In vivo evaluation of bioprinted prevascularized bone tissue

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Abstract
Bioprinting can be considered as a progression of the classical tissue engineering approach, in which cells are randomly seeded into scaffolds. Bioprinting offers the advantage that cells can be placed with high spatial fidelity within three-dimensional tissue constructs. A decisive factor to be addressed for bioprinting approaches of artificial tissues is that almost all tissues of the human body depend on a functioning vascular system for the supply of oxygen and nutrients. In this study, we have generated cuboid prevascularized bone tissue constructs by bioprinting human adipose-derived mesenchymal stem cells (ASCs) and human umbilical vein endothelial cells (HUVECs) by extrusion-based bioprinting and drop-on-demand (DoD) bioprinting, respectively. The computer-generated print design could be verified in vitro after printing. After subcutaneous implantation of bioprinted constructs in immunodeficient mice, blood vessel formation with human microvessels of different calibers could be detected arising from bioprinted HUVECs and stabilization of human blood vessels by mouse pericytes was observed. In addition, bioprinted ASCs were able to synthesize a calcified bone matrix as an indicator of ectopic bone formation. These results indicate that the combined bioprinting of ASCs and HUVECs represents a promising strategy to produce prevascularized artificial bone tissue for prospective applications in the treatment of critical-sized bone defects.

KEYWORDS
bioprinting, bone formation, endothelial cell, mesenchymal stem cell, vascularization

1 | INTRODUCTION

Bone transplantation represents an important option in the treatment of bone defects, caused by trauma, infection, or oncologic resection. In this context, autologous bone grafting is considered to be the gold standard. However, this procedure is associated with various drawbacks such as donor side morbidity, infection, chronic pain and limitations concerning the quantity and/or quality of bone for harvest (Calori, Mazza, Colombo, & Ripamonti, 2011; Gazdag, Lane, Glaser, & Forster, 1995). Bone tissue engineering is a promising alternative combining osteoconductive and/or osteoinductive scaffolds and autologous bone-forming cells, such as mesenchymal stem cells (MSCs).

MSCs can be isolated from various tissues such as bone marrow, adipose tissue, umbilical cord blood, and the gelatinous substance...
within the umbilical cord, called Wharton’s jelly (Amati et al., 2017; Beeravolu et al., 2017; Pittenger et al., 1999). MSCs show a high proliferative potential during in vitro cultivation, are able to self-renewal, and can differentiate in vitro as well as in vivo along the osteogenic, adipogenic, myogenic, and chondrogenic lineage (Pittenger et al., 1999; Witt et al., 2017). Especially in bone tissue engineering, MSCs have gained much interest in recent years, not only because MSCs can differentiate into osteoblasts, but also because autologous MSCs can be isolated without notable donor-site morbidity (Wakitani et al., 1994). Moreover, it has been shown in numerous animal studies that implanted MSCs support bone healing in critical-sized bone defects (Jones, Giannoudis, & Kouroupis, 2016; Kon et al., 2000; Koob et al., 2011).

However, in bone tissue engineering applications, it is also necessary to ensure sufficient vascularization of the transplant since transplantation of avascular artificial tissue will lead to hypoxic cell death of bone-forming cells. Vascularization of artificial implants can be achieved by coimplantation of endothelial cells (ECs) from different sources, such as human umbilical vein endothelial cells (HUVECs), primary human dermal microvascular endothelial cells (HDMECs) or stem cell derived ECs (Alajati et al., 2008; Freiman et al., 2018; Jain, Au, Tam, Duda, & Fukumura, 2005). It has been shown that implanted ECs can form functional blood vessels, which inosculate with the host circulatory system, and thereby support the engineered tissue with nutrients and oxygen, and also ensure removal of waste products (Alajati et al., 2008; Ben-Shaul, Landau, Merdler, & Levenberg, 2019; Roux et al., 2018; Steffens, Wenger, Stark, & Finkenzeller, 2009).

Recently, three-dimensional (3D) bioprinting techniques have been developed and can be considered to represent a progression of the classical tissue engineering approach (Ozbolat & Yu, 2013). In classical tissue engineering, cells are randomly distributed within the constructs, whereas in case of 3D bioprinting, cells can be printed with high spatial control of cell allocation within the 3D construct. This is especially important if more than one cell type is used to engineer an artificial tissue. Different printing methods have been adapted for bioprinting of mammalian cells. These methods include inkjet printing (e.g., drop-on-demand [DoD]), extrusion-based printing and laser-assisted printing (Murphy & Atala, 2014). For bioprinting, cells are dispersed into hydrogels to form a so called bioink. The hydrogels need to exhibit a high cytocompatibility, must be printable, and must provide a sufficient mechanical stability to the printed construct.

In recent studies, we have identified suitable hydrogels for extrusion-based bioprinting of human adipose-derived mesenchymal stem cells (ASCs) and DoD-based bioprinting of HUVECs (Trondle et al., 2019; Wehrle et al., 2019). In this context, we have shown in vitro that a multicomponent hydrogel with a thermal and enzymatic crosslinking mechanism due to gelatin and fibrin showed excellent biocompatibility toward ASCs. Hyaluronic acid and glycerol were added to the hydrogel (referred to as osteo-hydrogel in the following) to increase shape fidelity and printing uniformity and hydroxyapatite to support osteogenic differentiation of ASCs in vitro. Viability of ASCs after printing was more than 90% and the osteo-hydrogel was able to support osteogenic differentiation of ASCs in vitro (Wehrle et al., 2019). Similarly, we could show that HUVECs bioprinted via DoD using fibrin hydrogel and a high cell concentration showed a high cell viability after printing of more than 80%. Moreover, HUVECs displayed a time-dependent self-assembly of lumenized vascular structures in vitro (Trondle et al., 2019).

In this study, these two types of ASC and HUVEC laden hydrogels were bioprinted by extrusion and DoD, respectively, to produce blended constructs that were evaluated in vivo in a heterotopic ossification model in terms of bone and blood vessel formation.

2 | MATERIALS AND METHODS

2.1 | Cell culture

HUVECs were obtained from PromoCell and cultured in endothelial cell growth medium (ECGM; PromoCell). Media were supplemented with 1% penicillin/streptomycin (Pen/Strep) and 10% fetal calf serum (FCS; Biochrom). Cells from passages three to six were used in all experiments.

Tissue harvesting of ASCs was performed with the informed consent of the patients according to the Helsinki Declaration, and approved by the institutional ethics committee. ASCs were isolated from a 33-year old male donor using an established protocol of our group (Strassburg, Torio-Padron, Finkenzeller, Frankenschmidt, & Stark, 2016). In brief, subcutaneous fat tissue acquired during surgery was minced and incubated for 3 hr at 37°C with albumin phosphate buffer (phosphate-buffered saline [PBS], 3.5% BSA) containing 2 mg/ml collagenase type II (Sigma-Aldrich) to digest it. Afterwards, the solution was centrifuged (10 min, 250 g, room temperature) and the supernatant was removed. Subsequently, the cell pellet was resuspended in erythrocyte lysis buffer (17 mM Tris, 16 mM NH4Cl) and then centrifuged again. After filtering through a cell strainer and another centrifugation step, ASCs were resuspended in ECGM, (PromoCell) supplemented with 10% FCS and 1% Pen/Strep. ASCs were used for experiments at passage two to six. HUVECs and ASCs were maintained at 37°C with 5% CO2 in a humidified environment, changing medium twice a week. Splitting of cells was carried out when cells reached about 90% confluency.

In some experiments, HUVECs and ASCs were fluorescently labeled before bioprinting. HUVECs and ASCs were treated with 2 µM CellTracker-Red or CellTracker-Green, respectively (Thermo Fisher Scientific), for 30 min in ECGM, 10% FCS, 1% Pen/Strep at 37°C with 5% CO2. Thereafter cells were washed twice with PBS and incubated in ECGM, 10% FCS, 1% Pen/Strep until use.

For osteogenic predifferentiation, ASCs were incubated for 7 days in osteogenic differentiation medium based on Dulbecco’s modified eagle medium (DMEM; Thermo Fisher Scientific) supplemented with 10% FCS, 1% Pen/Strep, 10 mM β-glycerophosphate, 0.1 µM dexamethasone, and 50 µM ascorbic acid (all purchased from Sigma-Aldrich) with a medium change after 3 days.
2.2 | Preparation of hydrogels

2.2.1 | Fibrinogen for bioprinting HUVECs

Fibrinogen (Merck) was diluted using PBS to get a 10 mg/ml stock solution. Thereafter, recombinant angiogenic growth factors vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) and the protease inhibitor aprotinin (all from Sigma-Aldrich) were added. Final concentrations were fibrinogen (5 mg/ml), VEGF (1 µg/ml), bFGF (1 µg/ml), and aprotinin (500 µg/ml). HUVECs were added to this hydrogel to a final concentration of 1.5 × 10^7 cells/ml.

2.2.2 | Fibrin/gelatin/hyaluronic acid/glycerol (osteohydrogel) for bioprinting ASCs

This hydrogel was adapted from Kang et al. (2016) and modified with hydroxyapatite, aprotinin, and recombinant angiogenic growth factors to combine high cytocompatibility with printing uniformity and shape fidelity. Hyaluronic acid (Sigma-Aldrich) was dissolved in Dulbecco’s modified Eagle medium (DMEM) at 37°C overnight to gain a solution of 14 mg/ml. In addition, gelatin was dissolved in DMEM to gain a solution of 210 mg/ml. DMEM was also used to dilute glycerol (Sigma-Aldrich) to 50% (v/v). Fibrinogen (Merck) was diluted with PBS for a concentration of 50 mg/ml. Fibrinogen, gelatin, hyaluronic acid, glycerol and hydroxyapatite (Sigma-Aldrich) were combined and gently mixed in a 50 ml falcon tube and incubated on a tumbling rotator mixer (RMS Assistent, Hecht Glaswarenfabrik) at approximately 36 rpm for 1 hr at 37°C. Thereafter, recombinant angiogenic growth factors VEGF and bFGF and the protease inhibitor aprotinin were added manually with a 1 ml pipette. Final concentrations in this hydrogel were fibrinogen (20 mg/ml), gelatin (35 mg/ml), hyaluronic acid (3 mg/ml), glycerol (10% v/v), hydroxyapatite (10 mg/ml), VEGF (1 µg/ml), bFGF (1 µg/ml), and aprotinin (500 µg/ml). ASCs were osteogenically pre differentiated for 7 days and then added to this hydrogel to a final concentration of 4 × 10^6 cells/ml.

2.3 | Bioprinting of constructs

The ASC-containing osteo-hydrogel was extruded from a 2 ml syringe (Injekt Luer Lock, 4606701V, B Braun) using a piston-driven syringe pump. A parallel teflon-coated metal nozzle with 250 µm inner diameter (TF720100PK, Globaco) was used to form meandering lines. The maximum flow rate was adjusted to 3.8 µl/s for a printhead horizontal velocity of 4 mm/s. Overall, 12 layers were used to produce a cuboid structure sizing 10 mm × 10 mm × 5 mm. To enzymatically crosslink fibrinogen with thrombin a piezoelectric-driven DoD dispenser (PipeJet P9, Biofluidix) was used to dispense a 20 U/ml thrombin solution. An array of 10 × 10 droplets with a pitch of 1 mm was dispensed after every second layer of ASC-containing hydrogel. The droplet volume was approximately 50 nl. HUVECs were also printed by DoD in fibrinogen hydrogel in a grid like pattern with 3 × 3 lines per layer, each line comprising 51 droplets with a volume of 10 nl and a distance of 200 µm. Spacing between the lines was 3 mm. This pattern was printed after every second layer of ASC-containing hydrogel, in total five times. A short video demonstrating the extrusion- and DoD-based printing process is shown as Supporting Information (Video_S1_SuppInfo). The total printing time for one cuboid structure was about 15 min. The print design of the cuboid structure is shown in Figure 1a,b. Bioprinting was performed in a polycaprolactone (PCL) frame to facilitate handling of the constructs for the later transport to the animal facility, the location for the implantation. The bioprinted construct inside the PCL frame is shown in Figure 1c. After printing, the construct was cooled down for 15 min at 4°C to mechanically stabilize gelatin and hyaluronic acid. In the next step, the cube structure was transferred into a thrombin bath (20 U/ml) for 30 min. Finally, thrombin was replaced with cell culture medium (ECGM, 10% FCS, 1% Pen/Strep) containing 20 µg/ml aprotinin and the construct was incubated overnight at 37°C, 5% CO2 in a humidified atmosphere. The PCL-frame was removed before implantation of the construct. Negative controls were produced in the same way, omitting ASCs and HUVECs.

2.4 | Animal experiments

The constructs were implanted into dorsal subcutaneous pouches. A 4 to 6 weeks old SCID mice (C.B.-17-SCID, Charles River) served as recipients of the bioprinted constructs. German regulations for care and use of laboratory animals were met at all times. All experiments were approved by the animal care committee of the University of Freiburg. The animals were housed in the veterinary care facility of the University of Freiburg Medical Center. Animals were randomly assigned to one of the experimental groups (control: bioprinted construct without cells; ASC/HUVEC: bioprinted construct with ASCs and HUVECs). Three animals were used for each group and two constructs were implanted in each animal; one left and one right of the midline. Thus, each group consisted of six constructs (n = 6). Implantation of the constructs was carried out under general anesthesia by inhalation of 5% isoflurane and maintained by inhalation of 2.5% isoflurane. The bioprinted constructs were removed from the PCL container under sterile conditions. After dorsal skin disinfection, a full-thickness incision was made to create a subcutaneous pouch (approximately 2 cm²) on each side of the spine to accommodate the construct. Subsequently, the bioprinted constructs were implanted into the pouch. Finally, the wound was closed with interrupted 6-0 vicryl sutures. 12 days after implantation, the mice were killed and the constructs explanted.

2.5 | Histological and immunohistochemical analysis of constructs

Bioprinted constructs were fixed in 4% formalin and then dehydrated using a tissue processor (TP1020, Leica Biosystems). Subsequently,
the constructs were embedded in paraffin for paraffin sections. Serial sections were cut at 5 µm.

2.5.1 Alizarin-red staining

To evaluate the calcification of extracellular matrix, alizarin-red staining was performed. Therefore, alizarin-red (Sigma-Aldrich) was dissolved in water to create a 20 mg/ml solution. pH was adjusted to 4.2 using 5 M NaOH (Merck). Sections were deparaffinized, rehydrated and washed briefly in PBS. Afterwards, sections were stained for 2 min in alizarin-red solution followed by five washing steps with PBS. Subsequently the slides were air-dried and finally dipped in xylol (Fisher Chemical) and permanently mounted with Entellan (Merck).

2.5.2 Immunohistochemistry

Sections were deparaffinized and rehydrated using xylene, a descending ethanol series and deionized water. Antigen retrieval was performed by boiling slides in citrate buffer. Afterwards, endogenous peroxidase activity was blocked by 3% H2O2 followed by blocking of nonspecific bonding using 5% goat serum (Dako). For vimentin stainings, blocking was performed first with the Avidin/Biotin blocking kit (Vector laboratories) and then for additional 1 hr with the M.O.M. Mouse IgG Blocking Reagent (Vector Laboratories). The primary antibodies used were mouse anti-human vimentin (Dako; 1:100), mouse anti-human VE-cadherin (Santa Cruz Biotechnology, 1:50), mouse anti-human CD31 (Dako, 1:100) and mouse anti-human von Willebrand factor (vWF, Dako, 1:50). Antibodies were applied overnight at 4°C, or in case of anti-vimentin for 30 min at reverse transcriptase, in a humid chamber. After three washing steps with tris-buffered saline, Tween 20 (TBST; 50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 8.2), secondary antibodies were incubated for 45 or 10 min (for detection of vimentin) at room temperature. Secondary antibodies used were biotinylated anti-mouse IgG antibody from M.O.M Elite Peroxidase Kit (Vector laboratories) for the detection of vimentin and HRP-labeled anti-mouse antibody from EnVision+ Kit (Dako) for the detection of CD31, vWF, and VE-cadherin. In case of vimentin stainings, slides were incubated for 5 min with the Vectastain Elite ABC Reagent (Vector laboratories). After additional washing steps with TBST the slides were developed with 3,3'-diaminobenzidine (DAB; Vector Laboratories) or HistoGreen (Linaris). Thereafter, cells were counterstained with hematoxylin.

**FIGURE 1** CAD of 3D construct and macroscopic image of printing result. (a) CAD of cuboid construct with ASCs (brown strands) bioprinted in osteo-hydrogel via extrusion in meandering strands with 90° offset between individual layers. (b) Sliced model to show HUVECs (red dots) bioprinted in fibrinogen via DoD in form of grids (3 × 3 dotted lanes per layer). Thrombin (blue dots) was printed via DoD as crosslinking reagent to convert fibrinogen to fibrin. (c) Cuboid construct (10 × 10 × 5 mm³) bioprinted within a PCL-frame. (d) Bioprinted construct after removal of the PCL-frame. ASC, adipose-derived mesenchymal stem cell; CAD, computer-aided design; DoD, drop-on-demand; HUVEC, human umbilical vein endothelial cell; PCL, polycaprolactone [Color figure can be viewed at wileyonlinelibrary.com]
dehydrated, and finally permanently mounted with Entellan. Negative controls were treated the same way but omitting the primary antibodies and replacing it with 1% goat serum or M.O.M. Diluent Solution (Vector laboratories). For immunofluorescence studies, primary antibodies against human CD31 (Dako; 1:100) and mouse alpha smooth muscle actin (αSMA; cy3-labeled, Sigma-Aldrich; 1:100) were used. For detection of the CD31 antibody, a goat anti-mouse antibody conjugated with Alexa‐Fluor 488 (Invitrogen; 1:100) was used. Images of histological and immunohistological stainings were taken with an Axio Imager M2 microscope (Carl Zeiss Jena) at various magnifications.

2.6 | Quantification of human blood vessels

Quantification of blood vessels was done on paraffin sections stained with an antibody against human vWF. Microscopic images were taken at 200-fold magnification of 10 randomized areas per construct and the number of vWF-positive structures was determined manually. Numbers of vWF-positive structures per ROI were extrapolated to determine the microvascular density of human vessels per cm². Lumen diameters of human blood vessels were measured at the widest points, respectively. Values are given as mean value ± standard deviation from six constructs of the ASC/HUVEC-group (n = 6).

3 | RESULTS

3.1 | Bioprinting of ASCs and HUVECs

ASCs in osteo-hydrogel were printed by extrusion bioprinting, whereas HUVECs were bioprinted in the fibrinogen hydrogel via DoD, as described in Section 2. A combined printing approach was used to produce cuboid structures in the dimensions 10 mm × 10 mm × 5 mm. The computer-aided-design (CAD) model is shown in Figure 1 where ASCs have been printed in form of meandering strands with an offset of 90° between the layers (Figure 1a) and HUVECs in lattice shape with 3 × 3 dotted lines per layer (Figure 1b). The fibrinogen component of both hydrogels has been enzymatically crosslinked by DoD printing of thrombin. The structures were printed in a PCL frame for easier handling after printing and to facilitate transport of the bioprinted construct to the animal core facility, where implantations were performed. The cuboid structure that was
produced by the bioprinter inside the PCL frame is shown in Figure 1c. The construct after removal of the frame is shown in Figure 1d.

3.2 | Confirmation of the printing pattern

The spatial distribution of the cells was investigated in vitro after bioprinting. Analysis of paraffin sections of bioprinted fluorescently labeled ASCs and HUVECs by fluorescence microscopy confirmed the original predetermined print pattern (Figure 2a). The given print pattern of ASCs and HUVECs within the bioprinted construct could also be confirmed by immunohistochemistry by using antibodies against vimentin to detect ASCs (Figure 2b) and antibodies against VE-cadherin to detect HUVECs (Figure 2c).

3.3 | Bone formation in vivo

Next, in vivo experiments were performed to investigate whether the bioprinted ASCs produce bone-like extracellular matrix and whether bioprinted HUVECs are able to form blood vessels upon implantation. For this purpose, bioprinted samples (ASC/HUVEC), as well as negative controls (bioprinted constructs without cells) were implanted subcutaneously into SCID mice and retrieved after 12 days. As shown in Figure 3, ASCs were detectable within the bioprinted constructs by immunohistochemical staining of the paraffin section with a human-specific anti-vimentin antibody. Moreover, alizarin-red staining revealed the extensive production of a calcified extracellular matrix by ASCs, whereas the negative controls only showed unspecific background staining. This indicates that bioprinted ASCs are able to produce calcified bone matrix in vivo in this heterotopic ossification model.

3.4 | Blood vessel formation in vivo

To investigate whether the HUVECs within the bioprinted construct may form blood vessels in vivo, paraffin sections were stained with a human-specific antibody against the endothelial marker CD31. As shown in Figure 4a, no human blood vessels could be detected in the negative control. This result was expected and confirms the species-specificity of the used primary antibody. In contrast, human lumen-containing blood vessels could be detected in the bioprinted constructs containing ASCs and HUVECs. These blood vessels show different calibers ranging from 9.8 to 188.7 µm. Human blood vessels were also detectable within the paraffin sections in form of elongated structures. In this case, human blood vessels were coincidentally captured longitudinal. It could also be observed that some human blood vessels contained mouse erythrocytes in their lumen, indicating that these vessels were perfused by the host blood circulatory system. As indicated by double immunofluorescence studies with antibodies against human CD31 and against mouse αSMA, we could demonstrate that human blood vessels were encased and stabilized by mouse mural cells (Figure 4b). Human microvascular blood vessel densities were quantified based on immunostainings. Vessel densities were divided into three classes based on lumen diameters (≤ 10 µm; 11–40 µm and >40 µm). Quantification of blood vessel densities revealed a clear difference between small-sized vessels (lumen diameter ≤10 µm) and medium to large-sized vessels with a clear

**FIGURE 3** Visualization of ASCs and bone formation after 12 days in vivo. Bioprinted constructs with ASCs and HUVECs (ASC/HUVEC) and negative controls consisting of bioprinted hydrogels without cells (control) were stained with a human-specific antibody against vimentin to visualize ASCs (brown). Alizarin-red staining was performed for visualization of calcified bone matrix (red; scale bars 100 µm). ASC, adipose-derived mesenchymal stem cell; HUVEC, human umbilical vein endothelial cell. [Color figure can be viewed at wileyonlinelibrary.com]
overrepresentation of small-sized blood vessels, representing approximately 97% of all human blood vessels (Figure 4c).

4 | DISCUSSION

We have previously identified specific hydrogels that support various and crucial cell parameters of ASCs and HUVECs such as viability, differentiation, bone matrix formation and endothelial sprouting in vitro (Benning et al., 2017, 2018; Gutzweiler et al., 2017; Trondle et al., 2019; Wehrle et al., 2019). This led to the identification of a complex hydrogel consisting of fibrinogen, glycerol, hyaluronic acid, and gelatin that was described already by Kang et al. (2016). We have blended this hydrogel with hydroxyapatite to improve the mechanical stability of the hydrogel and to support osteogenic differentiation of ASCs and with the angiogenic growth factors VEGF and bFGF to support vasculogenesis.

In this study, we have bioprinted ASCs in the above-mentioned hydrogel (osteo-hydrogel) by extrusion-based printing and HUVECs in fibrinogen via DoD printing, to produce complex prevascularized bone constructs. These constructs were analyzed for bone and blood vessel formation upon subcutaneous implantation of the bioprinted
constructs in immunodeficient SCID mice. In this context, we have seen that ASCs were able to produce a calcified extracellular matrix in vivo, demonstrating that bone formation, mediated by bioprinted ASCs, is possible in a heterotopic ossification model. This further demonstrates that ASCs represent a promising cell source for tissue bioprinting, similarly as it was previously shown by other groups that ASCs support ossification and bone formation in vivo in classical tissue engineering application where ASCs were seeded randomly into scaffolds (Gu et al., 2014; Lin, Lin, Chiang, Chang, & Lee, 2019; Mohiuddin et al., 2019; Wagner et al., 2019). Moreover, since ASCs can be easily obtained by liposuction, they are generally an ideal source for personalized regenerative medicine applications.

MSCs from bone marrow have also been used for extrusion-based bioprinting of bone tissue (Cunniffe et al., 2017) and it was also shown that bioprinted adipose tissue-derived MSCs in a gelatin/alginate hydrogel were able to form bone tissue upon subcutaneous implantation (Wang et al., 2016). In our study, we have used hydroxyapatite to support osteogenic differentiation of ASCs in the complex osteo-hydrogel for extrusion-based bioprinting of ASCs and combined this approach with DoD-based bioprinting of a HUVEC-laden fibrin hydrogel, that has been shown previously to lead to lumen formation by self-organization in vitro, to support vascularization of the constructs.

It is still a matter of debate, whether bone regeneration is mediated by osteogenic differentiation of MSCs leading to osteoblasts that synthesize the bone matrix, or whether implanted MSCs support endogenous bone healing by the secretion of growth and differentiation factors, or by a combination of both mechanisms (Oryan, Kamali, Moshiri, & Baghaban Eslaminejad, 2017). However, the experimental design that was chosen in our study, strongly suggests that bone formation is due to the ability of ASCs to initiate a de novo synthesis of bone matrix. This is demonstrated by the fact that formation of calcified bone matrix was strictly dependent on the presence of ASCs in the implant and because bone matrix was produced at a heterotopic place within the animal where no natural bone is present.

In tissue engineering of bone, vascularization plays a crucial role since the blood supply is necessary for the formation of new bone in the context of endochondral ossification during embryogenesis, as well as during bone repair (Gerber et al., 1999; Peng et al., 2002). Therefore, it is necessary to develop vascularization strategies in bioprinting of living tissues. In this context, several groups have printed blood vessel-like channels with sacrificial hydrogels. After washing out the sacrificial material, these channels could be populated with ECs to generate blood vessel like structures with relatively high calibers (Kolesky, Homan, Skylar-Scott, & Lewis, 2016; Xu et al., 2018). However, this approach is limited to the printing resolution of the sacrificial material and therefore not suitable for producing small-scaled microvessels. Moreover, it requires significantly more technical effort than the approach presented in this study, exploiting the spontaneous self-organization of HUVECs into luminal structures.

In our study, we used high-density HUVEC-fibrinogen hydrogel solutions in combination with the angiogenic growth factors VEGF and bFGF for bioprinting dotted lines by DoD that form an endothelial prevascularization within the constructs before implantation. This approach focuses on self-assembly of HUVECs to generate blood vessels upon implantation. We have previously shown in vitro that this approach leads to the formation of lumenized vessel-like structures (Trondle et al., 2019). Based on these in vitro experiments, we have now tested bioprinted HUVECs for their ability to form functional blood vessels in vivo in a subcutaneous implantation model. After 12 days, we were able to find human lumenized blood vessels within the explants arising from implanted HUVECs. These human blood vessels had different calibers with a main emphasis on small-sized microvessels (lumen diameter ≤ 10 μm). Medium-sized (11-40 μm) and large-sized (lumen diameter > 40 μm) microvessels were less common. The bioengineered human vessels showed lumen diameters similar to native microvessels (Braverman & Keh-Yen, 1981). By using antibodies against αSMA, we could also show that human blood vessels were stabilized by the recruitment of mouse pericytes. This interaction between human microvessels and mouse mural cells has already been shown in HUVEC-seeded fibrin/matrigel implants that have been produced by using a classical tissue engineering approach (Alajati et al., 2008).

In our study, we have printed HUVECs in lattice shape with 3 × 3 dotted lines per layer and this pattern could be verified in vitro directly after printing. Interestingly, this pattern could no longer be found in the explants from the in vivo experiments. Instead, HUVEC-derived blood vessels appeared randomly distributed and heterogeneous concerning their diameters. This observation could be explained by the observed shrinkage of the bioprinted constructs during the in vivo experiment and the observed deformation of the constructs. The deformation is most likely due to the fact that the constructs were fused with the muscle fascia and skin of the mice. The movement of the mice can be expected to exert considerable mechanical force on the constructs, leading to deformation. Moreover, one should take into consideration that we have designed an artificial non-physiological endothelial pattern. It is conceivable that the original HUVEC design was remodeled in vivo by the host organism to build up a more physiological vascularature.

The approach to bioprint a prevascularized construct based on self-assembly of ECs was already published in the context of bioprinting of vascularized skin (Baltazar et al., 2019) and cardiac tissue (Maullari et al., 2018). In our study, we could show that bioprinted HUVECs were able to form blood vessels of different calibers in a combined construct with bioprinted ASCs, which were able to produce bone matrix. To the best of our knowledge, this vascularization strategy has not been published before in the context of bioprinted bone tissue.

5 | CONCLUSIONS

In summary, our experiments revealed that complex artificial bone tissue constructs of two different cell types and hydrogels can be printed with high spatial fidelity using a hybrid bioprinting technique.
After subcutaneous implantation of bioprinted constructs in immunodeficient mice, bone, and blood vessel formation can be detected due to the bioprinted ASCs and HUVECs, respectively. These experiments revealed that bioprinting of prevascularized artificial bone tissue based on human ASCs and HUVECs is feasible and may represent a promising strategy for prospective clinical applications in the field of regenerative medicine and tissue engineering. In particular, the observed self-organization of bioprinted ECs represents an appealing strategy to improve neovascularization, not only in bone, but in artificial tissues in general. In the future, combined bioprinting of soft cell compatible hydrogels with harder materials such as polycaprolactone or calcium phosphate cement should be considered to prevent deformation of the implanted constructs and to produce tissues with similar mechanical strength as natural bone.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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