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Engineered Composite Tissue as a Bioartificial Limb Graft

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Abstract

The loss of an extremity is a disastrous injury with tremendous impact on a patient's life. Current mechanical prostheses are technically highly sophisticated, but only partially replace physiologic function and aesthetic appearance. As a biologic alternative, approximately 70 patients have undergone allogeneic hand transplantation to date worldwide. While outcomes are favorable, risks and side effects of transplantation and long-term immunosuppression pose a significant ethical dilemma. An autologous, bio-artificial graft based on native extracellular matrix and patient derived cells could be produced on demand and would not require immunosuppression after transplantation. To create such a graft, we decellularized rat and primate forearms by detergent perfusion and yielded acellular scaffolds with preserved composite architecture. We then repopulated muscle and vasculature with cells of appropriate phenotypes, and matured the composite tissue in a perfusion bioreactor under electrical stimulation *in vitro*. After confirmation of composite tissue formation, we transplanted the resulting bio-composite grafts to confirm perfusion *in vivo*.

Keywords

Bioprosthesis; Mechanical Properties; Muscle; Bone graft

Introduction

In the United States, over 1.5 million people live with limb loss (1). Amputation is a severe socioeconomic challenge for most patients, causing emotional trauma equivalent to the loss of a family member (2–4). Therapeutic options after limb loss include reconstructive surgery using autologous tissue, or the use of prosthetic devices ranging from purely aesthetic prostheses to those with a focus on function (5). Although current prostheses are technically highly sophisticated devices, they only fulfill a minimum of physiologic function and many

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offer less than satisfactory aesthetics (5). The vast majority of patients consider the option of prosthesis, but amputees who suffer from large defects such as bilateral above elbow amputations adapt poorly and are usually dependent on others for personal care and hygiene (6). As a new approach, worldwide about 70 patients have received allogeneic hand transplants since 1998(7). Hand transplantation significantly improved the quality of life of upper limb amputees and eventually demonstrated hand function superior to that obtained with prosthetics (6, 8, 9). However, side effects and potentially life-threatening complications of long-term immunosuppression pose a significant ethical dilemma regarding this non life-saving reconstructive procedure (5, 9–11). A reduction of donor related risk factors, and elimination of long term immunosuppression would allow wider application of such reconstructive treatment options (6). Creation of an autologous, bioartificial forearm graft from patient derived cells would therefore be a valid alternative to allogeneic grafts. Cellular candidates to regenerate the required tissues such as muscle progenitor cells, endothelial progenitor cells, and mesenchymal stem cells can be isolated from patients (12–14). However, engineering of a composite tissue graft of the complexity of a hand or a forearm has been impossible to date due to the lack of appropriate scaffold materials to support the engraftment of several cell phenotypes and the formation of viable and functional tissue in its physiologic three dimensional context. A recent report of successful clinical implantation of acellular biological scaffolds into patients suffering from volumetric muscle loss underlines the huge potential of this principle for reconstructive surgery (15).

Using perfusion decellularization, we have shown that complex cadaveric organs can be rendered acellular, resulting in native extracellular matrix (ECM) scaffolds with intact tissue architecture that can be repopulated with cells to engineer functional tissue (16, 17). To investigate if these methods can be applied to complex composite tissues such as limb grafts, we isolated rodent and primate upper limbs, and perfused these with a sequence of detergent and washing solutions via the native vascular system. Perfusion decellularization led to the removal of cellular material in all respective tissue compartments, while retaining the mechanical properties of the musculoskeletal system. Repopulation of acellular composite tissue grafts with muscle progenitor, endothelial and mesenchymal cells resulted in formation of vascularized, muscle-like tissue within its native histological compartment. To enhance the formation of functional muscle-like tissue, we cultivated repopulated limb grafts in a biomimetic bioreactor system, including vascular perfusion and electrical stimulation. Finally, we tested functionality of engineered muscle in terms of isometric force measurement and patency of the vascular system by orthotopic limb transplantation.

Materials and Methods

Perfusion Decellularization

Research animals were cared for in accordance with the guidelines set by the Committee on Laboratory Resources, US National Institutes of Health, and Subcommittee on Research Animal Care and Laboratory Animal Resources of Massachusetts General Hospital. Male Sprague Dawley rats (Charles River Laboratories) were euthanized with 100 mg/kg ketamine (Phoenix Pharmaceutical) and 10 mg/kg xylazine (Phoenix Pharmaceutical) injected intraperitoneally. After systemic heparinization (American Pharmaceutical Partners)

through the IVC, the dissection of the skin of the whole upper limb allowed us to identify the brachial artery, the brachial vein and the nerve plexus. After dissecting the upper limb from the shoulder the brachial artery was cannulated with a prefilled 25G cannula (Luer Stubs, Harvard/Insteck) using a surgical microscope. Fasciotomies were performed before flushing the forearm with phosphate buffered saline (PBS). After flushing with 5ml PBS the isolated forearm was mounted into the organ chamber and perfusion was started with 1% SDS (Sigma) for up to 50h at a constant flow perfusion of 1 ml/min. This was followed by deionized water for 1h and 1h of perfusion with 1% Triton-X100 (Sigma). To wash out all debris, antibiotic-containing PBS (100 U/ml penicillin-G; Sigma, 0,25mg/ml streptomycin; Sigma and amphotericin B; Sigma) was used to perfuse the forearm for 124 h.

Recellularization of decellularized forearms

After washing with PBS for 124h, decellularized rat forearms were removed from the decellularization chamber and mounted in a biomimetic stimulation bioreactor system under sterile conditions. Prior to cell seeding, we perfused forearm matrixes with 37 °C oxygenated C2C12 growth medium for at least 1h at constant flow perfusion of 1 ml/min under standard culture conditions (37°C in 5% CO₂). The biomimetic stimulation bioreactor contains an organ chamber, which also serves as the main reservoir, in which the decellularized forearm is mounted. The bioreactor works as a closed-circuit system in which medium is perfused into the brachial artery by a constant flow pump (Ismatec). At day 0 we seeded the forearm matrix with 5×10^6 HUVECs by gravity infusion (100cm H₂O) into the brachial artery suspended in 75–100 ml Endothelial Cell Growth media (EGM-2 Bulletkit; Lonza). After a 60-minute static period to allow for cell attachment, we restarted perfusion. For regeneration of muscle tissue we injected a cell mixture of 10×10^6 C2C12 cells, 0.5×10^6 mouse embryonic fibroblasts and $X \times 10^6$ HUVECs suspended in 1 ml of growth medium through twenty injections in the compartments of the decellularized forearm with a 27-G needle and a 1-cc tuberculin syringe. After cell seeding we mounted the forearm in the biomimetic stimulation bioreactor and sutured sterile stimulation electrodes (Warner Instruments) to the wrist and to the elbow of the forearm. We applied no electrical stimulation in the first 5 days. Mechanical stimulation was provided by attaching the wrist of the forearm to a leverage, which was actuated by a hydraulic cylinder. Rate and extent of stretch could be adjusted via adjustment of the injection and withdraw volume and infusion speed of the syringe pump. We perfused the forearm with C2C12 growth media from day 0 to day 3. We changed the medium every 48 h. On day 3, we switched the medium to C2C12 differentiation medium supplemented with 3% horse serum and EGM2 bulletkit (Lonza). At day 6 we started electrical and mechanical stimulation by applying 6-ms pulses of 20 V and 1 Hz with a Grass S48 square pulse stimulator (Grass Technologies). Skin transplantation was performed as follows. Full thickness skin grafts were harvested from the upper extremities of deceased Sprague Dawley rats. After removal of access tissue, full thickness skin grafts were meshed to allow for better attachment and perfusion. After washing in PBS, full thickness mesh grafts were transplanted onto the engineered constructs using sutures and fibrin glue (Tisseel, Baxter). We maintained the matrix in culture for up to 21 days.

Cell Culture

Myoblasts—Mouse skeletal myoblasts (C2C12) were purchased from ATCC and expanded in DMEM (Gibco) supplemented with 10% FBS, and 1% HyClone Antibiotic Antimycotic solution (Thermo Scientific) on gelatin coated cell-culture plastic (BD Biosciences). Cells were cultured under standard culture conditions (37°C in 5% CO₂). Medium changed every other day. To avoid myotube formation, cells were passaged with 0.05% trypsin/EDTA (cellgro-25052CI) at 70 – 80% confluency. For C2C12 differentiation, we supplemented DMEM (Gibco) with 2% horse serum (Gibco) and 1% antibiotic antimycotic solution (10,000 units/ml penicillin, 10,000µg/ml streptomycin, and 25µg/ml Amphotericin B; HyClone). C2C12 were expanded until passage 6 – 10.

MEFs—Mouse embryonic fibroblasts were purchased from ATCC and cultured in DMEM, (Gibco) supplemented with 10% FBS and 1% Pen/Strep, (Sigma) until passage 4 – 6 on gelatin coated cell-culture plastic (BD Biosciences)

HUVECs—Primary human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and expanded in EBM2 endothelial cell media (Lonza) supplemented with EGM-2 bulletkit until passage 6 – 10 on gelatin coated cell-culture plastic (BD Biosciences).

Electrical Stimulation Bioreactor—We designed and custom built an electrical stimulation bioreactor based on our perfusion bioreactors. Decellularized forearms were mounted into the bioreactor by clamping the humeral head into a tissue clamp (Mueller, Germany), stabilizing the whole limb. Carbon rods were submerged into the culture medium for electrical field stimulation of the engineered graft. Electrical pulses were generated with a square pulse stimulator (Grass S48, Grass Technologies).

Microtomography—A high-resolution desktop micro-tomographic imaging system (µCT40, Scanco Medical AG, Bruttisellen, Switzerland) was used to assess cortical bone morphology and mineral density at the midshaft of the ulna and radius. Scans were acquired using a 16 µm³ isotropic voxel size, 70 kVP, 114 mAs, 300 ms integration time, and were subjected to Gaussian filtration and segmentation. Image acquisition and analysis protocols adhered to the JBMR guidelines for the use of µCT in rodents.

To enhance the contrast of decellularized and cadaveric muscle tissue, forearms were stained with 0.3% Phosphotungstic Acid (PTA; Sigma). For this purpose, isolated forearms were mounted in a perfusion chamber filled with 0.3% PTA dissolved in 70% ethanol and perfused via the brachial artery for 48 hours at room temperature. After staining, the specimens were scanned, mounted in 70% ethanol using a SkyScan 1173 high energy spiral scan micro-CT.

To highlight vascular conduits, angiography was performed on a decellularized forearm graft using a radiopaque silicone cast. (Microfil, Flow Tech Inc.) In brief, Microfil radiopaque silicone was injected into the brachial artery of a decellularized forearm submerged in PBS. After polymerization overnight, specimens were dehydrated through a series of increasing ethanol concentration and cleared by submersion in methyl salicylate (Fisher Scientific)

To assess cortical bone parameters, 100 transverse μ CT slices were obtained at the femoral mid-diaphysis using a 16 μ m isotropic voxel size. The ROI included the entire outer most edge of the cortex. Images were subjected to Gaussian filtration and segmented using a fixed threshold of 696 mgHA/cm³ (Figure 2). The following variables were computed: total cross-sectional area (bone + medullary area) (Tt.Ar, mm²), cortical bone area (Ct.Ar, mm²), medullary area (Ma.Ar, mm²), bone area fraction (Ct.Ar/Tt.Ar, %), cortical tissue mineral density (Ct.TMD, mgHA/cm³), cortical thickness (Ct.Th, mm), as well as maximum, minimum and polar moments of inertia ($I_{\max}$, $I_{\min}$, and J , mm⁴), which describe the shape/distribution of cortical bone.

Peripheral dual-energy x-ray absorptiometry (pDXA)—Peripheral dual-energy x-ray absorptiometry (pDXA, PIXImus II, GE Lunar Corp, Madison, WI) was performed on the bones to measure bone mineral density (BMD, g/cm³) and bone mineral content (BMC, g).

Mechanical testing of bone—Radii were mechanically tested in three-point bending using an electrical force materials testing machine (Electroforce 3230, Bose Corporation, Eden Prairie, MN). The bending fixture had a span length of 14 mm. The test was performed with the load point in displacement control moving at a rate of 0.03 mm/sec. All of the bones were positioned in the same orientation during testing with the anterior surface resting on the supports and being loaded in tension. Bending stiffness (EI, N-mm²), estimated modulus of elasticity (E, GPa), estimated bending strength (σ_{ult} , MPa), and fracture energy (mJ) were calculated based on the force and displacement data from the tests and the mid-shaft geometry measured with μ CT (see equations in Appendix 2). Fracture energy is the energy that that was required to cause the radius to fracture and it was calculated by finding the area under the force-displacement curve using the Riemann Sum method. Bending stiffness was calculated using the linear portion of the force-displacement curve. The maximum moment of inertia ($I_{\max}$) was used when calculating the estimated modulus of elasticity and bending strength.

Passive tension traction testing—Dissected rat limbs were mounted on a platform using tissue clamps (Mueller, Germany) and a tissue retractor, which allowed movement only in the wrist. Flexor carpi radialis and Extensor carpi radialis tendons were attached to an isometric force transducer (TRI202PAD, Panlab, Spain) using surgical sutures (6-0 silk), which was mounted on a Miniature Dovetail Stage (M-MT-AB2, Newport). Knots (4-0 monofilament, Ethicon) were placed in the skin to identify the radiocarpal and metacarpophalangeal joint. Tension was gradually increased from 0g to 25g using the micrometer screws of the Miniature Dovetail Stage and the degree of flexion respectively extension in the wrist was recorded using a digital camera (Nikon). Recording of the tension data was performed using Power Lab (AD Instruments). Analysis of the footage was done using ImageJ (NIH). Degree of flexion respectively extension was measured from the actual resting position of the joint presented as mean with STDEV; Statistical comparison between groups were performed using a two-tailed student's *t*-test; $n = 4$.

LC-MS/MS Proteomic Matrix Analysis—Excised gel bands of decellularized muscle were digested with trypsin. Peptide sequence analysis of each digestion mixture was performed by microcapillary reversed-phase high-performance liquid chromatography coupled with nanoelectrospray tandem mass spectrometry (LCMSMS) on an LTQ-Orbitrap Velos mass spectrometer (ThermoFisher Scientific, San Jose, CA). The Orbitrap repetitively surveyed an m/z range from 395 to 1600, while data-dependent MS/MS spectra on the twenty most abundant ions in each survey scan were acquired in the linear ion trap. MS/MS spectra were acquired with relative collision energy of 30%, 2.5 Da isolation width, and recurring ions dynamically excluded for 60 s. Sequencing of peptides was facilitated with the SEQUEST algorithm with a 30 ppm mass tolerance against a the rattus norvegicus subset of the Uniprot Knowledgebase supplemented with a database of common laboratory contaminants, concatenated to a reverse decoy database. Using a custom version of Proteomics Browser Suite (PBS v.2.7, ThermoFisher Scientific) peptide-spectrum matches (PSMs) were accepted with mass error <2.5ppm and score thresholds to attain an estimated false discovery rate of ~1%.

Histology—Decellularized rat forearms were fixed, decalcified, paraffin-embedded and sectioned as follows. Decellularized rat forearms were fixed in 5% formalin overnight. After fixation, decellularized rat forearms were decalcified with formic acid bone decalcifier (Immunocal, American Master Tech), for 24 – 48 h. After decalcification, rat forearms were washed for 3×30 minutes in PBS, and then transferred to 70% Ethanol. Then, decellularized rat forearms were paraffin-embedded and sectioned into 5- μ m sections following standard techniques. Specimens were then stained with Masson's Trichrome (American Master Tech), Russel-Movat's Pentachrome (American Master Tech) or H&E (American Master Tech) stain following the manufacturer's instructions and photographed on a Nikon Eclipse Ti-E microscope.

Immunohistochemistry—Specimens were fixed, decalcified, and sectioned as mentioned before. Then, paraffin sections were deparaffinized and rehydrated by two changes of Histo-Clear (National Diagnostics) for 5 min each, followed by a sequential alcohol gradient and rinsing in running tap water (solutions all from Fisher). Premade antigen retrieval solution (Antigen Unmasking Solution H-3300, Vector Laboratories) was used to do antigen retrieval. We heated the slides in antigen retrieval solution until the temperature reached 95°C for 20 minutes. After antigen retrieval, slides were allowed to cool down to room temperature. Slides were then washed in PBS at room temperature for 5–10 minutes. After washing in PBS, slides were blocked using dual endogenous enzyme-blocking reagent (Dako) for 5 minutes. After incubating the slides in PBS for another 5 minutes, antigen blocking was performed using 1% BSA (Sigma) in 1x PBS for another 30 minutes. After blocking, primary antibodies were added and incubated at 4°C overnight. A humidified chamber was used for all incubation steps. After washing in PBS, secondary antibody was added and incubated for 40 minutes at room temperature. Slides were washed in PBS for 5 minutes prior to Diaminobezidine (DAB) development. After DAB development slides were washed in deionized water and counterstained with hematoxylin following standard protocols. After dehydration, drops of Permount Mounting Media (Fisher Scientific) were added on the slide and covered with a coverslips.

For immunofluorescence, tissue sections were deparaffinized, rehydrated, and rinsed in PBS with 0.1% Triton X-100 for 15 minutes. Antigen retrieval was performed with 10mM sodium citrate, pH 6.0, at 95°C for 30 minutes. Sections were then blocked with 1% BSA in PBS for 1 hour at room temperature. Primary antibodies were applied in blocking buffer overnight at 4°C, followed by secondary antibodies at 1:400 dilution for 1 hour at room temperature. Secondary antibodies used were Alexa-Fluor anti-mouse 488 and anti-rabbit 594 (Invitrogen). Slides were mounted using DAPI-containing mounting media (Vector Labs), and images acquired using a Nikon Ti-E inverted fluorescent microscope.

Isometric force measurement—Isometric force measurement was performed on day 14–16 of in vitro culture and compared to rat neonatal forearm muscles. Individual native or engineered muscle fibers (n = 4) were attached to a force transducer (Model # 403A, Aurora Scientific) on one end and at the other end to a stainless steel pin between two carbon electrodes for electrical field stimulation. The tissue was suspended in an organ bath filled with Krebs Henseleit solution (Sigma Aldrich). The solution was oxygenated with 100% O₂ and maintained at 37°C using a heating bath circulator (Lauda E100, Germany). After applying pretension of 150–180 mg, the tissue was allowed to equilibrate for 10 minutes. The maximum contractile forces were measured at 120 Hz, 60 V, 50ms duration and 1,5 second train duration. Electrical stimulation was provided with a Grass S48 stimulator (Grass Technologies) and recording of the force measurement was performed using Power Lab (AD Instruments).

Morphometric measurement of myofibers—High-powered fields (20×) were selected from neonatal, adult or regenerated muscle of H&E or IF-stained sections (5 µm) (n = 3 in each group). Myofiber diameter was measured using ImageJ (NIH) and statistical analysis was performed using Microsoft Excel for Mac 2011 (mean ± SEM). All measurements were averaged per group to determine mean values ± SEM.

RESULTS

Perfusion decellularization of whole limb grafts

We harvested limb grafts from adult SD rats and perfused the tissue via the brachial artery with a 1% sodium dodecyl sulfate (SDS) based protocol. We noticed that tissue edema developed with decellularization, which led to increased compartment pressures and compromised perfusion. By performing fasciotomies before initiation of detergent perfusion, we were able to allow for radial tissue expansion without inhibiting perfusate flow. Since rats are loose skinned animals (their skin is loosely attached to underlying tissue and can slide and retract extensively), we decided to completely remove the skin of the forearm in the rat model. Low flow (0.6 to 0.8 ml/min) arterial perfusion was maintained with typical decellularization duration in the range of 24 to 50 hours (33h ± 14h). During the decellularization process, perfusion pressure oscillated between 20 to 185 mmHg. Over the course of several hours of detergent perfusion, the tissues remained intact on gross morphologic examination, while the muscle compartments became nearly translucent (Fig. 1a, Movie S1). This observation corresponded to removal of cellular tissue components and preservation of composite tissue architecture and extracellular matrix structure of muscles,

tendons, bones, ligaments, nerves, and blood vessels on histologic examination (Fig. 1b–e and Fig. 2a–d). The honeycomb patterned ECM on histological cross-section of decellularized grafts represent preserved endomysial sheets surrounding each single muscle fiber in native muscle (Fig 2a, b insets). Axial microtomographic imaging confirmed complete preservation of three-dimensional composite tissue architecture with decreased volume and signal intensity in soft tissues (Fig. 1h, i), but maintained cortical geometry and tissue mineral density of bone tissue (Fig. 2e, g). Additional analysis using peripheral dual-energy x-ray absorptiometry (pDXA), and three-point bending test were performed and revealed no significant effect of decellularization on mechanical, mineral or geometric bone characteristics (Fig. 2e–i). Thus, no weakening of the skeletal system occurs in decellularized tissue compared to native tissue. Microtomographic angiography confirmed presence of perfusable vascular channels throughout the entire graft flowing from larger to smaller vessels (Fig. 1j). Passive mechanical testing confirmed functional preservation of the entire skeletomuscular apparatus with intact osteotendinous junctions and maintained stability and full range of motion in joints of wrist, and digits (Fig. 2j). These findings are in line with our own experience with decellularization of heart muscle(17), and data on decellularized tendon (18), nerve (19), ligament-bone (20) and bone grafts (21) by other groups. Proteomic analysis confirmed the preservation of collagens and glycosaminoglycans within the muscle ECM. (Fig. 2k). Histological analysis and immunohistochemical staining demonstrated decellularization of all tissue compartments (Fig. 1b–e), leaving no motor proteins detectable (Fig. 2a, b), while preserving collagens (Fig. 1d, e and Fig 2c, d). Biochemical analysis showed that our decellularization protocol removed approximately 90% of the DNA content, while retaining 40% of the sulfated glycosaminoglycan (GAGs) content of native muscle tissue (Fig 1f, g). Hence, perfusion decellularization is applicable to composite tissue and produces acellular limb scaffolds with preserved anatomical structure and mechanical properties of the musculoskeletal system.

Biomimetic Culture

To determine if perfusion decellularized composite tissue matrixes could be repopulated with myogenic, vascular, and mesenchymal cells to form viable grafts, we designed a perfusion bioreactor enabling perfused long-term culture under sterile conditions (Fig. 3a). Since electrical stimulation has been described to improve the formation of functional muscle (22), we included carbon electrodes in the bioreactor for electrical field stimulation (Fig. 3a, A). Culture medium was perfused into the brachial artery at constant flow (1 – 1,5 ml/min) under standard culture conditions. To repopulate composite tissue scaffolds, we infused vascular endothelial cell into the brachial artery (Fig. 3b, 1) and injected skeletal myoblasts into the muscle ECM of selected muscles (Fig 3b, 2), and cultured the matrix for up to 21 days. In order to perform skin transplantation, we removed the reseeded constructs from the organ chamber under sterile conditions on day 10 and mounted it back after the grafting procedure to continue perfused organ culture (Fig 3b, 3).

Recellularization of acellular composite tissue scaffolds

To regenerate viable composite tissue grafts, we repopulated acellular composite tissue scaffolds with either C2C12 mouse myoblasts only (n=7), with a combination of C2C12 mouse myoblasts and mouse embryonic fibroblasts (n=20) for muscle regeneration, or with

a combination of C2C12 mouse myoblasts, mouse embryonic fibroblasts and human umbilical vein endothelial cells (HUVEC) for regeneration of re-endothelialized composite tissue (n=5). Finally, we performed skin transplantation onto the engineered composite tissue graft (n=5) (Fig. 3b, c). Regeneration of the different compartments was done stage by stage with specific subsequent culture periods. (Scheme Fig. 3 b) For repopulation of muscle, we tested two different seeding strategies. Seeding through vascular infusion of the cell population at constant high flow over the course of several hours resulted only in marginally distribution of myoblasts into muscle ECM with the majority of cells retained in the vascular conduit and therefore appeared not suitable for repopulation of muscle tissue. Injection of the cells into the muscle ECM resulted in good engraftment and muscle like tissue formation, however, the resulting mechanical trauma also disrupted ECM structure, therefore disturbing microcirculation and cell integration into the matrix, which led to areas of cell apoptosis at the injection site. Since vascular resistance increases with decellularization and decreases with re-endothelialization (23), we first seeded acellular matrixes with endothelial cells. Instillation of HUVECs through the brachial artery followed by 60 minutes of static culture led to homogenous lining of the vascular system (Fig. 3i) Engraftment efficacy of a single seeding step was $69\% \pm 15\%$ (n=4).

Next, we seeded C2C12 mouse myoblasts on day two of whole organ culture in co-culture with mouse embryonic fibroblasts or in tri-culture with fibroblasts and HUVECs by injection into the acellular muscle matrix of selected muscles. The injection of micro-depots using a surgical microscope resulted in good engraftment of the cells and eventually led to the formation of functional, muscle-like tissue (Fig. 3d–h). Since HUVECs deplete quickly in high glucose DMEM based media, we tested different media formulations for the maintenance of all cell types. We found low glucose DMEM supplemented with 10% FBS, 2% HEPES, VEGF, hydrocortisone, rhFGF-B, rhEGF, R3-IGF, ascorbic acid and penicillin (100 IE/mL) suitable for all cell types.

After cell seeding, we mounted the forearm construct back into the bioreactor and started perfusion after 60 minutes of static culture. Switching to a low-serum differentiation medium induced Myotube fusion. Electrical stimulation during growth and differentiation phase greatly enhanced cell alignment along endomysial sheets and eventually led to the formation of functional muscle tissue (Fig. 4a–c). Myofiber diameter was intermediate between neonatal and adult rat muscle tissue after 14 days of in vitro culture (Fig. 3f).

Finally, full thickness skin grafts were harvested from the upper extremities of donor rats and transplanted onto the engineered composite tissue grafts on day 10 of whole organ culture. Skin grafts were attached to the engineered tissue using sutures and fibrin glue.

To evaluate the distribution and phenotype of cells in the bioartificial forearm grafts, we performed histological analysis. Seeded cell types engrafted in their appropriate compartments (Fig. 3d–k). Myoblasts seeded into the acellular muscle matrix fused to multinucleated, striated fibers (Fig. 3h, right), aligned along the longitudinal axis and expressed α -actinin and myosin heavy chain (Fig. 3 g, h), as seen in native muscle tissue. HUVECs seeded into the brachial artery aligned along the vascular bed to form perfusable channels and expressed CD-31 (Fig. 3i).

Functional testing of engineered muscle

To determine whether engineered composite tissue grafts would contain functional skeletal muscle, we performed isometric force measurement on isolated muscles through electrical field stimulation (EFS) on day 14 to 16 of *in vitro* culture (Fig. 4a–c). Figure 4 b and c show a representative record of tetanic forces generated by engineered muscle tissue under EFS. The average peak isometric force was 18,7 μN $\pm$ 1,6 (n=4). The average specific force was 105 N/m² for engineered myofiber and 135 N/m² for rat neonatal muscle and therefore represented approximately 80% of the specific force of neonatal muscle (Fig. 4c).

Transplantation of engineered composite tissue grafts

To show that engineered composite tissue grafts can be used for surgical reconstruction after limb loss, we performed orthotopic limb transplantations. The native limb was exposed and amputated at the level of the mid-humerus while preserving the recipients upper arm muscles. Limbs were transplanted onto isogenic SD rats using a modified cuff technique for anastomosis of the vasculature. Graft brachial vessels were anastomosed to recipient axillary vessels, the brachial plexus approximated end-to-end and osteosynthesis was performed using an intramedullary rod (Fig. 4e, f). Biceps and triceps tendons were re-approximated to the recipient's biceps, respectively triceps. Upon unclamping of the artery, the vascular bed filled with blood (Fig. 4g, h). We inserted a pressure catheter in the radial artery to confirm pulsatility of blood flow (Fig. 4d). In addition to our passive tendon traction testing *in vitro*, we attempt to transplant a bioartificial hand graft at the level of the distal forearm to confirm preservation of mobility and the functional potential of the bioartificial graft *in-vivo*. After reconnection of the bone, we approximated flexor and extensor tendons to the recipients forearm muscles. Upon electrical stimulation of the recipients forearm muscles, the bioartificial graft performed a flexion movement in the wrist and metacarpophalangeal joints (Movie S 2).

To show that composite tissue perfusion decellularization can be scaled to clinically relevant size, we decellularized whole primate forearms (Fig. 5) at a constant perfusion pressure of 70 mmHg through the brachial artery. Similar to our rat experiments, we performed fasciotomies (Fig. 5a, dotted line) to allow for radial tissue expansion during the decellularization process but we did not dissect the skin. Histological examination showed the removal of cellular material in bone tissue compartments (Fig. 5b), muscle and tendons (Fig. 5c), the skin and subcutaneous tissue (Fig. 5d) and neurovascular bundles (Fig. 5e) with preservation of ECM ultrastructure after 7 days of detergent perfusion (Fig. 5a).

Discussion

Bioartificial composite tissue grafts, engineered using patient derived cells 'on demand', could become a tailored treatment option for patients suffering from volumetric tissue loss. Current treatments for the loss of composite tissue such as hand amputation are merely palliative. Although many challenges remain, we present a first step towards regeneration of a bioartificial composite tissue graft, by using the forearm as a proof of principle. Prior work in decellularization of isolated tissues such as tendon (24), bone(25), nerve (26), and muscle (27) inspired us to attempt to generate acellular scaffolds containing all of these tissues in

their physiologic context, while keeping the composite architecture and biomechanics of a graft as complex as a forearm intact. Moreover, the feasibility of using decellularized tissue for the treatment of soft-tissue loss was highlighted by a recent report of successful treatment of volumetric muscle loss in 5 patients using an acellular xenogeneic ECM scaffold (15). To accomplish this, we adapted a previously reported technique developed for heart (17), lung (16, 28), liver (29) and kidney (23) taking advantage of the native vasculature to deliver decellularization agents, hence allowing for intense penetration of the agents into the tissue compartments. Consistent with the collective experience with solid organs, we found that detergent decellularization of whole rat and baboon extremities removes cellular components throughout the entire graft and its diverse tissue compartments. Although it is hardly possible to remove all cellular components and nuclear fragments completely from a tissue (30), removal of DNA to an adequately low level is vital for avoiding adverse immune reaction. We confirmed decellularization of composite tissue grafts by biochemical and histological analysis. In our experiments, DNA content decreased by approximately 90 % to 350 ng/mg tissue, which is comparable to residual DNA content of decellularized kidney (31) and esophagus (32) in other studies.

Notably, a specific threshold amount of residual DNA that would facilitate a proinflammatory response against the scaffold is still unknown.(30) Moreover, other studies have shown that decellularized tissues with comparable residual DNA content such as esophagus (32) or liver tissue, decellularized with a similar protocol (33), are well tolerated in *in-vivo* implantation experiments and no adverse immune reactions are induced. Decellularization not only removes cellular components, but also causes a collateral loss of ECM components. Loss of ECM components increases with extended duration of decellularization (34), which subsequently alters the biomechanical properties and biochemical composition of engineered grafts. We therefore aimed to keep the decellularization process as short as possible, by allowing for a higher perfusion pressure; therefore, we were able to retain 40% of sulfated glycosaminoglycans in our experiments, which is in line with results of other studies (31).

Retaining the biomechanical properties of the composite tissue scaffold as well as the range of motion of joints during the decellularization process is a requirement for its function upon regeneration and dependent on the preservation of ECM components during decellularization. We performed Micro-computed tomography, peripheral dual-energy x-ray absorptiometry, and three-point bending analysis to evaluate the biomechanical properties of decellularized bone within the composite tissue graft. We found that perfusion-decellularization of the graft did not significantly affect the mechanical, mineral, or geometric characteristics of bone tissue. In order to perform movement, joints of the engineered composite tissue graft must stay flexible with a similar ease and range of motion compared to native joints. We performed a passive tension traction testing and found similar flexibility compared to the native tissue, suggesting that the decellularization had no negative influence on joint mobility.

In this initial work, we show that composite tissue scaffold can be recellularized with a mixture of myogenic, vascular, and mesenchymal cells by seeding each compartment separately. While cell seeding of perfusable compartments like the vascular system can be

accomplished by surface attachment of cells through infusion, resulting in a homogeneous distribution, seeding of dense tissues without physiological access poses numeral challenges. Injection of cells using small diameter cannulas under a surgical microscope allowed for targeted cell delivery to distinct muscle sheaths and enabled formation of contractile muscle with a similar morphology compared to native myofiber. In order to maintain viability of an engineered composite tissue graft, immediate reperfusion upon transplantation is necessary. We performed CT-angiography to highlight preserved vascular conduits after decellularization, which serve as a prerequisite for re-endothelialization and reperfusion in vitro. After seeding of endothelial cells and maturation in vitro, we observed the formation of perfusable vascular channels, which could withstand physiologic perfusion pressures upon transplantation.

In this work, we met numerous milestones towards the generation of a biomechanically intact composite tissue graft; however, there are several challenges that must be addressed from a translational point of view. First and foremost, neuronal ingrowth and integration into the recipient's nervous system is a prerequisite to gain ultimate functionality of an engineered limb graft.

Equal to allogeneic hand transplantation, axons of the recipient's nerve stumps have to regrow into the graft's nerve sheets in order to reinnervate sensory organs and muscles (35, 36). Therefore, peripheral nerve regeneration can only be achieved upon in-vivo implantation.

Restoration of intrinsic hand muscle function in hand transplant recipients shows that regeneration of neuromuscular junctions in a whole limb is possible (7). Moreover, regeneration of sensory nerves, resulting in restoration of discriminative sensation, can be observed in the same patients (7). Notably, decellularized nerve grafts are already in clinical use to bridge nerve gaps in sensory, motor and mixed nerves (26). However, such grafts are limited to bridging only short gaps in peripheral nerves. From our point of view, this might be due to their avascularity and the lack of support cells and growth factors. In support of this notion, recent studies have shown that vascularized nerve grafts are superior in restoring function compared to free nerve grafts (37, 38). A bioartificial composite tissue graft offers preserved neurovascular bundle anatomy, which might allow for neuronal ingrowth similar to allogeneic transplants. Long-term survival experiments will be required to enable peripheral nerve regeneration, and to ultimately proof whether regenerated composite tissue grafts can become fully functional. Second, injection of myoblasts in-vitro generates a considerable amount of matrix disruption, therefore disturbing microcirculation and cell integration into the matrix, which leads to areas of cell apoptosis at the injection site. The use of primary or iPS-derived mesangioblasts, which could be delivered systemically into an artery to regenerate muscle tissue (39), might be a promising cell candidate for in-vitro muscle regeneration prior to transplantation. Third, we transplanted engineered grafts only in short-term, non-survival procedures. We found that survival procedures at this early stage of limb regeneration would require extensive postoperative care, which is hardly possible in a rodent and would furthermore raise ethical concerns regarding animal welfare.

Conclusion

Our results demonstrate the feasibility of producing a complex whole limb scaffold containing preserved passive musculoskeletal apparatus, vasculature, and nerve sheets, which can be repopulated with cells of appropriate phenotype and orthotopically transplanted into a recipient. In contrast to solid organ transplants, allogeneic composite tissue grafts such as hand transplants are not fully functional at the time of transplantation. Recipient nerves have to regrow into the donor nerve sheaths, which serve as mere scaffold for the recipient's axons, to ultimately reinnervate the muscles and sensory organs within the skin (36). Bioartificial composite tissue grafts may be transplanted at an early stage of regeneration, and similar to allografts benefit from *in vivo* regeneration to enable further functional maturation.

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Fig. 1. Perfusion decellularization of isolated forearms

(a) Photograph of an isolated forearm, cannulated through the brachial artery for detergent perfusion, yielding acellular scaffolds after 35 hours. (b, c) Pentachrome stained serial cross-sections of native (left) and decellularized (right) whole forearms at different anatomic levels showing skin (s), tendons (t), bone (b) and muscle (m) tissue (b=mid-forearm, c=mid-hand. Insets show representative artery). (d) Masson's Trichrome stain of native (left) and decellularized (right) muscle tissue showing artery (a), vein (v), nerve (n) and muscle (m) tissue. (e) Masson's Trichrome stain of native (left) and decellularized bone showing cortical bone (CB) and medullary cavity (MC) (C) (f, g) Biochemical quantification of DNA (f) and glycosaminoglycan (g) content of native and decellularized muscle tissue. (n = 3 forearms, values are mean $\pm$ SD, asterisk indicating $P < 0.05$). (h-j) 3D microtomography reconstruction of soft tissue and bone in cadaveric (h) and decellularized (i) forearms and corresponding axial cross-sections at the mid-forearm level. White arrows indicating neurovascular bundles. (j) 3D reconstruction of CT-angiography of a decellularized forearm showing integrity of vascular conduits (white arrows).

Fig. 2. Protein content and biomechanical properties

(a–d) Immunohistochemical staining of native (top) and decellularized (bottom) tissue for alpha actinin (a), alpha skeletal myosin (b), collagen IV (c) and collagen X (d). Scale bar, 100 μm (e) 3D microtomography was used to analyze cortical geometry. (f–i) Peripheral dual-energy x-ray absorptiometry (pDXA) was performed to analyze bone mineral density and three-point bending analyses to determine mechanical properties after decellularization ($n = 3$). Error bars are SD. There were no significant differences between decellularized and native bone properties. (j) Passive mechanical testing of the musculoskeletal system before and after decellularization. Passive tendon traction was measured to determine mechanical properties of the passive musculoskeletal system after decellularization ($n=4$, values are mean $\pm$ SD, asterisk indicating $P < 0.05$). (k) Proteomic analysis of decellularized muscle matrix showing composition of preserved collagens and proteoglycans.

Fig. 3. Regeneration of composite tissue

(a) Schema of the functional bioreactor for electrical and mechanical stimulation. (b) Schema for composite tissue engineering. First, vascular endothelial cells are instilled into the vascular system of acellular composite tissue grafts. Second, myoblasts, fibroblasts and endothelial cells are injected into the muscle compartment on day 2 of whole organ culture. Third, full thickness skin grafts are transplanted onto engineered constructs on day 10 of in vitro culture. (c) Photograph of regenerated composite tissue graft and photograph of cross-sectional area of a regenerated forearm seeded with C2C12 mouse myoblasts, mouse embryonic fibroblasts, human umbilical vein endothelial cells (HUVEC), and skin transplantation (Right). Red dotted circles highlighting ulna and radius. Black dashed line is highlighting full thickness skin graft. (d) Masson's Trichrome stain of a regenerated whole flexor muscle after 20 days of culture. (e) Masson's Trichrome stain of regenerated muscle tissue surrounding nerve. High-power field showing aligned, multinucleated myofibers. (f) Comparison of myofiber diameter in regenerated, neonatal and adult rat muscle tissue ($n = 3$ muscles, values are mean $\pm$ SD, asterisk indicating $P < 0.05$). (g–h) Immunofluorescence stain for alpha actinin (g), myosin heavy chain (h), and high-power field of multinucleated,

striated myofiber (h, right). **(i)** Immunofluorescence stain for CD31 in re-endothelialized grafts in longitudinal low-power fields for overview and cross-sectional high power field to show perfusable channels (inset). **(j)** H&E stain of full thickness skin graft (s) with underlying regenerated muscle tissue (m). **(k)** Immunofluorescence stain for pan-cytokeratin (red) and myosin heavy chain (green). Green signal of stratum corneum is due to autofluorescence **(l)** Immunofluorescent stain for CD31 (green) and MHC (red) of regenerated, vascularized muscle. Nuclei counterstained with DAPI. Scale bars 100µm (g,h,i,k); 400µm (j); 500µm (e); 1000µm (d); 5000µm (c, right).

Fig. 4. Functional testing of engineered muscle and transplantation

(a) Photograph of engineered muscle after 16 days of organ culture and corresponding longitudinal section in Masson's Trichrome stain. Scale bar 2000µm. (b) Representative record of the tetanic contractile response of engineered muscle to pulse electrical field stimulation after 16 days of biomimetic organ culture. (pulse = 20V, 50ms). (c) Specific tetanic forces normalized for cross-sectional area for native (N) and regenerated (R) muscle (n = 4 muscles, values are mean ± SD). (e) Anastomosis of engineered composite tissue graft to the blood supply of adult SD rats. (d) Representative recording of intraoperative pressure curves measured in the radial artery of a engineered forearm graft. (f) X-ray image of recipient showing attached composite tissue graft with intramedullary rod (white arrowhead) and intravascular cuff (white arrow). (g) Photograph of explanted tissue showing re-perfused vascular tree (h) Immunohistochemical stain for CD31 on explanted tissue, showing perfusion of re-endothelialized vascular conduits (CD31, brown) with intravascular red blood cells. Scale bar, 100µm

Fig. 5. Perfusion decellularization of primate extremities

(a) Photograph of isolated primate limb. Dotted line represents incision line for volar fasciotomy. Photograph of primate forearm after 24h of decellularization (middle) and completely decellularized limb graft after 148 hours of perfusion decellularization. Perfusion pressure was maintained at 75 mmHg. (b) Representative H&E stain of the humerus. High-power field showing Haversian canal (HC) and acellular osteocyte-lacunae. (c) H&E stain of the cross-section of acellular muscle (m) with attached tendon (t) (separated by dotted line). High-power field showing a magnification of the same area in Masson's Trichrome stain (top) and acellular muscle in longitudinal section (bottom). (d) Russell Movat's Pentachrome stain of skin and subcutaneous tissue. (e) Russell Movat's Pentachrome of neurovascular bundle showing nerve (n), vein (v) and artery (a) with preserved elastic fibers (black) in the arterial wall. High-power fields are H&E stains of the same section and show absence of nuclei and preservation of ultrastructure with nerve fascicle (f) with endoneurium (en) surrounded by perineurium (pn). Arterial wall showing preserved tunica media (tm) and tunica adventitia (ta). Scale bar: 5 cm (a); 1000µm (b–e, low-power fields); 100µm (b–e, high-power fields)