3D Bioprinted muscle on a chip

A. Esteban Tezanos[1,2], M. Badiola Mateos[1,2], J. Samitier Martí[1,2,3*]

[1] Institute for Bioengineering of Catalonia (IBEC) - Barcelona Institute of Science and Technology, Barcelona, Spain

[2] University of Barcelona, Barcelona, Spain

[3] Centre for Networked Biomedical Research (CIBER-BBN and CIBER-NED), Madrid, Spain

*** Correspondence should be addressed to: jsamitier@ibecbarcelona.eu**

Abstract

Organs-on-a-chip enable good mimicking of pathophysiological conditions, multicellular architecture, tissue-tissue interfaces and physicochemical microenvironments in 2D and 3D. Skeletal muscle has a unique 3D structure that facilitates contraction. Biochemical, mechanical and electrical stimuli enable the differentiation of muscle cells into myocytes. However, its functionality highly depends on the microenvironment. The purpose of this project was to replicate muscle fibre mimicking physiological conditions through 3D bioprinting method in combination with cell-laden hydrogels and sacrificial moulding techniques. We first optimised the differentiation of C2C12 mouse myoblast into myocytes through biochemical stimuli and performed a viability test embedding cells in different combinations of ECM composites (Collagen I, Matrigel, and alginate). The optimal ECM composition was used to bioprint a muscle fibre. The results show the survival of myoblasts to printing process and adequate proliferation and differentiation afterwards. On the other hand, C2C12 myoblasts were transduced with a lentivirus containing Channelrhodopsine-2 (ChR2), differentiated and stimulated with light to induce contraction afterwards. C2C12 ChR2$^+$ cell line was successfully established, though we are still optimising light stimulation parameters to induce contraction. This study presents the basis of an in vitro model for biomedical studies that combines biochemical and optical stimuli, hydrogels and 3D bioprinting to enable successful mimicking of muscle tissue.

1. Introduction

The broad range of study models used for Biomedical applications includes animal models of human diseases, *in vitro* models (cell culture, bacteria, viruses and yeast) or modelling and simulation systems [1]. These models do not need to replicate exactly human conditions, though they should be reproducible and representative. Organs-on-a-chip consist in the fabrication of devices that replicate models on a microfluidic platform of sizes between millimetres and centimetres. They are used for relatively short (less than 1-month duration) pathophysiological process studies in structures of tens to hundreds of micrometres [2]. There are different techniques to design microdevices, but three-dimensional (3D) bioprinting is driving major innovations in the creation of human tissue-like structures. Mimicking physiologically relevant functional units *in vitro* would be a great advance in research of diseases with the possibility of translation to human studies and the creation of artificial organs [3]. Replication of skeletal muscle through 3D bioprinting is evolving exponentially both in Biomedical and Biorobotics fields.

Muscle tissue is formed by high density of cells embedded in an extracellular matrix (ECM), which is a set of materials such as proteins and carbohydrates that forms the interstitial (intercellular) environment[4]. The biochemical composition of the ECM is important to maintain the physical, topological, chemical and physiological properties of the sarcomere structure of the skeletal muscle. The characteristics acquired by the matrix trigger focal adhesions, affect cell morphology, movement and function [3,4]. Physiological characteristics of the ECM, which induce differentiation of myoblasts into myocytes and produce contraction, can be achieved with biochemical, mechanical and electrical stimuli. The integrity of muscle tissue relies in the adhesion among cells and between cells and the ECM [5].

Tissue engineering techniques such as Bioprinting and Optogenetics can be applied for the formation of *in-vitro* skeletal-muscle models. 3D Bioprinting creates spatially-controlled cell patterns in 3D preserving cell viability and function. It is a potential technique for engineering skeletal muscle cells based on the design of a 3D matrix that matches the physiological structure of muscle cells [5]. Optogenetics technique combines genetic manipulation and optical stimulation for cells excitation. Channelrhodopsine-2 (ChR2), a light-activated cation-selective membrane channel, can be used in skeletal muscle cells to induce contraction [6,7].

The proposed *in-vitro* model was to mimic skeletal-muscle physiology by making a non-cytotoxic 3D bioprinted structure that maintains cellular organization with relevant size, shape and structural integrity. The objectives of the proposed project were: to culture and differentiate myoblasts into myocytes, to 3D-bioprint cells into an ECM and to induce muscle fibre contraction by means of optogenetics.

2. Materials and methods

2.1. Cell culture and differentiation

C2C12 mouse myoblast (ATCC, CRL-1772), were seeded at a density of 2000cells/cm^2 in proliferation medium: DMEM, + 10 % FBS (Phoetal Bovine serum) + 1% Penicillin Streptomycin. Passages were made when cells reached 70% confluence and medium was changed every 2-3 days. To induce differentiation into myocytes, cells were seeded at 18.000 cells/cm^2 and changed to differentiation medium (DMEM + 5% HS, Horse Serum,

+ 1% Penicillin Streptomycin + 1% L-Glutamine + 2.5% HEPES) when they reached 80% confluence.

2.2. 3D cultures

Cells were seeded at a density of $1x10^6$ cells/ml embedded in a composite material (50% Collagen-I + 40% Matrigel + 10% medium + 0.25% alginate). For that purpose, the pellet of tripsinised C2C12 cells was resuspended in different ECM material compositions handling them on ice: **i)**Matrigel 75%, Collagen-I 25%; **ii)** Matrigel 25%, Collagen-I 75%; **iii)** Matrigel 50%, Collagen-I 40%, Medium 10%, Alginate 0,25%. The cell/gel solution was seeded in drops of 30µL and incubated for 10 minutes at 37ºC to gel the material. Then drops were covered with proliferation medium to maintain the culture. After 2 days in vitro (DIV), the proliferation medium was changed to differentiation medium. Viability of cells in 3D in each ECM composite was assessed checking the morphology and differentiation of cells in different layers every day during a week.

2.3. 3D bioprinting

BioCAD software was used to create the design to be printed, containing a rectangle-shape sacrificial mould compressing a muscle fibre inside (*Figure 1A*). C2C12 cells were tripsinised and the pellet was resuspended in the ECM material, loaded in the cartridge and incubated for 30 minutes to gel the material. Another cartridge was loaded with Pluronic-F127. The bioprinter (3D Discovery bioprinter, regenHU) was adjusted to print with both cartridges at the same time following the BioCAD design. The biocompatible sacrificial mould of Pluronic-F127 was printed in microextrusion mode adjusting pressure to 3,3 bar and feed rate to 7 ms. Cells/gel solution was printed in inject mode with a microvalve and the following parameters: pressure 0.5 bar, feed rate 10 ms. After bioprinting the whole design, it was incubated at 37ºC for 10 min to gel and then washed with proliferation medium (*Figure 1B*) and incubated at 27ºC for other 10 min, letting the sacrificial mould dissolve. Once the Pluronic-F127 structure was washed, the bioprinted fibre was covered with proliferation medium. After 3 days *in vitro*, the medium was changed to differentiation medium.

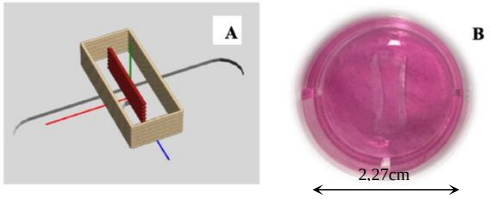

Figure 1*: Bioprinted skeletal-muscle model:* ***A)*** *BioCAD design containing a rectangle for the sacrificial mould and straight line inside for Cells/gel solution.* ***B)*** *3D structure outcome of muscle fiber after printing process.*

2.4. Establishment of C2C12 ChR2+ cell line

C2C12 cells were transduced with a ChR2 positive lentivirus containing YFP (Yellow fluorescent protein) sequence as a reporter (pLenti-EF1a-hChR2(H134R)-EYFP-WPRE, Addgene), kindly donated by Prof. del Río Fernández. The lentivirus was added during 24 hours, and transduced cells were purified through FACS (Fluorescent Activated Cell Sorting) obtaining C2C12 $ChR2^+$ cell line. Once C2C12 $ChR2^+$ cells were differentiated, it was applied light stimulus of 470 nm at the following frequency: 1 Hz (20 ms, 2000 pulses).

3. Results

Skeletal muscle cells cultured in two dimensions proliferated adhered to the surface acquiring a randomised configuration (*Figure 2A*). Biochemical stimuli facilitated myoblasts differentiation to myocytes. Physiological characteristics of skeletal muscle tissue, such as myotube formation and cell alignment (*Figure 2B*), were observed in 2D cultures.

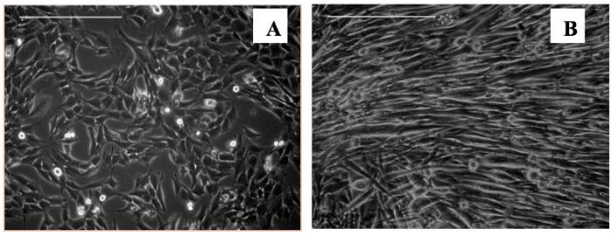

Figure 2*: C2C12 culture (scale bar, 300µm):* ***A)*** *Undifferentiated cells with fusiform morphology in random structure after 1 DIV.* ***B)*** *Differentiated aligned cells with myotube morphology after 7 DIV.*

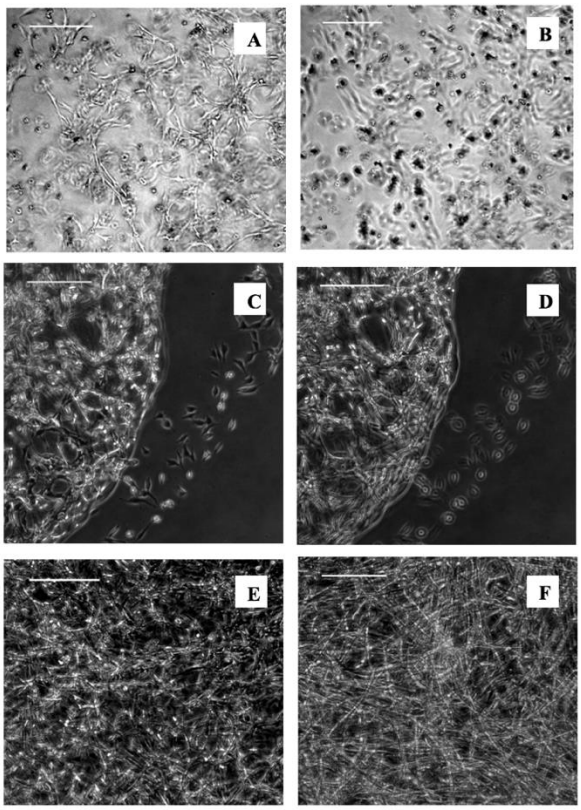

Figure 3*: C2C12 cells embedded in 3D composites of Matrigel and Collagen drops (scale bar 300µm):* ***A)*** *Cells live in high Matrigel concentration (75%) at the bottom of the drop.* ***B)*** *Cells die in high concentration of Matrigel at top layers of the drop.* ***C)*** *Cells live in ECM with high concentration of collagen (75%) at bottom layers of the drop.* ***D)*** *Cells live in highly concentrated collagen ECM at top layers of the drop.* ***E)*** *Cells live in 3D structure at ECM composed of Matrigel, Collagen and Alginate 0, 25% after 2 days in vitro.* ***F)*** *Differentiated cells live in ECM with Alginate 0,25% after 7 DIV.*

3D cultures viability tests showed that cells embedded in Matrigel survived only at the bottom of the plate, adhered to the surface at tested conditions (*Figure 3A* and *B*). While when they were cultured in Matrigel – collagen I mixture, they acquired 3D structure in suspension (*Figure 3C* and *D*). When they were embedded in more viscous composites (containing 0,25% alginate), cells proliferated and differentiated properly (*Figure 3E* and *F*).

3D bioprinted skeletal-muscle fibre model was successfully printed, achieving viability of cells after printing process with successful proliferation and differentiation afterwards (*Figure 4*).

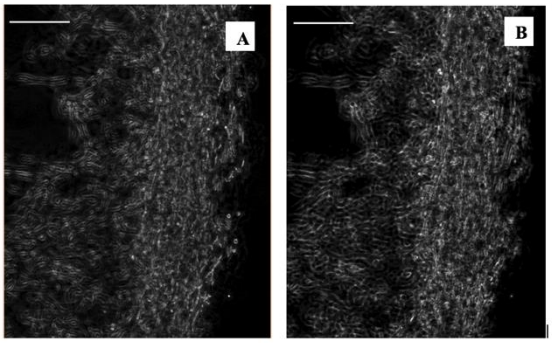

Figure 4*: Viability of bioprinted C2C12 cells after 2 DIV (scale bar 300 µm):* ***A)*** *Bioprinted C2C12 cells live at bottom layers.* ***B)*** *Bioprinted C2C12 cells live at the top layers.*

After 8 *DIV*, 6 days with differentiation medium, cells had differentiated into myocytes (*Figure 5*).

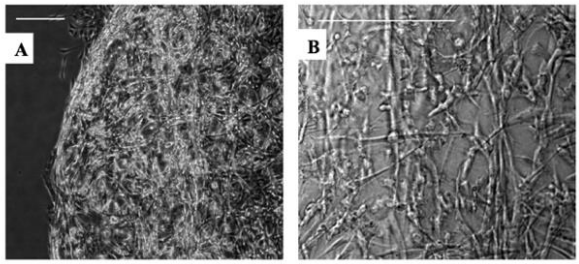

Figure 5*: Post- bioprinting stage (scale bar, 300 µm). Bioprinted C2C12 cells differentiated after 8 DIV, acquiring 3D mesh structure* ***(A)*** *and elongated morphology* ***(B)****.*

Transduction and purification of C2C12 cells generated the C2C12 ChR2^{+} cell line expressing YFP in the cell membrane (*Figure 6*).

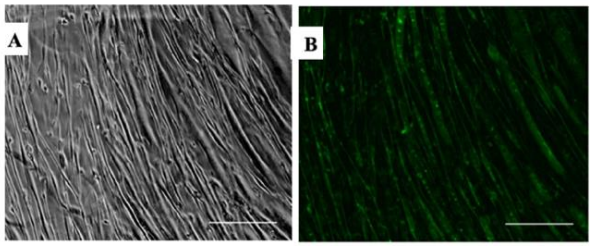

Figure 6: *C2C12 ChR2^{+} cells after 10 DIV (scale bar, 200 µm).* ***A)*** *Bright field image of myocytes.* ***B)*** *Cells expressing YFP in the membrane.*

4. Discussion

Skeletal muscle has a unique 3D sarcomere structure, required for its function of contraction. This structure is acquired with: uniform distribution of oxygen and nutrients, cell alignment, uniform tension and high density of cells [8]. The combination of 3D structures and biomaterials creates tissues capable of mimicking physiological conditions. This approach may satisfy the functional requirements of the cells as well as the structural requirements of the tissue.

Organs-on-chips have the potential to serve as a new platform enabling to identify and validate the efficacy, safety and viability of potential targets before clinical trials. Microfluidic devices that mimic organs require a complicate multi-step process of fabrication, such as photolithography among others [3]. This makes small differences among each device fabricated, whereas 3D bioprinting is an automatic technique that can create identical replicas. During bioprinting processes, cells are subjected to thermal and mechanical stress that might modify their behaviour, functionality and difficulty to vascularise. Furthermore, complex structures that are difficult to hold up and vascular systems that ensure the supply of oxygen and nutrients to cells are required to ensure long-term cell viability in 3D bioprinted models [8].

Almost all human tissues have complex combinations and gradients of ECM components, each with specific biological and mechanical influences [4]. 3D cultures were made to test the biocompatibility of materials with cells and to select the best cell/gel solution composition to mimic native ECM. The composition of materials where cells are cultured is very important because the physical surrounding plays an important role in cells behaviour [4]. Biochemical components can promote differentiation, inducing morphological changes by alteration of receptors [4]. One of the main challenges in the 3D bioprinting field is to find materials that are not only biocompatible but can also undergo printing process. Materials currently used in the field of tissue engineering are predominantly based on naturally derived polymers (including alginate, gelatine, collagen, chitosan, fibrin and hyaluronic acid) often isolated from animal or human tissues, or synthetic molecules (polyethylene glycol derivatives for example) [5]. Collagen-I is one of the predominant proteins in muscle tissue, located generally in strong and resilient tissues, and it has been crucial to replicate muscle tissue in tested conditions as it provides cells the adequate surface stiffness to proliferate. All naturally derived polymers require hydration in order to maintain elasticity and plasticity character that facilitates contraction. But bioprinting procedure requires the addition of a gelling agent (alginate 0,25%) to the ECM composition because the viscosity of the Matrigel-Collagen composite is very low. By adding alginate, we slightly increased the viscosity preserving cell viability. However, extrusion bioprinting of low viscosity materials still represent a challenge, as they cannot not hold up a structure until they gel. Sacrificial moulding technique facilitates compression of the bioprinted low viscosity structures (i.e. cell/gel solution) while it gels, avoiding spreading or spilling phenomenon of the ECM material after extrusion, and making possible to replicate physiological fibre structure.

Optogenetics technology enables the control of processes at millisecond scale and with cell specificity precision [7]. It represents a milestone for the available tools required for bioengineering. For this application, it is necessary to perform genetic manipulation of cells. When performing the transduction, if the virus is in contact with cells for a short time, it might not integrate its genotype. But if it is left for too long time, in some cases, it might integrate too many times or even harm cells. Once C2C12 $ChR2^+$ cells are differentiated, optical stimulation (2000 pulses of light of 20 ms) at 470nm enables non-invasive external control over contraction.

Future steps include the optimisation of optogenetics protocol and characterisation of bioprinted muscle fibres though immunostaining protocols, looking at skeletal-muscle differentiation markers.

5. Conclusions

Skeletal muscle tissue has a unique multicellular architecture and physicochemical microenvironments in 3D, both responsible for the generation of the mechanical contraction. The problems generally carried out in traditional culture assays enclose the physiological relevance, performance and long term survival. In 2D muscle cells lose cell-alignment and mechanical properties that facilitate contraction.

In this study we achieved: i) the differentiation of C2C12 myoblasts into myocytes after 7 days in vitro; ii) viability of C2C12 cells in 3D that create lattice structures in stead of proliferating adherent to the surface like in 2D structure; iii) the creation of bioprinted muscle fibre in 3D with an artificial ECM of Matrigel, Collagen-I and Alginate that contributes to cellular proliferation and functionality; iv) the establishment of C2C12 $ChR2^+$ cell line.

Organs-on-a-chip are helpful to mimic pathologies in physiological conditions *in vitro* and facilitate the development of treatments for diseases, diagnostic tools or new therapeutic methods. Creating models like bioprinted skeletal muscle represents a huge progress in tissue engineering for the advance in personalised and regenerative medicine. However, it still requires lots of years of research to bring these concepts into clinical trials.

6. Acknowledgments

First of all, thanks to the Faculty of Medicine of the University of Barcelona (UB) for selecting the project to the CASEIB price and to the Institute for Bioengineering of Catalonia (IBEC) where it has been performed. Express gratitude to the *Nanobioengineering* group, where the research is performed and the *Mollecular and cellular neurobiotecnology* group lead by Prof. del Río Fernández, that have kindly donated the virus. The Nanobioengineering SIC-BIO group is supported by the Commission for Universities and Research of the Department of Innovation, Universities, and Enterprise of the Generalitat de Catalunya (2014 SGR 1442). This work was partially supported by the MINDS project (TEC2015-70104-P) and BIOBOT (TEC2015-72718-EXP), awarded by the Spanish Ministry of Economy and Competitiveness and the CERCA program from Generalitat de Catalunya. CIBER-BBN is an initiative funded by the VI National R&D&i Plan 2008-2011, Iniciativa Ingenio 2010, Consolider Program, CIBER Actions and financed by the Instituto de Salud Carlos III with support from the European Regional Development Fund. Finally, thanks to previous CASEIB organizing committees for allowing generously to use their guides.

7. References

[1] Esch EW *et al.* Organs-on-chips at the frontiers of drug discovery. *Nature*, vol 14, sup 4, 2015, pp 248–260 (ISSN: 1474-1776).

[2] Bhatia SN, Ingber DE. Microfluidic organs-on-chips. *Nature biotechnology*, vol 32, sup 8, 2014, pp 760–772 (ISSN: 1087-0156).

[3] Uzel SGM *et al.* Microfabrication and microfluidics for muscle tissue models. *Progress in Biophysiics and Molecular Biology*, vol 115, sup 2-3, 2014, pp 279-293 (ISSN:0079-6107).

[4] Hinds S *et al.* The role of extracellular matrix composition in structure and function of bioengineered skeletal muscle. *Biomaterials*, vol 32, sup 14, 2011, pp 3575–3583 (ISSN: 0142-9612).

[5] Murphy S V, Atala A. 3D bioprinting of tissues and organs. *Nature biotechnology*, vol 32, sup 8, 2014, pp 773–785 (ISSN: 1087-0156).

[6] Nagel G et al. Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. *Proceedings of the National Academy of Sciences of the United States of America*, vol 100, sup 24, 2003, pp 13940–5 (ISSN: 0027-8424).

[7] Raman R et al. Optogenetic skeletal muscle-powered adaptive biological machines. *Proceedings of the National Academy of Sciences of the United States of America*, vol 113, sup 13, 2016, pp 3497–502 (ISSN: 1091-6490).

[8] Kolesky DB et al. 3D Bioprinting of vascularized, heterogeneous cell-laden tissue constructs. *Advanced Materials*, vol 26, sup 19, 2014, pp 3124–3130 (ISSN: 0935-9648).