Development of a bioreactor system for pre-endothelialized cardiac patch generation with enhanced viscoelastic properties by combined collagen I compression and stromal cell culture

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Abstract

Treatment of terminal heart failure still poses a significant clinical problem. Cardiac tissue engineering could offer autologous solutions for the replacement of non-functional myocardial tissue. So far, soft matrix construction and missing large-scale prevascularization prevented the application of sizeable cardiac repair patches. We developed a novel bioreactor system for semi-automatic compression of a collagen I hydrogel applying 16 times higher pressure than in previous studies. Resistance towards compression stress was investigated for multiple cardiac-related cell types. For scaffold prevascularization, a tubular cavity was imprinted during the compaction process. Primary cardiac-derived endothelial cells (ECs) were isolated from human left atrial appendages (HLAAs) and characterized by fluorescence-activated cell sorting (FACS) and immunocytochemistry. EC were then seeded into the preformed channel with dermal fibroblasts as interstitial cell component of the fully cellularized patch. After 8 days of constant perfusion culture within the same bioreactor, scaffold dynamic modulus and cell viability were analyzed. Endothelial proliferation and vessel maturation were examined by immunohistochemistry and transmission electron microscopy. Our design allowed for scaffold production and dynamic culture in a one-stop-shop model. Enhanced compression and cell-mediated matrix remodeling induced a significant increase in scaffold stiffness while ensuring excellent cell survival. For the first time, we could isolate HLAA-derived EC with proliferative potential. ECs within the central channel proliferated during flow culture, continuously expressing endothelial markers (CD31) and displaying basal membrane synthesis (collagen IV, ultrastructural...

ABBREVIATIONS: 3D, three dimensional; AA, atrial appendage; BIP, el, bovine serum albumin (BSA) polyvinyl alcohol essential lipids; CD, cluster of differentiation; CM, cardiomyocyte; CPC, cardiomyocyte progenitor cell; CSC, cardiac stem cells; DAB, 3,3'-diaminobenzidine; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco's modified eagle medium; EC, endothelial cell; ECM, extracellular matrix; EGM-2, endothelial growth medium 2; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; G', storage modulus; G*, complex shear modulus; G°, zero shear storage modulus; G°°, zero shear loss modulus; HAAEC, human atrial appendage endothelial cell; HDF, human dermal fibroblasts; HAAEC, human atrial appendage endothelial cell; HUT102, human umbilical vein endothelial cells; IMDM, Iscove's modified Dulbecco's medium; LAA, left atrial appendage; LVEF, left ventricular ejection fraction; MAP, magnetic-activated cell sorting; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PEG, polyethylene glycol; PI, propidium iodide; PSU, polysulfone; RAA, right atrial appendage; RPMI 1640, Roswell Park Memorial Institute 1640 medium; TEM, transmission electron microscopy; TIE2, angiopoietin-1 receptor; VECadherin, vascular endothelial cadherin; VSMC, vascular smooth muscle cells; WCM, whole cell mix.
analysis). After 7 days of culture, a complete endothelial monolayer could be observed. Covering cells aligned themselves in flow direction and developed mature cell–cell contacts.

**KEYWORDS**
cardiac tissue engineering, left atrial appendage, plastic compression, scaffold prevascularization

# 1 | INTRODUCTION

Heart transplantation is still considered the gold standard treatment for patients suffering from terminal heart failure (Carrier & Perrault, 2014). For years, Eurotransplant has reported increasing numbers of patients on the waiting list facing a nearly constant pool of available donor hearts (Eurotransplant International Foundation: Annual Report, 2014). Cardiac tissue engineering could provide a solution to this problem of organ shortage. Different strategies have been pursued to create functional heart muscle tissue primarily implementing either cell- or scaffold-based approaches (Haraguchi, Shimizu, Yamato, & Okano, 2012).

When considering scaffold-based strategies, collagen type I has proven an ideal scaffold for cardiac tissue engineering on numerous accounts (Perea-Gil, Prat-Vidal, & Bayes-Genis, 2015). Moreover, mechanical dehydration of collagen hydrogel could create a stiffened network to match the alternating strains imposed on the intercellular matrix by cardiomyocyte (CM) contractility. Following a distinct physicochemical model described by Hadjipanayi et al. (2011), plastic compression (PC) of collagen gels not only greatly enhances mechanical properties like stiffness and tensile strength but also has been shown to positively influence cell migration (Serpooshan et al., 2013) and proliferation of epidermal, corneal, and urothelial cells in complex 3D models (Ajalloueian, Zeial, Fossum, & Hilborn, 2014; Hu et al., 2010; Mi, Chen, Wright, & Connolly, 2010). Most groups so far used fixed weight plates with a maximum force generation of approximately 1.4 kN/m² (Serpooshan et al., 2013) for compression. There is a proportional interrelation of collagen pore size and fibroblast-induced gel contraction (Serpooshan, Muja, Marelli, & Nazhat, 2011) favoring higher compression rates for stable shape retention. Furthermore, according to Ghezzi, Muja, Marelli, and Nazhat (2011) increasing collagen fiber density exerts a positive effect on fibroblast growth induction. Hence, an elaborate PC protocol with high compression rates is justified for ideal scaffold construction.

Another major issue to date in cardiac tissue replacement is the immediate and long-term sustenance of engineered tissue upon implantation. For example, Kawamura et al. (2012) reported limited cell survival in human-induced pluripotent stem cell (hiPSC)-derived CM sheets after transplantation in a pig model of chronic myocardial infarction. Therefore, instant nutrient and oxygen supply through an intrinsically preformed vascular network is mandatory for complex tissues exceeding 200 μm in thickness (Benavides et al., 2015). Two domains must be considered when dealing with in vitro prevascularization: (1) tube/network formation and (2) endothelial lining. Regarding (1), capillarization can be accomplished by self-organizing effects of admixed endothelial cells in coculture (Sekine et al., 2008; Shimizu, 2014) or micropatterning within suitable scaffolds (Choi et al., 2007; Zheng et al., 2012). However, direct surgical anastomosis with a human host vasculature requires the provision of a larger connecting vessel inside the transplant. Vollert et al. (2014) successfully generated endothelialized, perfusable channels of up to 500 μm in diameter within their engineered heart tissue using alginate spacer tubes. Other groups followed a top-down approach by re-endothelializing decellularized porcine small bowel segments yielding full-size vascular networks which could be attached to host vessels via arterial and venous stubs (Andree et al., 2014; Mertsching et al., 2009; Schanz, Pusch, Hansmann, & Walles, 2010). In terms of (2), endothelial cell (EC) choice of origin is fundamental for graft-host and cell–cell communications. ECs are known to play a decisive role in allograft rejection, for example, via monocyte recruitment and T-cell stimulation (Al-Lamki, Bradley, & Pober, 2008), while at the same time exhibiting a certain tissue-specific heterogeneity concerning organ maintenance and cytokine responsiveness (Molema, 2010; Nolan et al., 2013). These data suggest an advantage using autologous, cardiac-derived ECs to optimize the cellular interplay for tissue engineered heart patch transplants.

We assumed that human left atrial appendages (HLAAs) could be harnessed for sufficient EC supply. These remnants of embryonic development can be accessed and excised easily during open or endoscopic heart surgery granting an additional benefit in reducing the incidence of postoperative cerebrovascular events, at least in patients with a low CHA2DS2-VASc score (Kato et al., 2015). In the past, mostly cardiomyocyte progenitor cells (CPCs) have been harvested from atrial appendages (AAs) fueling multiple animal and in-man studies. CPCs were tested for their potential of local cardiac repair after myocardial infarction in rats (Sakai et al., 1999), minipigs (Fanton et al., 2015), and humans (Chugh et al., 2012) yielding some promising results over the past two decades. In contrast, no efforts have been made so far to isolate stromal and EC populations of AAs, let alone introduce them to tissue engineering. Because coculturing of different cell types is known to exert positive effects on their self-organization potential (Czaik & Drake, 2015), it is conceivable that a proper mixture of endothelial and non-endothelial “support cells” could promote the growth and maturation of engineered cardiac tissues and a developing vascular network within.
In the present study, we aimed at building a scalable "cardiac-primed" scaffold with macrovascular features based on PC of collagen type I and LAA cell utilization. To this end, we designed a bioreactor providing both compactor and 3D culture housing. We then tested our compression method for rheological stability of the scaffold and for cytocompatibility with various cardiac cell types including LAA-derived EC, pericytes, and CMs. An imprinted central channel was lined with human atrial appendage endothelial cells (HAAEC) whereas the scaffold was seeded with human dermal fibroblasts (HDF) that contributed to scaffold remodeling. Culturing (HAAEC) whereas the scaffold was seeded with human dermal fibroblasts (HDF) that contributed to scaffold remodeling. Culturing under constant perfusion conditions forged a primitive nutrient channel was lined with human atrial appendage endothelial cells (HAAEC) whereas the scaffold was seeded with human dermal fibroblasts (HDF) that contributed to scaffold remodeling. Culturing under constant perfusion conditions forged a primitive nutrient vessel with metabolically active interstitial cells. Thus, this construct should lay an ideal foundation for CM and support cell seeding and sustenance.

2 | MATERIALS AND METHODS

2.1 | Construction of a multipurpose bioreactor

We aimed at constructing a one-stop-shop bioreactor that could house all procedures from scaffold creation to tissue sustenance. The reactor (Figure 1b) was manufactured from polysulfone (PSU), a translucent, heat-resistant and biocompatible material (Wintermantel & Ha, 2009). The bioreactor was made from single modules that could be stacked as needed: The core module had an outer diameter of 42 mm and an inner diameter of 24 mm. The interior consisted of two compartments, separated by a metal strainer. Separation height of compartments could be adapted by insertion of shims underneath the strainer. The upper compartment held a traversing needle with an outer diameter of 1.3 mm as a central placeholder. The lower compartment served as pressure compensation chamber containing 2 ml of medium fluid on default settings. The distance between strainer and needle was 9.8 mm. The default configuration was adapted to our experimental design yielding a reservoir volume of 3.3 ml and a distance to the needle of 5.8 mm. Inlet and outlet of both upper and lower compartments were attached to medium-perfused silicone connectors. To allow for initial collagen hydrogel compaction a fitted PSU cylinder was tightly connected on top of the core module during each compression cycle. After removal of the compression cylinder module, the core element was sealed by a matching lid featuring four sterile insertion ports for possible later manipulation.

2.2 | Cell isolation and differentiation

2.2.1 | Human dermal fibroblasts

HDF were isolated as previously described by Moll et al. (2013). In brief, skin biopsies were washed with phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA), connective tissue and fat were removed, and tissue was cut in strips of even size. Tissue was digested in dispase (2 U/ml; Thermo Fisher Scientific, Langenselbold, Germany) for 16 to 18 h at 4°C. Epidermal and dermal layers were separated with tweezers; dermal layers were cut into smaller pieces and digested for 45 min at 37°C in collagenase (500 U/ml; Serva, Heidelberg, Germany). Pieces were plated in Dulbecco's modified eagle medium (DMEM; high glucose; Thermo Fisher Scientific) with 10% FCS (Bio&SELL GmbH, Feucht, Germany). Outgrowing cells were cultured and split with Trypsin (Thermo Fisher Scientific) at some 80% confluency.

2.2.2 | Primary cardiac cells

HLAAs were excised during routine cardiac surgery with patients' consent and processed within 8 h. Cells were isolated as previously described (Messina et al., 2004; Smith et al., 2007) with minor modifications. Briefly, biopsies were washed with PBS, connective tissue was removed, and tissue was minced and partially digested with trypsin and collagenase A (f.c. 200 ng/ml; Roche Diagnostics Deutschland GmbH, Mannheim, Germany). Tissue pieces were plated on 1% gelatin-coated (w/v in ultrapure water; Serva) cell culture dishes and cultured in Iscove’s modified Dulbecco’s medium (IMDM; Thermo Fisher Scientific) with 5% FCS and 100 μM β-mercaptoethanol (Sigma-Aldrich, Taufkirchen, Germany). Within 3 weeks outgrowing cells were detached using Accutase (Thermo Fisher Scientific) resulting in a "whole cell mix" (WCM) fraction. WCM was MAC sorted for CD31 (Miltenyi Biotec, Bergisch Gladbach, Germany). The CD31-positive fraction was the labeled HAAEC. WCM, and CD31-negative cells were each seeded in cell culture flasks coated with 0.1% gelatin and cultured in IMDM with 5% FCS and 108.5 μM β-mercaptoethanol. HAAEC were seeded in cell culture flasks and cultured in VascuLife® VEGF-Mv (Lifeline Cell Technology, Frederik, MD, USA).

Isolated cells were stained and analyzed via flow cytometry. Cultured HLA-derivived cells were enzymatically detached and counted. 2 * 10^5 cells were stained for 30 minutes at 4°C for CD105, CD31, TIE2, and VE-cadherin using conjugated antibodies (mouse-anti-human CD105 [FITC conjugated; Ancell Corporation, Stillwater, MN, USA], mouse-anti-human CD31 antibody [APC conjugated; eBioscience Inc., San Diego, CA, USA], mouse-anti-human TIE2 antibody [unconjugated; Abcam, Cambridge, United Kingdom; secondary antibody donkey-anti-mouse Alexa 488], and VE-cadherin antibody [FITC conjugated; eBioscience Inc.]). For staining of vimentin (FITC conjugated; Bios antibodies, Woburn, MA, USA), BD Cytofix/Cytoperm (BD Biosciences, Franklin Lakes, NJ, USA) was used according to manufacturer’s protocol. Isotype controls were mouse IgG1 (FITC conjugated; eBioscience Inc.), mouse IgG1 (APC conjugated; BD Biosciences), and rabbit IgG (FITC conjugated; antibodies-online GmbH, Aachen, Germany). We used PBS containing a secondary antibody as negative control for TIE2. Stainings were analyzed using a FACSCalibur (BD Biosciences) and evaluated using FlowJo 10.0 (FlowJo LLC, Ashland, OR, USA).
Pericytes were differentiated from IMR90-4 cells (WiCell Research Institute, Madison, WI, USA) following an adapted protocol from Orlova, Drabsch, et al. (2014) and Orlova, van den Hil, et al. (2014). Briefly, IMR90-4 cells were plated on six-well plates coated with Matrigel (OMNILAB-LABORZENTRUM GmbH & Co. KG, Bremen, Germany) at a density of 2.5 * 10^5 cells per well. After 2 days of culture in mTeSR™1 (Stemcell Technologies Canada Inc., Vancouver, BC, Canada), cells were treated with daily medium change in mesoderm induction medium for 3 days: Bovine serum albumin (BSA) polyvinyl alchohol essential lipids (B(P)EL) was prepared as described in the original publication (Ng, Davis, Stanley, & Elefanty, 2008). Cells were cultured in vascular specification medium, B(P)EL containing hVEGF165 (50 ng/ml; New England Biolabs Ltd., Hitchin, United Kingdom), and TGF-β type I inhibitor SB431542 (10 μM; Tocris Bioscience, Bristol, UK), for 7 days. Cells were detached with Accutase and MAC sorted for CD31 as previously described. The CD31-negative cell fraction was incubated on 0.1% gelatin-coated cell culture flasks in EGM-2 (Lonza Group AG, Basel, Switzerland) for up to 80% confluency. For final differentiation, cells were cultured for 3 days in DMEM supplemented with 10% FCS, TGF-β1 (2 ng/ml; ProSpec Bio, East Brunswick, NJ, USA), and PDGFβB (4 ng/ml; PeproTech, Rocky Hill, NJ, USA). Fully differentiated pericytes were cultured in Pericyte Medium (PM; ScienCell Research, Carlsbad, CA, USA). Pericytes were analyzed via fluorescence-activated cell sorting (FACS) for CD146, PDGFβ, α-smooth muscle actin (αSMA), and NG2. We used mouse-anti-human CD146 (FITC conjugated; BD Biosciences), mouse-anti-human PDGFβ (PE conjugated; BD Biosciences), mouse-anti-human αSMA (unconjugated; Abcam; with secondary antibody donkey-anti-mouse Alexa 488), and mouse-anti-human NG2 antibodies (unconjugated; Abcam; with secondary antibody donkey-anti-mouse Alexa 488). For αSMA and NG2, cells were permeabilized and fixed using BD Cytofix/Cytoperm (BD Biosciences). Controls were stained with mouse IgG1 (FITC conjugated; eBioscience Inc.), mouse IgG2B (PE conjugated; eBiosciences Inc.), mouse IgG2A (unconjugated; Dako, Glostrup, Denmark; with secondary antibody donkey-anti-mouse Alexa 488), and mouse IgG1 (unconjugated; Dako; with secondary antibody donkey-anti-mouse Alexa 488).

### 2.2.4 | hiPSC-derived CMs

hiPSC-derived CMs were kindly provided by the Edenhofer group (Kadari et al., 2015). Cells had been differentiated from AR1034ZIMA hiPSC clone 1 and cultured in RPMI1640 (Thermo Fisher Scientific) with 2% B-27 supplement (Thermo Fisher Scientific), β-mercaptoethanol.
Collagen I was isolated following a protocol from Dieterich et al. (2002) with several modifications. Frozen tails of 8- to 10-week-old outbred rats (Charles River Laboratories Inc., Wilmington, MA, USA) were thawed in sterile PBS at room temperature and sterilized in 70% ethanol. Tails were cut open about 2/3, and the skin layer was pulled off with tweezers. Subsequently, tendons were sterilized in 70% ethanol. Tails were cut open about 2/3, and the skin layer was pulled off with tweezers. Subsequently, tendons were pulled from all tails, pooled, washed with PBS, and sterilized in 70% ethanol. Tendons were solved in sterile 0.1% acetic acid at a concentration of 6 mg/ml.

Fifteen milliliters of hydrated collagen gel were gently mixed 2:1 with gel neutralization solution (GNL; 231.5 ml 2 × ethanol. Tendons were solved in sterile 0.1% acetic acid at a concentration of 6 mg/ml.

2.3 Fabrication of cell-seeded PC collagen scaffolds

Collagen GNL mixture was poured into the bioreactor with minimized bubble formation and allowed to solidify for 1 h at 37°C. The compression system (Figure 4a) consisted of a fitted stainless-steel pestle, a linear motor (P01-37x120F-HP; NTI AG LinMot & MagSpring, Spreitenbach, Switzerland) and a scale (Navigator® XL; OHAUS Europe GmbH, Greifensee, Switzerland) for force level control. Motor and scale were connected to a SIMATIC HMI ET200 control panel (Siemens AG, Berlin, Germany) ensuring constant advance of the pestle (13 μm/s) and continuous pressure monitoring (maximum force applied 10.25 ± 5.97 N equalling 22.7 ± 13.2 kN/m²). After completion of the compression cycle, the bioreactor was connected to a medium reservoir with 30 ml of VascuLife via silicone tubing and transferred to a housing incubator. A constant medium flow of 0.1 ml/min was established using pumping tubes (inner diameter 1.52 mm; SC0744 PharMed BPT; Cole-Parmer GmbH) and a roller pump (Ismatec ISM 400 MS/CA 4-12; Cole-Parmer GmbH) attached to a SIMATIC HMI ET200 control panel. Patches were cultured at 37°C and 5% CO₂.

After 24 h, the medium reservoir was detached and stored at 37°C. The lower compartment of the reactor was closed, the central placeholder removed, and noncytotoxic silicone tubing was attached. HAAEC suspension was injected in a “pump and suck” manner retaining some 40 μl of a 1 * 10⁶ cells per milliliter suspension inside the channel. The tubing was closed and cells were allowed to settle for 1 h. The bioreactor was turned upside down for another hour to ensure ubiquitous EC attachment. The whole seeding procedure was performed twice to ensure adequate EC seeding density. After reconnection to the roller pump system patches were incubated under permanent perfusion conditions for a maximum of 7 days. Half of the reservoir medium was changed twice a week. At the end of the culture period, patches were gently removed from the bioreactor and further processed.

2.4 Examination of PC effects on mechanical properties and cell survival

To comprehensively cover viscoelastic properties of our PC collagen scaffold, we chose to determine storage and loss moduli (G’ and G’’) over Young's modulus. Rheological characterization was performed on freshly compressed patches (0D) and on patches cultured for 8 days (1 day of initial scaffold remodeling + 7 days of alleged channel endothelialization). Patches for both time points were manufactured with and without interstitial cells (HDF and control, respectively). For each setup (0D, 0DF, 8D, and 8DF), three patches were examined. Mean and SD were calculated from three submeasurements per sample. Mapping of samples was performed with a Physica MCR 301 oscillatory rheometer (Anton Paar, Graz, Austria), equipped with a 25 mm diameter flat plate geometry at 37°C with a gap of 1 mm. To prevent drying, the area around the sample was occasionally rinsed with distilled water.

An amplitude sweep (range 0.001–100% at 1 Hz with 30 read out points) was performed to determine the upper limit of the linear- viscous area (LVA) in the respective strain-modulus-diagram. The LVA in turn provided data on storage modulus G’ and loss modulus G’’. Where G’ followed Hookean law representing the elastic component of a viscoelastic fluid, G’’ constituted the viscous component according to Newtonian fluid dynamics.

Dynamic or complex shear modulus (G’’) was calculated from Equation 1 based on the relationship expressed in Equation 2:

$$|G'\,(\omega)| = \sqrt{G''\,(\omega)^2 + G''\,(\omega)^2}$$

(1)

$$G'\,(\omega) = G'\,(\omega) + iG''\,(\omega).$$

(2)

G’’ thereby closely resembles a Maxwell model of viscoelasticity with a serial setup of a pure viscous dampener and a pure elastic spring.

To determine the impact of compression on embedded cells, we digested freshly compressed patches (10 ml collagen, 5 ml GNL, and 1 * 10⁵ cells) with collagenase A (f.c. 1 mg/ml) for 1 h at 37°C. Cells treated with methanol for 30 min on ice (dead) and cells digested with collagenase A for 1 h at 37°C (live) served as controls. After their respective treatments, cells were suspended in PBS, stained with propidium iodide (PI; f.c. 3.5 μg/ml; Sigma-Aldrich), and analyzed via flow cytometry with a FACSCalibur (BD Biosciences). Counts were then corrected for collagenase A effects.

2.5 Qualitative MTT test of whole scaffolds

Viability testing of scaffold-incorporated cells was done via a qualitative MTT test. The cultured scaffold was taken out of the reactor and transferred into a 1 mg/ml MTT in VascuLife® VEGF-Mv solution. After 90 min of incubation at 37°C, the scaffold was washed thoroughly with PBS+ until no MTT residues were visible.

(f.c. 100 μM; Thermo Fisher Scientific), L-ascorbic acid (f.c. 0.5 mg/ml; Sigma-Aldrich), and 1% P/S.
2.6 | Microscopic characterization of isolated cells and cultured scaffolds

2.6.1 | Immunofluorescence

Isolated cells were grown on glass chamber slides coated with 1% gelatin and fixed after 24 h with icecold acetone/methanol (1:1). Cells were permeabilized for 5 min with 0.2% Triton-X in PBS with 0.5% Tween-20 (PBS-T) and blocked for 20 min with 5% donkey serum in antibody diluent (DCS Diagnostics, Hamburg, Germany). Primary antibodies were mouse-anti-human αSMA (Abcam), mouse-anti-human CD105 (Abcam), rabbit-anti-human Ki67 (Abcam), and mouse-anti-human VE-cadherin (FITC conjugated; eBioscience Inc.). Antibodies were incubated overnight. Secondary antibodies conjugated with Alexa fluorochromes (Invitrogen, Carlsbad, CA, USA) were used at a concentration of 5 μg/ml. After each step, cells were washed 3 × 5 min with PBS-T. Chamber slides were mounted with Moviol + 0.1% DAPI. Images were recorded with a Keyence BIOREVO BZ-9000 (Keyence Corporation, Osaka, Japan) and Leica TCS SP8 (Leica, Wetzlar, Germany) microscope, respectively. Scale bars were inserted with the Fiji plugin for ImageJ 1.51k (Wayne Rasband, National Institutes of Health, USA) (Rueden et al., 2017; Schindelin et al., 2015).

2.6.2 | Immunohistochemistry

Patches were fixed for 4 h in 4% PFA, dehydrated, and embedded in paraffin. Paraffin sections were cut to slices of 4 μm, deparaffinized using Roticlear® (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), rehydrated in descending ethanol dilutions, and washed in PBS-T for 5 min. Internal peroxidases were inactivated by incubation with 3% H2O2, sections washed 3 × 5 min in PBS-T, and blocked with 10% donkey serum in antibody diluent (DCS Diagnostics). Primary antibodies were mouse-anti-human CD31 (Dako) and rabbit-anti-human collagen IV (Abcam). To visualize bound antibodies IHC-Kit DCS SuperVision 2 HRP (DCS Diagnostics) was used according to manufacturer’s protocol. Counterstaining of nuclei was performed using Mayer’s hematoxylin (Morphisto GmbH, Frankfurt/Main, Germany). Sections were mounted with Moviol and images recorded with a Keyence BIOREVO BZ-9000 microscope (Keyence Corporation). Scale bars were inserted with the Fiji plugin for ImageJ 1.51k (Wayne Rasband, National Institutes of Health, USA) (Rueden et al., 2017; Schindelin et al., 2015).

2.6.3 | Cell labeling and fluorescent patches

Cells for fluorescence readout of endothelialization were stained with CellTracker™ Green and CellTracker™ Deepred according to protocol (Thermo Fisher Scientific). Cross sections of cultured patches were inserted into a self-built tissue chamber filled with PBS. Samples were analyzed using a Confocal TCS SP8 (Leica Microsystems) and pictures taken with LAS AF software (Leica Microsystems). Scale bars and 3D remodeling were done using the Fiji plugin for ImageJ 1.51k (Wayne Rasband, National Institutes of Health, USA) (Rueden et al., 2017; Schindelin et al., 2015).

2.6.4 | Transmission electron microscopy

For transmission electron microscopy (TEM) samples were fixed as previously published by Helimoebst, Frank, and Stigloher (2015) for 1 h in 2.5% glutaraldehyde. Preparation of fixed patches followed the same protocol. Briefly, samples were washed, incubated with 2% OsO4, and contrasted with 0.5% uranyl acetate as described by Reynolds (1963). Finally, samples were dehydrated in ascending ethanol dilutions and embedded in EPON® epoxy resin. Sections were contrasted with 2.5% uranyl acetate in ethanol and Reynolds lead citrate and subsequently covered with a carbon coat. Sections were then analyzed using a Jeol JEM-2100 transmission electron microscope at 200 kV (JEOL USA, Inc., Peabody, MA, USA). Scale bars were inserted with Fiji (Rueden et al., 2017; Schindelin et al., 2015).

2.7 | Statistics

Statistical tests and plotting were performed using Origin 2016 (OriginLab, Northampton, MA, USA) and Graphpad Prism 8.4.3 (Graphpad Software, San Diego, CA, USA), respectively. We used Student’s t-test or ANOVA with Tukey’s correction for multiple comparisons where appropriate. All values are given as mean ± standard deviation (SD).

3 | RESULTS

3.1 | Construction of a modular bioreactor for scaffold production and 3D cell culture in a one-stop-shop approach

We established a new, integrated bioreactor design to meet the demands of both scaffold fabrication and 3D cell culture for autologous prevascularized heart tissues. The bioreactor was built in a modular fashion: For initial PC of semi-fluidic collagen I, a linear motor-driven, stainless-steel cylinder was placed on top of the core module (Figure 1a,b). Before endothelialization, the needle was removed, and ECs were flushed into the central channel. After EC attachment, the patch was reconnected to the circulation tubules for dynamic culture perfusion. The internal space of the core module was divided by a metal strainer creating two internal compartments (Figure 1c). The individual height of each compartment could be adapted by insertion of shims underneath the strainer. The upper compartment served as housing for the scaffold including a stainless-steel needle as placeholder for the traversing endothelialized channel. The lower compartment was filled with cell medium for additional nutrient supply and hydrostatic pressure compensation.
3.2 | Automated PC generates superior matrix stability and does not impede cell survival of cardiac-associated cells

Major hurdles in the fabrication of apt cardiac tissue scaffolds are physiological stiffness and proper vascularization. Therefore, we designed our bioreactor to (1) allow for stiff yet elastic matrix production and (2) incorporate and dynamically culture all required cell types at the same time. We performed vibratory rheological measurements for quantification of cell-induced collagen alterations. Therefore, we compared $G'$ and $G''$ values of patches at two different time points: unincubated patches directly after compression (0 days [0D]) and patches that had been incubated for 8 days (8D). For each time point, two different settings were analyzed: patches consisting solely of collagen (0D and 8D) and patches with incorporated HDF (0DF and 8DF).

Patches at Day 0 (Figure 2, 0D and 0DF) showed no significant differences regarding storage and loss moduli or complex shear modulus ($G''$). Similarly, examination of control patches incubated with medium for 8 days without HDF (8D) showed no significant differences compared to the samples at Day 0. In contrast, patches including HDF that were cultured for 8 days (8DF), revealed high alterations of mechanical properties. With all rheological moduli significantly ($p < 0.05$) exceeding those of all other groups, stiffness rose even more in patches cultured with fibroblasts, indicating active HDF reconstruction of the collagen matrix.

Consecutively, patch dimensions were subject to artificial and time-dependent changes ($n = 20$). While patch diameter remained the same at all time points (2.4 cm), height was reduced by PC from 4.3 ($±0.2$) cm to 1.23 ($±0.14$) cm ($p < 0.01$). As a result of cellular remodelling effects, patch height was further decreased by approximately 0.8–1.0 cm around the central channel and 0.6–0.8 cm to the sides.

To evaluate the effect of compression on typical cardiac-associated cell types, we determined individual cell survival rates after compression. Freshly compressed patches were digested with collagenase A for 1 h, and the released cells were subsequently examined. FACS analysis revealed a type-specific survival of the cells tested (Table 1). While HDF, WCM, and CD31-negative cells showed high resistance towards the mechanical stress of compression, HAAEC and hiPSC-derived pericytes were more sensitive towards the process. hiPSC-derived CMs showed a 110% survival rate. This effect can be attributed to the correction of survival rates for collagenase A effects as CM were more sensitive towards the enzymatic treatment than to the compression itself.

3.3 | Human AAs are a reliable EC source for later autologous applications

To establish our prevascularized scaffold model, ECs were isolated from HLAAs. HLAA were excised during surgery, processed, and cells taken into culture (Figure 3a). Processed biopsies gave rise to WCM, a mixture of different cell types. We performed magnetic-activated cell sorting (MACS) for CD31 with WCM harvested from each specimen individually to obtain pure ECs. The resulting cell populations, CD31-negative cells and HAAEC as well as the unsorted WCM, were further investigated by FACS and immunofluorescence staining. Ki67 staining was applied to analyze the proliferative potential of all three cell populations from different donors (Table 2). On average, WCM showed the least proliferative activity. CD31-negative cells displayed medium Ki67 positivity while HAAEC held the highest number of proliferating cells. High SDs were presumably attributable to donor-specific variations.

In addition to Ki67, CD105 was visualized for fluorescent confirmation of HAAEC proliferative potential. HAAEC were also stained for vimentin assuming a cytoskeletal response towards cellular strain caused by atrial contractions. Both, CD31-negative cells and HAAEC, showed vimentin and CD105 expression, with an upregulation of CD105 in HAAEC. HAAEC but not CD31-negative cells expressed the EC markers CD31, TIE2, and VE-cadherin (see Figure 3b). WCM and CD31-negative cells were strongly positive for αSMA and less positive for CD105. In matching FACS findings, HAAEC stained stronger for CD105 and showed little positivity for αSMA (see Figure 3c). Isolation of other HLAA-derived cell types failed due to absence of unique markers, especially in case of fibroblasts and pericytes.

As the interplay of different cell types from the vessel microenvironment is crucial for endothelial sprouting and maturation, pericytes were differentiated from hiPSC and characterized using FACS analysis. About 75% to 90% of cells stained positive for CD146, PDGFRβ, αSMA, and NG2. For more information on the characterization of hiPSC-derived pericytes, please refer to Figure S2.

3.4 | Three-dimensional culture and constant perfusion create a primitive vessel capable of sufficient nutrient delivery

After removal of the central needle placeholder within the PC collagen matrix, HAAEC could be seeded onto the emerging channel surface. Continuous perfusion of the channel was initiated with VascuLife® endothelial growth medium at a rate of 1 ml/min and a surface shear stress of approximately 0.77 dyne/cm². The resulting flow corresponded to prior experiences at our lab showing good EC attachment and acceptable nutrient supply at the same time. Qualitative MTT tests after culture revealed excellent cell survival throughout the channel and within the patch (Figure 1d and S1).

Three series of patches were created to examine five time points of endothelial proliferation. Incubation of patches was stopped directly after seeding (0 h), after 24, 48, 72, and 168 h, respectively. At each level, the percental CD31-positive endothelial coverage of the channel surface was estimated by reference to six cross-sectional images. Hyperbolic curve fitting revealed a saturating growth over time (Figure 4c).

EC showed some metabolic activity by expression of collagen IV as early as 24 h after seeding (Figure 4b). After 7 days of incubation, the endothelial layer had little to no gaps in cross sections stained for
CD31. Collagen IV was abundant all over the patch indicating onset of additional high metabolic activity of interstitial HDF and could further be intensely detected just below the basal EC poles. For better visualization of the endothelial layer, cells labeled with CellTracker™ were used for patch generation. Three-dimensional reconstruction of confocal stacks revealed a closed monolayer of ECs exhibiting an elongated phenotype along the applied medium current (see Figure 4a).

We further evaluated endothelial cell–cell and cell–matrix contact formation using TEM after 7 days of culture. ECs were closely attached to each other creating spots resembling tight junctions (Figure 4d, red arrow), a desmosome (Figure 4d, pink arrow), and a hemidesmosome (Figure 4d, light blue arrow). Furthermore, signs of a primitive basal lamina production could be detected below the EC layer (Figure 4d, blue arrows).

### Table 1

| Cell type      | Survival of cells (%) |
|----------------|-----------------------|
| HDF            | 76.77 ± 5.20          |
| WCM            | 77.66 ± 10.42         |
| CD31-negative  | 83.36 ± 7.14          |
| HAAEC          | 68.36 ± 9.27          |
| CM             | 110.73 ± 9.70         |
| Pericytes      | 56.20 ± 5.40          |

Note: Effects of collagenase A were subtracted by adding any number of dead cells in live control to surviving cells in compressed patches. n = 3.

Abbreviations: CM, cardiomyocyte; FACS, fluorescence-activated cell sorting; HAAEC, human atrial appendage endothelial cells; HDF, human dermal fibroblasts; WCM, whole cell mix.

#### Discussion

In this study, we were able to devise a bioreactor system for combined PC of collagen I and subsequent 3D dynamic cell culture in a single integrated setup.

**FIGURE 2** Rheological examination of patches: (a) Vibratory rheological strain curves before and after 8-day incubation period, with and without fibroblasts. Two time points were examined: directly after compression (0D) and after 8 days of culture (8D). Shear modulus (G²; III) was calculated from elastic storage (G', I) and loss (G²; II). (b) Bar chart of G', G², and G* at 0.2 % strain. 0D, 0DF, and 8D patches showed no significant difference regarding G' or G². 8DF patches showed a significant increase in overall stiffness (*p < 0.05). (c) Table of measured values at 0.2% strain. Three patches per condition, each measured three times [Colour figure can be viewed at wileyonlinelibrary.com]
Collagen I has been broadly used for several tissue engineering applications. It is one of the most prominent proteins in various connective tissues throughout the human body and thus highly biocompatible with low immunogenicity. However, collagen I gels tend to be quite susceptible towards mechanical stress due to a high water content. Brown, Wiseman, Chuo, Cheema, and Nazhat (2005) published a static PC protocol for more stable constructs. PC has been shown to enhance mechanical properties of collagen gels (Hadjipanayi et al., 2011) and boost cell migration and proliferation in complex 3D models (Ajalloueian et al., 2014; Hu et al., 2010; Mi et al., 2010). Most articles published so far used static compression either under the constructs own weight (Brown et al., 2005; Cheema & Brown, 2013) or with a weighted plate (Drechsler et al., 2017; Hu et al., 2010; Witt et al., 2019). Our standardized, semi-automatic compression strategy yielded a considerably higher (about 16 times) compressive force than in previous reports by using a motor-driven approach instead of fixed weight plates (Serpooshan et al., 2013). While our method was able to limit further collagen shrinking due to time-dependent fluid loss, HDF seeding could still significantly enhance the viscoelastic properties of the scaffold. This matches with the findings of Ghezzi et al. (2011), who stated positive effects of

**FIGURE 3** Isolation and characterization of left atrial appendage cells. (a) Human left atrial appendages were excised during surgery; tissue was minced, partially digested, and plated. Cells grew out within 2 to 3 weeks. (b) CD31-MAC-sorted cells were analyzed by FACS. Isotype controls shown in blue. CD31-negative cells expressed vimentin and CD105 and showed negligible positivity for EC markers CD31, TIE2, and VE-cadherin. HAAEC expressed vimentin as well as CD31, TIE2, and VE-cadherin. HAAEC showed a more pronounced staining for CD105 compared with CD31 cells. (c) Immunofluorescence showed WCM and CD31-negative cells consisting mostly of αSMA-positive cells and containing slightly CD105-positive subpopulations. HAAEC strongly expressed CD105. CD, cluster of differentiation; FACS, fluorescence-activated cell sorting; TIE2, angiopoietin-1 receptor; VE-cadherin, vascular endothelial cadherin; HAAEC, human atrial appendage endothelial cells; WCM, whole cell mix. Scale bar: 100 μm [Colour figure can be viewed at wileyonlinelibrary.com]
fiber density on fibroblast growth. Because both storage and loss mod-
uli rose over time our PC collagen patch should retain enough pliability
despite superior stability. This in turn is a key element for later inpatient
use (Ghezzi, Marelli, Muja, & Nazhat, 2012). Consequently, we also
tested for surgical handling in a hands-on test of use. This in turn is a key element for later inpatient
uli rose over time our PC collagen patch should retain enough pliability
and paracrine networks. Thus, balanced coculture models are the pre-
mise for developing a fully grown myocardial patch. We postulated a
cell-specific resistance towards mechanical stress during our PC cycle.

As stated earlier, the complex interplay of all cardiac cell types is
required to create a unique environment of closely related mechanic
and paracrine networks. Thus, balanced coculture models are the pre-
mise for developing a fully grown myocardial patch. We postulated a
cell-specific resistance towards mechanical stress during our PC cycle.

Results of cell survival analyses indicated two distinguishable groups:
While interstitial cells such as HDF, CD31-negative cells, and CM dis-
played high compression resistance, vascular-associated cells, that is,
HAAEC and pericytes, were more sensitive. This matches general cell
culture findings: EC often exhibit a more sensitive behavior regarding
the processes of medium exchange, cell passing, and freezing/thawing than more resilient cell types. High survival numbers
of CM could be explained by their unique physiological properties.

Due to the demands of permanent contraction and relaxation CM
exhibit a suitable cytoskeleton and membrane configuration to
withstand substantial mechanical strains (Sequeira, Nijenhuis, Regan, & van der Velden, 2014). However, they are sensitive towards
collagenase A treatment, becoming obvious after correction of
survival rates for digestion effects.

Isolation of cardiac-derived cells, namely, cardiac stem cells (CSC),
has gathered more and more attention as first clinical trials of stem cell
therapy for myocardial infarction have been registered (Gyongyosi,
Haller, Blake, & Martin Rendon, 2018). In our work, we focused on
HLAA-derived cells. HLAs are a remnant of embryonic development
attached to the left atrium (Al-Saady, Obel, & Camm, 1999; Regazzoli et al., 2015). So far, murine LAA were used for isolation of cardiac pro-
genitor cells (CPC) (Leinonen et al., 2013). Approaches in human, for
example, isolation of a new subtype of CSC, utilized right AAs
(Konincx et al., 2013). Preparation of HLAA gave rise to a cellular mix
termed WCM which was divided in endothelial CD31-positive
(HAEC) and CD31-negative populations. In previously published pro-
tocols, cardiac-derived EC were isolated from ventricular tissue
(McDouall, Yacoub, & Rose, 1996; Nishida et al., 1993). Here, we
demonstrated isolation of HLAA-derived EC for the first time. Initial
characterization of HAAEC showed an expression of all typical EC
markers including a high expression of CD105 and vimentin. Due to a
limited number of donor cell sources, a proliferative analysis on all iso-
lated cell populations was performed on three randomly chosen donor
patients only. Each experiment used up all isolated cells and showed
large variations of individual proliferative potential in the examined
cell populations. However, each experiment yielded sufficient num-
ers of EC for scaffold seeding. As HAAEC showed the highest prolif-
erating activity in most HLAA-derived cultures, the observed scatter is
probably a donor-specific effect. Primary cardiac EC should contribute
to a cardiac-like primed scaffold and are therefore to be considered
the first choice for prevascularization. With further improvement of
isolation protocols and more detailed characterizations at our lab,
abundant autologous cells could be available for heart muscle patch
repair following primary cardiac surgery, for example, atrioventricular
valve reconstruction and coronary artery bypass grafting in ischemic
heart failure. Thus, HLAA are a promising cell source for individual car-
diac tissue engineering applications.

Perfused, EC-lined tubes in a three-dimensional collagen scaffold
were firstly published by the group of Joe Tien, though resembling
microvascular dimensions (Chrobak, Potter, & Tien, 2006). The group
used lithographically built channels with an inner diameter of about
120 μm within a collagen matrix. Seeding of human umbilical vein endo-
thelial cells (HUVEC) or human dermal microvascular endothelial cells
(HDMECs) led to a confluent monolayer that showed a strong barrier
function as well as responsiveness towards inflammatory stimuli. There
have been several experiments to engineer larger vessels, including a
scaffold-only as well as a cell-sheet-based approach. Data have been
concisely reviewed by Naito et al. (2011). Considering these publica-
tions, we have shown the first attempt of implementing a larger artificial
vessel within a 3D collagen network. Such a vessel could serve as a
direct connection of the patch to the host coronary system. This in turn
would warrant an uninterrupted oxygen and nutrient supply immedi-
ately after transplantation, which is crucial for an adequate sustenance
of all cardiac and cardiac-related cell types present within the patch.

It is known that medium or blood flow and thereby shear stress has
a significant impact on ECs. Blood flow is crucial for vessel development
during embryogenesis. Furthermore, shear stress influences survival of
EC by influencing several intracellular signaling pathways and eliciting
responses in cytoskeletal buildup, adhesion molecules, and membrane
molecules (Hahn & Schwartz, 2009). These findings suggest that proper
medium flow and the resulting shear stress should positively influence
growth and integrity of the endothelial layer. We chose a low flow
velocity of 1 ml/min to avoid initial flush detachment of the ECs. This
flow leads to a shear stress of some 0.77 dyne/cm². Most methods pub-
lished so far using microvascular networks with gravity-driven medium
flow reported a shear stress range from 0.1 (Zheng et al., 2012) to
5 dyne/cm² (Chan et al., 2014; Chrobak et al., 2006). HAAEC in our
model showed continuous proliferation over time and were metaboli-
cally active, suggesting appropriate dynamic culture conditions. Like-
wise, immunofluorescent 3D reconstruction of the channel highlighted
adaptation of HAAEC to the applied perfusion conditions. Cells

| TABLE 2 | Proliferative potential depending on donor: Ki67 immunofluorescence of three different donors cell populations 24 h after seeding |
|---------|---------------------------------------------------------------|
|         | WCM (%) | CD31-negative (%) | HAAEC (%) |
| Donor 1 | 27.24   | 44.15             | 39.23     |
| Donor 2 | 37.87   | 33.2              | 29.34     |
| Donor 3 | 21.1    | 18.99             | 50.31     |
| Overall | 28.46 ± 8.57 | 31.79 ± 12.16 | 39.33 ± 10.52 |

Note: Total and Ki67 positive nuclei were counted. All donors showed different proliferation activities causing overall high standard deviations. n = 3.

Abbreviations: HAAEC, human atrial appendage endothelial cells; WCM, whole cell mix.
FIGURE 4 Immunohistological timeline and transmission electron microscopy. (a) Schematic depiction of sample retrieval and orientation. $X =$ channel surface/endothelial layer, $Y =$ patch, and $Z =$ direction of medium flow. Original picture shows fluorescent-labeled cells within the patch after 7 days of endothelialization. A tilted 3D reconstruction of the channel surface shows the HAAEC layer (red) and interstitial CD31-negative cells (green). HAAEC exhibited an elongated shape along the flow direction of the medium. Scale bar: 100 μm. (b) CD31 DAB staining of patches after 24, 72, and 168 h of endothelialization. Endothelial cells proliferated over time and eventually covered most of the channel surface. Collagen IV DAB staining: HAAEC produced collagen IV, accumulating in subendothelial regions. Overall collagen IV expression rose over time as fibroblasts also showed expression. Scale bar: 200 μm. $n = 3$. (c) A time-dependent analysis of the endothelialization process of the channel surface revealed a saturating coverage curve (0 vs. 168 h: $p < 0.01$; figure showing fitted curve ± 95% CI). Due to extensive outliers, the 72 h time point was not included in the analysis. (d) TEM pictures of endothelial layer after 7 days showed a tight junction (red arrow) and a desmosome (pink arrow). Furthermore, a hemidesmosome (light blue arrow) and a partially formed basal lamina (blue arrow) could be observed. Scale bars: 500 (left) and 200 nm (right). CD, cluster of differentiation; DAB, 3,3'-diaminobenzidine; HAAEC, human atrial appendage endothelial cells; TEM, transmission electron microscopy; CI, confidence interval [Colour figure can be viewed at wileyonlinelibrary.com]
exhibited an elongated phenotype along the flow direction of the perfusing medium and developed differential cell junctions.

Type IV collagen is the most prominent protein in basement membranes. Its subunits, so-called protomers, are being built in the Golgi apparatus and secreted into the extracellular space, where collagen IV networks are formed by self-assembly (LeBleu, Macdonald, & Kalluri, 2007). HAAEC lining the central scaffold channel showed prominent expression of collagen type IV. Initial weak subendothelial deposits increased over time to form a strongly positive extracellular network surrounding the cells at Day 8. This matches findings of Bahramsoltani, Slosarek, De Spiegelaere, and Plendl (2014) who showed this increase of collagen IV expression and secretion over time under static conditions for different commercially available EC. Moreover, it was shown in vivo that high concentrations of collagen IV stabilized neo vessels and prevented vascular regression (Bonanno, Iurlaro, Madri, & Nicosia, 2000). The extremely high expression of collagen IV in shorter culture periods than in the report of Bahramsoltani et al. (2014) might also indicate a supporting role of dynamic medium flow. HAAEC might have higher expression rates as a reaction to shear stress.

TEM revealed an alleged formation of tight junctions and desmosomes between neighboring EC. A strong expression of collagen IV throughout the culture period indicated attempts of basal lamina formation. TEM pictures suggested formation of an extracellular matrix (ECM) as indicated by basal lamina residues. It is known that EC can express most components of the vascular basement membrane themselves, but the final assembly requires heterotypic cell–cell contacts (Davis & Senger, 2005).

5 | CONCLUSIONS AND OUTLOOK

We present a new method of manufacturing a large-scale collagen-based scaffold. Motor-driven PC of collagen I hydrogel yields a viscoelastic scaffold, more likely to resemble in vivo tissue stiffness. A traversing endothelialized channel and successful seeding of various cell types under dynamic culture conditions make this patch a versatile tool for complex tissue engineering efforts. Even though our choice of collagen work-up and cells aimed at constructing a cardiac repair patch, our principal strategy is not limited to this specific tissue type. The method could be adapted for a different organoid framework design. Possible modifications include but are not limited to changes in compression cycle length and force development, cell composition, and cytokine application due to the modular architecture of our bioreactor system.

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CONFLICT OF INTEREST

None declared.

ETHICS STATEMENT

Direct humanization for autologous transplantation without necessity of animal-based transplants. All tissues explanted under ethical vote of the university clinic Wuerzburg number 182/10.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

Figure S1. MTT test. This patch was cultured for eight days with 4 × 96 HAAEC spheroids each containing about 500 HAAEC. MTT test revealed blue spots showing surviving spheroids within the patch without any background staining.

Figure S2. FACS analysis of HiPSC-derived pericytes. Fully differentiated pericytes were analyzed by FACS. The figure shows an exemplary staining.

Figure S3. Surgical handling stability. The central channel of a freshly compressed patch was cut open (A) and subsequently readapted with four prolene interrupted sutures with USP 5–0 (B). Finally, bilateral manual pull by an experienced cardiac surgeon was applied using anatomical tweezers to prove the mechanical stability of the collagen patch adjacent to the suture line (C).

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