# Engineering of vascularized adipose constructs

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<td>Wiggenhauser, Paul; Rechts der Isar Medical Centre, Technische Universität München, Department for Plastic and Hand Surgery Müller, D; Rechts der Isar Medical Centre, Technische Universität München, Department for Plastic and Hand Surgery Melchels, Ferry; Queensland University of Technology, Institute of Health and Biomedical Innovation Egaña, J; Rechts der Isar Medical Centre, Technische Universität München, Department for Plastic and Hand Surgery Storck, K; Rechts der Isar Medical Centre, Technische Universität München, Department for Ear-Nose-Throat Mayer, H; Rechts der Isar Medical Centre, Technische Universität München, Department for Ear-Nose-Throat Leuthner, P; Rechts der Isar Medical Centre, Technische Universität München, Department for Ear-Nose-Throat Skodacek, D; Rechts der Isar Medical Centre, Technische Universität München, Department for Ear-Nose-Throat Hopfner, U; Rechts der Isar Medical Centre, Technische Universität München, Department for Plastic and Hand Surgery Machens, H; Rechts der Isar Medical Centre, Technische Universität München, Department for Plastic and Hand Surgery Staufenmaier, R; Rechts der Isar Medical Centre, Technische Universität München, Department for Ear-Nose-Throat Schantz, Jan; Rechts der Isar Medical Centre, Technische Universität München, Department for Plastic and Hand Surgery</td>
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Brisbane/München, 5 May 2011

Subject: Manuscript submission to *Cells and Tissue Research*

Dear Professor Unsicker,

With this manuscript we would like to present the results of our research on *Engineering of vascularized adipose constructs*.

Adipose tissue engineering forms a viable approach for sustainable regeneration and reconstruction of soft tissue defects, such as after mastectomy. In this work we assessed the suitability of two scaffold types for adipose tissue engineering. Prototyped polycaprolactone scaffolds were selected as they present a promising opportunity for automated fabrication of patient-specific scaffolds. Both scaffold types showed abundant adipose tissue formation and neo-vascularization. The relatively high stiffness of the polycaprolactone scaffolds compared to that of the softer polyurethane foams did not impede adipogenesis considerably, however it did result in superior shape stability.

Our methods were chosen for their clinical relevance and applicability: adipose tissue presents an abundant and easily obtainable cell source, and the vessel-loop technique guarantees copious neo-vascularization.

We believe that our research makes for a significant step towards the preparation of functional and viable, well-vascularized adipose constructs.

Yours sincerely,

F.P.W. Melchels
K. Storck, H. Mayer, P. Leuthner, D. Skodacek, and R. Staudenmaier
Fig. 1: Scaffold geometries (A) PCL-scaffold having a regular, interconnected strut architecture (B) SEM photograph showing smooth surface of a strut (C) µCT reconstruction of PCL scaffold (D) PU-scaffold showing a foam structure with randomly formed cavities (E) SEM photograph indicating limited interconnectivity (F) µCT reconstruction showing randomly organized trabecular network.

Scale bars represent 1mm in A, C, D, E, F and 500µm in B

169x102mm (200 x 200 DPI)
Fig. 2: Cell-seeded PCL (top row) and PU (bottom row) scaffolds after 14 days of culture (A) SEM images show continuous cell layers covering the whole scaffold surface (x indicates a cell layer detached by drying of construct) (B) Live/dead staining shows viable cells green and dead cells red (white arrow) (C) shows HE staining of control group with cells growing regularly on scaffolds (x indicates the PU scaffold is not seeded on all edges) (D) Oil Red staining of adipose constructs where orange lipid droplets (white arrow) mark cells differentiated to adipogenic lineage. Scale bars represent 100µm

172x63mm (200 x 200 DPI)
Fig. 3: Quantification of adipogenesis in vitro showing a significantly higher fraction of differentiated hAPCs on PU-scaffolds compared to PCL scaffolds (* indicates significance p<0.05)
Fig. 4: Macroscopic images showing implantation of PCL (A) and PU (C) scaffolds in femoral region (B) PCL showed macroscopic increase of blood vessels after four weeks in vivo whereas (D) PU changed in shape and was encapsulated by abundant fibrous tissue (white arrow). Scale bars represent 1mm.

82x84mm (200 x 200 DPI)
Fig. 5: HE-stained sections of PCL (upper row) and PU (lower row) (A) Small areas of fat tissue (black arrow) were observed after two weeks (B) Large areas showed ring-like structure of mature fat cells (black arrow) after four weeks (C) Fibrin was efficiently degraded on unseeded PCL control scaffolds (D) PU sections show untouched areas of fibrin (black arrow) The scaffolds disintegrated due to the use of solvents in histological processing and are therefore represented as empty spaces in the histological sections. Scale bars represent 100µm 170x66mm (200 x 200 DPI)
Fig. 6: Quantification of adipogenesis in vivo (A) PU scaffolds showed higher relative areas of fat tissue (*) indicates significance p<0.05) (B, D) Brown staining of peroxidase labeled antibodies proving human origin of fat cells after four weeks in vivo (C, E) Corresponding negative controls. Scale bars represent 100µm.
Fig. 7: Quantification of neovascularization (A) No statistical difference in number of blood vessels between scaffold types, time-points or seeded vs unseeded (B) Fibrin matrix on an unseeded PCL scaffold is degraded after four weeks in vivo (C) Seeded PCL scaffold after 4 weeks in vivo with vessels following the surface (+) of the construct and sprouting vertically into its centre (black arrow) (D, E) Unseeded PCL (D) and PU (E) scaffold after four weeks in vivo with blue DAPI-stained cell nuclei found in central (vessel loop marked by white arrow) and peripheral areas of both scaffolds (reactive tissue formation). Anti-SMA shows vascular structures (+) in the center originating from the vascular bundle (white arrow) and stretching towards the periphery of the scaffold. # indicates residual fibrin clots with no cellular invasion and x indicates scaffold. Scalebar represents 100µm

170x81mm (200 x 200 DPI)
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<th>Scaffold</th>
<th>Elastic modulus (kPa)</th>
<th>Porosity (%)</th>
<th>Avg. pore size (mm)</th>
<th>Avg. wall thickness (mm)</th>
<th>Spec. surface area (mm(^{-1}))</th>
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<tr>
<td></td>
<td>gravimetry µCT</td>
<td></td>
<td></td>
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<tr>
<td>PU</td>
<td>13.5 ± 4.6</td>
<td>92.8 ± 1.0</td>
<td>0.56</td>
<td>0.045</td>
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<td>PCL</td>
<td>17.5 ± 3.3 (\times)10(^3)</td>
<td>57.9 ± 0.4</td>
<td>0.42</td>
<td>0.46</td>
<td>3.03</td>
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Table 1: Physical properties of scaffolds determined by compression testing, gravimetry and μCT

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Fig. 27: Quantification of neovascularization (A) No statistical difference in number of blood vessels between scaffold types, time-points or seeded vs unseeded (B) Fibrin matrix on an unseeded PCL scaffold is degraded after four weeks in vivo (C) Seeded PCL scaffold after 4 weeks in vivo with vessels following the surface (+) of the construct and sprouting vertically into its centre (black arrow) (D, E) Unseeded PCL (D) and PU (E) scaffold after four weeks in vivo with blue DAPI-stained cell nuclei found in central (vessel loop marked by white arrow) and peripheral areas of both scaffolds (reactive tissue formation). Anti-SMA shows vascular structures (+) in the center originating from the vascular bundle (white arrow) and stretching towards the periphery of the scaffold. # indicates residual fibrin clots with no cellular invasion and x indicates scaffold. Scalebar represents 100µm.
Engineering of vascularized adipose constructs

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Keywords

Adipose tissue engineering, polycaprolactone, polyurethane, vessel loop, neovascularization

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Abstract

Adipose tissue engineering offers a promising alternative to the current surgical techniques for the treatment of soft tissue defects. It is a challenge to find the appropriate scaffold that not only represents a suitable environment for cells but also allows fabrication of customized tissue constructs, particularly in breast surgery. We investigated two different scaffolds for their potential use in adipose tissue regeneration. Sponge-like polyurethane scaffolds were prepared by mold casting with methylal as foaming agent, whereas polycaprolactone scaffolds with highly regular stacked-fiber architecture were fabricated with fused deposition modeling. Both scaffold types were seeded with human adipose tissue derived precursor cells, cultured and implanted in nude mice using a femoral arteriovenous flow-through vessel loop for neovascularization. In vitro, cells attached to both scaffolds and differentiated into adipocytes. In vivo, neovascularization and adipose tissue formation were observed throughout both constructs after two and four weeks, with neovascularization being comparable in seeded and unseeded constructs. Fibrous tissue formation and adipogenesis were more pronounced on polyurethane foam scaffolds than on polycaprolactone prototyped scaffolds. In conclusion, both scaffold designs can be used for adipose tissue engineering effectively.
Introduction

Plastic surgeons frequently use flap techniques to cover soft tissue defects with autologous tissue. Autologous flaps are based on skin, fat and muscle (Cordeiro 2008; Kanchwala et al. 2009). The use of free non-vascularized fat grafts is limited by shrinkage, fibrosis and oil cyst formation (Fagrell et al. 1996; Kononas et al. 1993; Nguyen et al. 1990; Smahel 1989). Vascularized fat grafts on the contrary show good results, such as the deep inferior epigastric perforator flap, which is a skin and fat graft that is removed from the lower abdomen and transferred to the chest to reconstruct a breast after mastectomy without the sacrifice of any of the abdominal muscles. The use of such flaps is limited by long operation times, risk of thrombotic events and prediction of transplanted flap shape (Cordeiro 2008; Tachi et al. 2005). Moreover, the harvesting of a flap creates a donor site defect with the risk of infection, instability and wound healing disorders (Hijjawi et al. 2010; Rozen et al. 2009).

Methods have been investigated to avoid donor site morbidity. Historically, techniques of tissue expansion and flap prefabrication were used to improve vascularization and to obtain more tissue that could be used to cover a defect (Exner 2003; Morrison et al. 1997). Also, chamber models creating an artificial space that is to be naturally filled with fat tissue in vivo have been investigated (Dolderer et al. 2007; Hofer et al. 2003; Morrison, Penington 1997). The latest surgical approach is called lipofilling: the injection of autologous fat obtained from non-traumatic liposuctions to increase the quantity of subcutaneous fat of the face (Coleman 1997), which has improved volume stability when enriched preadipocytes are additionally injected (Yoshimura et al. 2008).

Beside all surgical procedures, adipose tissue engineering is a promising approach for soft tissue regeneration. Using a poly(lactide-co-glycolide) scaffold and preadipocytes, adipose tissue was generated in vivo for the first time in 1999 (Patrick et al. 1999). Since that time researchers have mainly focused on three aspects of adipose tissue engineering: scaffolds for volume maintenance
(Electronic Supplementary Information, Table S1), matrices for supporting microenvironment, and neovascularization strategies (Tanzi et al. 2009). Moreover, different precultivation and seeding strategies have been studied (Flynn et al. 2000; Weiser et al. 2008).

The use of human adipose tissue derived precursor cells (hAPCs) is preferred to the use of mature fat cells. Mature fat cells have inferior viability, low expandability and inferior volume stability (Tanzi and Fare 2009). Experimentally, hAPCs are more resistant to stress and hypoxia (Nguyen, Pasyk 1990). The easy harvesting via liposuctions and the ability of proliferation and adipogenic differentiation predestines hAPCs as the ideal cell source of adipose tissue engineering (Schreml et al. 2009; Van et al. 1977; Van et al. 1982).

In this study, we compared two different scaffold types for adipose tissue engineering, which differ in physiochemical properties as well as pore architecture. One scaffold type is prepared from soft polyurethane (PU) by mold casting with methylal as foaming agent, resulting in a tortuous network of interconnected spherical pores. It has adjustable porosity, pore size and biodegradability (Eyrich et al. 2007; Wiese et al. 2005), and can accumulate precursor cells in its cavities. This expectedly leads to a high adipogenic potential (von Heimburg et al. 2001).

The other scaffold type is Osteomesh; a polycaprolactone (PCL) scaffold with highly regular stacked-fiber architecture prepared by fused deposition modeling (FDM). FDM is an additive manufacturing method (also referred to as rapid prototyping or solid freeform fabrication) that allows for the fabrication of biodegradable porous structures of virtually any design, potentially obtained from medical imaging techniques (Melchels et al. 2011). Osteomesh is FDA approved, intensively tested with mesenchymal precursor cells and has a fully interconnected network of large pores, which facilitates cell invasion and blood vessel ingrowth (Huang et al. 2004; von Heimburg et al. 2001; von Heimburg, Zachariah 2001). We hypothesize that these two scaffold designs follow a different path of adipose tissue engineering; invasion for the open PCL structure vs. accumulation for the tortuous PU foam (Tanzi and Fare 2009).
To create a viable adipose construct, sufficient oxygenation and nourishment is needed. Vascularization is highly developed in native adipose tissue. There are multiple experimental approaches to rebuild the native vascularization of tissue: vessel loop methods, precursor cell induction, templates of acellular blood vessels and artificial vessels (Borges et al. 2003; Carraro et al. 2008; Lokmic et al. 2007; Macchiarini et al. 2004; Mertsching et al. 2005; Tanaka et al. 2003). Some in vivo methods are based on hypoxia-mediated neovascularization (Kotch et al. 1999; Morrison, Penington 1997; Tanaka, Sung 2003). The vessel loop model is favored for this study because it has been intensively tested and immediately feasible. Moreover, plastic surgeons are familiar with the surgery of small vessels and this method can thus be transferred to clinical application. According to Tanaka, a flow-through vessel loop provides the best vascularization among vessel loops (Tanaka, Sung 2003).

This study evaluated the potential for adipose tissue engineering for soft, sponge-like PU scaffolds and more rigid prototyped PCL scaffolds. The study was performed in vitro and in vivo focusing on adipose tissue formation and neovascularization.

Methods and Materials

Scaffolds

Polyurethane (PU) foam was obtained en bloc from Polymaterials (Polymaterials, Kaufbeuren, Germany). It is a highly crosslinked, completely aliphatic polyetheresterurethane mainly based on PCL with 5% of polyethylene glycol for increased hydrophilicity. Similar to PCL, it degrades slowly by hydrolysis (about 2 years to collapse of porous structures) (Eyrich, Wiese 2007; Wiese and Maier 2005). Rapid prototyped porous polycaprolactone (PCL) mesh was purchased from Osteopore (Singapore). osteomesh® is a sheet (50 mm x 40 mm x 1 mm) of 4 layers of PCL strands deposited by FDM and was received sterile. Blocks of approximately 8x8x15 mm were cut out of the PU sponge material and slabs of 8x8x1 mm were cut out of the PCL sheets for
characterization by μCT and mechanical testing. First, the specimens were scanned on a Scanco μCT40 at 8 μm resolution, employing 55 kV and 145 μA with 250 ms exposure time. Afterwards, the specimens were subjected to compression testing on an Instron 5848 MicroTester at a nominal strain rate of 30 %/min.

For cell culture and in vivo experiments, disk-shaped scaffolds of 6 mm diameter and 1 mm height were prepared as follows. PCL scaffolds were cut out of the sheet with a sterile biopsy punch (6 mm Acupunch, Xiomedics, Unterhaching, Germany) in the biosafety cabinet. PU foam was frozen in liquid nitrogen and cut into 1 mm thick plates from which disk-shaped scaffolds were punched in a similar way as the PCL scaffolds. The PU scaffolds received were autoclaved in PBS (H15-002, PAA Laboratories, Pasching, Austria) at 120°C.

**Isolation and culture of human adipose tissue derived precursor cells**

All human adipose tissue derived precursor cells (hAPCs) were obtained from liposuction aspirates. All tumescent liposuctions were performed with the patients’ informed consent and with approval from the local ethics committee at the Department of Plastic Surgery at “Rechts der Isar Medical Center” (Klein 1990). Isolation was performed according to Schantz and Ng. (Schantz et al. 2004) In short, tissue was digested with 0.075% Collagenase A (10103586001, Roche Diagnostics, Penzberg, Germany) for 30 min. Cells were cultured with Dulbecco’s modified eagle medium (E15-883), 10% fetal calf serum (A15-152), 1% antibiotic/antimycotic drugs (P11-002) all purchased from PAA Laboratories (Pasching, Austria). Adipogenic differentiation medium (PT8002) was obtained from Lonza (Lonza Group, Walkersville, MD, USA). All cells were used in passage 1.

**Cell seeding and culturing in the scaffold**

All scaffolds were seeded with hAPCs within a fibrin matrix to improve cell attachment. The fibrin contained aprotinin to stabilize the fibrin. Fibrinogen (F8630) and aprotinin (A1153) were purchased from Sigma-Aldrich (Steinheim, Germany). Thrombin (Tissucol Duo S Immuno
from Baxter, Vienna, Austria) was used to coagulate the fibrin. The concentration of aprotinin was 10000 KIU per ml of PBS and the concentration of fibrinogen was 100 mg per ml aprotinin solution. Thrombin was buffered in CaCl$_2$ at 5 IU/ml. After trypsinization, hAPCs were diluted in the thrombin solution at 50·10$^6$ cells/ml. Then, 20 µl of fibrinogen solution was mixed with 20 µl thrombin-cell suspension (1·10$^6$ cells) and seeded on each scaffold. Cell-seeded scaffolds were cultured at 37 °C and 5% CO$_2$. On day 1 adipogenic differentiation was induced and differentiation medium (containing insulin, dexamethasone, IBMX and indomethacin) was replaced every third day.

**Characterization of cell-seeded scaffolds**

On day 14 of culture, two PCL scaffolds and two PU scaffolds were rinsed with PBS and fixed with 4% glutaraldehyde, followed by dehydration through an ethanol gradient and CO$_2$ critical point drying. Empty and cell-seeded scaffolds were fastened on double-sided carbon tape and sputtered with gold-palladium. Images were obtained by a Hitachi 3500-N SEM (Hitachi High-Technologies Europe GmbH, Krefeld, Germany) at 10kV. Two other scaffolds of each type were washed and stained with Live/Dead staining according to the manufacturer’s instructions (L-3224, Invitrogen, Carlsbad, CA, USA), and were analyzed by confocal laser scanning microscopy (CLSM 510 Meta, Carl Zeiss, Oberkochen, Germany).

**Quantification of adipogenesis in vitro**

To prepare lipophilic Oil Red staining for assessing adipogenesis, Oil Red-powder (O0625, Sigma-Aldrich, Steinheim, Germany) was dissolved in 99% isopropanol (3 mg/ml) and diluted 3:2 in deionized water. On day 17 of culture, eight cell-seeded constructs (4 of each type) were washed with PBS, fixed with neutral buffered 3.7% formalin overnight and stained for 1 hour with the Oil Red solution. Scaffolds were rinsed twice with tap water, counter-stained with hematoxylin according to Mayer (T865, Carl Roth, Karlsruhe, Germany) for 15 min, again rinsed with tap water twice and stored at 4 °C overnight. The stained constructs were visualized with a
transillumination microscope (Eclipse TE2000S, Nikon, Tokyo, Japan). The differentiation percentage was defined as the ratio of differentiated cells (indicated by bright orange stained lipid droplets) to the total number of cells (indicated by the cell nuclei stained dark blue by hematoxylin). The presented data were averaged over two independent and randomly chosen microphotographs from each of two constructs, which was repeated for three donors to obtain statistically relevant results (in total 12 images per condition). Two PCL and two PU constructs were cell-seeded and cultured in non-adipogenic medium to serve as negative controls.

Assessment of adipogenesis in vivo

Twelve female nude mice (NU/NU, Charles River, Wilmington, MA, USA) of 6 to 7 weeks old were used with permission of the Bavarian ethics committee. After sedation (Midazolam, Medetomidin and Fentanyl), a 10 mm long incision was made in the skin of the femoral region. Tissue was dissected and femoral artery and veins mobilized. The scaffolds were placed between the inguinal ligament and hiatus adductoris. The femoral superficial artery and veins were placed upon the scaffold, keeping the perfusion of the distal leg intact. This method is comparable to a flow-through vessel loop (Tanaka, Sung 2003). Next, a 6 mm broad silicone rubber strip (FA Gyrus medical GmbH, Tuttlingen, Germany) was wrapped around the vessels and the scaffold to separate them from surrounding tissue. The incision was closed with interrupted 6-0 prolene sutures (EH7406H, Johnson & Johnson, New Jersey, USA) and the mice recovered quickly.

PU and PCL scaffolds (6 each) were cell-seeded and cultured in vitro (as above) for 12 days, implanted for 2 weeks or 4 weeks in left legs, with non-seeded controls (only fibrin) in the contralateral side. After explanation, constructs were fixed with 3.7% formalin, dehydrated and coated with paraffin wax using a tissue processor (ASP200S, Leica, Wetzlar, Germany). Constructs were vertically sliced to 7 µm, deparaffinized manually (Roti-Histol 6640, Carl Roth, Karlsruhe, Germany), rehydrated with a decreasing series of ethanol and stained with eosin
(X883) for 15 min and hematoxylin (vide supra). Slides were imaged by transillumination microscopy and the relative area of adipose tissue was determined using ImageJ imaging software (National Institute of Health, USA). Furthermore, all blood vessels that showed red erythrocytes within the lumen were counted. Values are based on 4 microphotographs from each of 3 mice per experimental condition.

MHC-I staining was performed on seeded and unseeded scaffolds. Slides were submerged in 10mM TRIS/1mM EDTA (pH=9) at 100 °C for 10 min using a household microwave (R-239IN-A, Sharp Electronics Europe, Hamburg, Germany). Slides cooled down at room temperature for 20 min and were rinsed with PBS. Endogen peroxidase was blocked with 3 % H2O2 for 5 min. Slides were rinsed with PBS and blocked with 10 % BSA (T8442, Carl Roth, Karlsruhe, Germany) at room temperature for 1 h. First, antibody mouse-anti-human-MHC-I (ma1-19151, Dianova, Hamburg, Germany) was added in a 1 % BSA solution and slides were incubated at 4 °C overnight. Slides were rinsed with PBS and second peroxidase-labeled antibody (115-035-003, Jackson Immuno Research Laboratories West Grove, PA, USA) was added in a 1 % BSA solution and incubated at 37 °C for 1 h. Slides were rinsed with PBS and incubated with DAB solution (D3939, Sigma-Aldrich, Steinheim, Germany) for 5 min. Afterwards, slides were rinsed with PBS, counterstained with hematoxylin for 30 s, rinsed with tap water, mounted and visualized with the microscope.

Anti-smooth muscle actin (anti-SMA) staining was performed on unseeded scaffolds. Antigen demasking was done with TRIS buffer and a household microwave analogously to MHC-I staining. Membranes were permeabilized using 0.1 % Triton-100 for 5 min and blocked with BSA as described above. First, antibody mouse-anti-smooth-muscle-antigen (ma1-06110, Thermo Scientific Rockford, IL, USA) was added in a 1 % BSA solution and slides were incubated at 4 °C over night. Slides were rinsed with PBS and second fluorescent goat-anti-mouse-antibody (Alexa Fluor 568, Invitrogen, Carlsbad, CA, USA) was added in a 1 % BSA solution and incubated
at 37 °C for 1h. Slides were rinsed with PBS and counterstained using DAPI Prolong Gold solution (P36931, Invitrogen, Carlsbad, CA, USA), mounted and visualized.

**Statistics**

All statistical analyses are Anova-one-way tests performed with SigmaPlot 10 (Systat Software, San Jose, California), using a significance threshold of p=0.05.

**Results**

**Characterization of scaffolds**

The scaffolds were subjected to compression testing, gravimetric analysis and µCT, of which quantitative outcomes are presented in Error! Reference source not found. The PU and PCL scaffolds differ in surface chemistry, pore architecture and mechanical properties. The PCL scaffolds are three orders of magnitude stiffer than the PU scaffolds, as a combined result of stiffer bulk material, lower porosity and higher degree of interconnectivity of the struts. The lower porosity of the PCL scaffolds also implies less volume available for tissue ingrowth and tissue formation. The average pore size is in the same range for both scaffold types, and both are far above the minimum pore size of 120 µm was postulated to be favorable for adipose tissue engineering (von Heimburg, Zachariah 2001). However, the pore structure of the PCL scaffolds is characterized by higher interconnectivity in the form of open channels, whereas the PU foam is more tortuous with small and randomly distributed open windows between the pores Fig. 1.

**Assessment of in vitro adipogenesis**

To evaluate the interaction of hAPCs and scaffolds, cell attachment, distribution and viability were studied. Constructs were visualized by SEM 14 days after seeding. Cells were growing in continuous layers on both PCL and PU scaffolds, covering the complete surface and growing into the scaffold pores as well (Fig. 2). Confocal laser scanning microscopy (CLSM) imaging with
Live/dead staining revealed layers of viable cell on PCL scaffold. On the PU some dead cells can be discerned amongst predominantly viable cells. HE staining showed blue cell nuclei regularly distributed on scaffolds of both types, with uninterrupted cell layers on the PCL scaffolds. The PU scaffolds also exhibited continuous cell layers, although hAPCs did not grow on all edges of the scaffold (Fig. 2 C and D). The cocktail of differentiation growth factors used in the culture medium for 17 days led to the formation of lipid droplets large enough to visualize using a transillumination microscope (Fig. 2D). Constructs in control medium showed no lipid droplet formation. The percentage of differentiated cells (i.e. cells with visible lipid droplets) was calculated and is presented in Fig. 3. On both scaffolds the prevalence of differentiated cells was high, with a significantly higher degree of differentiation on the PU scaffolds compared to the PCL scaffolds.

**Assessment of in vivo adipogenesis**

All mice tolerated the constructs well and did not develop limitations in leg movement or significant foreign body reactions. Fig. 4 shows photographs taken of implantation and explantation after four weeks. Both constructs changed from a transparent appearance (of the pore space) to an overall opaque appearance, due to a smooth fibrous capsule surrounding the construct. The fibrous tissue formation was much more pronounced on PU scaffolds than the PCL scaffolds, with large fibrous invasion along the vascular pedicle of the vessel loop (indicated by the arrow in Fig. 4D). Furthermore, the wrapping of the silicone rubber strip and the fibrous encapsulation changed the shape of the PU construct from straight to slightly bent. Both seeded scaffolds showed an increase in blood vessels. Fig. 5 shows representative images of seeded and unseeded PCL and PU constructs after two and four weeks in vivo. After four weeks, sections showed the typical ring-like morphology of fat tissue. Multiple areas of coherent adipose tissue were found on both PCL and PU scaffolds.
Non-seeded control constructs showed formation of fibrous tissue and blood vessels, with fibrin resorption proceeding increasingly from week two to four. Both PCL and PU constructs showed formation of fibrous tissue around the constructs. However, cell invasion into the fibrin matrix and blood vessel formation was more homogenous in PCL constructs, as PU scaffolds showed areas of untouched fibrin (Fig. 5 arrow) after both two and four weeks. Fat cells could be identified in micrographs by their typical ring-like morphology and the empty fat vacuole in the middle of the cell, enabling quantification of the area of fat tissue using image analysis software. The relative area of adipose tissue increased from week two to week four. PU constructs had larger relative areas of adipose tissue than PCL constructs, with a significant difference after two weeks but not after four weeks (Fig. 6A). To prove the human origin of the formed fat tissue, representative slides were stained with anti-human-MHC1 antibodies. This allowed the identification of human cells within constructs. Seeded constructs showed positive detection of human cells within the fibrin matrix, whereas controls showed no brown color of peroxidase reaction (Fig. 6B-E). To quantify neovascularization, blood vessels were counted on all slides. Blood vessels were identified by a ring or tubular structure, with only structures lined with red blood cells were regarded as functional blood vessels. All constructs showed substantial ingression of neovasculature, independent of PCL or PU scaffold material (Fig. 7). Unseeded controls showed even slightly higher neovascularization than cell-seeded constructs, but not statistically significant. Sections of constructs showed many blood vessels on and parallel to the surface of the construct. From these surface-bound vessels, new capillaries sprouted that penetrated into the scaffolds.
Discussion

Patrick et al. were the first to study adipose tissue engineering in vivo (Patrick, Chauvin 1999). Since that time researchers have followed different approaches to tissue engineering fat, using different types of cells and carrier materials. Pre-adipocytes have proven a good cell source; the injection of pre-adipocytes suspended in fibrin glue, led to long term stable adipose tissue (Torio-Padron et al. 2007). However, scaffolds are required for maintaining volume and facilitating cell invasion and ingrowth of vasculature. In this study, we seeded adipose precursor cells incorporated in a fibrin matrix in two different scaffold types; PU foams vs. PCL prototyped scaffolds. PCL and PU differ in mechanical properties, degradation profile, and possibly in cellular interaction and in vivo behavior.

PCL has been used with murine embryonic precursor cells to engineer fat tissue (Kang et al. 2007), but to the best of our knowledge, this is the first study that evaluates the potential of PCL scaffolds with human adipose tissue derived precursor cells for adipose tissue engineering. PCL is a relatively stiff and slowly degradable polymer that can be easily processed into scaffolds, has been tested intensively and is approved in the form of Osteomesh by the FDA for tissue engineering (Woodruff et al. 2010). Despite its proven suitability for the engineering of rigid tissues, this study shows good results for adipose tissue engineering. The material invoked only a mild inflammatory response with intermediate fibrous tissue formation, which resulted in strong neovascularization. This was backed up by our histological findings, which suggest the ingrowth of blood vessels inside the fibrous capsule around the construct followed by sprouting into the scaffolds. Inflammatory processes are known to invoke blood vessel formation (Campa et al. 2010; Carmi et al. 2009; Presta et al. 2009; Sato et al. 2010). The differentiation of precursor cells depends on forces and substrate stiffness (Engler et al. 2006; McBride et al. 2008; Tanzi and Fare 2009), with stiff materials such as PCL being more favorable for regenerating rigid tissues such as bone (Gleeson et al. 2010). We observed different rates of
adipose tissue formation in vivo: 5±0.4% for PCL vs. 9±2.5% for PU constructs. Apart from seeding and cell accumulation effects, material stiffness is the most likely reason of this observation. Another advantage of the PCL material is shape stability; the low stiffness of the PU material also led to those scaffolds being easily deformed, even wrapping the silicon strip too tightly around the construct. Furthermore, PU scaffolds induced more fibrous tissue formation, without additional neovascularization as compared to the PCL scaffolds. Eventually, the ideal scaffold material for adipose tissue engineering could have a stiffness which is intermediate to PCL and PU, or ideal scaffolds may be composites of stiffer materials for shape-stability and more flexible materials as cell substrate. PCL could be chemically modified to decrease its stiffness and degradation time, making it more suitable for adipose tissue engineering. PCL takes about two years to degrade (Lam et al. 2009), whereas six months would be sufficient time for fat tissue to develop fully (Weiser, Prantl 2008). Ideally the modified PCL would still be processable with FDM, allowing the preparation of individually customized, anatomically correct scaffolds.

Scaffold geometry (the pore architecture in particular) had a large impact on cell seeding, adipogenic differentiation, and fat tissue formation. PCL scaffolds were prepared by FDM, thus being homogenously organized with precise bars and struts, with highly interconnected porous channels. The PU foam on the contrary showed a more random network of spherical pores, formed by the expanding gas during fabrication. The interconnections between the cavities are smaller in size, making it a more tortuous pore network. The wide interconnected pores of the PCL scaffolds caused many of the cells to sink through during the seeding process, with only few cells attaching to the scaffold and growing on top of the fibrin matrix. In the PU foams, hAPCs were seeded with lower loss, and accumulated particularly on the lowest points of the cavities. This created aggregates with high local densities of cells, which stimulates adipogenic differentiation as compared to the monolayer of cells growing on the struts of the FDM scaffold (von Heimburg, Zachariah 2001). The increased differentiation rate that was observed in PU
foams after *in vitro* culture (34% for PCL vs. 42% for PU) as a result of this, is also the most likely cause for the higher amounts of fat formed *in vivo* (Weiser, Prantl 2008). Differentiated fat cells with single lipid droplets as seen at day 17 of *in vitro* culture start to enlarge and accumulate fat *in vivo*, forming increasingly large vacuoles of fat after two and four weeks. Thus, a scaffold that traps aggregates of cells in its pores is favored over a very open mesh for cell seeding as well as differentiation and fat accumulation.

Neovascularization plays an important role in adipogenesis. Blood vessel formation was investigated on seeded and unseeded constructs. Unexpectedly, there was no difference between PCL and PU scaffolds, or between seeded and unseeded scaffolds. A higher rate of blood vessel formation was expected in seeded constructs due to hypoxia-induced secretion of vascular endothelial growth factor by the seeded cells (Dolderer, Abberton 2007; Hofer, Knight 2003; Kotch, Iyer 1999; Pugh et al. 2003; Spanholtz et al. 2010; Tanaka, Sung 2003). Moreover, it was expected that highly interconnected PCL scaffolds would better facilitate blood vessel ingrowth than tortuous PU foams, but no difference was observed here either. It appears that the light foreign body inflammatory reaction, in combination with the vessel loop technique, induces strong vascularization of either type of constructs, with or without pre-seeded cells.

In summary, PU foam scaffolds and prototyped PCL scaffolds are both good candidates for adipose tissue engineering. The tortuous pore network with limited interconnectivity in the PU scaffolds facilitates cell seeding and improves *in vitro* adipogenic differentiation by accumulating hAPCs in pores, also leading to increased fat accumulation *in vivo*. Unexpectedly, the tortuous pore network does not impede vascularization or compromise cell viability when used in combination with a vessel loop, at the small scale we employed here. The stiff PCL scaffolds were superior in preventing shape-stability, and induced less fibrous capsule formation. Also, they showed more consistent outcomes, as the variability from sample to sample is lower. Neither of the two scaffold types is ideal for adipose tissue engineering, but lessons have been learned to
create optimized scaffolds in the future. Furthermore, the clinical application will also decide on the strategy to be followed. For example, PU scaffolds could be used to fill holes or subcutaneous skin defects after de novo creation of fat tissue. Contrarily, PCL scaffolds allow long term stability in shape and could therefore be used for the reconstruction of more demanding defects with respect to shape, e.g. breast reconstruction.

**Conclusions**

Both PU foam scaffolds and prototyped PCL scaffolds can be used for adipose tissue engineering. The tortuous network of cavity-pores of the PU foam facilitates cell seeding and stimulates adipogenesis, without compromising cell viability. The high stiffness of PCL compared to PU does not lead to much lower differentiation and fat formation rates, but does ensure shape-stability and in addition shows better tissue tolerance. Using the vessel-loop technique, scaffolds were readily invaded by newly formed blood vessels, independent of the presence of cells or the material or architecture of the scaffold.

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