A 3D Bioprinted Model to Study Osteogenic Differentiation of Primary Mesenchymal Stem Cells

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Abstract

Primary cells from healthy and diseased donors are becoming more acceptable tools for studying disease biology and developing new cell-based assays. Similarly, in the bioprinting field, the use of primary human mesenchymal stem cells (hMSCs) is considered a gold standard for the initial evaluation of bioink compatibility and the bioprinting process by determining the viability and functionality of cells. In addition, hMSCs are used in biofabrication for regenerative medicine research, including bone tissue regeneration, because of their potential to differentiate into different cell lineages. Bone is a specialized connective tissue that provides rigidity and support to soft tissues. The main challenge in fabricating bone tissue is effectively recapitulating the *in vivo* microenvironment that are seen *in vivo*. Here, we report the development of a 3D bioprinted model to study osteogenic differentiation of hMSCs. These cells were mixed with GelMA Fibrin and bioprinted in multi-well plates using the BIO X, where the bioink was crosslinked with both UV light and thrombin. Following 21 days of culturing in osteogenic or control media, the printed constructs were analyzed for osteogenic differentiation by Alizarin Red S staining and qPCR. The method described here can easily be scaled up and translated to other primary cell systems to develop novel cell-based assays or to study bone regeneration.

Introduction

Mesenchymal stem cells (MSCs) are stromal cells that have the potential to self-renew and differentiate into many different lineages, including chondrogenic, osteogenic, adipogenic and neural (Figure 1), when cultured in the recommended differentiation medium. MSCs can be harvested from multiple tissue sources, including umbilical cord, bone marrow and adipose tissue. Human MSCs (hMSCs) are characterized by the expression of several surface markers, including CD29, CD44, CD105 and CD166. However, hMSCs do not express CD14, CD34 or CD45 markers.

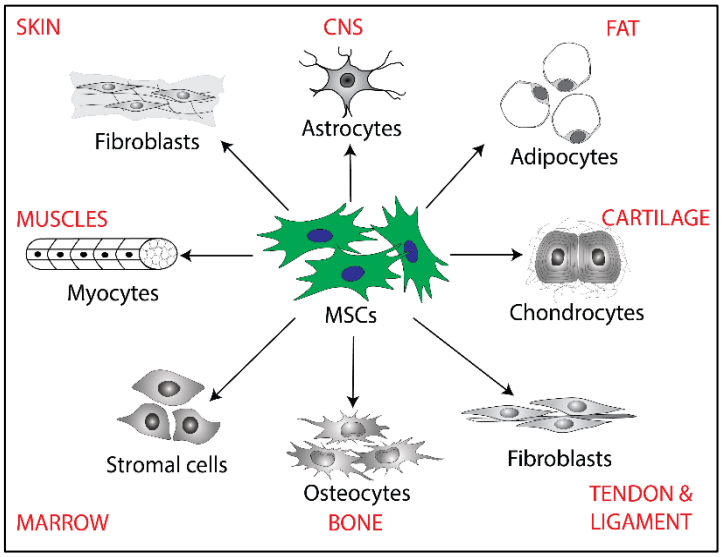

Figure 1. *Differentiation of human mesenchymal stem cells (hMSCs). Normal human bone marrow-derived MSCs can differentiate into different cell lineages, including the adipogenic, chondrogenic and osteogenic lineages when cultured in the recommended differentiation medium.*

Because they are able to self-renew and differentiate, hMSCs are often used for regenerative medicine research, including biofabrication or bioprinting. Substantial progress has been made to regenerate bone tissue using 3D bioprinting (Ashammakhi, 2019; Adepu, 2017). Bone is a specialized connective tissue that provides rigidity and support to its surrounding soft tissues while maintaining the osteogenic potential of the stem cells in its inner core. Different types of scaffold and bioinks have been tested to fabricate bone. The main challenge faced by researchers when fabricating bone tissue is trying to effectively recapitulate the microenvironments that are seen *in vivo*. Gelatin methacrylate (GelMA),

a natural polymer, and its derivatives have been used to fabricate bone tissue using a 3D bioprinter (Yue, 2015). GelMA can be crosslinked with UV light, enabling the stiffness of the printed tissue to be tuned. To mimic native bone tissue, many studies also incorporate different cell types, such as angiogenic cells to induce vasculature. Prof. Ali Khademhosseini's group recently published a study in which they created a bioprinted model designed to emulate bone tissue. To print this construct, hMSCs and human umbilical endothelial cells (HUVECs) were mixed in a modified GelMA bioink that exhibited excellent osteogenic differentiation of hMSCs and calcium deposition in the printed tissue (Byambaa, 2017). In the study, the incorporation of HUVECs and vascular endothelial growth factor exhibited a better osteogenic differentiation of the printed tissue due to the formation of microvascular networks.

In this study, we used primary hMSCs and HUVECs mixed with GelMA Fibrin bioink to bioprint 3D constructs that were subsequently crosslinked with both UV light and thrombin. After 21 days of culturing in osteogenic or control media, the printed constructs exhibited signs of osteogenic differentiation as determined by Alizarin Red S staining and qPCR. The method described here can easily be scaled up and translated to other primary cell systems to develop novel cell-based assays or to study bone regeneration.

Materials and methods

Cell preparation

Primary bone marrow-derived hMSCs (Lonza, PT-2501) and HUVECs (Lonza, C2519A) were cultured according to the supplier's instructions. Briefly, hMSCs were maintained in MSCBM Basal Media (Lonza, PT-3238) supplemented with MSCGM SingleQuots Kit (PT-4105). HUVECs were maintained in EBM-2 basal medium (CC-3156) supplemented with EGM-2 SingleQuots Kit (CC-4176). The cells were grown at 37°C in 75 cm^2 tissue culture flasks in a humidified incubator with 5% CO_2. The cells were harvested using Accutase and washed in Dulbecco's phosphate-buffered saline (DPBS) before they were used in the biofabrication process.

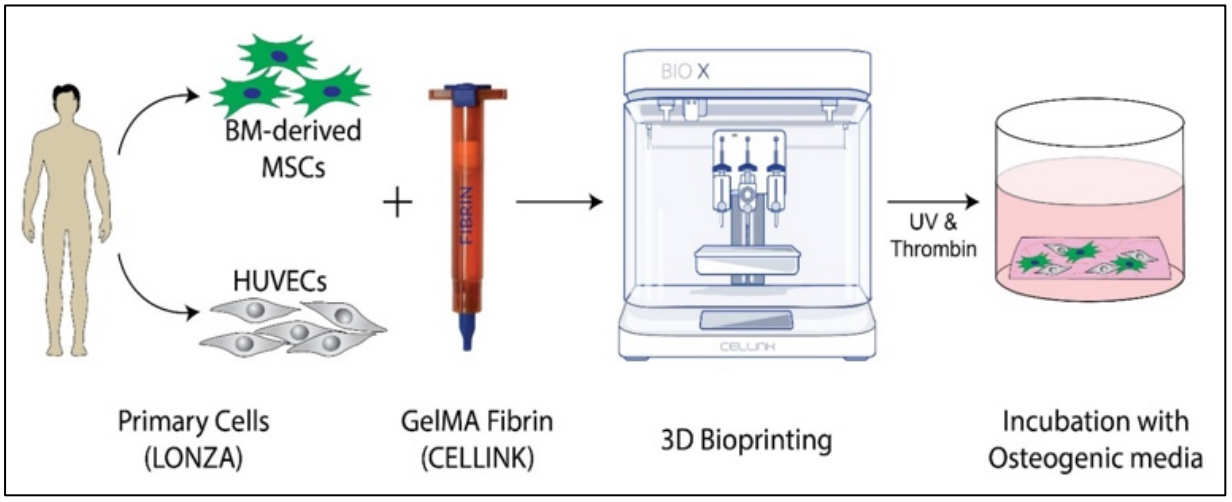

Figure 2. 3D bioprinting with hMSCs and HUVECs. Primary hMSCs and HUVECs were mixed with GelMA Fibrin bioink and dispensed using the BIO X. The 3D printed constructs were then crosslinked with both UV and thrombin. Long-term cultures of the constructs were maintained in either control growth media or osteogenic differentiation media for 21 days.

Bioink preparation and bioprinting

The overall bioprinting process shown in Figure 2 was followed (read the full protocol). GelMA Fibrin bioink (CELLINK, IKG106L3030-M) was placed in a 37°C incubator for 10 minutes before mixing with cells. HUVECs and hMSCs were mixed at a 1:3 ratio, co-pelleted, and resuspended in the bioink (CELLINK, IKG106L30301) at a 1:9 ratio (cells to bioink, v/v) to reach a total of $4x10^6$ cells/mL of bioink-cell mixture. The bioink and cells were mixed gently between two syringes and then dispensed in a 48-well plate as a thin disk through a connected 22G nozzle (CELLINK, NZ4220005001) using the Temperature-controlled Printhead (at 23°C) on the BIO X (printbed at 18°C). The 3D constructs were then photocrosslinked for 15 seconds at a distance of 3 cm. The constructs were incubated overnight with MSCGM growth media supplemented with 10 U/mL thrombin and 25% EGM-2 growth media. On the following day, thrombin-containing media was replaced with either control growth media (MSCGM:EGM-2, 3:1) or osteogenic growth media (Lonza, PT-3924/4120) (Osteogenic media:EGM-2, 3:1). The media was refreshed every 2 to 3 days for 21 days.

Viability assay and imaging

3D bioprinted constructs were taken out at different time points (Days 1, 7, 14, 21) and stained with Calcein and propidium iodide following standard protocol. Cell viability was determined by fluorescent imaging of the constructs with green and red channels. Images were analyzed using ImageJ software and presented as a normalized viability score using GraphPad Prism 8.0.

Alizarin Red assay and quantification

Alizarin Red S (ARS) staining kit was purchased from ScienCell (#8678) and the manufacturer's protocol was adapted for 3D culture for qualitative and quantitative analysis. The 3D constructs from the control and osteogenic differentiation groups were washed 3 times with DPBS. The constructs were fixed in 4% paraformaldehyde for 20 minutes at the room temperature. The fixative solution was aspirated, and the constructs were washed 3 times with diH_2O. Next, 1 mL of 40 mM ARS solution was added to each well and the samples were incubated at room temperature for 40 minutes with intermittent shaking. The dye was removed, and the samples were washed 5 times with diH_2O. A few stained samples were observed under a bright-field microscope, and images were taken with a color camera. The remaining samples were stored at -20°C for quantitative evaluation as described below.

Frozen samples were thawed on ice and transferred to 1.5 mL centrifuge tubes. Next, 800 µL of 10% acetic acid was added to each sample, which was then triturated with a P200 pipette to break the constructs into suspension. The cell suspension was vortexed and incubated at room temperature for 30 minutes. Samples were then placed on a heating block set at exactly 85°C for 10 minutes. The samples were then transferred on ice for 5 minutes followed by centrifugation at 13,000 rpm for 25 minutes. The supernatant was transferred to a 96-well plate to measure optical density at 405 nm (OD405) and compared with a standard curve as described in the manufacturer's protocol.

Table 1. Primer list used for qPCR to evaluate osteogenic gene expression.

GENE	FORWARD PRIMER	REVERSE PRIMER
Col1	5'-GTC ACC CAC CGA CCA AGA AAC C-3'	5'-AAG TCC AGG CTG TCC AGG GAT G-3'
CD31	5'-TCT ATG ACC TCG CCC TCC ACA AA-3'	5'-GAA CGG TGT CTT CAG GTT GGT ATT TCA-3'
RUNX2	5'-GGT TAA TCT CCG CAG GTC ACT-3'	5'-CAC TGT GCT GAA GAG GCT GTT-3'
OCN	5'-TCA CAC TCC TCG CCC TAT TG-3'	5'-TCG CTG CCC TCC TGC TTG-3'
ALP	5'-GGA CAT GCA GTA CGA GCT GA-3'	5'-GCA GTG AAG GGC TTC TTG TC-3'
GAPDH	5'-GCT CTC TGC TCC TCC TGT TC-3'	5'-ACG ACC AAA TCC GTT GAC TC-3'

Total RNA Isolation, cDNA preparation and qPCR

The 3D constructs from both the control and osteogenic differentiation groups were treated with Cell Collect G (CELLINK, LR020000) to harvest cell pellets. Cells were lysed and total RNA was eluted in DNAse/RNAse-free water using a Quick-RNA Miniprep kit (ZYMO Research, #R1054) and following the manufacturer's protocol. To remove inhibitors, total RNA was precipitated following a standard sodium acetate protocol and resuspended overnight in nuclease-free water. Total RNA was stored at -80°C until used in a reverse transcription (RT) reaction.

To generate cDNA library, an RT Kit (Thermo Fisher Scientific) was used. A three-step RT reaction was performed following the manufacturer's protocol: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes. cDNA samples were stored at -20°C until used. To compare osteogenic gene expression, a SYBR Green-based quantitative polymerase chain reaction

Table 2. Thermocycler program for qPCR.

Step	Temp	Duration	Cycles
UDG Activation	50°C	2 min	Hold
Initial Denature	95°C	2 min	Hold
Denature	95°C	15 sec	40X
Anneal/Extend	60°C	1 min	
Store	8°C	∞	Hold

(qPCR) protocol was followed, using a commercially available MasterMix kit (Applied Biosystems, 4309155). The primer sequences of the target genes were previously published (Byambaa, 2017) and are listed in Table 1. For each reaction, 500 nM of forward and reverse primers, 10 µL of 2X SYBR MasterMix, 100 ng cDNA, and nuclease-free water were mixed together to a final volume of 20 µL. The reactions were performed on an Open qPCR thermocycler (CELLINK, D16110022870) using a program listed in Table 2.

Results and discussion

Cellular morphology and viability

To determine the impact of the osteogenic differentiation medium, morphology and viability of the cells were determined using bright-field (Figure 3A) and fluorescent microscopy (Figure 3B-C). There were no significant differences observed when the cells were grown in either control or osteogenic medium. Calcein and PI staining showed more than 80% of viability in all the conditions at Days 1, 7, 14 and 21. A subtle difference in cell morphology was observed when the printed constructs were treated with either control or osteogenic media. As shown in Figure 3C, cells were stretched in the control group whereas they were more connected to each other in the osteogenic treatment group.

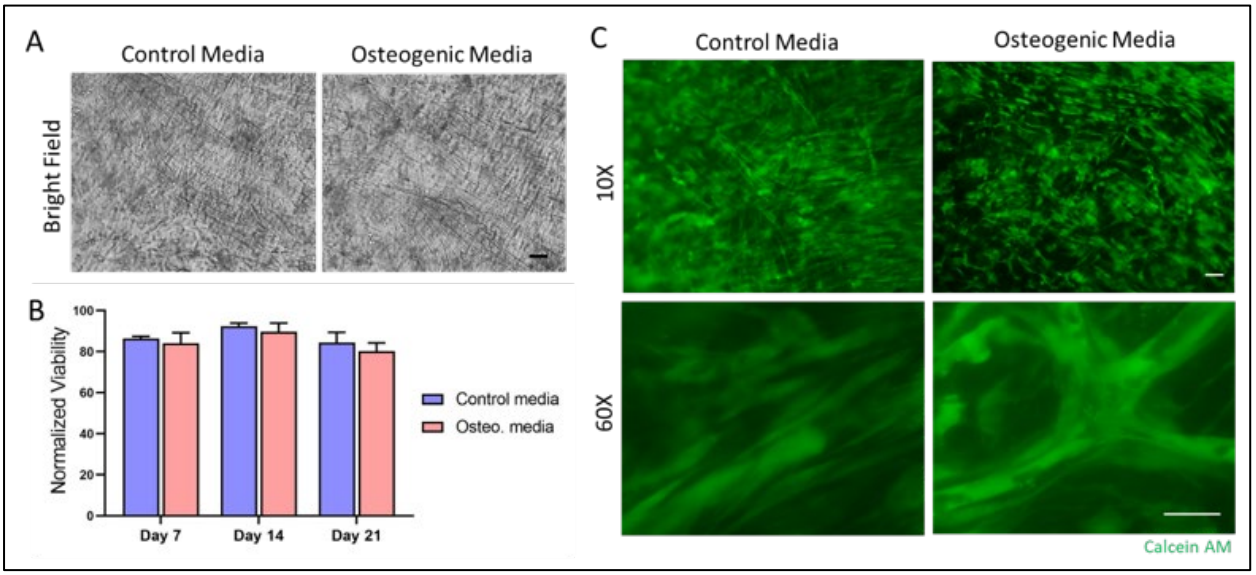

Figure 3. Morphology and viability of bioprinted MSCs. (A) Bright-field images of the printed constructs were taken with 10X objective lens. (B) Viability of the printed cells were determined by staining with Calcein and PI. Viability scores were normalized to Day 1 data and presented as percentages. (C) Representative images of the printed cells stained with Calcein are shown. Scale bars = 10 µm.

Alizarin Red S staining and quantification

Alizarin Red S (ARS) is an anthraquinone dye widely used to evaluate mineral (calcium) deposition in cell culture. Here, we adapted an ARS staining protocol for 3D bioprinted construct and evaluated the dye retention by qualitative imaging and quantitative measurement. As shown in Figure 4A, the control samples did not retain any ARS dye after washing with dH_2O. However, patches of calcium deposition (blue arrows) in red were observed in the osteogenic differentiation group. In the next step, ARS dye was extracted from the samples and the concentration of ARS was measured with a plate reader. Figure 4B showed increased retention of ARS in the samples that were treated with osteogenic differentiation media.

Gene expression

Next, we determine the differences in the gene expression between the control and treated groups following a standard SYBR Green-based qPCR method. One angiogenic (CD31) and 4 osteogenic (Col1, RUNX2, OCN and ALP) genes were tested. GAPDH was used as a housekeeping gene for normalization purposes.

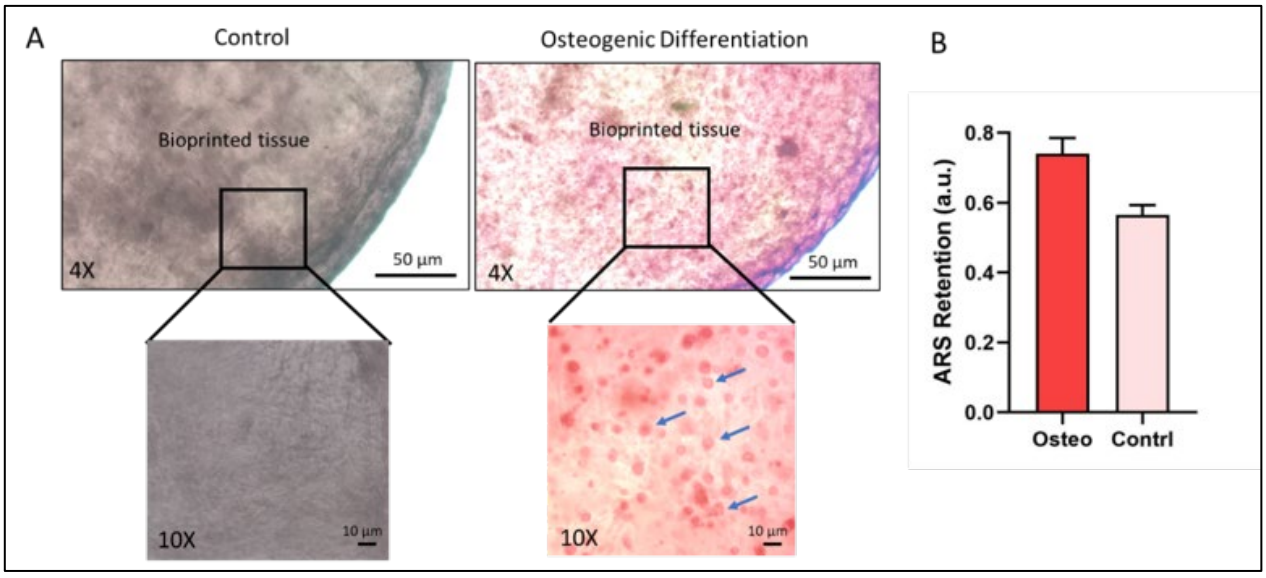

Figure 4. Osteogenic differentiation. (A) On Day 21, 3D bioprinted constructs were stained with Alizarin Red S stain and images were taken with a color camera at 4X and 10X magnification. Blue arrows indicate areas of mineral (calcium) deposition. Scale bars are as indicated in individual images. (B) Three samples from each treatment group were stained with ARS and the retention of the dye was measured using a plate reader. Comparative ARS retention was presented after normalizing to the OD_{405} of 40 mM standard solution.

As shown in Figure 5, there was no significant difference in CD31 gene expression between the groups. However, all the osteogenic genes were overexpressed in the osteogenic treatment group compared to the control group. Most notably, OCN gene expression was 6 times more prevalent in osteogenic differentiation group. Consistent with previous studies, these osteogenic genes are overexpressed in osteogenic differentiated MSCs (Wang, 2009; Byambaa, 2017) as these genes are critical for bone formation and regeneration. Although not explored in this project, a recent study by Byambaa (2017) showed that medium perfusion through the bioprinted construct enhances the expression of these osteogenic genes in differentiating MSCs.

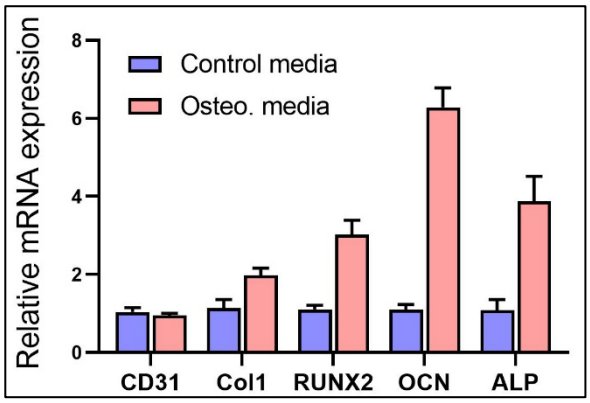

Figure 5. Quantification of gene expression by qPCR. Differences in gene expression were determined by SYBR Green-based qPCR method. Relative expression of mRNA was determined by comparing the Ct value and presented after normalizing with GAPDH expression.

Conclusions and future direction

- The 3D bioprinted model presented here can be used to study osteogenic differentiation of human bone marrow-derived MSCs. These primary cells exhibited excellent calcium deposition within the matrix and osteogenic differentiation-specific gene expression.
- GelMA Fibrin bioink provides necessary support and perfusion of nutrients to facilitate osteogenic differentiation of human primary MSCs.
- A modified ARS staining protocol can be adapted to study mineral deposition in 3D bioprinted constructs.
- Future direction includes identification of microvascular networks formation by HUVECs and their impact on long-term culture of the bioprinted bone tissue.

References

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