Advanced In Vitro 3D Models to Investigate iPSC Pluripotency and Capillary Network Formation of HUVECs

Josefin Blell, MSc, Shubhankar Nath, PhD,
Christen Boyer, PhD, and Itedale Namro Redwan, PhD
CELLINK, Gothenburg, Sweden

Abstract

A co-culture of human umbilical vein endothelial cells (HUVECs) and human dermal fibroblasts (HDFs) and a monoculture of induced pluripotent stem cells (iPSCs) were separately embedded in a selection of biomaterials for 7 days. Cellular markers from these 3D culture systems were compared to 2D cultures using immuno-fluorescence imaging and demonstrated the importance of using cell-relevant material. It was also concluded that the culture time for HUVEC-HDF co-cultures was an important factor to obtain complex structures. Extended culture times in 3D lead to the observation of proangiogenic structures, a phenomenon only observed in 3D. The iPSCs formed clusters, remodeled their cellular assembly and formed torus-like structures when cultured in 3D as opposed to 2D., The iPSCs also expressed the pluripotency markers OCT4 and NANOG in 3D. That the iPSCs can retain their pluripotency in 3D cultures enables scientists to design more advanced experiments in which differentiation of the stem cells can be performed after embedding in 3D.

Introduction

3D bioprinted and co-cultured organoids have gained much attention recently due to the differences in cellular self-assembly and reorganization throughout 3D matrices. Monoculture organoids tend to aggregate in 3D to maximize adhesion and minimize energy, while co-cultures reorganize based on differences in cell-cell adhesion (Foty, 2005; Napolitano, 2007). In general, different cell types also significantly impact each other during cellular reorganization. For example, endothelial progenitor cells alone do not form vascular tissues, but when co-cultured with human dermal fibroblasts (HDFs) or smooth muscle cell types, vascular endothelial tubes can form in porous biomaterials or bioinks (Unger, 2007). A study using human umbilical vein endothelial cells (HUVECs) showed that these cells cannot form endothelial cell lumen without the presence of fibroblasts which highlights the importance of co-culture systems (Newman, 2011).

During the study, relevant markers for HUVEC cultures were analyzed. Cluster of differentiation 31 (CD31) is an endothelial cell-specific marker used to monitor endothelial differentiation, self-assembly and angiogenesis in both 2D and 3D cultures. CD31 is expressed on the surface of HUVECs and is known to self-organize within organoids (Wu, 2004). Zonula occludens-1 (ZO-1) is a cytoplasmic protein that acts as a scaffolding molecule and is a component of tight junctions in epithelial and endothelial cells. The ZO-1 amino terminus is capable of binding to claudins and α-catenin/cadherins, and the carboxyl terminus can interact with the actin cytoskeleton (Itoh, 1997). It is shown that in epithelial cells, ZO-1 is necessary for lumen formation in 3D. In aligned HUVEC 3D cultures, ZO-1 plays an important role in the formation of functional endothelium with tight junctions (Kang, 2018).

Pluripotency markers OCT4, SOX2 and NANOG play a critical role in maintaining the stemness of embryonic stem cells as well as induced pluripotent stem cells (iPSCs). Evidence suggests that changes in their expression pattern control the cell fate during differentiation and development (Wang, 2012). For example, OCT4 regulates and interacts with the BMP4 pathway to specify different developmental fates. High levels of OCT4 enable self-renewal in the absence of BMP4 but specify mesendoderm in the presence of BMP4. Low levels of OCT4 induce embryonic ectoderm differentiation in the absence of BMP4 but specify extraembryonic lineages in the presence of BMP4. SOX2 represses mesendoderm differentiation, while NANOG represses embryonic ectoderm differentiation (Rizzino, 2016). This demonstrates the importance of maintaining these pluripotency markers when embedding iPSCs in a 3D matrix or culturing in 2D.

Materials and methods

Cell preparation

HUVECs were cultured in large vessel endothelial cell medium with growth supplements (Cellworks, ZHM-2953) with the addition of 0.1% gentamycin/amphotericin antibiotics (Gibco, R-015-10). HDF (neonatal) were cultured in fibroblast growth medium with supplements (Promocell, C-23010). iPSCs were cultured according to Cellartis's culturing protocol. iPSCs were cultured on Synthemax II-SC substrate (Corning, 3535) coating and detached with Versene solution (Gibco, 15040-033). The medium used was Cellartis DEF-CS 500 Xeno free medium (Takara Bio, Y30045) with additives (Takara Bio, Y30042) and added 1% gentamycin/ amphotericin (Gibco, R-015-10).

Bioink preparation and bioprinting

For HUVEC-HDF bioprinting, GFP-labeled HUVEC cells were mixed with HDFs in a 1:1 ratio and mixed with bioink, Matrigel Matrix (Corning, 354234) or GelMA (CELLINK, IK305102), supplemented with fibronectin at 2 million cells/mL with a 9:1 ratio of bioink to cells. The bioink and cells were mixed between two syringes and then dispensed through a connected 22G nozzle (CELLINK, NZ4220005001). The GelMA-based samples were photocrosslinked for 15 seconds at 3 cm distance and Matrigel-based samples were thermally crosslinked for 20 minutes at 37°C before medium was added. The constructs were cultured in the HUVECs medium which was changed three times a week.

For iPSCs bioprinting, the cells were mixed in at 1 million cells/mL in a 10:1 ratio of bioink (Matrigel or GelMA supplemented with laminin 521 (GelMA+LN521)) to cells (v/v) between two syringes and dispensed through a 20G nozzle (CELLINK, NZ4200005001). GelMA was photocrosslinked for 5 to 10 seconds, Matrigel was thermally crosslinked. The medium was changed daily.

In general, all samples were bioprinted as droplets in untreated 96-well plates (VWR, 7342781) using the BIO X (CELLINK, D16110020717) and cultured in the presence of 100 µL of corresponding growth medium. All photocrosslinking was performed with a 405 nm light module. 2D cells were cultured on chambered cell culture slides (Falcon, 354108). All the cultures were maintained at 37°C and 5% CO_2. Cells were cultured for either 5 to 14 days or 3 to 4 days in 3D or 2D, respectively, and further analyzed for expression of different markers using immunohistochemistry (IHC).

Immunostaining

The 3D constructs were fixed with 4% paraformaldehyde (in $CaCl_2$ 50 mM) for 5 to 6 hours and then dehydrated with ethanol and xylene. The constructs were infiltrated and embedded with paraffin before sectioning to 5 µm thick sections using a microtome. The sections were fixated on a microscope slide and deparaffinized in xylene and ethanol. The samples were prepared for antibody staining by being boiled in antigen retrieval buffer and then treated with a blocking solution. The 2D cells were fixed for 1 to 3 hours, permeabilized with 0.5% Triton-X 100 and treated with blocking solution. Primary antibody solutions were added to all the slides and incubated overnight at 4°C. After washing with PBS, the sections were treated with secondary antibodies for 1 hour at room temperature. The cell nuclei were counterstained with NucBlue Fixed Cell ReadyProbes Reagent (Invitrogen, R37606). Coverslips were mounted on the slides and attached with Fluoromount-G (Invitrogen, 00-4958-02). The localization of the secondary antibodies was analyzed with a fluorescent confocal microscope (ZEISS LSM 710 NLO) with the same acquisition parameters.

Results and discussion

HUVEC-HDF co-culture: To determine the impact of different matrices on HUVECs and HDFs co-culture, differences in morphology were first characterized using fluorescence microscopy. The cells growing in Matrigel and GelMA Fibronectin formed dense networks and showed elongated phenotype, whereas cells in 2D exhibited less elongated and more rounded morphology (Figure 1). Moreover, the cells in the 2D images revealed that the GFP-HUVECs were in few epithelial clusters.

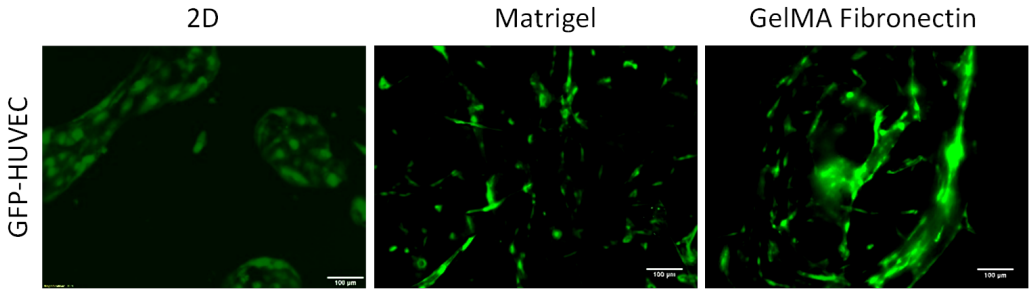

Figure 1. GFP-labeled HUVECs (green) in co-culture with HDF in 2D monolayer (Day 3) and in 3D culture on Matrigel (Day 7) and GelMA Fibronectin (Day 14). Scale bar = 100 µm.

ZO-1 staining: In addition to cell morphology, tight junctions play a crucial role in the regulation of endothelial barrier function. To this end, immunostaining was performed for the tight junction-associated protein ZO-1 to analyze its cellular distribution in 2D and different 3D matrices. As shown in Figure 2 (top panel), a high level of ZO-1 was detected in GelMA Fibronectin samples on Day 15. Several sprouts were also observed in the GelMA Fibronectin sample, indicating the ability of these cells to form capillary-like structures. Interestingly, the ZO-1 staining in 2D was not restricted to HUVECs only but could also be detected in HDFs, suggesting that a 3D environment containing appropriate ECM components is necessary for maintaining the structure as well as spatial and temporal expression of proteins in different cell lines.

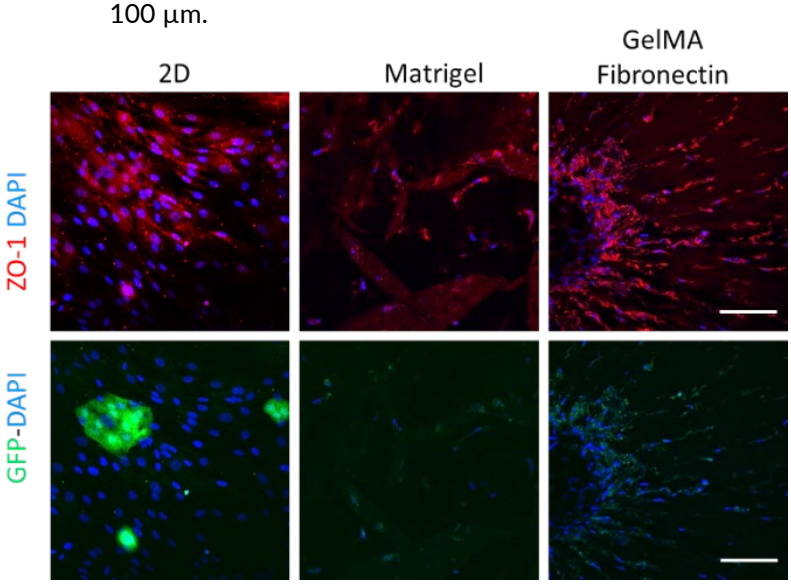

Figure 2. GFP-HUVEC and HDF co-culture stained for ZO-1 (red) and DAPI (blue) in 2D (Day 3) and in Matrigel (Day 8) and GelMA Fibronectin (Day 15). Scale bar = 100 µm.

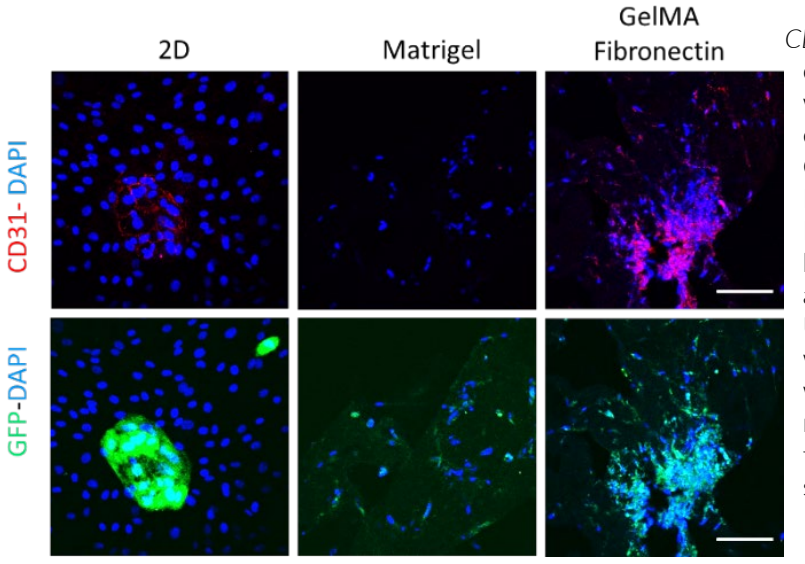

Figure 3. GFP-HUVEC and HDF co-culture stained for CD31 (red) and DAPI (blue) in 2D (Day 3) and 3D Matrigel (Day 8) and GelMA Fibronectin (Day 15). Scale bar = 100 µm.

CD31 staining: To further characterize the co-culture, immunofluorescence staining was performed for CD31, a specific endothelial marker. Not surprisingly, CD31 expression was restricted to HUVECs only in both 2D and GelMA Fibronectin Day 15 as can be visualized by overlapping HUVECs expressing GFP and CD31/DAPI image panels (Figure 3). Unfortunately, very low levels of CD31 were detected in Matrigel Day 8 cultures, which could be explained by the very low number or lack of HUVECs cells from the field of view as indicated by the GFP signals (Figure 3).

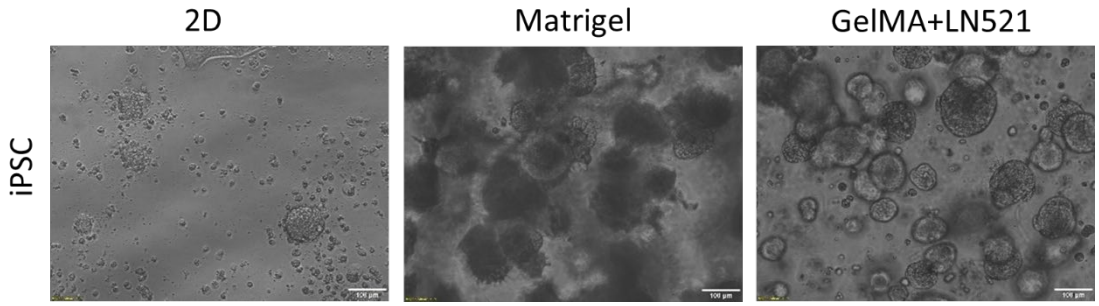

Figure 4. Bright-field images of iPSCs after 4 days of culture in 2D and 7 days in 3D Matrigel or GelMA+LN521. Scale bar = 100 µm.

Next, the morphology of iPSC cells grown in 2D and 3D clusters was investigated using bright-field microscopy, as shown in Figure 4. Spheroids of different sizes were observed in Matrigel as well as in GelMA+LN521. In addition, the expression of three pluripotency markers OCT4, NANOG and SOX2 was evaluated.

The pluripotency marker OCT4 was expressed to different extents in all conditions as shown in Figure 5 (top panel). In 2D, the highest signal intensity was observed at the perimeter of the cell cluster. Whereas in Matrigel, the signal intensity was distributed evenly over the entire sample. Very similar expression patterns were observed for NANOG-stained samples in Figure 5 (middle panel). A longer exposure time was required to visualize the SOX2 expression in the 2D samples illustrated in Figure 5 (bottom panel). In addition to the expression of the pluripotent markers, a torus-like morphology was observed for the cell clusters in GelMA+LN521, and most predominantly in Matrigel. These hollow clusters resemble neural rosettes or early mesodermal structures (Muratore, 2014). However, further analysis is needed to confirm any potential differentiation since the iPSCs retain the expression of OCT4 and NANOG for all conditions where cell aggregates were observed. These pluripotency markers are not pan-repressors for all fates of differentiation, but each control differentiation toward specific lineages. For example, NANOG represses embryonic ectoderm differentiation with little effect on other lineages, and SOX2 represses mesendoderm differentiation (Wang, 2012). The transcription factors interplay carefully to retain the cells in pluripotent state and small fold changes in the expression of one factor compared to the others can lead to differentiation (Rizzino, 2016). The small changes observed with immunofluorescence were not quantitative but demonstrated the retention of pluripotency markers even after embedding and 3D bioprinting, opening new opportunities for differentiation after the 3D bioprinting process.

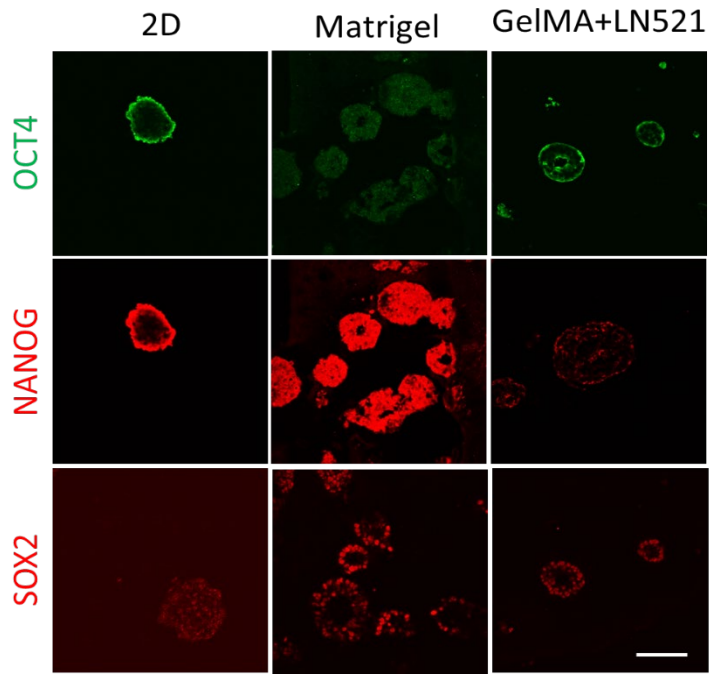

Figure 5. OCT4, NANOG and SOX2 expression in iPSCs after 4 days of culture in 2D and 7 days in 3D Matrigel or GelMA+LN521. All images were captured using the same acquisition parameters except for the 2D image of SOX2 which has been enhanced for visualization. Scale bar = 100 µm.

Conclusions and future direction

3D bioprinting exhibits multiple advantages over 2D cell cultures, including the precise geometrical arrangement of multicellular constructs that can better recapitulate the native 3D human physiology. Cells self-assemble based on external signals from surrounding cells and the environment. This important phenomenon is aiding in the understanding of embryogenesis, angiogenesis, wound healing, tumor formation and may also potentially assist in the development of new drugs and routes for the vascularization of bio-artificial organs in the future. The following were concluded from this study:

- A 3D environment is crucial for the development of complex structures and the network formation of HUVECs.
- The iPSCs form clusters, remodel their cellular assembly and form torus-like structures when cultured in 3D. A similar rearrangement of the iPSCs is not observed in the 2D controls.
- In 2D as well as 3D, the iPSCs express the pluripotency markers OCT4 and NANOG, whereas the SOX2 expression is lower in 2D than in 3D.
- That the iPSCs can retain their pluripotency in 3D cultures enables scientists to design more advanced experiments where differentiation of the stem cells can be performed after embedding in 3D.

References

1. Foty RA, Steinberg MS. The differential adhesion hypothesis: a direct evaluation. *Developmental Biology*. 2005; 278(1): 255–263. DOI:10.1016/j.ydbio.2004.11.012.
2. Napolitano AP, Chai P, Dean DM, Morgan JR. Dynamics of the self-assembly of complex cellular aggregates on micromolded nonadhesive hydrogels. *Tissue Engineering*. 2007; 13(8): 2087–2094. DOI:10.1089/ten.2006.0190.
3. Unger RE, Sartoris A, Peters K, et al. Tissue-like self-assembly in cocultures of endothelial cells and osteoblasts and the formation of microcapillary-like structures on three-dimensional porous biomaterials. *Biomaterials*. 2007; 28(27): 3965–3976. DOI:10.1016/j.biomaterials.2007.05.032.
4. Newman AC, Nakatsu MN, Chou W, et al. The requirement for fibroblasts in angiogenesis: Fibroblast-derived matrix proteins are essential for endothelial cell lumen formation. *Molecular Biology of the Cell*. 2011; 22(20): 3791–3800. DOI:10.1091/mbc.E11-05-0393.
5. Wu X, Rabkin-Aikawa E, Guleserian KJ, et al. Tissue-engineered microvessels on three-dimensional biodegradable scaffolds using human endothelial progenitor cells. *American Journal of Physiology. Heart and Circulatory Physiology*. 2004; 287(2): H480–7. DOI:10.1152/ajpheart.01232.2003.
6. Itoh M, Nagafuchi A, Moroi S, Tsukita S. Involvement of ZO-1 in cadherin-based cell adhesion through its direct binding to alpha catenin and actin filaments. *The Journal of Cell Biology*. 1997; 138(1): 181–192. DOI:10.1083/jcb.138.1.181.
7. Kang B, Shin J, Park H-J, et al. High-resolution acoustophoretic 3D cell patterning to construct functional collateral cylindroids for ischemia therapy. *Nature Communications*. 2018; 9(1): 5402. DOI:10.1038/s41467-018-07823-5.
8. Wang Z, Oron E, Nelson B, et al. Distinct lineage specification roles for NANOG, OCT4, and SOX2 in human embryonic stem cells. *Cell Stem Cell*. 2012; 10(4): 440–454. DOI:10.1016/j.stem.2012.02.016.
9. Rizzino A, Wuebben EL. Sox2/Oct4: A delicately balanced partnership in pluripotent stem cells and embryogenesis. *Biochimica et Biophysica Acta*. 2016; 1859(6): 780–791. DOI:10.1016/j.bbagrm.2016.03.006.
10. Muratore CR, Srikanth P, Callahan DG, Young-Pearse TL. Comparison and optimization of hiPSC forebrain cortical differentiation protocols. *PLoS One*. 2014; 9(8): e105807. DOI:10.1371/journal.pone.0105807.

Contact us

U.S. phone:
(+1) 833-235-5465

Email:
sales@cellink.com

European phone:
+46 31-128 700

Website:
www.cellink.com