.5 million Americans per year and command a market of more than 1 billion dollars in repair and regenerative therapies.¹ The current therapies are based on various types of autografts, allografts, or synthetic bone grafts. Unfortunately, current treatments can result in host rejection, improper vascularization, incomplete healing, or life threatening complications from surgery. New efforts have focused on bone grafts generated using tissue engineering techniques. Many groups have utilized bone marrow-derived human mesenchymal stem cells (hMSCs) to grow tissue grafts on a variety of natural and synthetic beads.^{2–4} hMSCs have the ability to differentiate quickly into osteogenic cells and have been characterized as strong immunomodulators and paracrine activity regulators, which could lead to robust *in vivo* function post implantation.⁵

Initially, static conditions were used to culture bone tissue grafts.^{6–8} However, these fail to deliver adequate nutrient supply and remove waste products, and can lead to poor tissue formation, necrosis, and incorrect cell migration.^{7,9} Therefore, the need for a dynamic culture environment is imperative for the *in vitro* formation of functional bone grafts. Bioreactors provide increased control over environmental parameters such as media flow and oxygen distribution. Perfusion bioreactors, by enabling the continuous and circular flow of media and

oxygen through the perfusion chamber, have demonstrated improved mass transport inside scaffolds and upregulation of important osteoblastic markers.^{10–14} Such bioreactors mimic *in vivo* environments, where human bone tissue is subjected to two mechanical stimuli during development or regeneration: fluid shear strain and physical tissue stress.¹⁵

Notably, our group has shown that the tubular perfusion system (TPS) bioreactor, which comprises a perfusion chamber where the cells and scaffolds are cultured, a medium reservoir, a tubing circuit, and a peristaltic pump, maintains cell viability at the center of grafts and enhances osteogenic differentiation of hMSCs compared to static culture conditions.^{16–18} Computational modeling of steady-state oxygen concentrations throughout an alginate bead cultured under static and dynamic conditions illustrated that oxygen concentration fell to 0.03 and 0.15 mM oxygen, respectively, suggesting that greater oxygen supply to encapsulated cells will maintain their viability and function.¹⁶ Additionally, simplified COMSOL modeling of the fluid flow pattern in the TPS bioreactor growth chamber indicated velocities as high as 3.5 cm/s given a 3 mL/min flow rate.¹⁶ This shear stress is applied to the surface of the alginate bead, triggering several mechanotransduction receptors on the cell membrane surface of encapsulated hMSCs, ultimately leading to augmented osteogenic differentiation.¹⁹

Furthermore, we have investigated such mechanical stimulation within the local environment of the alginate bead after 2–3 weeks of dynamic culture and discovered that the effects of shear stress on the surface, while localized to a volume close to the surface, encourage the release of paracrine factors, that in turn, affect the response of the cells in the core of the bead.²⁰ Leveraging the dynamic culture of these versatile scaffolds, we aggregated the small alginate beads into a larger construct and demonstrated continued viability and function of the encapsulated cells.²¹ More importantly, TPS bioreactor cultured cells were able to induce increased bone formation after implantation into a rat critical sized bone defect.²² These positive outcomes allow us to further build upon our system, especially in the application of fabricating high volume tissue constructs.

Multiple studies have demonstrated positive effects of dynamic perfusion bioreactor culture on osteogenesis for bone tissue-engineered bone grafts.^{14,23–27} To date, tissue-engineered bone constructs cultured in dynamic conditions using the indirect perfusion bioreactors have been fabricated up to a volume of 10.7 cm³.^{23,24} However, many systems are currently limited by the size of the culture chamber and inefficient supply of oxygen and nutrients to critical defect sized grafts.

The goal of this study is to engineer a scale-up of a 1 inch bone graft to a full-size, superior portion of an adult human femur (200 cm³). We successfully demonstrate that the TPS bioreactor system can support cell viability and function throughout the entire engineered tissue. This work signifies a major step in tissue engineering by creating high volume bone constructs that could help regenerate entire bones. Further, the scalability of the TPS bioreactor could expedite fabrication of other whole organs and tissues that would otherwise require multiple systems and strategies. To our knowledge, this is the first time a tissue-engineered bone construct of such size has been fabricated in the laboratory.

Materials and Methods

Human mesenchymal stem cell culture

Bone marrow-derived hMSCs (passage ≤5) for use in the 1-inch bone construct were purchased from Lonza and cultured in a growth media containing High Glucose Dulbecco's modified Eagle's medium with L-Glutamine (Gibco), supplemented with 10% fetal bovine serum (Invitrogen), 1% v/v penicillin/streptomycin (Gibco), and 0.1 mM nonessential amino acids (Invitrogen) following the manufacturer's protocol with a media change every 2–3 days, and passaged every 6–7 days. hMSCs (passage 4) for use in the large femur mold study were purchased from RoosterBio, Inc. and cultured in the accompanying high performance media kit from RoosterBio. To acquire the necessary cell numbers, we cultured the cells in several 2- and 10-stack cell culture flasks from Corning CellSTACK (Sigma). Cells were passaged on day 3 and cultured for an additional 5 days. All cells were cultured at 37°C and 5% of CO₂. The osteogenic media was formulated as previously described²⁸ by supplementing growth media with 100 nM dexamethasone (Sigma), 10 mM β-glycerophosphate, and 173 μM ascorbic acid (Sigma). Before using hMSCs from the two cell sources, their CD biomarker analysis were compared to ensure positive for known hSMC marker expressions such as CD 105, CD 166,

CD 90, and CD70, along with negative expression of CD 14, CD 34, and CD45. Lastly, osteogenic differentiation of both types of hMSCs was compared over a 21-day differentiation period to verify that there was no statistical difference in osteogenic gene and protein expressions (data not shown).

Cell encapsulation

hMSCs were mixed into 2% w/v alginate (Sigma) solution and used to make 3 mm diameter alginate beads by adding the mixture dropwise (flow rate of 1 mL/min) into a suspension of 0.1 M calcium chloride (Sigma) and stirring for 10 min (100,000 cells/bead). The 1 inch construct utilized 20 million cells in 200 alginate beads, while the femur-shaped construct required 720 million cells in 7200 alginate beads.

Design and 3D printing of femur mold

A human femur render was obtained from the open source online database GrabCAD. The file was imported into SolidWorks and the superior half of the femur was isolated. An outward extrusion of the composite resulted in a hollow construct with a wall thickness of 2 mm. Cylindrical pores were then placed at an approximate density of 1 hole per 2.83 mm² surface area throughout the surface of the mold. Finally, the femur shell mold was split into six pieces for 3D printing. Cuts were made on the transverse and sagittal planes of the femur mold. The construct was printed out of MED610 material using an Objet500 Connexin 3D printer (Stratasys) courtesy of the Sheikh Zayed Institute for Pediatric Surgical Innovation at Children's National Health System, Washington, D.C. Post printing, the femur shell was sutured together using medical grade sutures (Ethicon), and sterilized in 70% ethanol and under UV light.

TPS bioreactor assembly

For the culture of the 1 inch bone construct, the bioreactor was set up as described previously.²⁰ Briefly, a 1-inch platinum-cured silicone growth chamber was loaded with 200 hMSC-seeded and 200 acellular alginate beads and connected to the tubing circuit and media reservoir. The flow was driven by an L/S Multichannel Pump System (Cole Parmer) at a flow rate of 20 mL/min and media was changed every 2 days. The cells were cultured for 18 days in the 1 inch construct before cell analysis.

Similarly, to fabricate the human femur bone graft, the 3D printed femur shell mold was filled with 7200 hMSC-loaded alginate beads and placed inside a 10-inch (25.4 cm) long, 4-inch (10.16 cm) diameter culture chamber (MSC Industrial Supply). The cell-seeded alginate bead filled mold was cultured with the femur head downward and the femur shaft upward in the culture chamber, as seen in Figure 2d, right. Acellular alginate beads were placed in the surrounding void space of the culture chamber to ensure uniform media flow throughout the chamber by providing roughly the same resistance to flow as the hMSC-loaded alginate beads inside the femur shell mold. Custom-made reducing connectors were 3D printed and attached to either end of the chamber, and the remainder of the bioreactor was set up as described above. Media flow was driven at a rate of 240 mL/min to maintain velocities and shear stresses previously shown to enhance osteogenic differentiation of hMSCs in the TPS bioreactor.²²

At the end of the 8 day differentiation period, the femur mold was removed from the growth chamber, injected with liquid alginate, and submerged in a solution of 0.1 M CaCl_2 in hMSC growth media to aggregate the alginate beads into a single construct. hMSCs were isolated from specific sections of the construct (Fig. 2d) and used for subsequent analyses. Three samples from each group were taken ($n=3$).

Viability assay

Cell viability was assessed along the length of the femur construct using a fluorescent Live/Dead assay (Invitrogen) following standard protocols. Beads from each designated section were placed in 48-well plates and incubated in 2 mM ethidium homodimer and 4 mM calcein AM (Molecular Probes) for 30 min. Fluorescent images were then taken of the entire bead using a fluorescence microscope (Axiovert 40 CFL; Zeiss) equipped with a digital camera (11.2 Color Mosaic; Diagnostic Instruments).

Immunohistochemistry

Antigens were retrieved by exposure to steam composed of Tris base and ethylenediaminetetraacetic acid (EDTA) buffer (pH=8) containing TWEEN 20 for 15 min. Samples were blocked and then stained with the primary antibodies to detect bone morphogenic protein-2 (BMP-2) and alkaline phosphatase (ALP; Abcam), respectively. Protein presence was visualized with a 3,3'-diaminobenzidine tetrahydrochloride (DAB) chromogen. Samples were counterstained with hematoxylin, dehydrated, and cleared. Negative control slides were stained using the same protocol, omitting the primary antibody.

Real-time quantitative polymerase chain reaction

hMSCs from each section (head, trochanter, middle, shaft, and inner and outer shells) were isolated from alginate beads by dissolution in EDTA for 30 min at 37°C and a cell pellet was formed by centrifugation. The RNeasy Plus Mini Kit (Qiagen) was used to isolate total RNA from hMSCs encapsulated in alginate beads using following standard protocols. Total RNA was quantified using a Nanodrop Spectrometer (Thermo Scientific). Isolated RNA was then reverse transcribed to cDNA using a High Capacity cDNA Archive Kit (Life Technologies). Quantitative real-time polymerase chain reaction was performed by combining the cDNA solution with a Universal Master Mix (Life Technologies), along with oligonucleotide primers and Taqman probes for *ALP* and *BMP-2*, and compared to the endogenous gene control glyceraldehyde 3 phosphate dehydrogenase (*GAPDH*; Life Technologies). The reaction was performed using a 7900HT real-time PCR System (Applied Biosystems) at thermal conditions of 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C. The relative gene expression level of each target gene was then normalized to the mean of the *GAPDH* in each group. Fold change was calculated using the $\Delta\Delta\text{CT}$ relative comparative method as described previously²⁹ and represented in comparison to day 0 static control results. Samples were completed in technical triplicates and standard deviations are reported ($n=3$).

Statistical analysis

Each analysis was performed in triplicate ($n=3$). Statistical significance was determined by one-way analysis of

variance and Tukey's multiple-comparison test. A confidence interval of 95% ($\alpha=0.05$) was used for all analyses. Mean values of triplicates and standard deviation error bars are reported on each figure and relevant statistical relationships.

Results

Effect of location on osteogenic differentiation in dynamically cultured 1 inch bone graft

The TPS bioreactor has previously been used to culture hMSC-loaded alginate beads for the fabrication of bone constructs with a volume of $\sim 2.5 \text{ cm}^3$. However, to demonstrate the ease of use of the off-the-shelf components of the system, and assess location-based osteogenic differentiation of hMSCs, we expanded the growth chamber dimensions to result in a 12.8 cm^3 construct. After 7 days of dynamic culture in the TPS bioreactor, the alginate beads were aggregated into a single construct using 2% alginate and then cross-linked in additional CaCl_2 (Fig. 1a). On days 1, 4, and 7, beads from the periphery and interior of the construct were analyzed for viability (Fig. 1c) and expression of early osteogenic marker *ALP* (Fig. 1d) and late marker osteopontin (*OPN*; Fig. 1e) gene expression. Fluorescent staining indicated homogenous distribution of cells throughout the individual beads and continuous viability of cells in both experimental groups throughout the study. The expression of *ALP* mRNA increased as expected on day 7 compared with day 1 expression, yet there was no statistical difference between cells cultured in the peripheral and interior beads (Fig. 1d). Similarly, *OPN* mRNA expression remained consistent between groups over 7 days of osteogenic differentiation (Fig. 1e). These results indicated no significant difference in osteogenic differentiation of hMSCs cultured in the TPS bioreactor regardless of position in the growth chamber.

Culture of human femur using osteogenic differentiated hMSCs

Creating a bone graft of sufficient size has been a major obstacle in the field of bone tissue engineering. Utilizing 3D printing technology, we created a hollow mold of the superior half of a human femur (Fig. 2a). The printed femur was 22.86 cm in length, 10.16 cm in width at its widest point (femur head to trochanter), and had an internal volume of 200 cm^3 . We created 1 mm holes throughout the 3D femur shell, resulting in a 62.5% porosity (with an average density of one hole per 2.81 cm^2) to allow sufficient media and oxygen flow throughout the interior. The pieces of the femur mold were sutured together and filled with 7200 hMSC-loaded alginate beads (Fig. 2b). After 8 days of dynamic culture, the femur mold was removed from the growth chamber and the cultured beads were aggregated into a single construct using 2% alginate cross-linked in 100 mM CaCl_2 supplemented hMSC growth media (Fig. 2c). Post aggregation, the bone construct was 20 cm in length, and 8.9 cm in width (femur head to trochanter). We first divided the composite into four sections (femur head, trochanter, middle, and femur shaft), followed by an additional inner core and outer shell for each section to analyze the cells' viability and osteogenic differentiation throughout the construct over the culture period.

FIG. 1. Fabrication of 1-inch bone graft. **(a)** Encapsulation of hMSCs in 2% alginate solution results in the formation of spherical alginate beads. They were dynamically cultured in the TPS bioreactor setup with adjustable growth chamber, media reservoir, and peristaltic pump. After 7 days of culture, the alginate beads were aggregated into a single construct (1" diameter, 1" height). **(b)** Schematic showing cross-sectional view of alginate beads in 1" construct categorized into either interior beads or peripheral beads for analysis of cell viability and function. **(c)** Fluorescence staining of interior and peripheral alginate beads on days 1, 4, and 7 depicting live (green) and dead (red) cells. Results indicated that cells remained viability throughout the graft with no visible differences between interior and peripheral culture locations. Scale bar represents 1000 μ m. **(d)** Gene expression of ALP and OPN mRNA on days 1, 4, and 7. While there was an increasing trend of ALP mRNA expression by day 7, there was no statistical difference between the expression of interior and peripherally cultured cells. **(e)** Similarly, no difference was observed in OPN mRNA expression on all three time points ($n=3$, $p<0.05$). ALP, alkaline phosphatase; hMSC, human mesenchymal stem cell; OPN, late marker osteopontin; TPS, tubular perfusion system.

Fluorescent staining of live (green) and dead (red) cells indicated that the majority of cells remained viable after 8 days of dynamic culture in the TPS bioreactor (Fig. 3a). There were no qualitative differences visible between cells from the inner core or outer shell of the construct, indicating that sufficient amounts of nutrients and media were supplied throughout the width of the engineered femur. Immunohistochemical staining for ALP and BMP-2 protein (in brown, cells in blue) on day 8 showed no visible differences between early and late protein markers expressions (Fig. 3b, c). However, when examining mRNA expression of the same markers on day 8, cells cultured in the inner (dashed line, 25.2-fold) and outer (solid line 39.7-fold) shells of the

shaft section in the femur mold expressed a significantly greater amount of ALP and BMP-2 compared with other day 8 sections and day 0 static control cells (Fig. 3d, e). Average values of each femur section (depicted as solid bars) demonstrated that there was no significant difference between ALP mRNA expression of inner and outer shells of the femur head, trochanter, and middle sections of the femur.

Similarly, expression of BMP-2 mRNA showed a statistically significant increase in cells cultured in the inner (dotted line) and outer (solid line) shell of the femur shaft compared with the other sections on day 8 and the day 0 static control. Additionally, the average BMP-2 expression on day 8 in the femur head, trochanter, and middle sections

FIG. 2. Design, fabrication, and culture of human femur graft. **(a)** Solidworks CAD rendering of superior half of adult human femur. The mold was 22.86 cm in length, 10.16 cm in width at its widest point (femur head to trochanter), and had an internal volume of 200 cm³. There were covered 1 mm holes throughout the hollowed mold with an average density of one hole per 2.81 cm². **(b)** Image of 3D printed femur mold after it was filled with alginate-encapsulated hMSC beads (light pink color). **(c)** Image of aggregated alginate construct after 8 days of dynamic culture in TPS bioreactor. Its parts were categorized as femur head, trochanter, middle, or shaft. **(d)** Image of TPS bioreactor setup in incubator with growth chamber (circled in blue) containing femur mold. Schematic on right depicts TPS assembly with femur mold inside growth chamber and showing the direction of flow from the femur head-trochanter toward the femur shaft.

were significantly greater than in the day 0 static control cells. Yet, with the exception of the femur shaft, there were no differences in *BMP-2* expression seen between cells cultured in the inner or outer shells of the femur on day 8.

Discussion

Many bioreactor systems are limited in the size of the tissue that can be fabricated due to lack of oxygen that reaches the center of the graft, leading to cell necrosis. To solve this issue, our system utilizes smaller alginate beads (3 mm in diameter) that can be aggregated into a single construct of any size after dynamic culture in the TPS bioreactor (Fig. 1a). The TPS bioreactor allows enhanced osteogenic differentiation of hMSCs by applying fluid shear stress on the surface of the beads.^{14,20} The culture and fabrication of the 1 inch bone graft, which is similar in diameter size to the shaft of a human femur, was the next step toward creating critically sized and clinically relevant tissue constructs for the regeneration of bone. Most importantly, this pilot study confirmed continuous viability of

cultured cells throughout the culture chamber over 7 days, demonstrating that oxygen and nutrient supply was not different in the periphery or interior of the culture chamber (Fig. 1b, c). This overcomes a major hurdle in tissue engineering, wherein cells cultured at the center, or core, of the graft experiences necrosis due to the consumption of oxygen by cells at the periphery of the graft. Additionally, osteogenic differentiation of hMSCs, as indicated by increased expression of *ALP* and *OPN* mRNA expression, proved to be similar in cells cultured throughout the chamber (Fig. 1d, e). Therefore, an expansion of the TPS bioreactor growth chamber does not affect viability or osteogenic differentiation of hMSCs. In addition, we were able to demonstrate the ability to create large aggregates of alginate beads after their dynamic culture. The technique of utilizing additional alginate to fabricate a single construct resulted in a solid hydrogel composite that was easily handled and could be transferred into a defect site.

While the TPS bioreactor enables great flexibility in the size and length of the tubing circuit, the cylindrical shape of the tube defines the resulting architecture of the construct

FIG. 3. Osteogenic differentiation of hMSCs in adult human femur mold. **(a)** Fluorescence staining of hMSCs after 8 days of dynamic culture in the TPS bioreactor. Live (green) and dead (red) cells are shown in inner and outer shells of the femur head, trochanter, middle, and shaft sections of the construct. It is visible that the majority of cells remain viable and that no qualitative differences are observed between the cultured sections, indicating sufficient oxygen and nutrient supply throughout the construct. Scale bar represents 200 μ m. **(b, c)** Immunohistochemical staining of ALP and BMP-2 protein expression, respectively, of all experimental groups. Cells are stained in dark blue and protein in brown. No visible differences are seen between experimental groups in either inner or outer culture location, indicating homogenous differentiation of hMSCs over 8 days of dynamic culture. **(d, e)** Average ALP and BMP-2 mRNA expression on day 8 compared to static day 0 control (blue bars). Gene expression of cells cultured in the inner and outer shell are depicted by a dashed and solid line, respectively. Average ALP expression demonstrated no statistical difference between cells cultured in the femur head, trochanter, or middle sections of the femur compared to static day 0. However, an average of 32.4-fold increase of ALP mRNA was observed in the femur shaft. Gene expression of BMP-2 showed statistically significant increase on day 8 in all experimental groups compared with the static day 0 control. Additionally, BMP-2 gene expression was ~900 times greater in the femur shaft on day 8, which was significantly greater than expression in all other groups. Markers * and ** indicate statistical significance compared with control ($n = 3$, $p < 0.05$).

cultured in the growth chamber. In recent years, 3D printing technology has emerged as a leading technological innovation, especially in medical applications. It has allowed the creation of complex structures with precise architecture and consistency. In this work, 3D printing techniques helped

fabricate an accurate model of an adult femur, the largest bone in the human body (Fig. 2a). This demonstrates not only the potential of the technology, but also its capability to remove size limitations in the tissue engineering field. Additionally, the ability to print patient-specific molds based

on magnetic resonance imaging or computed tomography (CT) scans leads to more successful patient-specific care and tissue regenerative treatments.

By printing a porous 3D human femur, we were able to ensure fluid flow into and throughout the construct. During the dynamic culture in the TPS bioreactor, the femur mold was cultured with the head and trochanter at the inlet of the growth chamber (Fig. 2d, right). This portion of the femur held the majority of the cell-loaded beads and therefore needed to receive the most oxygenated media as it circulated via gas permeable tubing from the media reservoir to the inlet at the bottom toward the outlet at the top of the growth chamber. Additionally, to ensure uniform flow throughout the chamber, acellular alginate beads filled the void space surround the femur mold, as depicted by the pink patterned background in schematic 2d. After aggregation of alginate beads into a single construct, it was divided into further segments for viability and gene expression analysis.

On day 8, cell viability remained high in both the inner and outer shell of each section (Fig. 3a), with no visible differences observed. This is an important and momentous achievement for any large tissue engineering construct. The advantage of dynamically culturing small alginate beads in the TPS bioreactor before aggregation and implantation is most evident with the high cell viability result. Additionally, the immunohistochemical staining of early osteogenic marker ALP and osteogenic growth factor BMP-2 indicated only subtle differences in protein expression between the experimental groups on day 8, with slightly greater staining on femur shaft samples. Therefore, the cells are experiencing similar culture environments, which produce homogenous expression of protein throughout the construct.

The average mRNA expression of *ALP* (Fig. 3d, blue bars) showed no significant differences between the femur head, trochanter, or middle sections compared to day 0 static control cells. However, a significant increase in expression was observed in cells cultured in the inner and outer femur shaft (26.2- and 39.7-fold change, respectively), compared with the static day 0 control. A similar pattern was seen in the *BMP-2* mRNA expression, in which the inner and outer shell of the femur shaft expression was statistically greater than in the other groups on day 8. In addition, the femur head, trochanter, and middle sections express significantly greater *BMP-2* mRNA compared with day 0 static control (3.8-fold, 3.2-fold, and 4.8-fold, respectively). We hypothesize that this substantial increase in *ALP* and *BMP-2* may be attributed to growth factor production and uptake from cells from the inlet and the outlet of the growth chamber.

In particular, we believe that there is a growth factor gradient created during unidirectional flow in the growth chamber, which is generated when growth factors are released from cells cultured at the inlet and taken upstream by the fluid flow to cells cultured at the outlet, where they enhance functions like osteogenic differentiation. We have been able to show diminishing effects of this phenomenon when periodically alternating the direction of the flow inside the growth chamber. Therefore, we believe the direction of the flow can influence the expression of osteogenic markers in differentiating hMSCs. While the expression of osteogenic mRNA was much higher in cells cultured in the femur shaft, we observed trends of increased *BMP-2* expression over 8 days of dynamic culture in all sections of the graft. It is also interesting to note that the outer shell of the femur

construct expressed greater amounts of osteogenic mRNA in almost all groups on day 8. This could be seen as an advantageous benefit of the culturing system, in which the outer shell experiences relatively accelerated development to form the compact and stiffer cortical bone tissue, leading to structural support for the growth of the interior trabecular bone, which is spongy and weaker in mechanical strength.

Conclusion

Although this study has demonstrated major strides toward developing a bone graft fit for clinical application, there are still limitations that need to be addressed. This one-time proof of concept provides invaluable information about the ability to create a high volume engineered bone construct, yet additional studies with longer culture periods will generate more data and knowledge on the capabilities of the system. Although bone-marrow derived mesenchymal stem cells have been used in broad tissue engineering applications, a limitation in their osteogenic differentiation has been found when used in a bone defect composed of cells of neural-crest origin compared with cells of mesoderm origin.³⁰ Therefore, the stem cell origins and final defect destination need to be considered when designing a bone tissue engineering graft. Additionally, the current setup utilizes alginate hydrogels as a cell deliver and culturing environment, owing to the natural polymer's known noncytotoxic and bio-inert properties. However, its lack of mechanical strength makes it nonideal for future bone tissue engineering applications without further additions or modifications. Therefore, we plan to utilize a scaffold sleeve carrier made from a polymers such as poly(propylene fumarate), which has mechanical properties close to bone,³¹ that can be utilized to 3D print the femur shell and then be directly implanted into the defect site after aggregation of the alginate beads.

A drawback of the current scaffold material includes the lack of cell-cell contact between differentiating hMSCs in separate alginate beads during initial dynamic culture. With the aggregation of scaffolds using additional alginate, we hope that cells will be able to migrate within the constructs after *in vivo* implantation. More importantly, to bring this technology from bench to bedside, a vascular network will be vital; without it, the encapsulated cells at the core of the construct will not survive after implantation into the patient. Like many organs, bone contains an intricate vasculature that maintains viability throughout the tissue. Therefore, we are developing techniques to incorporate vascular networks within the engineered bone tissue for improved incorporation into the defect and surrounding tissue. With these next steps, we anticipate the fabrication of a fully functional, size and patient-specific tissue-engineered bone construct that can be dynamically cultured in the TPS bioreactor and then directly implanted into a bone defect.

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Author Contributions

B.N.B.N. and J.P.F. designed the study. B.N.B.N., H.K., R.A.M., and J.M.E. contributed to the collection of experimental data. B.N.B.N., H.K., and J.P.F. analyzed the data. B.N.B.N., H.K., R.A.M., and J.P.F. contributed to the writing of the article.

Disclosure Statement

The authors claim no financial conflict of interests. J.P.F. is an inventor on an invention disclosure and patent application for the tubular perfusion system (TPS) bioreactor.

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