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A potential future Fontan modification: preliminary *in vitro* data of a pressure-generating tube from engineered heart tissue

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Key question

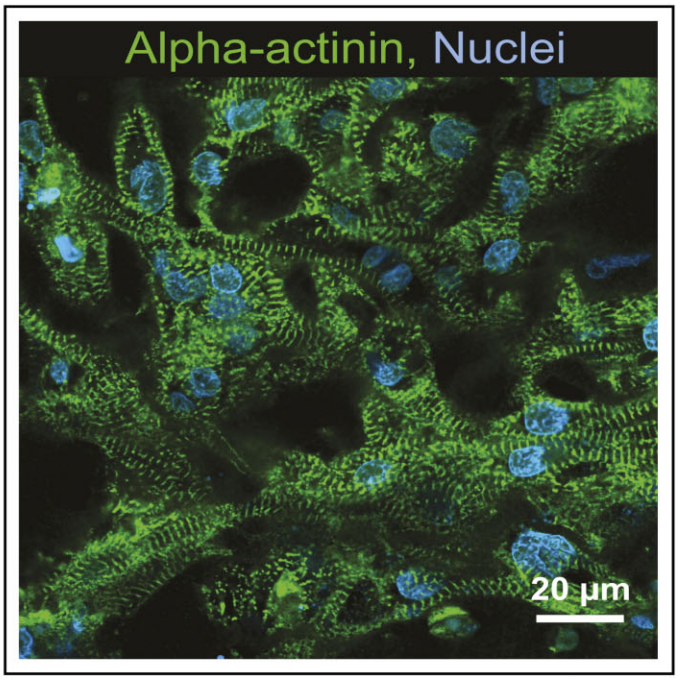
Is it possible to generate a contractile subpulmonary neo-ventricle to support the univentricular circulation in the future?

Key finding(s)

A pressure-generating tube from engineered heart tissue was created using human inducible pluripotent stem cells.

Take-home message

The present study lays the foundation for generating a tissue-engineered subpulmonary neo-ventricle supporting the univentricular circulation.



Abstract

OBJECTIVES: Univentricular malformations are severe cardiac lesions with limited therapeutic options and a poor long-term outcome. The staged surgical palliation (Fontan principle) results in a circulation in which venous return is conducted to the pulmonary arteries via

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passive laminar flow. We aimed to generate a contractile subpulmonary neo-ventricle from engineered heart tissue (EHT) to drive pulmonary flow actively.

METHODS: A three-dimensional tubular EHT (1.8-cm length, 6-mm inner diameter, ca. 1-mm wall thickness) was created by casting human-induced pluripotent stem cell-derived cardiomyocytes (0.9 ml, 18 mio/ml) embedded in a fibrin-based hydrogel around a silicone tube. EHTs were cultured under continuous, pulsatile flow through the silicone tube for 23 days.

RESULTS: The constructs started to beat macroscopically at days 8–14 and remained stable in size and shape over the whole culture period. Tubular EHTs showed a coherent beating pattern after 23 days in culture, and isovolumetric pressure measurements demonstrated a coherent pulsatile wave formation with an average frequency of 77 ± 5 beats/min and an average pressure of 0.2 mmHg. Histological analysis revealed cardiomyocytes mainly localized along the inner and outer curvature of the tubular wall with mainly longitudinal alignment. Cell density in the center of the tubular wall was lower.

CONCLUSIONS: A simple tube-shaped contractile EHT was generated from human-induced pluripotent stem cells and developed a synchronous beating pattern. Further steps need to focus on optimizing support materials, flow rates and geometry to obtain a construct that creates sufficient pressures to support a directed and pulsatile blood flow.

Keywords: Univentricular heart • Single ventricle • Fontan circulation • Engineered heart tissue • Subpulmonary neo-ventricle • Tissue engineering

ABBREVIATIONS

CM	Cardiomyocyte
EHT	Engineered heart tissue
hiPSC	Human-induced pluripotent stem cell
ID	Inner diameter
PVR	Pulmonary vascular resistance
WT	Wall thickness

INTRODUCTION

Besides primary heart transplantation, the Fontan principle in all its variations is the only surgical treatment option for patients with single-ventricle anatomy [1, 2]. Nevertheless, compared to children after biventricular repair, Fontan patients have significantly increased morbidity and mortality [3]. With the systemic and pulmonary circulation connected in series, blood flows passively into the pulmonary circulation. The flow is consequently dependent on the pulmonary vascular resistance (PVR) and the (diastolic) function of the systemic ventricle. PVR is an important prognostic factor for a good functional outcome after Fontan completion, and despite the multifactorial nature of the so-called 'failing Fontan', a high PVR represents a significant risk factor for long-term failure. Reasons for an increase in PVR are the lack of shear stress-induced release of endothelium-derived nitric oxide from the pulmonary vascular endothelium, limited capillary recruitment and eventually a reduced pulmonary vessel growth resulting from the laminar flow [4, 5].

There is evidence from a chronic porcine model of cavopulmonary connection that even weak pulsatile stimuli (micropulsatility) have positive effects on vascular tone, endothelial function and the development of pulmonary arterio-venous malformations [6]. Against this background, maintaining residual antegrade pulsatile flow in the sense of leaving the pulmonary valve or aortopulmonary connection partly open during cavopulmonary shunt operations is a recognized approach [7].

To overcome the existing limitations of laminar pulmonary flow in the Fontan circulation, we ultimately aim to create a contractile subpulmonary neo-ventricle using cardiac tissue engineering techniques. As a first step, our group published a feasibility study demonstrating cell survival and tissue maturation after implantation of engineered heart tissue (EHT) in a rat model [8]. For the present study, we hypothesized that it is possible to generate a pulsatile tubular EHT from human-induced pluripotent stem cells (hiPSC) as the first step in creating a potential neo-ventricle that could improve pulmonary circulation in patients with univentricular hearts.

MATERIALS AND METHODS

Ethical statement

The underlying work with hiPSC was approved by the Ethical Committee of the University Medical Center Hamburg-Eppendorf (Az. PV4798/28.10.2014 and Az. 532/116/9.7.1991).

Cardiomyocyte differentiation

Cardiomyocytes (CMs) were differentiated from hiPSC (UKEi1-A). Stem cell culture and cardiac differentiation were conducted as previously described, based on cultivation in spinner flasks in an embryoid body format [9]. Cell populations used for tubular EHT generation consisted of >90% troponin T-positive cells.

Generation of tubular engineered heart tissues

Tubular EHTs were generated from CMs and fibrinogen/thrombin as previously described with an adapted master mix protocol (Supplementary Material, Table S1) and a new self-generated casting system [10]. Constructs were cast around a custom-made silicone perfusion tube (Supplementary Material, Fig. S1) that was horizontally integrated into a polytetrafluoroethylene-based perfusion chamber (Fig. 1A). Two stainless steel adapters connected the perfusion tube with a silicone tubing system [inner diameter (ID): 3 mm, wall thickness (WT): 1 mm] that was coupled to a small peristaltic pump (0–150 ml/min) and a beaker flask (50 ml) serving as a medium reservoir (Fig. 1B). The perfusion chamber can be disconnected from the tubing system with 2 three-way valves, if necessary. The culture medium (Supplementary Material, Table S2) was changed every second day. The perfusion system was started 24 h after EHT casting with a pulsatile flow.

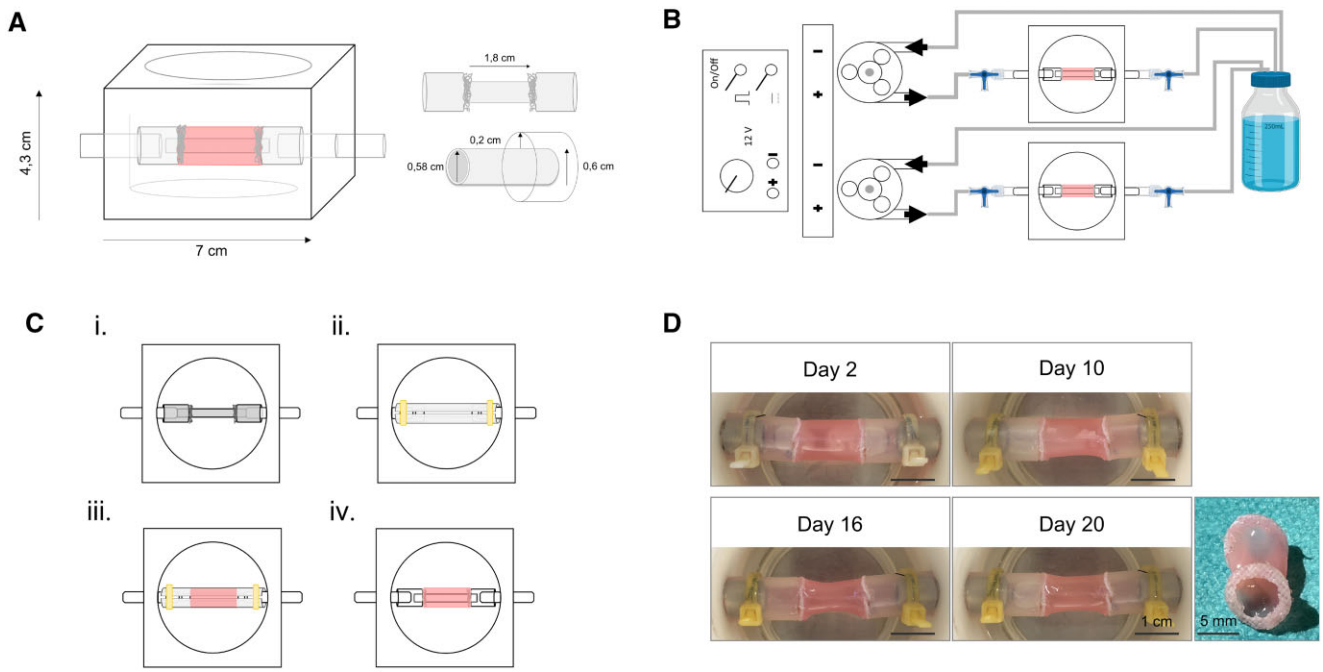


Figure 1: Casting and cultivation of tubular engineered heart tissues in a pulsatile perfusion system. (**A**) Left: schematic drawing of the perfusion chamber (side view) with the horizontally integrated perfusion tube and the tubular engineered heart tissue (depicted in red). Right: diameters of the silicone perfusion tube. (**B**) Schematic drawing of the casting procedure of tubular engineered heart tissues: (i) the perfusion tube (depicted in grey) was connected to the perfusion chamber (view from above); (ii) outer silicone cover was fixed around the perfusion tube to generate the casting mold; (iii) the master mix was cast into the mold; and (iv) the outer silicone cover was removed from the perfusion tube after solidification of the master mix. (**C**) Illustration of the pulsatile perfusion system (tubing, guiding pulsatile flow is depicted in grey lines). (**D**) Photographic images of a tubular engineered heart tissue in the perfusion chamber, taken over a 23-day culture period demonstrating tissue remodelling over time. Right: tubular engineered heart tissue after removal from the perfusion setting.

The flow rate was set at 10 ml/min, the frequency at 83 beats/min, resulting in an intraluminal pressure of ∼20 mmHg. Tubular EHTs were cultivated under sterile conditions for 23 days.

Casting of tubular engineered heart tissues

A custom-made form was used for EHT casting (Fig. 1C). The silicone perfusion tube was connected to the perfusion chamber. A silicone tube with a larger diameter (ID: 8 mm, WT: 1 mm, length: 4 cm) was placed around the perfusion tube and fixed to the perfusion chamber to create a tube-shaped casting mould. The fibrinogen-based master mix of 878 µl (Supplementary Material, Table S1), containing 18 mio CMs/ml, was mixed briefly with 27 µl thrombin (Sigma Aldrich, St. Louis, USA) and pipetted into the mould between the silicone perfusion tube and the outer silicone cover, through a narrow opening on top. After solidification at 37°C, the outer silicone cover was carefully removed and the medium was filled into the perfusion chamber. Tubular EHTs were cultivated at 37°C, 40% O_2, 7% CO_2 and 98% relative humidity.

Histology

After 23 days, EHTs were fixed in Histofix (Roth, Karlsruhe, Germany) for histological analysis.

Fixed EHTs were either embedded in 4% agarose to obtain vibratome tissue sections (100 µm) or paraffin. Primary antibodies were visualized with a multimer technology-based detection kit (Roche, Mannheim, Germany) or a fluorochrome-labelled Alexa-conjugated, secondary antibody (Thermofisher, Waltham, USA). Microscopic images were taken on an Axioskop 2 microscope (Zeiss, Oberkochen, Germany). Confocal images were acquired with an LSM 800 (Zeiss). Dystrophin-stained sections were imaged with a Nanozoomer (Hamamatsu, Hamamatsu, Japan) whole slide scanner.

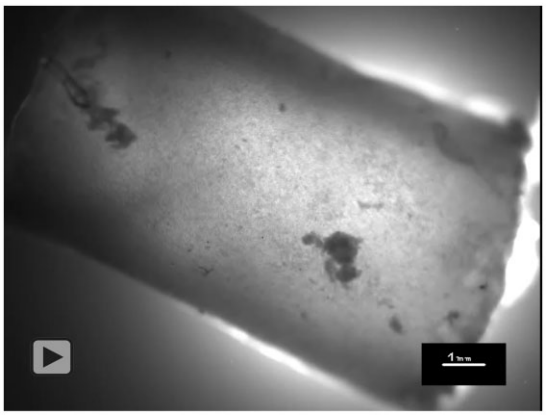
Histological assessment. Sarcomere length was measured in confocal images of alpha-actinin stained sections (30 sarcomeres per image; $n = 3$ images per EHT/$n = 3$ EHTs; 40× magnification).

Functional analysis of contractility

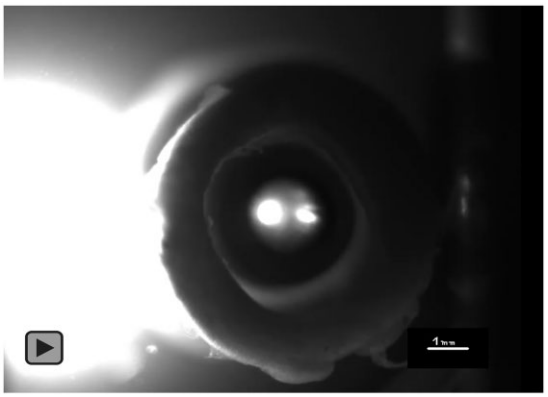
Contraction kinetics were analyzed as recently described [11]. The setting was modified for tubular EHTs. In short, 23-day-old tubular EHTs were carefully removed from the silicone perfusion tube and incubated in 25 mM HEPES-buffered culture medium (Supplementary Material, Table S2) for 2 h prior to the experiment. For the measurement, EHTs were placed on a 6-well culture dish positioned inside a gas- and temperature-controlled transparent chamber (37°C, 5% CO2). Wall deflection of the EHTs was recorded video-optically with a motorized camera and analyzed with an automated figure recognition algorithm. Force was measured in relative units (arbitrary units). Electrical pacing of tubular EHTs was conducted as previously described (6 V, 4 ms) [12].

Functional analysis of pressure recordings

The pressure was measured with a pressure transducer connected to a bridge amplifier (FMI, Seeheim, Germany) and



Video 1: Tubular engineered heart tissue unpaced, side view.



Video 2: Tubular engineered heart tissue unpaced, top view.

Powerlab8 (ADInstruments, Oxford, UK). LabChart5 software (ADInstruments) was used for acquisition.

Pressure measurement of tubular EHTs. Luminal pressure generation of the tubular EHTs was recorded after disconnection of the perfusion chamber from the perfusion system, with the pump flow turned off (at room temperature). The pressure was measured inside the silicone perfusion tube, with the tubular EHT assembled around it (≙ isovolumetric). To investigate whether pressure was retained by the material of the perfusion tube, the compliance of the material at low pressure (<1 mmHg) was determined. To do this, the pressure was applied by volume change on the tubular system from the outside. It was measured and recorded using a catheter for rodents (Transonic, Ithaca, USA) and the LabScribe2 software. Simultaneously, we measured the pressure inside the silicone perfusion tube with a pressure transducer (Supplementary Material, Fig. S2A).

Burst pressure measurement. It was impossible to directly assess burst pressure because the EHT tube could not be integrated into a closed system without the inner silicone tube. We, therefore, inserted a self-constructed balloon into the tubular EHT and measured pressure with increasing volume load in the balloon. The balloon catheter was generated based on the model for Langendorff experiments [13] and was connected to a force transducer. Double distilled H_2O was administered gradually into the balloon in 30-µl steps until the balloon was filled. The pressure was recorded after every volume increase. The same experiment was repeated without the EHT to investigate the compliance of the balloon material. With this setup, it was only possible to apply a pressure of up to 25–30 mmHg on the EHT because above this threshold the balloon was fully filled and retained most of the additional pressure (Supplementary Material, Fig. S3).

Statistics

Cultivation of tubular EHTs within the pump setting was conducted in at least 3 biological replicates. For each experiment, the number of replicates (n) is listed in figure legends. Statistical analysis and graphic illustrations were generated using Graph Pad Prism 9 (GraphPad Software, San Diego, USA). Data were expressed as the mean and standard deviation.

RESULTS

Generation of tubular EHTs using a pulsatile perfusion system

Pulsatile perfusion system. A pulsatile perfusion system (Fig. 1C) was generated to cultivate tubular EHTs cast around a thin silicone membrane in a horizontal setting. The cultivation system consisted of a custom-developed control unit driving a microcentrifugal pump, generating flow rates ranging from 0 to 150 ml/min in a continuous or pulsatile fashion (10–100 beats/min). The tubular EHTs were cast around a custom-made silicone tube (outer diameter: 6 mm, WT: 200 µm) attached to a custom-made culture vessel. Velcro rings were glued to both ends of the perfusion tube, preventing tissue detachment during cultivation (Fig. 1D). A pulsatile flow mode (10 ml/min, frequency 83 beats/min, maximum systolic pressure: 20 mmHg) was used for cultivation. Oxygen partial pressure (pO_2) was measured in all medium compartments of the perfusion setting, indicating sufficient gas diffusion into the system during cultivation, compared to control (Supplementary Material, Fig. S1B).

Assessment of tubular EHTs during cultivation. Tubular EHTs (1.5 cm length, ID 6 mm, WT ca. 1 mm, 1.8×10^7 CMs/ml) could be cultivated with the abovementioned setting. Tubular EHTs remodelled by showing moderate compaction around the silicone tube and remained stable in size and shape over a culture period of 23 days (Fig. 1D). Initial microscopical contractions could be observed between days 6 and 10 after casting. Spontaneous macroscopic contractions started between days 8 and 14 in a synchronized manner with a regular beating pattern (Fig. 4B and Videos 1 and 2). Glucose consumption increased simultaneously over time, indicating CM viability and maturation (Supplementary Material, Fig. S4). After 23 days of culture, tubular EHTs were removed from the perfusion system for physiological and histological analysis (Fig. 1D).

Morphological evaluation of tubular EHTs

Histological analysis demonstrated that CMs mainly localized along the inner and outer curvature of the tubular wall, whereas CM density was lower in the middle part of the tubular wall (Fig. 2A and B). CMs expressed the atrial (MLC2a) and ventricular (MLC2v) isoform of the myosin light chain (Fig. 2B) and mainly aligned longitudinally along the tubular wall. Whereas most CMs

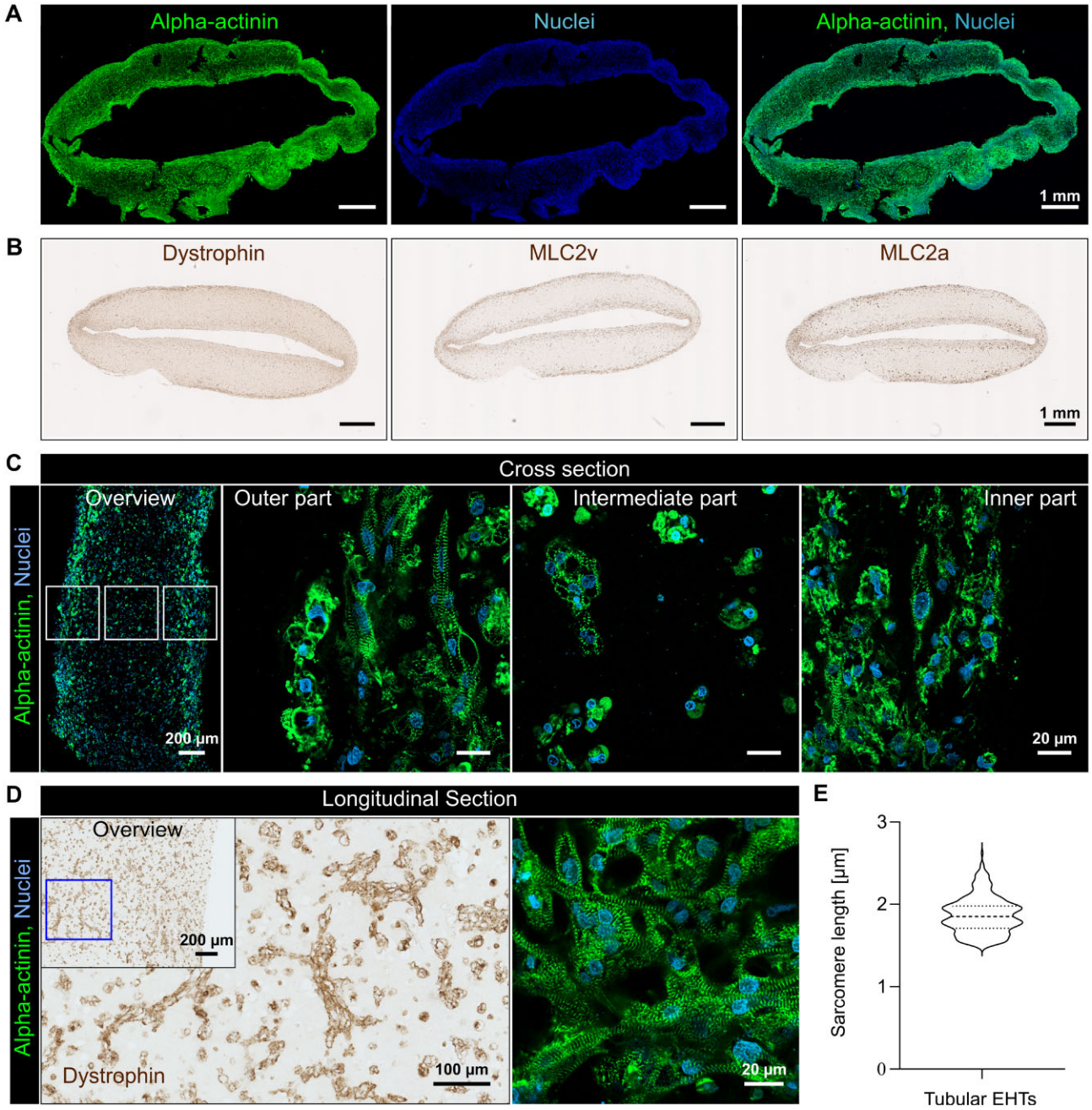


Figure 2: Histological characterization of cardiomyocyte alignment, localization and maturity in tubular engineered heart tissues. (**A**) Cross-section of a tubular engineered heart tissue stained for alpha-actinin (green) and nuclei (blue) in low magnification. (**B**) Cross-sectional view of a tubular engineered heart tissue, stained for (from left to right) dystrophin, myosin light chain, ventricular isoform and myosin light chain, atrial isoform. (**C**) Cross-sectional view of a tubular engineered heart tissue stained for alpha-actinin (green) and nuclei (blue), low magnification. Insets of 3 parts of the tubular wall are shown in high magnification. (**D**) Longitudinal section of a tubular engineered heart tissue, dystrophin-stained section and alpha-actinin-stained section (higher magnification). (**E**) Quantification of sarcomere length in tubular engineered heart tissues (n = 3 engineered heart tissues, >50 sarcomeres per engineered heart tissue).

were still immature, some CMs showed a mature, elongated phenotype (Fig. 2C and D). The average sarcomere length was calculated at 1.9 µm (Fig. 2E).

Functional evaluation of tubular EHTs using pressure analysis

After initiation of macroscopic contraction, tubular EHTs generated forces that deflected the silicone perfusion tube. Pressure measurements showed a regular pulsatile pressure curve (n = 3, Fig. 3A). The average spontaneous beating frequency was 77 ± 5 beats/min. Maximum systolic pressure (measured inside the silicone perfusion tube) varied between the 3 EHTs, ranging between 0.09 and 0.29 mmHg, with an average maximum pressure of 0.2 mmHg (Fig. 3B). To evaluate whether the material of the perfusion tube retained pressure, we simultaneously obtained measurements inside and outside the perfusion tube while applying pressure from the outside. Even though there was a minimal difference (on average 0.09 mmHg), this effect

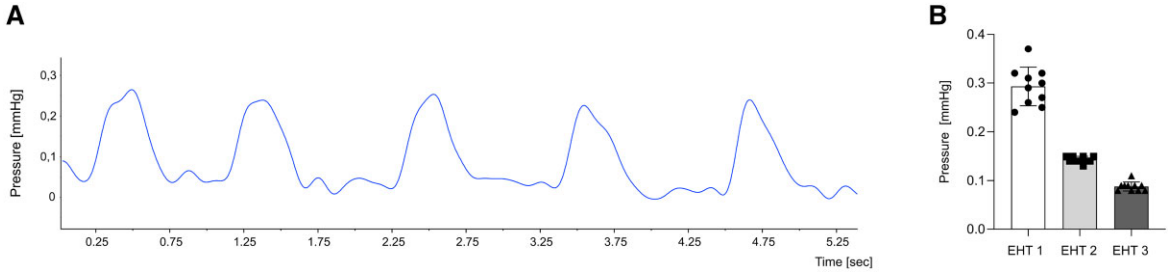


Figure 3: Functional analysis of pressure development in tubular engineered heart tissues. (**A**) Pressure measurement of a tubular engineered heart tissue inside the perfusion tube (n = 3, representative measurement shown). (**B**) Maximum pressure measurement of 3 tubular engineered heart tissues (n = 3). Each data point represents 1 tubular engineered heart tissue. The pressure was averaged from at least 10 individual pressure peaks.

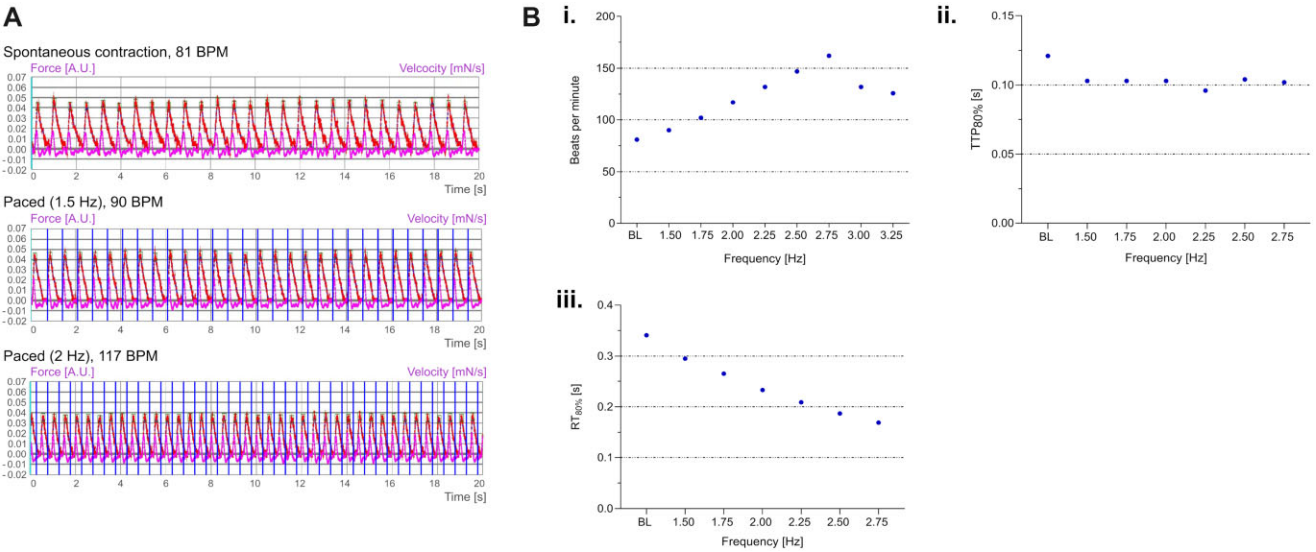


Figure 4: Physiological analysis of contractility in tubular engineered heart tissues. (**A**) Pictogram demonstrating the frequency of a tubular engineered heart tissue during contractility analysis (n = 2). Top-to-bottom: spontaneous contraction. Paced condition at 1.5-Hz stimulation. Paced condition at 2-Hz stimulation. Blue lines indicate electric impulses. (**B**) Stimulation-frequency analysis of a tubular engineered heart tissue with contractility measurements (n = 1). Contraction peaks were analyzed for (i) beats per minute, (ii) contraction time (time to peak at 80% peak height, time to peak 80%) and (iii) relaxation time (relaxation time at 80% of peak height, relaxation time 80%) at different frequencies (1.5, 2, 2.25, 2.5, 2.75), independent of absolute force values.

was negligible (Supplementary Material, Fig. S2B). After removal from the perfusion setting, the EHT could withstand a burst pressure of up to 25–30 mmHg (the maximal force that could be analyzed in our setting) without bursting (Supplementary Material, Fig. S3).

Functional evaluation of tubular EHTs using contractility analysis

After 23 days in culture, tubular EHTs remained stable and showed a continuing spontaneous beating pattern outside the perfusion setting (Fig. 4A and Videos 1 and 2). Contractility was analyzed under spontaneous beating conditions and electrical stimulation (Fig. 4A and Videos 1 and 2). Tubular EHTs followed the electrical pacing up to 2.75 Hz (Fig. 4B-i). Contraction time (time to peak at 80% peak height, time to peak 80%) did not change with increasing beating frequency (Fig. 4B-ii), whereas relaxation time (relaxation time at 80% peak height, relaxation time 80%) showed a physiological decrease (300 ms at 1.5 Hz to 170 ms at 2.75 Hz stimulation, Fig. 4B-iii).

DISCUSSION

The ultimate goal of the approach is to design a contractile neoventricle for potential clinical use in patients with univentricular hearts. For this, a construct capable of developing pressures of at least 5–10 mmHg, withstanding burst pressures of ∼25 mmHg, and eventually generating a directed pulsatile flow into the pulmonary circulation is needed. As a very first step, we intended to generate a miniaturized, stable tubular EHT that could be used for prospective preclinical transplantation studies. We developed a novel system to fabricate tubular EHTs based on a fibrin-containing matrix. We focused on (i) establishing a setting that allows generating and culturing tubular EHTs, (ii) evaluating tissue stability and integrity and (iii) analyzing tissue functionality.

Limitations

A significant limitation of EHT generation using hydrogels is tissue stability [14]. EHT constructs are usually limited in WT during cultivation due to dependency of nutrient and gas supply by diffusion [15]. Notably, compared to other tubular

approaches (WT: <500 μm, ID: ca. 2 mm) [16], our setting differed in thickness and stability of the construct walls (WT: ca. 1000 μm) and the comparatively large ID (6 mm). However, cellular localization was not continuous throughout the tubular wall but mainly along with the border areas of the tissue, as seen in thick EHTs before [12, 17].

It has been shown that the cultivation of EHTs under perfused conditions may enhance cell viability and maturity within the constructs and thus contractile output [18, 19]. This is intuitive as it recapitulates embryonic development, whereby haemodynamic flow supports heart morphogenesis [20]. However, the high shear stress acting on the tissue appears to compromise CM viability and maturity, even though higher flow rates increase oxygen and nutrient supply [21]. In this study, human tubular EHTs were cast around a thin (∼200 μm) silicone perfusion tube that shielded CMs from direct flow, thus allowing the application of intraluminal pressure (20 mmHg) during cultivation. It has been shown before that mechanical strain during culture is crucial for maturation and can improve cell alignment and force generation [12, 22]. In the present study, many CMs within the construct showed signs of immaturity like the lack of elongated nuclei, low sarcomere length or the expression of MLC2a. Nevertheless, almost 50% of cells within the construct expressed MLC2v, the ventricular isoform, indicating ongoing maturation [23]. We generated tubular constructs that can produce intraluminal pressure (average: 0.2 mmHg, max. 0.3 mmHg) against a water column, with deflection of a thin (200 μm) silicone membrane. The pressure measurement had a significant limitation, as it was only possible to be conducted at room temperature without a sufficient gas supply. However, pressure generation was not in a clinically relevant range to significantly improve the outcome of patients with univentricular hearts. For this reason, prospective investigations need to focus on enhanced and directed contractile output. To ensure that the structural integrity of the tubular EHTs is likely to be maintained under pressure conditions in the univentricular circulation, we simulated *in vivo* conditions by exposing the tissue to increasing pressures, acting on the luminal side of the tube. The tissue could be exposed to pressures of at least 25–30 mmHg, which is above the physiological increased central venous pressure in the Fontan circulation. In addition, the incorporation of valves at both ends of the construct would be necessary to produce a directed flow and enhance the biomechanical strain acting on the tissue. Functional tissue-engineered tricuspid valves could be made from decellularized tissue [24], or three-dimensional printed biomaterials [19] and might be a potential solution to improve cell alignment and contractile output.

Human tubular EHTs showed persistent spontaneous contraction after removal from the perfusion setting. The contraction direction was mainly longitudinal along the tubular wall, consistent with CM alignment in the histological analysis. We assume that CM alignment would be rather circumferential along force lines produced by a cyclic stretch of the pulsatile flow since it was shown that CMs in EHTs align parallel to the mechanical stretch direction *in vitro* [25]. One suggestion for subsequent experiments could be electrical training during cultivation, which showed increased cell distribution, maturation and higher force development in previous studies [12]. Tubular EHTs followed pacing frequencies up to 2.75 Hz (165 beats/min), which was lower than for small stripe EHTs [12] but higher compared to similar tubular constructs (up to 2 Hz) [16, 26]. This could be advantageous after implantation for the regulation of contraction with a pacemaker to synchronize it with the heart, as a natural coupling to the heart might be difficult after transplantation. The relaxation and contraction times showed a physiological response similar to values reported for EHTs before [11].

CONCLUSION

In summary, our construct may be the basis to reach our overall goal: a pressure-generating neo-ventricle that can actively propel blood towards the pulmonary arteries in palliated patients with total cavopulmonary connection. However, to have a relevant impact on the clinical outcome of patients with single ventricle circulations, many limitations need to be overcome. Our primary constraint is the low-pressure build-up in the construct, likely influenced by unfavourable CM alignment and maturation. Future approaches need to focus on optimizing the *in vitro* conditions to increase the directed contractile output of the tissue-engineered construct.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *EJCTS* online.

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Conflict of interest: none declared.

Data Availability Statement

The data underlying this work are available in the article and its online supplementary material.

Author contributions

Maria Köhne: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Visualization; Writing—original draft. **Charlotta Sophie Behrens:** Data curation; Investigation. **Tim Stüdemann:** Data curation; Investigation. **Constantin von Bibra:** Data curation; Investigation. **Eva Querdel:** Data curation; Investigation. **Aya Shibamiya:** Data curation; Investigation. **Birgit Geertz:** Investigation. **Ida Hüners:** Data curation; Investigation. **Jakob Olfe:** Data curation; Investigation. **Stefan Jockenhövel:** Writing—review & editing. **Michael Hübler:** Data curation; Project administration; Resources. **Thomas Eschenhagen:** Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources;

Software; Supervision; Validation. **Jörg Siegmar Sachweh:** Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation. **Florian Weinberger:** Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Visualization; Writing—original draft. **Daniel Biermann:** Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Writing—original draft; Writing – review & editing.

Reviewer information

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