Vascularization of Human Embryonic Stem Cell-derived Cardiac Tissues

Lara Filipa Ferreira Mil-Homens | lara.mil-homens@tecnico.ulisboa.pt

Instituto Superior Técnico, Lisboa, Portugal

October 2018

Abstract

Cardiovascular diseases can leave permanent lesions and cardiac repercussions, especially concerning extensive cardiomyocyte death and regression of coronary microvasculature, as a result of heart failure, myocardial infarction or ischemia/reperfusion episodes. In the past few decades, several approaches aiming to restore the native function in damaged hearts have been attempted, including through the generation of tissue-engineered cardiac constructs that are intended to repopulate the injured cardiac muscle with a new group of contractile cells. However, the major limitation to these constructs has been their inadequate vascularization and reperfusion *in vitro* that highly limits their long-term efficacy and viability, particularly in thick and complex tissue-constructs. Moreover, the paracrine cross-talk between endothelial cells and cardiomyocytes also plays a critical role in improving tissue functionality, which can further promote the tissue-engineered construct survival. In the present work, a fluidic device able to support the co-culture between cardiomyocytes, entrapped in a fibrin matrix, and endothelial cells, both derived from human sources, was successfully established. In this currently optimized fluidic device, it was possible to generate several human beating cardiac constructs with endothelial cells co-presented in their networks, establishing significant ground work for further formation of a vascular system throughout the entire construct.

Key-words: Vascularization, Tissue Engineering, Cardiomyocytes, Endothelial Cells, Organ-on-a-Chip, Co-culture.

Introduction

Cardiovascular Diseases

Cardiovascular diseases include a range of conditions that affect the structures and/or function of the heart and blood vessels and are among the leading causes of morbidity and mortality in the worldwide nations. Although some pharmacological experiments have led to some improvements in patients affected by cardiovascular diseases, heart transplantation remains to be the gold-standard treatment. Due to the low availability of transplantable hearts, engineered cardiac constructs are one of the most promising alternatives to restore the native myocardial function. Furthermore, accurate prediction of proarrhythmic side effects of drugs in cardiovascular-related preclinical trials would also highly benefit from improved human heart models, since most of these studies are currently performed using animal models which naturally exhibit substantial genomic and physiological differences [1].

Cardiomyocyte Differentiation and Immaturity

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), have the ability of unlimited self-renewal and intrinsic potential of differentiation into all the specialized cells, including heart cells, with a high cardiac differentiation efficiency compared to other proliferative cell types (e.g. bone marrow stem cells) [2]. Hence, constituting the most promising cell source for the generation of functional human cardiomyocytes. Nevertheless, hPSC-derived cardiomyocytes are often grown on standard two-dimensional (2D) contexts, that barely recapitulate the native cardiac environment, ending up by inducing an immature state in the newly differentiated-cardiomyocytes [3]. This immature state can negatively affect their functional properties, limiting their envisioned use for regenerative purposes, as well their ability of providing predictive drug responses. In order to address these limitations, tissue-like three-dimensional (3D) environments that can provide adequate structural cues [3], by properly mimicking the surrounding environment of cardiomyocytes *in vivo*, and medium supplementation with triiodothyronine (T3) hormone [4], insulin-like growth factor-1 (IGF-1) [5] and galactose [6], that provide pivotal biochemical cues to cardiomyocytes, have been intensively explored in the last years in order to further the maturation of these cells *in vitro*.

Cardiac Tissue Engineering Strategies

Several approaches can be pursued in order to generate cardiomyocytes embedded in a 3D-structure *in vitro*, being the most common to make use of the endogenous ability of cardiomyocytes to naturally assemble and form functional

syncytium, ally to engineering techniques to form those constructs with the intended size, geometry and orientation [7]. Among these strategies, hydrogel encapsulation methods are probably the most resorted technology to generate engineered heart muscles *in vitro* due to their structural similarities to the extracellular matrix (ECM) and intrinsic ability to support cell adhesion and growth [8].

Hydrogel Encapsulation Methods

The technology behind hydrogel methods require essentially four main factors, that can be summarized in: heart cells (or cardiomyocytes), solutions of gelling natural products or hydrogels, a casting mold and an anchoring support [9].

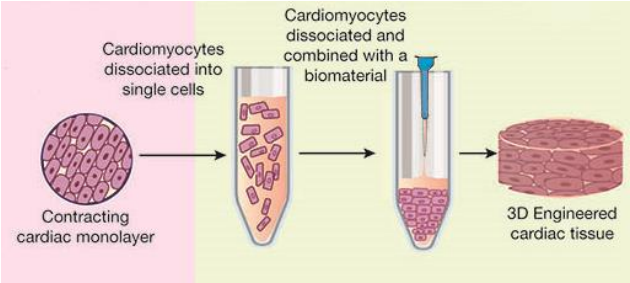

Figure 1: Schematic illustration of a 3D-engineered cardiac tissue obtained from a hydrogel-based method. The hPSC-committed cardiomyocytes are combined with the hydrogel and placed in a casting mold that will confer the 3D profile to the developing tissue. Adapted from [10].

Shortly, the liquid hydrogel is used to entrap the cells during its gelation time in the 3D shape provided by the casting mold; while the anchoring points allow the growing tissue to stay fixed to the casting mold [7]. Additionally, the anchoring points expose the cells to a continuous mechanical strain since the cells are constantly generating force (of contraction) against the structures to where they are attached [7]. This mechanical strain is recognized as one of the main advantages of this approach since it allows the cardiomyocytes to align parallel to the force lines, resembling their alignment in the myocardium, and largely contributing to their development and maturation *in vitro* [7]. Besides these four main factors, the supplementation with horse serum (HS) is also a crucial point when culturing engineered heart tissues (EHTs), since the withdrawal of this component leads to a rapid decline in the contractile activity of cardiomyocytes that culminates in the complete absence of a beating activity over time for still unknown reasons; whereby EHTs depend on the continuous presence of horse serum at 10% (v/v), like fully investigated by Eschenhagen et al. [11].

Types of Hydrogels

Natural and synthetic hydrogels are both suitable for tissue engineering purposes due to their soft and viscoelastic properties that closely mimic the native cardiac tissue [12]. Nevertheless, natural hydrogels are biodegradable, non-cytotoxic as some synthetic polymers and are naturally compatible with cell adhesion [12]. In the last few years, between natural hydrogels, fibrin(ogen) has been increasingly elected, due to its faster gelation time that highly contributes to a more homogenous cellular distribution among the gel [13], nonlinear elasticity that is expressed in a higher elastic modulus when exposed to shear stress, high softness that contributes to cardiomyocytes with higher motility [14] and easy synthesis *in vitro* by the thrombin-catalyzed conversion of fibrinogen into fibrin [13]. Along with that, the rate of degradation of fibrin (fibrinolysis), by the active fibrinolytic components present in the horse serum and by the increased production of ECM proteins as matrix metalloproteinases (MMPs) by the cardiomyocytes [15], can easily be controlled *in vitro* by the addition of antifibrinolytic agents such as the aprotinin, that significantly decreases the concerned rate [13].

The Importance of a Vascular System

However, the generation, and posterior scaling-up, of fibrin-based cardiac tissues, as any other 3D-engineered tissue, is hampered by the lack of an intrinsic vascular system able to assure constant flows of oxygen and nutrients to the tissue, as well as escape routes to eliminate carbon dioxide and other cellular wastes, in order to assure its long-term viability and functionality [16]. The vascular system is composed of several blood vessels that are formed *in vivo* in a process known as vasculogenesis, that initially involves the coalescence of mesoderm-derived endothelial cells to form the primary lumen structure of blood vessels, followed by the recruitment of other cell types (e.g. mesenchymal stromal cells), to form the final and mature structure of a blood vessel, under the influence of paracrine signaling from endothelial cells (ECs) [17].

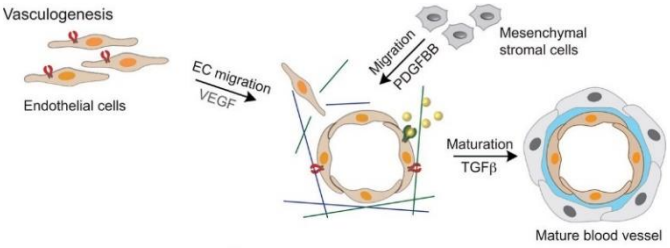

Figure 2: Schematic illustration of the vasculogenesis process. Under the influence of the vascular endothelial growth factor (VEGF) signaling pathway, endothelial cells (ECs) start to migrate and form tubular-like structures. Afterwards, signaling molecules secreted by the ECs recruit the remaining cells required for the obtention of a mature blood vessel. Adapted from [17].

Vascularization Strategies

Several approaches aiming to promote vascularization *in vitro* have been developed in the last few years, from where can be highlighted co-culture systems between the intended tissue to be vascularized and endothelial cells [18]. These approaches rely on the endogenous ability of endothelial cells to self-assembly in lumen-like structures, especially in the presence of angiogenic factors (e.g. vascular endothelial growth factor (VEGF)), since interesting factors that induce angiogenesis *in vivo* (the formation of new blood vessels from pre-existing ones) stimulate the sprouting-like of endothelial cells *in vitro* [18].

Co-culture between Endothelial Cells and Cardiomyocytes

Cardiac endothelial cells are in close contact with cardiomyocytes in the human myocardium, not only just to ensure an efficient physiological transport of nutrients and oxygen, but also to allow a local communication between these cells through several paracrine signaling [19]. The intrinsic communication pathways established between these cells, specifically through physical cellular interactions and diffusion of signaling molecules (growth factor delivery) are in part responsible for promoting the growth of vasculature [19]. Moreover, the concerned cross-talk is also highly beneficial for the cardiomyocytes, since mediators secreted by endothelial cells also positively influence cardiomyocytes survival, development and spatial organization, consequently promoting their maturation *in vitro* [19].

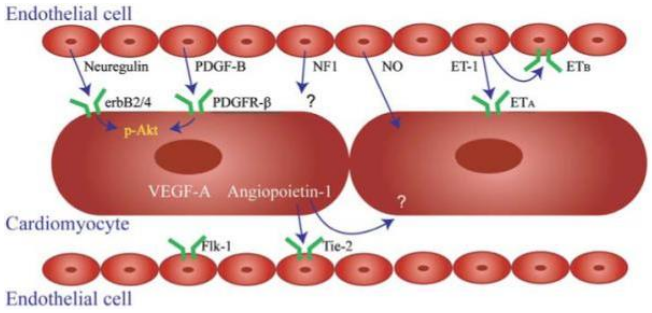

Figure 3: Schematic illustration of the endothelial-cardiomyocyte cross-talk through several paracrine and autocrine signaling mechanisms. Endothelial cells (ECs) secrete signaling mediators that control cardiomyocytes development, survival and contraction; whereas cardiomyocytes secrete angiogenic mediators that influence the ECs survival and assembly. Autocrine signaling involved between cardiomyocytes and endothelial cells also influences the development of a normal cardiac function. Adapted from [19].

Most of the times, these kind of approaches is combined with microfluidic techniques to direct and guide the growth of vasculature *in vitro* [18].

Microfluidic Systems

Considering microfluidic systems, the most used approach consists in the generation of negative molds of the desired vascular pattern that will lately be used as molding templates for the chosen polymer (usually, PDMS), in a process called replica molding [18]. The vascular pattern is usually designed to present conformational and geometrical aspects relevant to the concerned tissue *in vivo*, since cells cultured in the absence of their native environment tend to lose their specific tissue physiology [20]. For this ability, this type of technologies can also be referred as “organ-on-chip platforms”.

Glass over PDMS

However, despite fabricating PDMS microfluidic devices being a relatively simple and inexpensive process, there are several drawbacks linked to this polymer, for instance, its propensity to swell [21], poor cell adhesion [22] and proneness to adsorb compounds such as drugs [23]. On the other hand, although glass-based microfluidic devices are made from fused silica, turning the process expensive and complex, its high transparency, resistance to several solvents, dimensional stability, low auto-fluorescence, well-understood surface chemistry [24] and good cell adhesion, makes it a gold standard for several applications, overcoming the major limitations associated to the PDMS.

Project Motivations

The present work focus in the development of a novel fluidic device able to support the co-culture between fibrin-based cardiac tissues and endothelial cells, both derived from human sources, aiming to further promote the formation of a primary vascular network throughout the cardiac tissue, in order to enable its prolonged culture *in vitro*. The device was also projected to allow the scaling up of the bioengineered construct in a future phase, physiologically possible when performed alongside with the integration of the vascular system, conferring a distinct advantage over other fluidic devices. This innovative approach can largely contribute to the emerging cardiovascular regenerative medicine field, as well to complement current research work regarding the study of key cellular interactions that are associated to tissue vascularization.

Materials and Methods

hESCs Maintenance and Differentiation towards Cardiomyocytes

The HES3-NKX2-5-eGFP line was cultured and maintained in Essential 8™ medium (Gibco™) on 6-well plates (Greiner) coated with 5 µg/mL of vitronectin (Gibco)™ in DPBS (Gibco™). The differentiation towards cardiomyocytes was promoted by plating the hESCs in BSA Polyvinylalchohol Essentials Lipids medium (BPEL) [25] supplemented with 20 ng/mL of bone morphogenetic protein 4 (BMP4) (R&D Systems), 20 ng/mL of Activin A (Miltenyi) and 1,5 µM of CHIR99021 (Axon Medchem) on a 6-well format coated with 83 µg/mL of Matrigel (Corning) in DMEM-F12 medium (Gibco™). The medium was then refreshed at day 3 with BPEL medium together with 5 µM of XAV-939 (R&D Systems) and 50 µg/mL of Matrigel (optional). At day 6 and 9 of differentiation, the medium was refreshed solely with fresh BPEL medium. hESC-committed cardiomyocytes started beating around day 10 of differentiation.

Generation of the Fibrin-based Cardiac Tissues

At day 13 of differentiation, the medium was refreshed with a non-commercially available and unpublished cardiomyocyte medium (CM medium) containing T3 hormone, IGF-1 and galactose (all from Sigma-Aldrich®) to improve the maturation of the hESC-derived cardiomyocytes (hESC-CMs) *in vitro*. At day 16, the monolayer-derived cardiomyocytes was dissociated using 1xTryplE-Select (Gibco™) for 10 minutes at 37°C and plated in the CM medium supplemented with 10% (v/v) of horse serum (Gibco™). To generate the fibrin-based cardiac tissues, a reconstitution mixture was prepared on ice containing the

following components (final concentrations after optimization): 10% (v/v) of bovine fibrinogen (Sigma-Aldrich®) (stock solution: 5 mg/mL in NaCl 0,9%), 10% (v/v) of Matrigel, 10% (v/v) of CM medium (2x), cell suspension at 4×10^7 cells/mL (remaining volume to address the volume of tissue in preparation) and 1% (v/v) of aprotinin (Sigma-Aldrich®) (stock solution: 25 µg/mL in water (Gibco™)). The 2x CM medium was added to restore the isotonic conditions of the medium, since the fibrinogen is dissolved in a saline solution. Afterwards, for each 150 µL of reconstitution mixture prepared, 1 µL of thrombin (Sigma-Aldrich®) (stock solution: 100U/mL in a 0,1% (w/v) BSA (Bovostar) solution in DPBS) was added to the mixture.

hiPSC-derived Cardiac Endothelial Cells Maintenance

The hiPSC-derived cardiac endothelial cells, generated in house by a group's collaborator, were cultured and maintained in BPEL medium supplemented with 50 ng/mL of VEGF (Miltenyi) and 5 µM of SB431542 (Tocris Bioscience), henceforth addressed as BPEL+VEGF+SB medium, for expansion purposes, in 6-well plates previously coated with 50 µg/mL of fibronectin (Sigma-Aldrich®) in DPBS. For co-culturing purposes, cells were kept in a non-commercial and unpublished combination of a BPEL-derived with a CM-derived medium (BPEL+CM), also supplemented with VEGF and SB, and with 100 ng/mL of IGF-1 (Sigma-Aldrich®) and 10% (v/v) of HS, named as BPEL+CM+VEGF+SB+IGF+HS medium. The hiPSC-derived cardiac endothelial cells were passaged using a 1xTrypLE-Select solution for 3 minutes at 37°C and at a seeding density of $1{,}5 \times 10^5$ cells/mL.

Fluidic Device

A novel fluidic device aiming to support the vascularization of hESC-derived fibrin-based cardiac tissues was projected and optimized along this project. Shortly, the designed fluidic device is comprised by three modules: an upper PDMS reservoir in where the endothelial cells can be plated, a middle triple-layered stack with several holes drilled along it where the fibrin-based cardiac tissues will be seeded; and a layer of PDMS with engraved channels that in a general way has the same purpose as the reservoir layer.

The Design

The envisioned fluidic device, aiming to combine multilayered systems with microfluidic techniques, is completely brand-new and pioneer for the author's knowledge, hence its design and final dimensions were progressively adapted and optimized along the project according to the gradually obtained results (see Chapter "Results and Discussion").

The Fabrication

Triple-layered Stack Fabrication

Commercial 76 x 26 x 1 (mm) glass slides (Thermo Scientific Menzel™) were used to produce the first (top) layer of the triple-layered glass stacks, while 24 x 50 x 0,15 (mm) glass coverslips (Thermo Scientific Menzel™) were used to produce the second (middle) and third (bottom) layer of the designed glass stack. In its turn, plastic (vinyl) coverslips (Fisherbrand™) presenting 22 x 22 x 0,2 (mm) as external dimensions were chosen as raw materials for the second (middle) and third (bottom) layers of the plastic stack; while casted poly(methyl methacrylate) (PMMA) slides presenting 2 mm of thickness were chosen to be used as first (top) layer of the plastic stack. The dimensions of the glass and PMMA slides were adapted to the dimensions of the glass and plastic coverslips, respectively, before the assembling of the layers. The drilling process in the glass layers was provided by a collaborator from the Techno Center for Education and Research (TCO), the University of Twente (The Netherlands), while the plastic layers were drilled by the author using regular drillers with the appropriate steel bits.

Reservoir Fabrication

The PDMS reservoir was obtained after punching, using a commercial puncher (Robbins Instruments), several holes in the desired positions of a thin layer of cured PDMS (0,2 mm thick). The PDMS was obtained by mixing the pre-polymer and the curing agent (Sylgard 184 Silicone Elastomer Kit, Dow Corning®, Midland, MI, USA) in a 10:1 (w/w) ratio and cured at 67°C for 2 hours.

Channel Fabrication

The channel layer was obtained after peeling-off a cured PDMS layer molded from the negative mold of the channels, obtained resorting to a CNC-milling machine (Model 5410, Sherline Products Inc., Vista, CA), that was held together using a metal clamp against a flat PMMA block with the same external dimensions and with a 4 mm hole to allow the injection of the PDMS.

The Assembly

Assembling the Triple-layered Stacks

The assembly of the triple-layered stacks was initially performed using an autoclaved 10% (w/v) gelatin solution in DPBS from porcine skin powder (Sigma Aldrich®) between each layer, followed by the placement of the stack in the fridge at 4°C to enable the solidification of the gelatin. In a later stage of the project, the assembly of the stacks was carried out using NOA 81 (Norland Products Inc.) that was cured using a UV-LED system at full power ($400\ mW/cm^2$) for 10 seconds, 7 times, in every position of the stack. Afterwards, the stack was placed in an agitated beaker (VWR) with deionized water (Milli-Q®, Merck Millipore) to ensure that any remaining of liquid NOA81, and therefore non-cured, could be released from the assembly.

Assembling the Reservoir and Channel Layer to the Triple-layered Glass Stack

The reservoir and the channel layer, both made of PDMS, were assembled into the triple-layered glass stack through a plasma treatment process. For that, and one at a time, the PDMS

surfaces were place inside of a plasma cleaner (PDC-002-HP, Harrick Plasma) along with the triple-layered glass stack at a pressure of 500 mTorr and for about 45 seconds. The plasma treatment introduces polar silanol groups (Si-OH) in the PDMS surface and OH groups in the glass surface, allowing the irreversible bonding of both surfaces by Si-O-Si bonds that are formed when the surfaces are brought together [26].

Cell Casting in the Fluidic Device

Casting the Fibrin-based Cardiac Tissues in the Triple-layered Stacks

For glass stacks, 2 µL of the prepared reconstitution mixture and for plastic stacks, 4 µL of the mixture, were added to each intended casting mold in a timeframe of 1 minute, corresponding to the period in which the newly formed fibrin is still accessible.

Casting the Endothelial Cells in the Reservoir and Channels

Silane and Glutaraldehyde Functionalization

The PDMS walls of the reservoir and channels are significantly hydrophobic, and the hydrophilicity conferred by the plasma treatment to these surfaces is only temporary, which is a limitation for cell adhesion, since the latter is highly favored by surface wettability [22]. To address this issue, a surface functionalization process using (3-aminopropyl)triethoxy silane (APTES) (Sigma-Aldrich®) and glutaraldehyde (GA) (Merck Millipore) was performed in the channels and reservoir to enable the immobilization of fibronectin on their walls, prior to the seeding of the endothelial cells, since fibronectin highly favors the adhesion of these cells [22]. For that, and right after the plasma treatment, the PDMS channels and reservoir were infused with a 3% (v/v) APTES solution in deionized water and incubated at room temperature for 5 minutes, followed by their washing with ethanol 70% (v/v) (Azlon). Afterwards, both surfaces were infused with a 10% (v/v) GA solution in 1xDPBS and incubated for another 5 minutes at room temperature. After the incubation period, both surfaces were washed with distilled water, dried with an air gun and used to be coated with the fibronectin solution (50 µg/mL in DPBS).

Endothelial Cells Plating

The hiPSC-derived cardiac endothelial cells were dissociated using the same protocol for cell passaging and seeded at 5×10^5 cells/mL (after optimization) in BPEL+VEGF+SB or BPEL+CM+VEGF+SB+IGF+HS medium in the reservoir and/or in the channels according to the purpose of the ongoing experiment.

Endothelial Cells Staining

The hESC-derived cardiomyocyte line has two reporter genes, namely an enhanced green fluorescent protein (eGFP) and a fusion protein α-actinin-mRuby, overexpressed in the cell line through Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) mediated gene editing. Since the hiPSC-derived cardiac endothelial cells are not genetically labeled and to enable their visualization under the fluorescence microscopy, the dissociated endothelial cells were labeled in suspension with a Vybrant DiD® cell-labelling solution (Molecular Probes™), before seeding in the fluidic device and according to the manufacturer's protocol. This dye barely overlaps the emission spectrum of the fluorescent reporter genes that mark the cardiomyocytes.

Microscopy

All the micrographs referring to bottom views were taken using an inverted microscope (Nikon Eclipse Ts2) connected to a computer (Dell) with the Nikon's NiS Elements Software installed; whereas the micrographs concerning top views were taken using a sterile upright microscope (Leica) and a microscope smartphone camera adaptor (Carson). Fluorescence micrographs were also taken using the inverted microscope or the confocal microscope (Nikon Confocal A1).

Flow Cytometry

A flow cytometer (MACSQuant VYB, Miltenyi Biotech) was used to quantify the population of endothelial cells in the control medium (BPEL+VEGF+SB) and in the medium formulated in house to co-culture endothelial cells and cardiomyocytes (BPEL+CM+VEGF+SB+IGF+HS). For that, dissociated cells from both mediums were resuspended in FACS buffer (0,5% BSA (w/v) (Sigma-Aldrich®) plus 2 mM of EDTA (Gibco™)) and passed through a cell strainer. Then, the cells were pelleted, resuspended in FACS buffer with the Monoclonal Anti-Human-CD31-APC Antibody (eBioscience™) and incubated for 30 minutes at 4°C. Afterwards, the cells were washed, resuspended in FACS buffer and run in the flow cytometer along with the negative samples (not stained with the antibody) in order to allow the further validation of the flow cytometry model.

Results and Discussion

Triple-layered Stack Concept and Design

The hESC-derived cardiomyocytes, and also the fibrin-based cardiac tissues, are anchorage dependent relying on the adherence to supportive structures to ensure their normal development and survival. Having that in mind, a triple-layered stack with holes with different diameters drilled along its layers, henceforth addressed as working setups, was thought out in order to promote the establishment of several anchoring points to where the cardiomyocytes could attach (Figure 4).

Figure 4: Cross view of a triple-layered stack, highlighting their anchoring points (black contours). The green and pink layers (top and bottom sheets) that compose the triple-layered stack present holes with a higher diameter than the blue layer (middle layer) allowing the formation of anchoring structures.

Initially, the triple-layered stacks were envisioned to be made from glass, due to the optimal adhesion and optical properties of this material, but since the drilling in these surfaces needs to be provided by a specialized glass technician, turning the fabrication of the glass stacks in a time and cost-limiting step, its replacement by plastic materials was brought to analysis. The initial design projected for the triple-layered plastic stacks, including relevant dimensions for its construction, are properly discriminated in Figure 5.

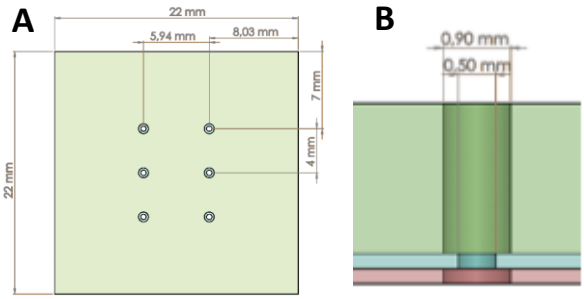

Figure 5: A) Top view of the initial projected triple-layered plastic stack and its corresponding dimensions. **B)** Cross view of a working setup composing a triple-layered plastic stack and associated diameters. The colors of the layers concern the same subjects as Figure 4.

Studying the Effects of Growing Fibrin-Based Cardiac Tissues in Triple-layered Plastic Stacks Assembled with Gelatin

In the first set of experiments, the goal was to assess the viability of using triple-layered plastic stacks to culture fibrin-based cardiac tissues, in order to validate the underlying proof of concept. Since gelatin has been described as an attractive polymer for tissue engineering approaches due to its biological origin, biodegradability, wide availability, cost-efficiency, non-antigenicity and easy handling [27], it was chosen as first assembling material for bonding the stack layers. Fibrin-based cardiac tissues were initially generated using a cell density of 2×10^7 cells/mL and using half of the ratio of thrombin reported in Materials and Methods Section, given its proportionality to the amount of cells intended to be entrapped in the fibrin network. The cardiomyocyte population composing the casted tissues was able to spread out inside the fibrin matrix over time of cultivation (Fig. 6A and 6B – white arrows), which was expected since fibrin is a naturally occurring hydrogel that inherently promotes cell adhesion and spreading [7]. Additionally, the degradation of the fibrin gel, as a result of the plasminogen present in the culture medium and of the increased cellular production of ECM proteins, was evident after a few days and observable by the continuous decrease of the size of the tissues (Fig. 6A and 6B – green arrows) [15]. Even though the degradation rate of fibrin is remarkably slowed, and consequently controlled, by the use of the antifibrinolytic agent aprotinin, it is never stopped even when using aprotinin concentrations way higher than the ones used in this project [14]. The continuous remodeling and degradation of the hydrogel highly contributed for an increase between the proximity of the cells, that enabled the increasing establishment of novel intercellular interactions, that at day 4 post-seeding were translated in the appearance of several beating areas inside the construct.

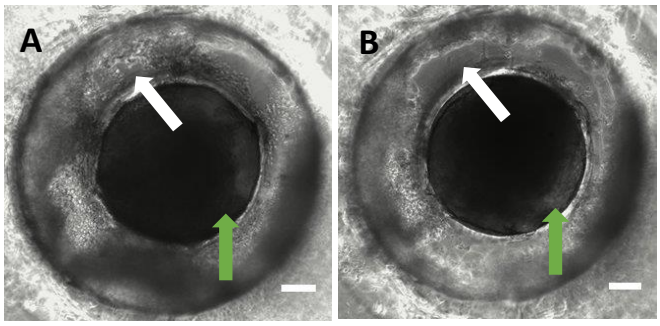

Figure 6: Micrographs taken to a same developing tissue casted in a triple-layered plastic stack, assembled with a gelatin solution, at **(A)** day 4 and **(B)** day 10 post-seeding. Both micrographs correspond to bottom views of the working device. Scale bars: 100 µm.

Despite the promising results, it was not visible a synchronously contracting tissue englobing all the cells. A possible reason for the incomplete remodeling could be the cell density used not being adequate, meaning that, there might not be enough cells sufficiently close to each other to establish novel intercellular connections; hence, the cell density should be increased, in order to rise the amount of contacting cells. Besides that, the results obtained were not always reproducible, in fact, most of the times the casted tissues ended up by being undeveloped either because they were disrupted by air bubbles that were trapped inside the gelatin, and were released afterwards due to the hydraulic pressure exerted by the culture medium (Fig. 7A), or they were formed with a really low cell concentration, due to the infiltration and dispersion of the tissues through the stack layers caused by the low sealant properties offered by the gelatin (Fig. 7B).

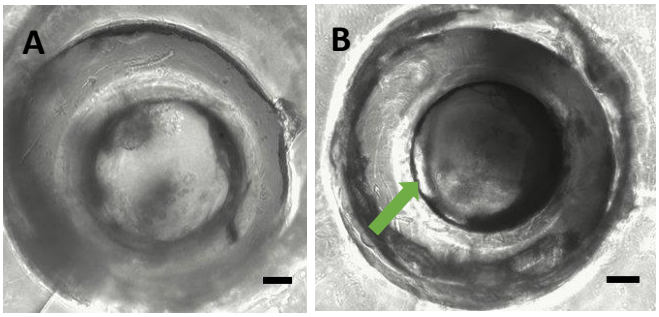

Figure 7: Micrographs taken to different tissues casted in a triple-layered plastic stack, assembled with a gelatin solution, at **(A)** day 4 and **(B)** day 8 post-seeding. Both micrographs correspond to bottom views of the working device. Scale bars: 100 µm.

Considering all these hardly controllable events, and in order to try to obtain reproducibility in the further studies, a new alternative to gelatin was brought to scope, specifically the use of the optical adhesive NOA81. Besides the problems associated to the assembling agent, it is also visible from micrograph 7B (green arrow), that overtime, the degradation of the tissue was so prominent that, in some cases, its size was getting smaller than the middle hole, causing a progressive lack of physical support to the tissue. Having that into consideration, it was decided to increase the outer diameters from 0,9 mm to 1,2 mm.

Studying the Effects of Growing Fibrin-Based Cardiac Tissues in Triple-layered Stacks Assembled with NOA81

NOA81 is a UV-curable liquid photopolymer that presents several characteristics really valued for microfluidic operation, namely, its entire transparency and low auto-fluorescence; its good chemical resistance to organic solvents, allowing the cleaning and consecutive re-utilization of chips assembled with it; its impermeability to oxygen and water vapor, so even if any air bubbles get trapped on its surface, they would never be released from there, since neither the NOA81 or the plastic/glass are permeable to air; its stability during surface treatments (e.g. plasma treatment); its biocompatibility after being cured and finally its good adhesion to plastic/glass materials without showing any leakage for up to months [28]. After the first experiments performed using NOA81 as assembling agent it was possible to conclude that the material was being toxic for the cells, largely hampering their remodeling into a functional tissue (Fig. 8A and 8B). As a matter of fact, even contractions of small cell clusters only started after 8 days in culture, long after of what was observed in the previous experiments with gelatin, with the remaining cells being assumable dead.

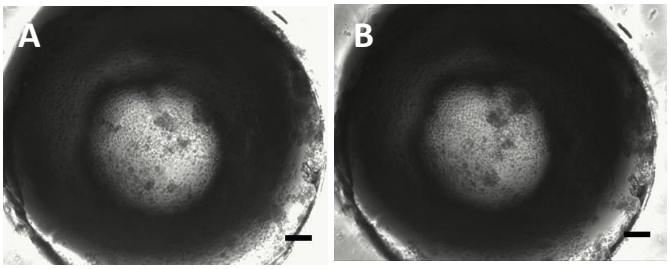

Figure 8: Micrographs taken to a same tissue casted in a triple-layered plastic stack, assembled with NOA81, at **(A)** day 8 and **(B)** day 11 post-seeding. Both micrographs correspond to bottom views of the working device. Scale bars: 100 µm.

Since the use of NOA81 has been successfully reported in multiple cell culture approaches, it came to our evidence that the current implemented curing step (one single application of UV-light for 10 seconds at $400\ mW/cm^2$) could not be effective enough, hence from this point forward a more aggressive curing step was implemented, performed as described in the Materials and Methods Section. Besides that, it was also increased the cell density to the double (4×10^7 cells/mL) in order to attempt to improve the remodeling of the cells, as hypothesized before, followed by the increase of the ratio of thrombin, also to the double (1 µL of thrombin/ 150 µL of final volume) to answering to the current higher cell trapping demand. In these optimal conditions, the cardiomyocytes started beating spontaneously and at the same pace as part of a synchronous tissue, englobing all the cells (Fig. 9A and 9B) and not only enclosing some major cell clusters as in previous experiments with gelatin (Fig. 6A and 6B), at day 4 after the casting and continued for several days until the end of the experiment (at day 15). After the successful optimization of the culture of fibrin-based cardiac tissues inside of triple-layered plastic stacks, the bonding to the channel layer was set as the next goal.

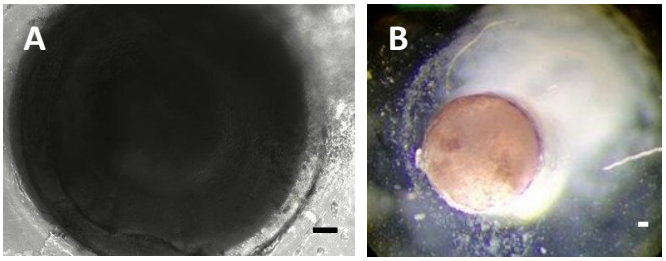

Figure 9: Micrographs taken to a same tissue that composes a triple-layered plastic stack, assembled with NOA81, at day 4 post-seeding. Micrograph A reports to a bottom view while micrograph B reports to a top view of the tissue. Scale bars: 100 µm.

A set of different approaches was tested to attempt the desired bonding, namely plasma treatment, rolling a thin layer of PDMS and toluene, or PDMS, or NOA81 only, or by directly pipetting and curing NOA81 on top of the channel surface. Neither of these techniques yielded successful results in providing the irreversible bonding of the PDMS to the bottom vinyl surface of the stack. Since the exact chemical composition of the vinyl layer is unknown, it was not possible to make assumptions of the causes behind the failure of these techniques as well rethink new approaches. For all these reasons, the use of triple-layered plastic stacks, although being more practical for drilling related motivations, was set aside, believing that the bonding of the PDMS to the glass surfaces would be easily accomplished by plasma treatment, as reported by numerous authors, for instance by Katzenberg et al. [29]. Taken into consideration that the channels projected for the plastic stacks were not used for further experiments, as well the methods attempted to its bonding (excluding the plasma treatment process), detailed information regarding these subjects was dismissed from this document. Similar to the triple-layered plastic stacks but adapted to the higher dimensions of the glass layers, a triple-layered glass stack was fabricated according to the projected design of Figure 10.

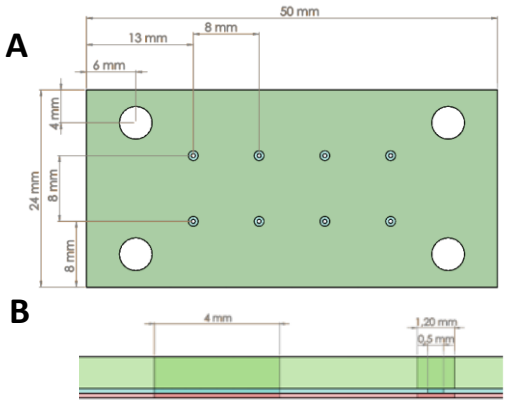

Figure 10: A) Top view of the projected triple-layered glass stack and its corresponding dimensions. **B)** Cross view of a triple-layered glass stack and associated diameters. The green, blue and pink layers illustrate the first (glass slide), second (glass coverslip) and third (glass coverslip) layers of the concerned stack, respectively, from a top to bottom perspective.

The holes with 4 mm of diameter (Fig. 10B), henceforth addressed as “inlets” and “outlets”, were drilled to allow the subsequent communication to the channels, since both modules present the same external dimensions. The design of

the channel layer intended to be bonded to the triple-layered glass stack is presented in Figure 11.

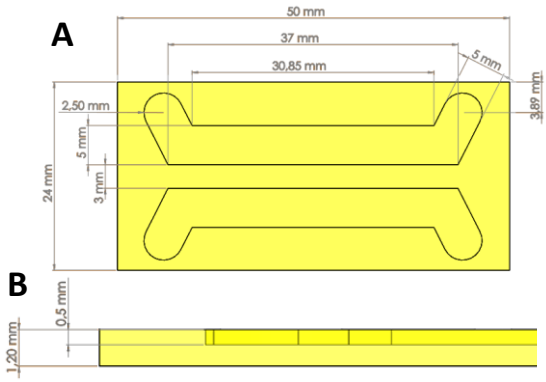

Figure 11: A) Top view of the channel layer intended to be assembled to the triple-layered glass stack and its corresponding dimensions. **B)** Cross view of the channel layer and associated thicknesses.

The depth (0,5 mm) and width (5 mm) of each channel were carefully established so its hydraulic resistance could be inferior to the hydraulic resistance of the casting molds [30]. Otherwise, after applying a fluid into the channel, it would be immediately diffused through the casting molds (since the fluid flows through the way that offers a lower resistance) instead of being diffused through the channels; contributing for the disruption of the seeded tissues. Additionally, plastic stoppers able to fit in the 4 mm holes of the glass stack were also fabricated, in order to restrain or isolate the channel medium from the remaining. As expected, the assembling of both modules was easily accomplished by plasma treatment. Afterwards, the glass stacks assembled with NOA81 and previously bonded to the channel layer were assessed in order to validate their usage. From Figure 12, it is visible that the cells were able to perform an extensive and complete remodeling around the middle hole that led to the formation of a spontaneously and synchronously contracting tissue-construct 4 days post-seeding. Moreover, the engineered fibrin tissues were able to acquire a stable circular shape, not seen until this stage, as the cardiomyocytes were getting aligned along the multiple radial force lines to which they are being subjected due to their anchorage.

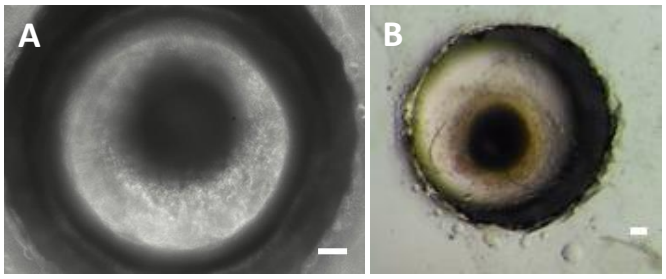

Figure 12: Micrographs taken to different tissues that compose a triple-layered glass stack, assembled with NOA81, at **(A)** day 4 and **(B)** day 12 post-seeding. Micrograph A reports to a bottom view, while micrograph B reports to a top view of a tissue. Scale bars: 100 µm.

After the procedure for culturing fibrin-based cardiac tissues inside the fluidic device being properly optimized, the next stage of the project was focused on the co-culture with hiPSC-derived cardiac endothelial cells, to further attempt to promote the vascularization inside these engineered-constructs.

Co-culture with Endothelial Cells in the Chip

The first set of experiments concerning this matter were performed by seeding endothelial cells inside the channels, 7 days post-seeding of the tissues, at a seeding density of 1×10^5 cells/mL (proportional cell monolayer density to the one observed in 6-well plates) in their regular medium (BPEL medium), while the tissues were maintained in the CM medium. After seeding, it was clear that the cell density used was not enough to enable the formation of a monolayer (Fig. 13A) and beyond that, along the days in culture, the endothelial cells start dying, whereas the surviving ones where exhibiting morphological signs of induced stress, identified by the loss of their round-shaped morphology to a more elongated one (Fig. 13B). It was also noticeable an increase in the EC-EC communications (Fig. 13B), probably because the endothelial cells were trying to adapt their collective architecture to minimize the sensed stress [31], admittedly brought by their contact with the CM medium due to lack of proper sealing of the inlets and outlets. Parallel to that, the bottom-placed tissues, that are directly exposed to the BPEL medium, also started being negatively affected after a couple of days post-seeding of the endothelial cells, by starting to exhibit lower beating frequencies.

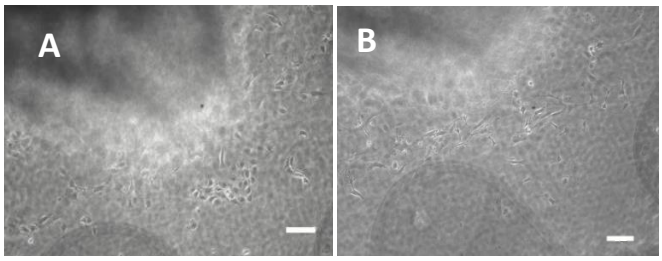

Figure 13: Micrographs taken at **(A)** day 1 and **(B)** day 5 post-seeding of the endothelial cells in the chip device to a same region of a channel. Both micrographs correspond to bottom views. Scale bars: 100 µm.

These morphological results clearly revealed the extreme importance of finding a matching medium suitable for co-culturing cardiomyocytes and endothelial cells in the same device. Thus, a combination of a BPEL-derived with a CM-derived medium (BPEL+CM+VEGF+SB+IGF+HS medium), was established after several experiments that revealed the positive influence of the components that comprise this medium over the cells, and whose final formulation was initially tested in a standard culture of endothelial cells alone. The endothelial cells cultured in this medium (Fig. 14B) were almost completely identical, morphologically speaking, to the ones cultured in their control medium (Fig. 14A), exhibiting a formed monolayer with cells grouped in a visible cobblestone-like structure and in their majority round-shaped; giving in this way the first morphological evidences of the viability of using this newly formulated medium to culture, at least, endothelial cells.

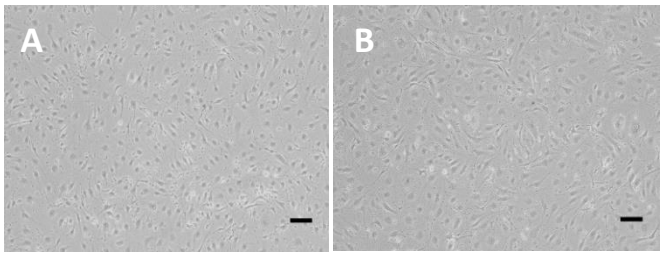

Figure 14: Micrographs taken at day 7 post-seeding to a culture of hiPSC-derived cardiac endothelial cells in **(A)** BPEL+VEGF+SB (control medium) and **(B)** BPEL+CM+VEGF+SB+IGF+HS (medium matching). Scale bars: 100 µm.

In addition to the morphological analysis, the efficacy of using this medium was also evaluated and quantified though fluorescence analysis of endothelial cells previously stained with the anti-human-CD31-APC conjugated antibody by flow cytometry. CD31 is believed to be one of the main specific markers for endothelial cells [32] and from the results of the flow cytometry model, it was observable that even a higher percentage of cells were positive for the CD31 marker in the matching medium (Fig. 15B) than in the control medium (Fig. 15A), admittedly presenting an endothelial identity, giving the second validation proof of the viability of using this medium to culture endothelial cells.

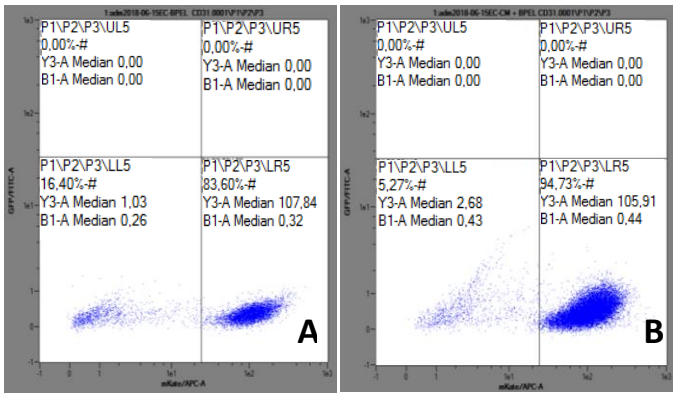

Figure 15: Flow cytometry analysis of endothelial cells stained with the anti-human-CD31-APC conjugated antibody and cultured in **A)** BPEL+VEGF+SB medium and **B)** BPEL+CM+VEGF+SB+IGF+HS medium. Gating for Allophycocyanin (APC-A) (x axis).

After having enough evidences of the applicability of this medium to culture endothelial cells, its viability for sustaining the culture of cardiomyocytes was also assessed. For that, the medium created in house was used as the only culture medium in the device from post-seeding of the cardiac tissues and endothelial cells (after optimization seeded at 5×10^5 cells/mL in the reservoir (Figure 16)), both casted at the same day.

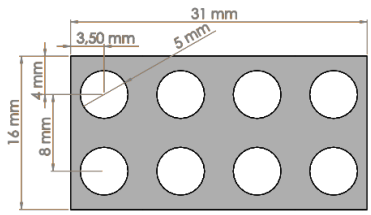

Figure 16: Top view of the reservoir layer and its corresponding dimensions.

In this experiment, the cells were seeded in the reservoir instead on the channels, in order to allow the subsequent imaging using the confocal microscope since the channel layer is too thick to enable this procedure. Remarkably, the developing fibrin-based cardiac tissues started beating as a whole construct at day 4 post-seeding in this newly formulated medium, at a synchronous pace, showing the positive effect of this medium over the cardiomyocytes; therefore, giving the final validation proof of the viability of using this medium not only to culture endothelial cells, but cardiomyocytes as well. Moreover, the tissue engineered-cardiac constructs were kept under culture for longer than one month, without displaying any signs of induced stress or morphological instability, assumable due to the cardiomyocyte-endothelial paracrine signaling that greatly can contribute to enhance the tissue survival *in vitro* [19]. It was also clear that the endothelial cells density used, enabled the formation of a monolayer of these cells around the tissues (Fig. 17A) as well in their superficial layer (Fig. 17B).

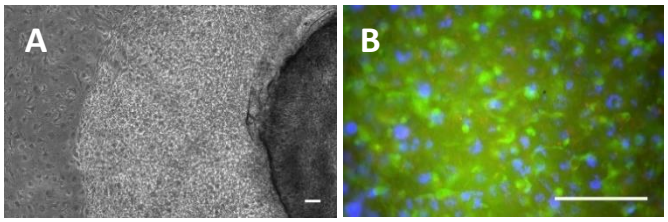

Figure 17: Micrographs taken at day 4 post-seeding of the endothelial cells and cardiac tissues. **(A)** Brightfield micrograph concerning a view of an extremity of a tissue seeded on top with endothelial cells. **(B)** Overlapping of several fluorescence micrographs taken to the endothelial cells, cardiomyocytes and sarcomeres planes, at the center of a tissue, to which were applied a blue, green and red color filter, respectively, using the Fiji Software. Both micrographs correspond to top views. Scale bars: 100 µm.

Two days after the previous results, so at day 6 post-seeding, it was noticeable that the population of endothelial cells composing the top of the tissues was drastically diminished (Fig. 17B and 18A). Multiple explanations can be attributed to this event, like the possible reduced lifetime of the fluorescent dye, simple cell death or the expectedly migration of the endothelial cells into the tissue. The remote hypothesis of the lifetime of the fluorescent dye being decreasing rapidly was immediately withdrawal after imaging the extremity of the reservoir, since it was still possible to observe nicely stained endothelial cells in this region (Fig. 18B).

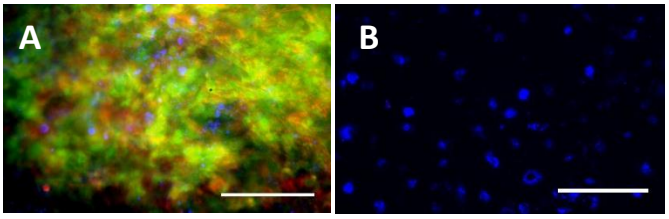

Figure 18: Micrographs taken at day 6 post-seeding of the endothelial cells and cardiac tissues. **(A)** Overlapping of several fluorescence micrographs taken to the center of a tissue. **(B)** Fluorescence micrograph taken to the extremity of the reservoir. The color filters applied concern the same subjects as in Figure 17. Both micrographs correspond to top views. Scale bars: 100 µm.

To analyze the hypothesis of the recruitment of the endothelial cells into the concerned tissues, a confocal microscopic

analysis was performed to their center. After the reconstruction of the 3D-structure of the tissue, resorting to Fiji Software, through the generation of a Z-stack projection from the images obtained from the confocal system, it was possible to observe planes that englobed cardiomyocytes (and endothelial cells), followed by planes that continued to englobe endothelial cells, indicating that the endothelial cells have indeed migrated into the tissue. The migration of the endothelial cells may be explained by the supplementation to the medium with VEGF that is a critical inducer of the endothelial cell migration, mainly through the activation of the phosphatidylinositol 3-kinase (PI3K) and the GTPase Rac-1 pathways [33]. When the VEGF is added to the culture medium, endothelial cells can sense it and start slowly to migrate in random directions [34].

Conclusions and Future Perspectives

In this project, a novel fluidic device able to support the culture of hESC-derived fibrin based cardiac tissues, by providing the right cues for its development in a spontaneously and synchronously contracting construct, was successfully accomplished. Moreover, the established fluidic device can easily support the scaling up of the engineered constructs through several practical ways. To attempt to promote the long-term survival of the tissues through the integration of a vascular system, a co-culture approach englobing hiPSC-derived cardiac endothelial cells was also efficiently brought to the working device. This approach resulted in the migration of the endothelial cells into the tissue, possibly due to the supplementation of the medium with a pro-angiogenic factor, namely the VEGF, providing ground work to further develop the sprouting and formation of vascular-like structures throughout the tissues that could lately be perfused with culture medium, allowing the survival of increasingly thicker constructs. For attempting that, several approaches can be followed *in vitro*, including co-culture systems with fibroblasts, pro-angiogenic factors gradients and/or flow and pressure gradients.

References

[1] H. F. Lu *et al.*, “Engineering a functional three-dimensional human cardiac tissue model for drug toxicity screening,” *Biofabrication*, vol. 9, no. 2, p. 25011, May 2017.

[2] G. J. Scuderi and J. Butcher, “Naturally Engineered Maturation of Cardiomyocytes,” *Front. Cell Dev. Biol.*, vol. 5, p. 50, May 2017.

[3] R. R. Besser, M. Ishahak, V. Mayo, D. Carbonero, I. Claure, and A. Agarwal, “Engineered Microenvironments for Maturation of Stem Cell Derived Cardiac Myocytes,” *Theranostics*, vol. 8, no. 1, pp. 124–140, 2018.

[4] Y.-K. Lee *et al.*, “Triiodothyronine Promotes Cardiac Differentiation and Maturation of Embryonic Stem Cells via the Classical Genomic Pathway,” *Mol. Endocrinol.*, vol. 24, no. 9, pp. 1728–1736, Sep. 2010.

[5] C. E. Rupert and K. L. K. Coulombe, “IGF1 and NRG1 Enhance Proliferation, Metabolic Maturity, and the Force-Frequency Response in hESC-Derived Engineered Cardiac Tissues,” *Stem Cells Int.*, vol. 2017, pp. 1–13, 2017.

[6] C. Correia *et al.*, “Distinct carbon sources affect structural and functional maturation of cardiomyocytes derived from human pluripotent stem cells.”

[7] M. N. Hirt, A. Hansen, and T. Eschenhagen, “Cardiac Tissue Engineering: State of the Art,” *Circ. Res.*, vol. 114, no. 2, pp. 354–367, Jan. 2014.

[8] I. M. El-Sherbiny and M. H. Yacoub, “Hydrogel scaffolds for tissue engineering: Progress and challenges.,” *Glob. Cardiol. Sci. Pract.*, vol. 2013, no. 3, pp. 316–42, 2013.

[9] F. Weinberger, I. Mannhardt, and T. Eschenhagen, “Engineering Cardiac Muscle Tissue: A Maturating Field of Research.,” *Circ. Res.*, vol. 120, no. 9, pp. 1487–1500, Apr. 2017.

[10] E. Morgan and E. Lipke, “Direct Production of Engineered Cardiac Tissue within Hybrid Biomaterials.” .

[11] T. Eschenhagen, M. Didié, F. Munzel, P. Schubert, K. Schneiderbanger, and W.-H. Zimmermann, “3D engineered heart tissue for replacement therapy,” *Basic Res. Cardiol.*, vol. 97, no. 7, pp. 1–1, May 2002.

[12] Z. Li and J. Guan, “Hydrogels for Cardiac Tissue Engineering,” *Polymers (Basel).*, vol. 3, no. 2, pp. 740–761, Apr. 2011.

[13] E. Cholewinski, M. Dietrich, T. C. Flanagan, T. Schmitz-Rode, and S. Jockenhoevel, “Tranexamic Acid—An Alternative to Aprotinin in Fibrin-Based Cardiovascular Tissue Engineering,” *Tissue Eng. Part A*, vol. 15, no. 11, pp. 3645–3653, Nov. 2009.

[14] A. Hansen *et al.*, “Development of a Drug Screening Platform Based on Engineered Heart Tissue,” *Circ. Res.*, vol. 107, no. 1, pp. 35–44, Jul. 2010.

[15] C. J. Connon and I. W. Hamley, *Hydrogels in Cell-based Therapies*. 2014.

[16] E. C. Novosel, C. Kleinhans, and P. J. Kluger, “Vascularization is the key challenge in tissue engineering,” *Adv. Drug Deliv. Rev.*, vol. 63, no. 4–5, pp. 300–311, Apr. 2011.

[17] K. M. Park and S. Gerecht, “Harnessing developmental processes for vascular engineering and regeneration,” *Development*, vol. 141, no. 14, pp. 2760–2769, Jul. 2014.

[18] M. Lovett, K. Lee, A. Edwards, and D. L. Kaplan, “Vascularization Strategies for Tissue Engineering.”

[19] P. C. H. Hsieh, M. E. Davis, L. K. Lisowski, and R. T. Lee, “Endothelial-cardiomyocyte interactions in cardiac development and repair.,” *Annu. Rev. Physiol.*, vol. 68, pp. 51–66, 2006.

[20] A. D. van der Meer and A. van den Berg, “Organs-on-chips: breaking the in vitro impasse,” *Integr. Biol.*, vol. 4, no. 5, p. 461, 2012.

[21] J. N. Lee, C. Park, and G. M. Whitesides, “Solvent Compatibility of Poly(dimethylsiloxane)-Based Microfluidic Devices,” *Anal. Chem.*, vol. 75, no. 23, pp. 6544–6554, Dec. 2003.

[22] S. Kuddannaya, Y. J. Chuah, M. H. A. Lee, N. V. Menon, Y. Kang, and Y. Zhang, “Surface Chemical Modification of Poly(dimethylsiloxane) for the Enhanced Adhesion and Proliferation of Mesenchymal Stem Cells,” *ACS Appl. Mater. Interfaces*, vol. 5, no. 19, pp. 9777–9784, Oct. 2013.

[23] B. J. van Meer *et al.*, “Small molecule absorption by PDMS in the context of drug response bioassays,” *Biochem. Biophys. Res. Commun.*, vol. 482, no. 2, pp. 323–328, Jan. 2017.

[24] C.-W. Chi, A. R. Ahmed, Z. Dereli-Korkut, and S. Wang, “Microfluidic cell chips for high-throughput drug screening,” *Bioanalysis*, vol. 8, no. 9, pp. 921–937, May 2016.

[25] E. S. Ng, R. Davis, E. G. Stanley, and A. G. Elefanty, “A protocol describing the use of a recombinant protein-based, animal product-free medium (APEL) for human embryonic stem cell differentiation as spin embryoid bodies,” *Nat. Protoc.*, vol. 3, no. 5, pp. 768–776, May 2008.

[26] A. Note, “Plasma Treated PDMS for improved bonding performance of microfluidic devices.”

[27] S. A. Sell, P. S. Wolfe, K. Garg, J. M. McCool, I. A. Rodriguez, and G. L. Bowlin, “The Use of Natural Polymers in Tissue Engineering: A Focus on Electrospun Extracellular Matrix Analogues,” *Polymers (Basel).*, vol. 2, no. 4, pp. 522–553, Nov. 2010.

[28] “Microfluidic device for chemical and mechanical manipulation of suspended cells,” *J. Phys. D Appl. Phys*, vol. 51.

[29] F. Katzenberg, “Plasma-bonding of poly(dimethylsiloxane) to glass,” no. 60, 2005.

[30] K. W. Oh, K. Lee, B. Ahn, and E. P. Furlani, “Design of pressure-driven microfluidic networks using electric circuit analogy,” *Lab Chip*, vol. 12, no. 3, pp. 515–545, 2012.

[31] S. Ohta, S. Inasawa, and Y. Yamaguchi, “Alignment of vascular endothelial cells as a collective response to shear flow,” *J. Phys. D. Appl. Phys.*, vol. 48, no. 24, p. 245401, Jun. 2015.

[32] L. Liu and G.-P. Shi, “CD31: beyond a marker for endothelial cells,” *Cardiovasc. Res.*, vol. 94, no. 1, pp. 3–5, Apr. 2012.

[33] Y. Wang *et al.*, “Regulation of VEGF-induced endothelial cell migration by mitochondrial reactive oxygen species.,” *Am. J. Physiol. Cell Physiol.*, vol. 301, no. 3, pp. C695-704, Sep. 2011.

[34] H. Gerhardt, “VEGF and endothelial guidance in angiogenic sprouting.,” *Organogenesis*, vol. 4, no. 4, pp. 241–6, Oct. 2008.