Bio-printing of living organized tissues using an inkjet technology

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Abstract

This study is directed towards developing a novel and versatile method for creating complex tissue structures that use simultaneous ink-jetting of multiple cell types. To demonstrate this concept, 3 different cell types, consisting of human amniotic fluid-derived stem cells (hAFSC), smooth muscle cells (SMC), and bovine aortic endothelial cells (EC), were printed by a thermal inkjet printer to form a heterogeneous cell configuration. Each of the printed cell types maintained viability, proliferation, phenotypic expression, and normal physiological function within the complex structures in-vitro. The bio-printed constructs were able to survive and mature into functional tissues with adequate vascularization in vivo. These findings demonstrate that fabrication of complex hybrid tissue structures that require multiple cell types can be achieved by using the inkjet technology.

Introduction

Living tissues maintain an inherent multi-cellular heterogeneous structure. Rebuilding of such complex structure requires subtle arrangements of different types of cells and extracellular matrices (ECM) at their specific anatomical target sites. Although enormous technological advances have been made to date, reconstitution of a functional complex tissue structure, consisting of multiple cell types has not been achieved. Inkjet printing technology has been proposed as a tool for building applicable biomaterials. Recently, single cells have been printed to demonstrate the feasibility of printing viable cells[1]. Although the capability of inkjet printing of viable single cells has been reported[2], simultaneous printing of multiple cell types to build viable heterogeneous cellular structures has not been demonstrated to date. This study investigates whether cell inkjet printing method could be employed to deliver multiple cell types to predetermined target sites for the creation of functional complex tissue structures for application *in vivo*.

Materials & Methods

Cell source and cell labeling

Human amniotic fluid-derived stem cells (hAFSC), canine bladder smooth muscle cells (dSMC), and bovine aortal endothelial cells (bEC) were used in the study. To identify each cell type from another, 2 different fluorescent dyes, PKH67(red) and PKH26(green) were used to label dSMCs and bECs, respectively, and hAFSC was transfected with lacZ retrovirus which can express -galactosidase and exhibit blue under X-gal staing, prior to inkjet printing.

Fabrication of hybrid cell structures

HP DeskJet 550C printers and inkjet cartridge (HP51626A) were modified by the methods previously described[1]. In order to build 3D structures, z-axis module with a controlled elevator chamber was added to the modified printers. After trypsinizing, cell pellets of each cell type were collected and re-suspended in $CaCl_2$ solution with a final concentration of $2\text{-}3\times10^6$ cells/ml. Print suspensions of each cell type were loaded into different ink cartridges. For a model system to generate multiple cell hybrid structures, a "pie" pattern was designed using Microsoft PowerPoint to program the printer. The designed "pie" was divided into 3 different parts and each area was designated for predetermined cell type in the final structure.

A thin layer of the alginate-collagen composite was applied onto a sterile coverslip. By using the modified HP printer, each type of cells suspended in $CaCl_2$ was over-printed successively into the designed locations of the "pie" structure. This procedure was then repeated for one more time. Another thin layer of the collagen-alginate composites was applied onto the previously gelled layer for the subsequent printing of other cell types with $CaCl_2$. A 3D "pie" structure entrapped with various cell types in different locations was generated through the process. To demonstrate the printer also can print different biomaterials, a collagen "pie" containing 3 different color dyes was also prepared.

To evaluate viability and proliferative ability of the printed 3D constructs, as well as to analyze phenotypic and physiological function of each cell type used in the "pie" constructs, individual 3D samples containing each cell type were prepared by the same method used for the hybrid cellular "pie" constructs.

Viability and proliferative ability of the printed cell constructs

The viability of the constructs containing dSMCs was evaluated by a two-color fluorescence live/dead assay using calcein AM and ethidium homodimer (EthD-1) (Molecular probes, OR). Proliferative ability of the printed constructs contained bECs was tested using the mitochondrial metabolic (MTT) activity assay.

Phenotypic analyses

Each cell type was printed onto a neutralized collagen gel and cultured in their respective culture media. The fluorescence-labeled antibodies, including anti-αSMC actin (Vector), anti-CD31 (Santa Cruz), and OCT3/4 (Santa Cruz), were used to confirm the expression of SMC, EC, and AFSC cells, respectively.

Functional evaluation

(1) Electrophysiology of smooth muscle cells

Conventional whole-cell patch clamp configurations were used for electrophysiological recordings from dSMCs before printing and after printing at 0 and 4 hours, and at 7 days. Whole cell currents were amplified with an Axopatch 200B, and digitized

with a Digidata 1322A (Axon Instruments, CA). The resting membrane potential of intact cells was measured immediately after rupture. To record K+ channel currents, a brief depolarization protocol was applied. Cells were held at -70 mV and the membrane potential was stepped from -60 to 60 mV in 10 mV increments with 200 ms of pulse duration.

(2) Intracellular calcium mobilization of endothelial cells within the printed constructs

Global calcium concentrations in bECs were measured on the cells with and without the interaction of bio-printing after 7 days of culture. The printed samples with bECs were incubated with the calcium-sensitive fluorescent dye fura-2 AM (Molecular Probes), for 60 minutes. Fura-2 ratio images were obtained using a computer controlled monochromator excitation light source (TILL-Photonics, Germany) and a cooled CCD camera with exposure control (SensiCam, Till-photonics). During excitation at 340 or 380 nm, emission at 510 nm was collected and 340/380 ratio images were generated with background subtraction following each treatment. As control, bECs were seeded onto a glass bottom dish and also evaluated after 7 days of culture.

(3) Osteogenic differentiation of amniotic fluid derived stem cells within the printed constructs

After printing, the constructs containing hAFSC were cultured in a defined osteogenic differentiation medium, which was composed of DMEM medium with FBS, dexamethasone, β-glycerophosphate, and ascorbic acid-2-phosphate. After 45 days of culture, calcium production from the printed constructs cultured in the osteogenic differentiation medium was measured by alizarin red staining. The staining results obtained for the printed hAFSC constructs in the osteogenic differentiation medium were compared with controls which were cultured in the hAFSC maintaining medium.

In-vivo study

The printed sample constructs were subcutaneously implanted into athymic mice. After 4 and 8 weeks, implants were scanned by an MRI scanner (Bruker-BioSpin, Germany), and histological evaluation was performed on the retrieved implants. All animal experiments were performed according to ACUC protocols at Wake Forest University Health Sciences.

Results

Fabrication of viable multi-phenotypic 3D hybrid constructs

The hybrid cell constructs with a "pie" shape were generated through direct and simultaneous inkjet printing of 3 different living cell types (SMC, EC, AFSC), together with $CaCl_2$, into different target locations on the uncrosslinked alginate-collagen composites. A complete 3D viable "pie" constructs containing the 3 cell types was observed under fluorescent and light microscopy 1 day after printing, as shown in Figure 1 (A). PKH 26 green dye stained bECs, PKH 67 red dye stained dSMCs , and lacZ transfected hAFSCs (exhibited blue with X-gal staining) were clearly examined and they were found to register into their respective pre-determined areas. The morphologies at a higher magnification of the fluorescently labeled dSMCs and bECs and the X-gal stained hAFSCs within the "pie" construct are shown in Figure 1 (B), (C), and (D), respectively. Figure 1 (E) shows a 3-D collagen "pie" with different color dyes, demonstrating the capability of the inkjet printers to print different biomaterials as well as multiple cell types

Viability and cell proliferation of the printed constructs

Figure 2(A) shows the mean viability of the dSMCs within the printed 3D constructs at 4 different time points as follows: before printing, and after printing for 4 hours, 1 day, and 7 days. It was found that after 4 hours the mean viability of the printed samples was approximately 5% to 10% less than that of the control group (cultured dSMCs). After 1 and 4 days of culture, the mean viability of the printed constructs increased gradually, and by 7 days the mean viability value was close to that of the controls. Figure 2(B) shows the proliferative ability of the bECs within the printed constructs at 4 different time points: 0 (immediately), 1, 4, and 7 days after printing. As shown in Figure 2(B), compared with the samples at day 0 (immediately after printing), the printed group had a smaller cell number 1 day after printing. After 4 and 7 days of culture, however, the bECs accelerated their proliferation rates within the printed samples and exhibited an increased cell number. After 4 days of culture, the printed samples had a similar proliferation pattern in the MTT curve as the controls, which bECs were cultured in a standard tissue plate.

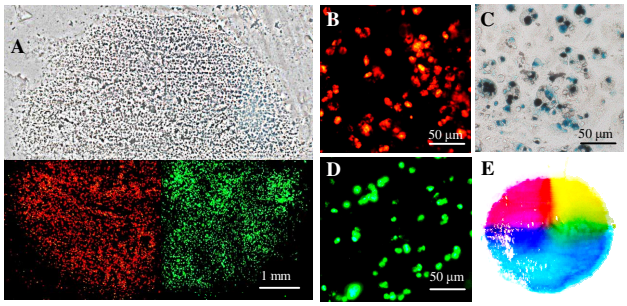

Figure 1. *(A) Fluorescent microscopic image of a viable 3-D cell constructs with a specific "pie" shape 1 day after culture. The cells that appear red are dSMCs labeled with pKH 67 dyes; the cells that appear blue are lacZ transfected hAFSCs with X-gal staining; the cells that appear green are bECs labeled with pKH 26 dyes. The enlarged images show the morphologies of dSMC (B), hAFSCs (C), and bECs (D). (E) A 3-D collagen "pie" with different color dyes.*

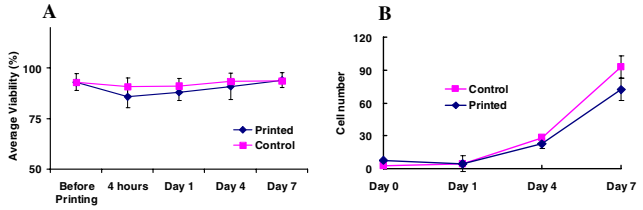

Figure 2. *Viability (A) and proliferative ability (B) of the printed 3D constructs. Viability of dSMCs within the printed constructs was evaluated by live/dead assay and compared with the non-printed samples (the control) (n=10). Proliferative ability of bECs within the printed constructs were analyzed by mitochondrial metabolic activity (MTT) assay and compared with the non-printed samples (the control)* (n=4).

Phenotypic analysis

Immunocytochemistry for printed dSMC, bEC, and hAFSC was performed after 2 days of culture (Figure 3). dSMCs were identified with α-smooth musle actin, bECS stained postiveivly for anti-CD31 (PECAM) antibodies, and hAFSC identified with anti-OCT 3/4. The immunocytochemical data indicates the preservation of dSMC, bEC, and hAFSC phenotypes after being printed.

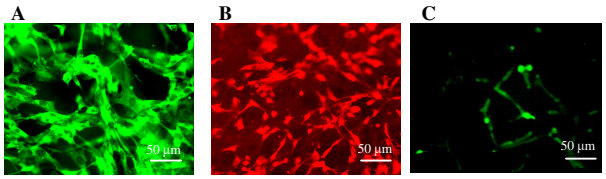

Figure 3. *Fluorescent microscopy of printed dSMCs (A), bECs (B), and hAFSCs (C) onto a collagen gel after being stained with their respective cell specific antibodies. (A): The printed dSMCs were stained positively with soomth muscle α action (green). (B): The printed bECs expressed CD-31 antibodies, a common epithelial cell specific maker (red). (C): The nuclei of hAFSCs were stained in blue by OCT 4, one of the most recognized markers used for the identification of ES cells.*

Functional evaluation

(1) Electrophysiology of smooth muscle cells within the printed constructs

Potassium (K) channels are thought to be the key regulators of membrane potential in many smooth muscles. Hyperpolarization or depolarization of the membrane influences transmembrane Ca^{2+} flux, and subsequently, regulates the tone of smooth muscles, including bladder smooth muscle. In this study the voltage clamp model was used to analyze the potassium currents of the dSMCs under four different conditions. Outward currents were evoked by stepping from -60 mV to 60 mV in 10 mV increments, and the prominent outward K currents were detected in each condition. Figure 4(A) shows the summary of the current-voltage relationships expressed by mean outward current under four different conditions. As shown in Figure 4(A), dSMCs after printing in 0 hour, 3 hour, and 7 days exhibited similar patterns in the mean current-voltage relationships as the cells before printing. These data suggest that dSMCs are able to maintain normal electrophysiological characteristics after the printing in different time periods, although there were slightly diminished outward currents in the cells after the printing.

(2) Intracellular calcium mobilization of endothelial cells within the printed constructs

It has been demonstrated that the spatial and temporal dimensions of Ca^{2+} signaling play an important role in physiological functions of endothelial cells, such as regulating the generation of nitric oxide. Vascular tone is affected by vasorelaxation factors released from endothelial cells, i.e., nitric oxide and endothelium-derived hyperpolarizing factor, whose production and release are known to depend on intracellular Ca^{2+} concentration ($[Ca^{2+}]i$). The peptide bradykinin is an endothelium-dependent vasodilator for most blood vessels. Therefore, bradykinin-induced $[Ca^{2+}]i$ response measured with fura-2 becomes a useful tool that can determine the physiological functions of endothelial cells. Figure 5(B) shows the measurements of intracellular Ca2+ concentration ([Ca2+] i) in printed endothelial cells and non-printed (the control). The bradykinin-induced increases in [Ca2+]i are similar between the printed bEC cells and the controls.

(3) Osteogenic differentiation of amniotic fluid derived stem cells within the printed constructs

The calcium production and nodule formation in the printed constructs under osteogenic differentiation culture were analyzed by alizarin red S staining that gives red staining image for calcium salt crystallization. As shown in Figure 4©, after 45 days culture, a strong alizarin red staining was apparently seen in the printed samples. Moreover, nodule formation was also observed, as shown in darker zones within the samples. The alizarin red staining results suggest that hAFSCs within the 3D printed constructs are able to retain their capability to differentiate into specific cell lineages under appropriate conditions.

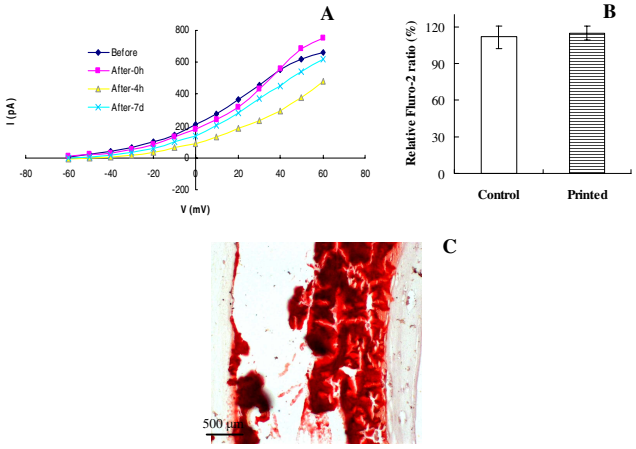

Figure 4. *(A) Mean current-voltage relationships of steady-state K+ outward currents obtained from the dSMCs within the printed constructs at 4 different time points: before printing and after printing in 0 and 4 hours, as well as 7 days. (B) Measurements of intracellular Ca2+ concentration ([Ca2+]) for bECs within the printed constructs: mean percentages of the relative Furo-2 ratios for the printed bECs and non-printed bECs (the control). Alizarin red staining of the hAFSC-printed 3D constructs in the osteogenic differentiation medium (C) after 45 days of culture.*

In-vivo analyses

Survival, maturation, and vascularizaton of the printed 3D constructs *in vivo* were evaluated following subcutaneous implantation of the printed samples. Figure 5(A) and (B) show Magnetic Resonance Imaging (MRI) of the bEC-printed samples and the scaffold-only implant (the control) after 8-week implantation, respectively. After the gadolinium (Gd) contrast agent was injected intravenously into the mouse, the contrast enhancement was visualized within the bEC-printed implants, which indicates the presence of vascular network within the implanted tissue. However, a minimal enhancement was observed within the control implants. The MRI imaging data suggests that EC-printed implants formed functional vasculatures which integrated into the existing vasculatures. Figure 5(C) and (D) show gross examinations of the bEC-printed samples and the controls retrieved after 4-week implantation, respectively. The bEC-printed implants exhibited a more abundant vasculature than the controls (the scaffold only implant). A higher magnification image of the vasculature on the surface of the cell printed implants is shown in Figure 5(E). Figure 5(F) shows Haematoxylin and Eosin (H&E)

staining of the cross-section of the bEC-printed implants, and small blood vessels were seen within the bEC-printed implants. These data indicate that adequate vasculatures were formed within the printed implants.

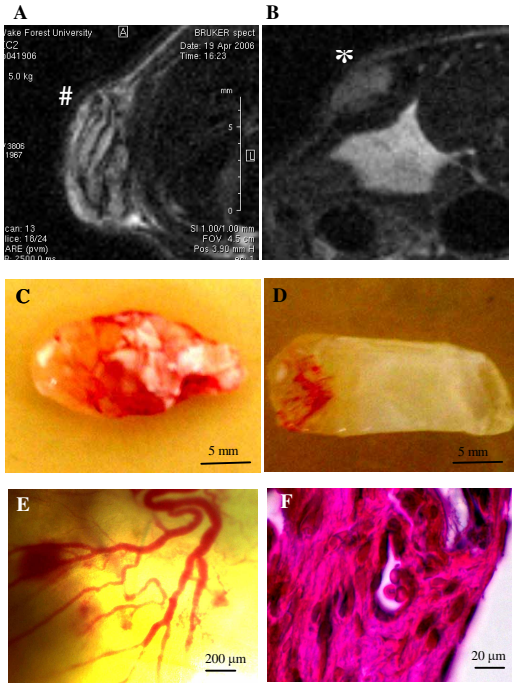

Figure 5. *In-vivo analyses of the bEC-printed cell constructs. MRS scanning of the printed sample (A) and the control (scaffold-only implant) (B) after 8-wk implantation. Gross examination of the printed samples (C) and the control (scaffold-only implant)(D) after 4-wk implantation. Microscopic images of surfaces (E) of the bEC-printed implants (E). H&E staining of the cross-section of the bEC-printed implants (F).*

Discussion

Organs or tissues exhibit complex multi-material, multi-cellular heterogeneous structures. More than 200 types of cells exist in the human body and each of them plays a specific role in its respective anatomic sites. Building such a complex structures with specific cells in their respective sites for functional replacement of diseased tissues has been a challenge. Various technological applications, including cell patterning and tissue fabrication methods, such as photolithographic techniques[3] and microfludic channels[4], have been attempted to effectively deliver living cells into designed space. However, none has shown success, due to the difficulty in delivering multiple cells into the 3 dimensional target space simultaneously.

In this study an ink jet-based technique has been developed to address this challenge. A 3D hybrid cell construct with a "pie" shape configuration containing 3 different cell types was generated by using the inkjet printing technique. The printer used in the study is based on a drop-on-demand mechanism, in which ink is ejected only after receiving a demand signal from the computer followed by execution by the printer. In this study, a pre-designed pattern, in a "pie" configuration was generated by a computer, which enabled the delivery of 3 different cell types into their respective target locations, leading to the formation of a complex functional tissue system. Each cell type retained their phenotypic and functional characteristics. Furthermore, all of the implanted constructs formed by the inkjet printing technique were able to survive, mature and form adequate vascularization.

Conclusion

To our knowledge, this is the first report that demonstrates the generation of multi-phenotypic hybrid 3D constructs by using the inkjet printing technology. This study shows that viable three-dimensional heterogeneous constructs with multiple cell types can be created by printing multiple cells with cross-linkers into alginate-collagen composites layer-by-layer. The printed cells are able to survive and proliferate within the 3D constructs, and are able to maintain normal cellular properties and function in their spatially registered regions *in vitro*. *F*urthermore, the cell-printed constructs are able to survive and mature to tissues with adequate vascularization when implanted *in vivo*. These findings demonstrate that it is possible to create complex tissues that require multiple cell types and ECM materials by using the ink-jet technology.

References

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Author Biography

Tao Xu received his BS in polymeric materials from Beijing University of Aeronautics & Astronautics (1994) and his PhD in bioengineering from Clemson University, SC (2005). Since then he has worked in Wake Forest Institute for Regenerative Medicine, NC. His work has focused cell and organ printing and their applications in regenerative medicine. He received a 2004 Materials Research Society Graduate Student Gold Award for his contribution in engineering functional cardiac tissues using the inkjet technology.