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# Growing blood vessels in space: Preparation studies of the SPHEROIDS project using related ground-based studies

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ABSTRACT

Endothelial cells (ECs) grow as single layers on the bottom surface of cell culture flasks under normal (1*g*) culture conditions. In numerous experiments using simulated microgravity we noticed that the ECs formed three-dimensional, tube-like cell aggregates resembling the intima of small, rudimentary blood vessels. The SPHEROIDS project has now shown that similar processes occur in space. For the first time, we were able to observe scaffold-free growth of human ECs into multicellular spheroids and tubular structures during an experiment in real microgravity. With further investigation of the space samples we hope to understand endothelial 3D growth and to improve the *in vitro* engineering of biocompatible vessels which could be used in surgery.

## 1. Introduction

A few years ago, we detected that some types of endothelial cells (ECs) form tube-like structures when cultured on a random positioning machine (RPM) [1,2]. This observation was of great interest, because it opened up new possibilities to study neovascularization, which plays a crucial role for instance in tumor development. Gaining a deeper understanding of the underlying mechanisms in neovascularization further helps to engineer blood vessels that can be used in hand surgeries, plastic reconstructive surgeries and transplantations for example. RPMs represent one of several tools available to simulate microgravity (μg) on ground (1*g*). The working principle of the RPM is based on gravity vector averaging to zero [3]. The typical RPM system comprises two gimbal-mounted frames, which are each driven independently by motors. Through dedicated algorithms, the samples placed on the inner frame are constantly reoriented, such that the gravity vector is distributed in all directions over time. Thus, from the sample's point of view, the constantly reorienting gravity vector's trajectory converge toward zero over time. However, 1*g* is always acting on the sample at any given instant. It is assumed that the gravity vector needs to point in a specific direction for a minimal period of time in order to allow biological systems, like cells, to adapt to the gravity vector. But if the gravity vector constantly changes its orientation, the cells will lose the sense of direction and thus experience a state similar to μg (removed gravity vector). Therefore, the frame rotations shall be faster than the biological process studied [4]. However, the rotation cannot be too fast,

*Abbreviations:* Clinostat, (CN); endothelial cell, (EC); extracellular matrix, (ECM); Gene Ontology, (GO); human microvascular endothelial cell, (HMVEC); human saphenous vein endothelial cell, (HSVEC); human umbilical vein endothelial cell, (HUVEC); International Space Station, (ISS); microgravity, (μg); paraformaldehyde, (PFA); Periodic acid-Schiff, (PAS); Random Positioning Machine, (RPM); Rotating Wall Vessel, (RWV); three-dimentional, (3D); vascular endothelial growth factor, (VEGF)

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as centrifugal forces will become effective [5]. Therefore, the RPM is typically used to examine slow processes, which are observed at least on the timescale of hours. It is obvious that an RPM treatment introduces additional forces to the samples through the unique moving pattern. If the RPMs are operated within certain boundaries however, these disturbing forces can be reduced to a minimum [6]. We therefore belive that an RPM treatment, applied with caution, can be called "simulated microgravity". The question remains, whether real μg can induce the same tube-like structure formation of ECs that were cultivated on the RPM. To answer this question, the ESA-SPHEROIDS project was launched. This project included an extensive phase of preparation regarding the selection of the type of ECs capable of tube-like structure formation even under the stressful situation and special cell culture conditions during a spaceflight. In addition, a hardware was prepared that guaranteed a proper culturing of human cells in space.

Two independently engineered hardware types were available in our team for a space mission to the ISS or a space experiment on an unmanned satellite/spacecraft. The first hardware (SimBox/CellBox), developed by Airbus Defence and Space (Friedrichshafen, Germany), holds a cell culture chamber with a volume of 13.5 mL. Below the cell culture chamber, two fluid tanks are mounted. One of them acts as a reservoir for fresh media in concert with being a waste compartment, the other one stores the fixative and is the compartment for the waste media of the second pumping procedure. Both, the fresh media compartment and the fixative compartment can hold up to 10 mL of a liquid and are used to exchange the fluid in the cell culture chamber [7]. The special feature of the tank is an internal silicon membrane, which very effectively allowed the storage of different fluids inside the same compartment without any cross-contamination. When the fresh media is pumped into the cell culture chamber, the waste fluid is simultaneously guided into the empty space on the other side of the membrane. In this way dead volume was avoided. Besides the fixed cells, the supernatant can be used for analyses.

The other hardware (now called SPHEROIDS hardware) was designed and constructed by RUAG Space (Nyon, Switzerland). One unit holds one fixative chamber, two cell culture chambers, two fresh media compartments and two waste compartments. Each cell culture chamber has a growth area of 9 $cm^2$ providing a total of 18 $cm^2$ per unit. The completed unit, including electronics, has a dimension of 10 × 10 × 10 cm [8] (Fig. 1).

Both hardware types are built of biocompatible materials and can be handled easily by the experimentators. However, the CellBox hardware is more compact. It provides a larger growth area but lacks the possibility of a complete media exchange. It has proven to be suitable for cancer cell cultures in space [7,9,10]. The SPHEROIDS hardware is designed with supernatant chambers, which are empty during the missions start. A complete exchange of media and also a complete flushing with fixative is possible. As these characteristics appeared important for tissue engineering of tubular structures in space, the SPHEROIDS hardware was chosen for this experiment.

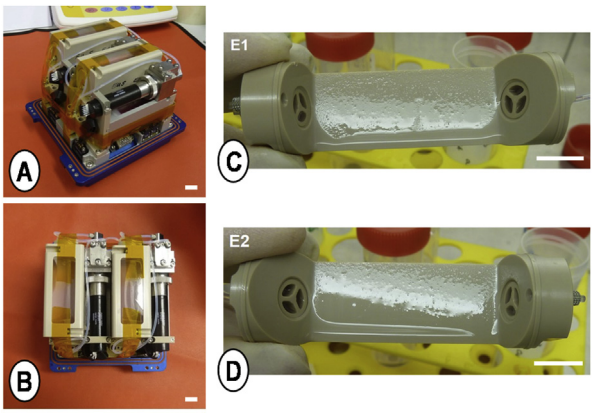


**Fig. 1.** Sample retrieval from the SPHEROIDS hardware. (A) Side view and (B) top view on one hardware unit removal of the cover. The two cultivation chambers (E1 and E2) are installed at the top of the unit. Samples in both chambers are fixed with PFA. (C + D) View onto the growth area of the cultivation chambers. The 'goose pimples-like' structures on the material surfaces are adherent EA.hy926 cells. Scale bars: 1 cm.

## 2. Material and methods

### 2.1. Cells

EA.hy926 [11] cells were cultivated in RPMI 1640 medium (Invitrogen, Eggenstein, Germany) containing 2 mM L-glutamine (Thermo Fisher Scientific, Waltham, US-MA) and 10% FCS, 100 U/mL penicillin and 100 μg/mL streptomycin (all Biochrom, Berlin, Germany).

Human saphenous vein endothelial cells (HSVECs) and human microvascular endothelial cells (HMVECs) were grown in advanced EC growth medium purchased from Provitro (Berlin, Germany).

All cell types grew in 75-$cm^2$ tissue culture flasks (Sarstedt, Nümbrecht, Germany) at 37 °C and 5% humidity.

### 2.2. Tube formation test on the RPM

In order to test the tube formation capability of the three cell types, the monolayer cells were scrapped off the bottom of the culture flask and suspended in RPMI 1640 medium. Subsequently, $10^6$ cells were added to each of sixteen 25 $cm^2$ tissue culture flasks, respectively and incubated under normal gravity conditions at 37 °C until they reached 70% confluence. Afterwards, eight culture flasks, completely filled air-bubble-free with culture medium, were either mounted on a table top RPM [6] kept within a commercially available incubator or set in the incubator next to RPM. The cell culture procedure was continued as indicated below.

### 2.3. Spaceflight experiment

For preparing the spaceflight experiment, EA.hy926 cells were detached from the bottom of the culture flask, and suspended in RPMI 1640 medium. Then, $10^6$ cells were seeded in each of the 16 cultivation chambers (RUAG Space, Nyon, Switzerland) of the SPHEROIDS hardware recently described [8] (Fig. 1). VEGF-A was added to one half of the culture chambers. As fixatives either paraformaldehyde (PFA) or RNA*later* (for RNA-sequencing at a later time) were used. During the 7-day-experiment, the nutrient solution was not refreshed, whereas it was changed for the 14-day-experiment.

### 2.4. Cell characterization

Cells cultured on the RPM were studied by phase contrast microscopy. For visualization of the three-dimensional (3D) aggregates, tube-like structures fixed with 4% PFA at the end of the culture period, indicated below, were collected from spaceflight cultures. Afterwards, they were embedded in paraffin and subjected to Periodic acid-Schiff (PAS) staining, performed according to routine protocols [12]. Then the coverslips were mounted and the structures were evaluated using a Zeiss Axiovert wide field microscope (Oberkochen, Germany).

### 2.5. Pathway analysis

To investigate and visualize the original localization and the mutual interactions of proteins coded by genes which were more than 4-fold up or down-regulated, we used the relevant UniProtKB entry numbers in the Elsevier Pathway Studio® v.11 software (Elsevier Research Solutions, Amsterdam, The Netherlands). The method was described earlier [13,14]. A STRING analysis was performed entering the names/

**Table 1**
Selected articles addressing the mechanisms of 3D growth of human ECs exposed to simulated μg.

| Cells | Device, Duration | Findings | Ref. |
|---|---|---|---|
| HUVECs | CN, 1 d | Angiogenesis is mediated through PI3K-Akt-eNOS signalling pathway | [15] |
| HUVECs | CN, 1 d | iNOS up-regulation AP-1↓, NF-κB↓ | [16] |
| HUVECs | CN, 1 d | Enhanced autophagosome formation, LC3↑, beclin-1↑ | [17] |
| HUVECs | CN, 2 d | Induction of autophagy via HDM2-p53-mTOR pathway | [18] |
| HUVECs | CN, 1–2 d | eNOS↑, posttranslational modifications of caveolin-1 | [19] |
| HUVECs | RWV, 1 d | Angiogenesis through RhoA-dependent rearrangement of the actin cytoskeleton | [20] |
| HUVECs | RWV, 3 d | hsp70↑, IL-1α↓, rapid remodelling of the cytoskeleton, actin↓ | [21] |
| HUVECs | RWV/RPM, 2–4 d | Alterations of the actin cytoskeleton, NO synthesis | [22] |
| HUVECs/ HMVECs | RWV, 4 d | Up-regulation of hsp70 | [23] |
| HUVECs | RWV, 8 d | Prostacyclin↑, NO↑ | [24] |
| HUVECs | RPM, 1 h-3 d | Increased cell migration | [25] |
| HUVECs | RPM, 1 d | *ICAM1*↑ | [26] |
| HUVECs | RPM, 1 d | *ICAM1*↑ in TNF-α-stimulated ECs | [27] |
| HUVECs | RPM, 1–2 d | "Anti-inflammatory phenotype", *NOS3*↑, *CAV1*↑, *CAV2*↑ | [28] |
| HUVECs | RPM, 1–3 d | Alteration in cytoskeleton structure and mechanical properties | [29] |
| HUVECs | RCCS[a], 1–4 d | Increase of mTOR and Apaf-1, *miR-22*↑, *SRF*↓, *LAMC1*↓ | [30] |
| HUVECs | RPM, 4 d | Secretome analysis: RANTES↑, eotaxin-1↑, IL-1α↓, IL-8↓, bFGF↓ | [31] |
| EA.hy926 | RPM, 2 h | Angiogenesis via the cGMP-PKG-dependent pathway | [32] |
| EA.hy926 | RPM, 2 h | Increased migration caused by actin modulation and NO release | [33] |
| EA.hy926 | RPM, 4–24 h | Induction of apoptosis, ECM proteins, ET-1, TGF-β. *NOS3*↓. IL-6↑, IL-8↑ NF-κB p50↑ and p65↑. bFGF protects ECs from apoptosis. | [34] |
| EA.hy926 | RPM, 4–72 h | Increase of ECM protein (collagen type I, fibronectin, osteopontin, laminin, Flk-1), altered cytoskeletal components. Cell-protective influence of VEGF. | [35] |
| EA.hy926 | RPM, 1–15 d | 3D growth, increase of ECM proteins (osteopontin↑, fibronectin↑) | [36] |
| EA.hy926 | RPM, 5–7 d | Secretome analysis: VEGF↓, bFGF↓, TNFRSF5↓, TNFSF5↓, ICAM-1↓, TNFR-2↓, IL-18↓, C3↓, VWF↓. PKCα plays a key role in tube formation | [37] |
| EA.hy926 | RPM, 5–7 d | Gene array analysis: 1625 differentially expressed genes (*VWF*↑, *CXCL8*↑, *IL6*↑, *HMOX1*↑) | [38] |
| EA.hy926 | RPM, 5–28 d | Tubes: integrin β1↑, α-tubulin↑, laminin↑, fibronectin↑ | [1] |
| EA.hy926 | RPM, 35 d | Secretome analysis: Suggested key proteins of spheroid formation (VEGF↑, LCN2↑, IL-6↑, IL-8↑, MCP-1↑, VCAM-1↑, ICAM-1↑, fibronectin↑, RANTES↑) | [39] |
| EA.hy926/ HMVECs | RPM, 5–7 d | Proteome analysis: Proteasomes and ribosomal proteins may play a role in tube formation | [2] |

↑ = up-regulation/increase, ↓ = down-regulation/decrease.
[a] RCCS = rotary cell culture system.

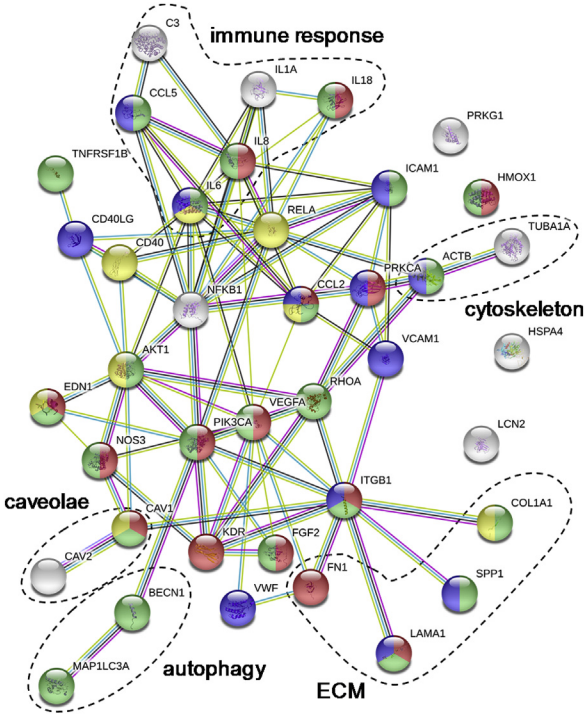


**Fig. 2.** Interactions of proteins influenced in ECs under μg conditions (STRING analysis). Contributions to biological processes (GO) such as blood vessel development (red), cell adhesion (blue), response to mechanical stimulus (yellow) and response to external stimulus (green) are indicated with different colours. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

entry numbers of proteins found in the literature references [10–34] using the STRING platform (https://string-db.org/).

## 3. Current knowledge from ground-based studies

Experiments using ground-based devices such as clinostats (CNs), rotating wall vessels (RWVs), or the RPM provide the basis for research in real μg and pave the way for rare and cost-intensive spaceflight projects like SPHEROIDS. Table 1 summarizes the previous research that was performed with human ECs on μg simulation devices.

The exact mechanism of endothelial 3D growth in simulated μg is still unknown. Fig. 2 shows a STRING analysis of EC proteins that are known to be affected by μg according to the literature summarized in Tab. 1. Alterations of the actin cytoskeleton were reported often and corroborate the theory of cytoskeletal gravisensing in eukaryotic cells [40]. Taking into account their corresponding Gene Ontology (GO) biological processes, proteins such as RelA, ET-1, CD40, Akt, caveolin-1, MCP-1, or interleukin (IL)-6 are modulated as reaction to mechanical stimuli (Fig. 2, yellow). They are metabolically connected to factors for cell adhesion (Fig. 2, blue) and blood vessel formation/angiogenesis (Fig. 2, red). Studies with human umbilical endothelial cells (HUVECs) indicated, that angiogenesis could be mediated via the PI3K-Akt-eNOS pathway and Rho signalling [15,18]. Possible key proteins of endothelial spheroid formation that were identified in EA.hy926 cells in RPM experiments build an interaction network between extracellular matrix (ECM), cytoskeleton, immune response and other cellular processes. It seems that μg intervenes deeply into cell function and metabolism finally resulting in an altered growth behaviour and the formation of tubular structures.

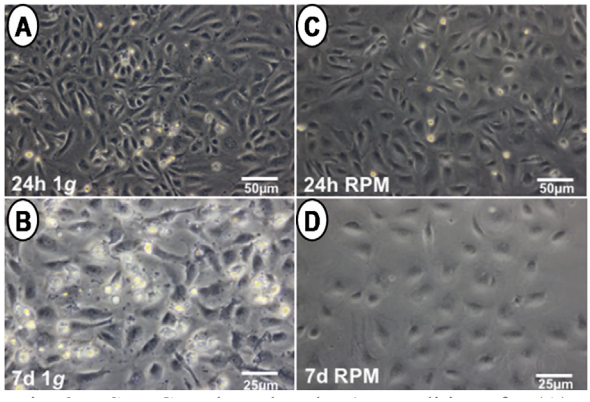


**Fig. 3.** HSVECs cultured under 1*g*-conditions for (A) 24 h and (B) 7 d or on the RPM for (C) 24 h and (D) 7 d.

## 4. Results

### 4.1. Selection of the most suitable cell line

We were looking for the cells, which could be expected to give the most appropriate answers in our experimental setup. According to the literature, a number of studies investigating ECs exposed to simulated µg have been published (as shown in Table 1). They were performed on HUVECs (Table 1, [15–31]) and EA.hy926 cells (Table 1, [1,2,32–39]). The studies have accumulated profound data about the behaviour of these cells under simulated µg conditions. In addition, they indicated various proteins and cellular factors playing a role in adjustment of ECs to randomization of the gravity vector. However, it should be kept in mind that HUVECs are cells derived from the endothelium of veins of the umbilical cord and EA.hy926 are hybrid cells generated by fusing HUVECs with the cancer cell line A432 [11]. As mentioned above, we were interested in studying normal adult ECs. We thus investigated human saphenous vein endothelial cells (HSVECs) and human microvascular endothelial cells (HMVECs). HSVECs are normal adult ECs. Hence, they appeared to be most appropriate to gain relevant knowledge. However, these cells are very sensitive (see Fig. 3).

Already after 24 h dead cells became visible in the culture flask incubated under static 1*g*-conditions (Fig. 3A). Their number increased up to the 7th day (Fig. 3B). On the RPM, the cells remained adherent during the first 24 h (Fig. 3C). After 7 d only adherent cells were seen, but no floating 3D aggregates (Fig. 3D). In addition, their number had decreased considerably. Hence, the data of the culturing of HSVECs showed that these cells do not form 3D aggregates and may be too sensitive for the stress of a spaceflight.

Afterwards, we searched for an alternative cell type representing adult healthy ECs and studied the EA.hy926 cell line and HMVECs. Both formed tube-like structures [2]. The formation was fast and the tubes were completed after 7 d (Fig. 4). However, HMVECs were also very sensitive. After 7 d under static 1*g*-conditions, a considerable number of dead or apoptotic cells can be found. In addition, HMVECs constantly need a temperature of 37 °C and die quickly when the temperature falls below 30 °C. Therefore, they are not suitable for a longer culture under ambient temperature in the Dragon capsule traveling to the ISS. In contrast, EA.hy926 cells appeared very tough without much apoptosis and presented some resistance to lower temperature, which made them the tool of choice.

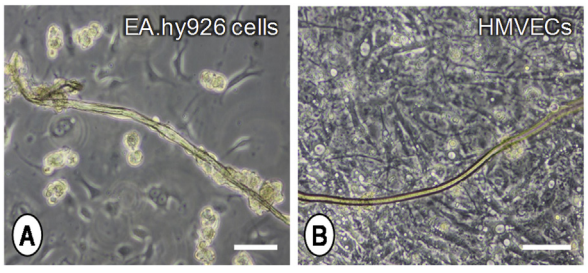


**Fig. 4.** Tubular structures formed by (A) EA.hy926 cells and (B) human microvascular endothelial cells after 7 d on an RPM. Scale bars: 100 µm.

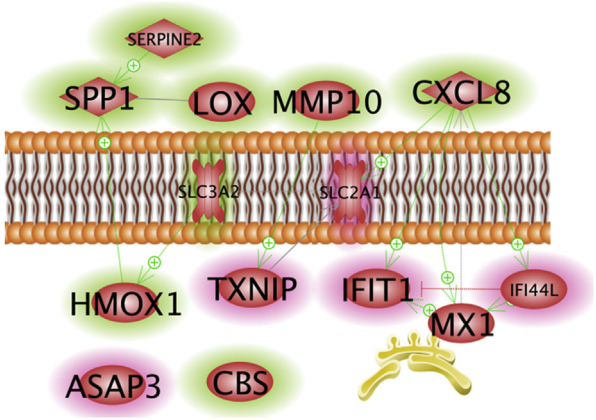


**Fig. 5.** Mutual interaction of proteins coded by genes which had been up- (green rim) or down-regulated (red rim) during a 5- or 7-day-exposure to the RPM. The icons at the upper edge indicate genes coding for extracellular proteins. The icons at the lower edge indicate cytoplasmic proteins. The yellow one indicates the endoplasmatic reticulum. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

### 4.2. In silico analysis

After the preparatory experiments we decided to use the EA.hy926 cell line for a first approach. These cells form tube-like structures (Fig. 4A) and survive a few days of ambient temperature below 30 °C [1,8,37]. We had characterized these cells very well [2,38]. From a genetic point of view 26 genes were of high interest, because they were either down- or up-regulated more than 4-fold. In addition, a few of them were genes whose products were known to be involved in the formation of 3D aggregates of human ECs as wells as of human cancer cells. Fig. 5 shows that the genes of the interacting osteopontin, protein-lysine 6-oxidase, and heme oxygenase 1 [37,41,42], were up-regulated, while those of interferon-induced proteins (IFIT1, IFI44L, MX1) were down-regulated. The latter proteins contribute to the activity of ISG15, which we recently found to be involved in breast cancer spheroid formation [43].

### 4.3. Tube-like aggregate formation during spaceflight

The cells seeded in the flight hardware were flown to the ISS and stayed there for 2 and 9 d at 37 °C as described in Ref. [8], before they were fixed and stored for the return. All eight experimental units were returned successfully. After the sample collection from the flight hardware units (Fig. 1), we detected 3D cell aggregates, which assembled after the launch of the experiment until their fixation approximately 5 and 12 d later (Fig. 6).

We investigated these aggregates emphasizing the morphology of the single cells. Fig. 5 shows a representative tube-like structure. It looks similar to the intima constructs, which we had detected in a preparatory study on the RPM [1] and confirmed these results obtained using a device designed to create conditions of µg on Earth.

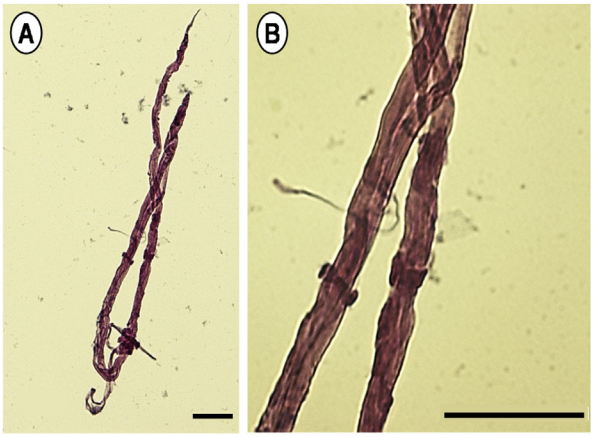


**Fig. 6.** Tube-like structure of human ECs harvested from the SPHEROIDS hardware after 12 d in space. (A) Periodic acid-Schiff (PAS) staining ( × 200) (B) The enlarged detail view of the same tube ( × 200) with subcellular structures. Scale bars: 35 µm.

## 5. Discussion

Extensive studies were performed on cultured ECs over the last years using the RPM (Table 1). These studies have shown that ECs are highly sensitive to random positioning, which induced 3D cell aggregation as well as an up-regulation of several growth factors and ECM components [38], but also initiated apoptosis in the EA.hy926 human endothelial cell line [35]. Further, it has been reported that $CD34^+$ human umbilical cord blood progenitors trans-differentiated into a vascular endo-thelial cell phenotype and continued growing into 3D structures inside a RWV [44].

Based on the data collected during the preparation of the spaceflight, we decided to use the EA.hy926 cell line for this study, although they may not be the optimal model to obtain the desired information in a first approach. At least, these cells form tube-like structures (Fig. 4A) and survive a few day period of ambient temperature below 30 °C. Hence, we expected that the cells show whether they behaved in space like they did on the RPM, forming tube like structures.

Especially the 3D growth behaviour is of interest, as it could be a versatile tool for angiogenesis studies *in vitro*. SPHEROIDS was not the first experiment culturing human ECs in real µg [45–47], but 3D growth was observed for the first time. The flight hardware enabled growth of EA.hy926 cells as tube-like structures in space, similar to previous results using an RPM. This suggests that among other environmental difference between space and the RPM, µg may be the main trigger for 3D growth. The lack of sedimentation and convection phenomena in µg enables cells to arrange themselves in 3D aggregates, that allow simulating the way cells are organized *in vivo*. This has provided more insights into the morphological and functional behaviour of ECs and allowed to collect valuable results on the pathophysiological morphologies and functions of ECs that could not be obtained through experiments in a normal terrestrial lab.

## 6. Conclusion

SPHEROIDS was not the first EC experiment in space, but 3D growth in real µg was observed for the first time. A specially developed hardware enabled growth of EA.hy926 cells as tube-like structures, which resemble the aggregates formed on the RPM. This confirms that µg may be the main trigger for 3D growth, although there are other environmental differences between space and the RPM. Hence, it appears that the lack of sedimentation facilitates the formation of 3D aggregates, simulating the way cells organize themselves inside the human body.

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