Engineered 3D-Vessels-on-Chip to study effects of dynamic fluid flow on human induced pluripotent stem cell derived endothelial cells

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Chapter six:

General discussion

Discussion

Combining differentiated pluripotent stem cells (hiPSCs) with Organ-on-Chip (OoC) technology has the potential to improve *in vitro* disease modelling and drug discovery[1]. Currently, standard cell-culture models, even when based on hiPSC derivatives from patients, still fail to capture many aspects of human physiological reality, missing important information for adequate drug-effect and toxicity prediction[2]. This failure can be partly attributed to the cell culture conditions, that typically consist of single cell-types, grown on two-dimensional culture plastic that has viscoelasticity properties that differ by orders of magnitude from that in real tissue[3]. Because of this, *in vitro* cell cultures miss (or have inappropriate) intracellular communication, cellular orientation and the biomechanical stimulation necessary for correct cellular phenotype and drug response[4,5]. By modifying the extracellular environment to mimic aspects of real tissue *in vitro,* models based on OoC-technology can improve the predictive value of cultured cells for disease modelling and drug discovery compared to the standard in vitro assays[6].

Despite this potential of OoCs, widespread use of this technology is still limited because it is highly multi- and interdisciplinary, with the challenges of combining advanced engineering, with complex and highly skilled stem cell biology by a single end-user[7].

As discussed in this thesis, hiPSC can give rise to all cells of the human body but in the context of OoCs, hiPSC-derived endothelial cells (hiPSCs-ECs) are of particular interest as a cellular lining to microfluidic channels, as they provide a way of mimicking (diseased and healthy) blood vessels[8]. This thesis focused on the development of 3D vascular models that combine hiPSC-derived vascular cells (both ECs and adjacent pericytes or smooth muscle cells) and haemodynamic forces. These mechanical stimuli are essential for revealing a "true" EC-phenotype close to that of ECs in vessels of the human body[9]. The perfusable 3D-vessels-on-chip (3D-VoCs) described in this thesis were developed to bring OoC technology closer to the end user; therefore, they were developed to be scalable, reproducible and compatible with the biology carried out in laboratories modelling vascular disease and examining responses to drugs.

As described in **chapter 1**, hiPSC-ECs have many advantages compared to the primary and immortalized endothelial cell lines currently used, not least their batch-to-batch consistency[10]. However, their phenotype is immature and they resemble foetal rather than adult vessels in their (functional) properties and gene profile[11]. Improving their biology to mimic more mature vessels, requires the introduction of realistic functional parameters such as fluidic flow and biomaterials with realistic viscoelastic properties and structures[12]. Different methods are being developed to generate 3D vascular models. However, many methods rely on specialized equipment, reagents

and extensive know-how. Only a few methods are scalable, support controlled-perfusion and are easy to implement within biologically oriented laboratories.

Development of scalable 3D-VoCs compatible with controlled perfusion.

In **Chapter 2** we therefore described a scalable method to fabricate 3D-VoCs scaffolds, compatible with controlled perfusion. The method uses a microfluidic technique called Viscous Finger Patterning (VFP). VFP exploits a phenomenon called the Saffman–Taylor instability that occurs when a viscous fluid is displaced by a less viscous fluid on a microfluidic scale[13]. The less viscous fluid displaces the viscous hydrogel faster at the centre of the fluidic-channel than at the boundary-wall, resulting in a circular lumen inside the hydrogel. In essence, this protocol is the sequential pipetting of 2 fluids in a microfluidic channel and easily caried out by a biologically-trained end-user, without the use of highly specialized equipment. The microfluidic devices are easy to fabricate as they only require a microfluidic channel with a square flow area.

VFP was first used to generate 3D-VoCs by Bischel et al.[14], who demonstrated that the method was not only simple to perform but also allows the formation of straight-, curved- and even bifurcated 3D-VoCs. Furthermore, sequential patterning of different layers of hydrogel supports the development of multi-layered lumens and different tissue specific cell types can be added to the hydrogel, demonstrating the flexibility of the methodology[15-17]. However, when implementing the protocol proposed by Bischel et al., we found that the resulting lumens have a tapered shape, i.e. a significant narrowing of the luminal diameter occurs along the length of the sample, as shown in **chapter 2**. This tapering of the lumen generates a gradient of wall-shear stress (WSS) when perfused, making it difficult to control haemodynamics and interpret results. We therefore sought methods to optimize luminal shapes such that they were compatible with perfusion studies. We demonstrate that by simply extending the entry length of the microfluidic-channel during VFP, the resulting lumens were uniform in diameter over their whole length. This scaffold is therefore capable of generating equal shear stress in the sample when perfused. This small adjustment to the original protocol resulted in lumens suitable for perfusion studies, with comparable quality as those generated with (needle)template-based approaches but retaining the scalability and ease of the original work.

Controlling OoC perfusion

A major obstacle in the use of perfusable OoC platforms is the scalability of controlled, unidirectional perfusion for extended periods of time. When medium refreshment is the only requirement, using gravity alone is sufficient to replenish the nutrients inside the microfluidic devices[18,19]. However, when

biomechanical forces are important, more control is needed. Positive displacement pumps like syringe and peristaltic pumps are the standard for controlled perfusion as they are intuitive and straightforward to set-up. To increase throughput, OoCs can be connected via fluidic-networks to perfuse simultaneously using a single pump which is referred to as "multiplexing". However as described in **chapter 3**, positive displacement pumps have limitations when used for the perfusion of OoCs. Due to the lack of control of mechanical stress in the OoCs, these positive displacement pumps can be detrimental to experimental success. Cell debris formation from dead, dying or detached cells, often occurs in OoC devices and can block microfluidic channels, damaging fragile (engineered) tissue structures. Furthermore, dimensional variation in OoCs does not allow for accurate control of mechanical parameters when multiplexing samples. This is because individual sample variation changes flow distribution and alters the intended mechanical stress, limiting the throughput per fluidic perfusion unit. **Chapter 3** describes a fluidic perfusion platform for the controlled and continuous recirculation of fluid in OoCs. This platform addresses 3 key limitations of the currently available systems when used for the perfusion of OoC.

Firstly, by regulating perfusion with a controlled pressure difference across the OoCs, rather than controlling the flowrate, multiplexing of samples using a single perfusion setup becomes possible. Because perfusion parameters of the individual samples are not changed when other samples have variable dimensions, intact samples are protected from unwanted mechanical stress when others become obstructed.

Secondly, pressure driven fluidic systems often require complex fluidic circuits using actively controlled valves to switch fluidic direction to generate continuous unidirectional flow[20]. To simplify the recirculation, we developed a passively controlled hydraulic analogue to a Graetz-rectifier bridge, used in electrical AC/DC converters. The cell culture medium can be recirculated indefinitely by simply switching the pressure from the medium reservoirs when the liquid levels run low. This fluidic circuit can easily be implemented with inexpensive, off-the-shelf components allowing scaling of experiments with fewer electric components.

Finally, we also demonstrated that thermal mass-flowrate-sensors generally used in Lab-on-a-Chip devices are not suitable for long-term OoC perfusion. Due to fouling of the sensor when making measurements in cell culture medium, readouts become less accurate resulting in lower measured than reality. This inaccuracy has implications for the interpretation of the applied mechanical stimulation and should be avoided. This sensor fouling also hinders use of long term recirculation algorithms as pressure driven recirculation requires switching from one direction to another. We found that by using pressure sensors to measure the liquid level of the medium

reservoir, the precise timing of recirculation can be determined in a fail-safe manner. By combining these developments with commercially available pressure controllers, we were able to develop a flexible, controlled and unidirectional long-term perfusion platform that can be used for different of OoC devices without modification.

Multiplexing of 3D-VoCs with intrinsic variation

An important feature for the adaption of OoC for biomedical research is the ability to replicate experimental condition for multiple samples simultaneously. As already mentioned, blood flow is a vital physiological variable for blood vessels as the biomechanics resulting from blood flow are key modulators of cellular signal transduction and have to be taken into consideration for modelling a cellular response[21,22]. By scaling the flowrate with the luminal diameter, these biomechanical variables can be scaled and different physiological conditions can be modelled in tractable models. However as described in **chapter 4**, 3D-VoCs have an intrinsic lumen diameter variation due to method of fabrication, sample handling and cellular response[23-25]. Also, sample deformation when subjected to an applied force combined with a mural-cell response, referred to as (vascular) compliance, contribute to this (intended) diameter variation [26]. This intrinsic variation hinders multiplexing as it does not allow accurate control of the mechanical parameters when perfused. Standardising experimental conditions in each channel would then require either (i) individual pumps, set at flow-rates corrected for sample diameter or (ii) only select channels with similar diameters, which limits throughput significantly since not all samples, and valuable resources, can be used for experiments. **Chapter 4** describes a solution to bypass intrinsic variation of luminal diameters within 3D-VoCs to enable multiplexing perfusion and increasing sample numbers in a single perfusion experiment. We described a fluidic circuit board (FCB) specifically designed to standardise WSS for 3D-VoCs for a wide range of luminal diameters and ensure this is equal in all channels. The FCB relies on a tailored, fixed hydraulic resistance that scales the flowrate in lumens within a range of possible luminal diameters. The induced flow-rate of an individual 3D-VoC will generate a comparable WSS when equal pressure is applied. By allowing diameter variance rather than focusing on reducing it, more samples can be addressed using the same set of perfusion parameters and therefore allows for increasing the throughput per FCB. Using the perfusion platform we developed in **chapter 3,** the internal pressure can also be controlled, allowing independent regulation of the circumferential stress and the WSS. This can be used, for instance, to model effects of high (blood) pressure compared to low using the same WSS. Using this platform, we demonstrated that the morphology of hiPSC-ECs is influenced by both parameters as expected. By mimicking flow conditions of a vein, the cells adjusted their cytoskeleton accordingly. Future investigation of specific biomarkers will be

needed; however, the system presented can become a valuable tool to promote and investigate different endothelial phenotypes in various physiologically relevant conditions.

Controlling the formation of a perfusable capillary network

The vascular model as discussed above, is an easy-to-fabricate 3D-VoCs which can be used for various purposes. However, the model does oversimplify important aspects of the structural and biomechanical properties of the capillary vasculature. For instance, peripheral blood cells have the same dimensions as the capillary diameter. As a result, they are deformed when passing through the capillary[27,28]. The resulting biomechanical forces on both the peripheral and endothelial cells are therefore not solely the result of continuous fluid flow. Furthermore, mural-cell interaction involves complete wrapping around the endothelial capillary, which cannot be achieved with large lumens.

The most accessible method to engineer a complex microvascular is cellular self-assembly. Endothelial cells can be mixed with mural and tissue specific cells inside a hydrogel where they form a capillary bed by themselves. However, these capillary beds all have different dimensions, which makes downstream analyses laborious as matching haemodynamic stimulation with phenotype becomes location depended[29]. Also, it has been shown that adding different cellular components not only influence the cellular responses but also influences the formation and dimensions of the network formed[30]. Controlling and equalizing the haemodynamic forces becomes impracticable which adds additional experimental variation between conditions. Furthermore, the majority of the self-assembled vessels are in fact larger than *in vivo* capillaries[29,30].

Chapter 5 describes a method to engineer and control the dimensions of a perfusable complex capillary network. To engineer capillary vessels with a predefined network, we use on-chip guided cellular self-assembly. To generate these scaffolds, we used a commercial system (called Primo, from Alveole) for UV-hydrogel patterning to construct a vascular capillary-network. To guide the hiPSC-ECs into the pre-determined perfusable format we patterned a rectangular channel with a fixed height of 50 µm composed of gelatine Gelatin methacryloyl (GelMA) inside a microfluidic device. GelMA is a widely used UV-curable hydrogel where mechanical and chemical properties can be tailored to specific needs by varying the UV-dose, concentration or premixing with other UV curable ECM components.

Using these hydrogel scaffolds we discovered a remarkable behaviour of hiPSC-ECs. When the cells are located in a channel with a width larger than 50 µm, they form an EC monolayer which covers both the walls and the ceiling of the fluidic channel. However, when the hydrogel channels were

smaller than 30 µm, the hiPSC-ECs that were seeded did not form vertical walls of endothelium but instead formed round, tubular structures.

This result suggests that by confining ECs, a predefined capillary network can be engineered. Compared to alternatives of generation true capillary structures like 2-Photon laser-ablation[31] or laser-cavitation[32] the used method is less technical complicated and easy to scale, as working designs can also be patterned using mask-lithography.

However some limitations need to be addressed before the protocol is advantageous for *in vitro* modelling. Due to the small and confined culture environment the fragile cell structures can be easily over-stressed and therefore the fluidic handling needs to be optimized. It is therefore desirable to employ a Fluidic Circuit Board to automize and control the fluidic handling, so that operator manipulation can be minimized or circumvented[33].

Altogether, the advantages of the methods presented in this thesis bring OoC technology closer to biologists as end-users. We developed simple protocols to generate 3D-VoCs with various dimensions from 350 µm all the way down to even the smallest capillary with a diameter of 10 µm. The VoCs developed were all reproducible to produce, perfusable and implementable in biology lab, supporting steps towards the development of better *in vitro* models of vasculature. Importantly, the methodologies were all scalable in production, with up to 24 samples in one run by a single operator. This allows the simultaneous generation of sufficient technical replicates from a single experiment for different structural an functional readouts.

Future perspectives

The methods described in this work allow for the development of complex and physiologically relevant *in vitro* vascular models. Nevertheless, further refinement of the technology will be necessary before widespread adoption of OoC-technology is likely. A selection of points that particularly need refining are discussed in the following sections.

Microfluidic handling

Advances in microfabrication allow the miniaturization of cell culture devices, reducing the amount and volume of reagents and cells required, allowing better control of mechanical stress and the application of chemical gradients. However, miniaturization increases the complexity of manual handling. For instance, preventing evaporation of cell culture medium inside small devices is important as it can change concentrations of culture medium components very quickly. Furthermore, the simple insertion of a normal pipette-tip inside a fluidic channel can generate enough mechanical stress to damage delicate cellular structures.

Automated liquid handling systems could help address these limitations; however, this would require the standardization of microfluidic interfaces and

at present, no current standard can fit all purposes. Different OoCs also require different interfaces to connect with external pumping systems for example. Devices such as the Translational Organ-on-chip Platform (TOP), currently being developed in the University of Twente, is one example of how one interface can be used to accommodate many different chip devices and systems which can be very helpful in bringing the technology to the end users[34]. The transition of the TOP system to a commercial project is eagerly awaited by many since it offers substantial flexibility in chip design yet does not require customised solutions to regulate fluidic flow.

Use of new Biomaterials

Although the models presented in this thesis do not use (living) animal models, reagents derived from animals are still widely used for the production of the necessary hydrogels. For instance, the extracellular matrix Collagen I, is generally extracted from bovine- or rat-tails and GelMA is derived from pigs. Matrigel, also an important component of many hiPSC differentiation protocols and assays, is extracted from sarcomeres induced in mice. Since these extracellular matrix components are animal derived, they are accompanied by batch-to-batch variation which means that assays often require re-optimisation when a new batch is used[35,36]. Recombinant alternatives are often too expensive to be a feasible alternative for the generation of hydrogels, or they are ineffective as replacements. Recent advances in the development of biomaterials indicate that these should now be further explored. Materials like polyethylene glycol (PEG) linked with RDG (or other sequence)peptides that recognize receptors[37,38] on the cell surface can engineered on the basis of animal free- and chemically defined reagents and will increase the reproducibility and availability of essential OoC reagents[39].

Use of genetically encoded reporter lines

hiPSC-derived cells are beginning to change the field of not only stem cell biology as such but also drug discovery and disease modelling. This is in part because of their ability to generate all cell types of the human body and in part because they can be derived from patients with genetic or somatic disease; they thus may encompass a complete patient genotype. New developments in gene targeting and the introduction of transgenes has also provided opportunities to generate genetically encoded reporter lines, either to identify specific cell types[40] or to measure functional parameters such as calcium ion flux and action potentials of electrically active cells [41,42]. Complex models can be generated and imaged in real time using these fluorescent readouts of cell type or function in 3D making it possible to collect information during experiments rather than only terminally, at the end-point of the experiment[40,43,44].

Using OoCs to accelerate drug discovery

Envisioning a future where all drugs are tested in a patient-specific model in a personalised way prior administration is already tempting but may only be a long-term outcome of the type of research described here and only for selected conditions. Nevertheless, there are more immediate and practical applications of OoC technology. For tissues that are closer to those in organs of the body because they are cultured in OoC devices, opportunities arise to study physiology and disease in humans at the cellular level.

Multiplexed devices using patient-genotypes, such as described in this thesis, will allow parallel analysis of drug or cellular responses with the number of biological replicates needed for robust conclusions without the risk for patients. Furthermore, if fluid flow-through can be collected and the cells can be extracted it will be possible to obtain multi-omics data parameters to feed *in silico* predictions of disease pathways and drugs that could be used to target them[45,46]. OoCs can link and validate all databases of Genomics, Transcriptomics, Proteomics and Metabolomics to answer the important question: how can we improve human health? OoCs can be a stepping stone in the right direction.

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