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Sustained regeneration of high-volume adipose tissue for breast reconstruction using computer aided design and biomanufacturing

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ABSTRACT

Adipose tissue engineering offers a promising alternative to the current breast reconstruction options. Here we investigated patient-specific breast scaffolds fabricated from poly(D,L)-lactide polymer with pore sizes >1 mm for their potential in long-term sustained regeneration of high volume adipose tissue. An optimised scaffold geometry was modelled *in silico* via a laser scanning data set from a patient who underwent breast reconstruction surgery. After the design process scaffolds were fabricated using an additive manufacturing technology termed fused deposition modelling. Breast-shaped scaffolds were seeded with human umbilical cord perivascular cells and cultured under static conditions for 4 weeks and subsequently 2 weeks in a biaxial rotating bioreactor. These *in vitro* engineered constructs were then seeded with human umbilical vein endothelial cells and implanted subcutaneously into athymic nude rats for 24 weeks. Angiogenesis and adipose tissue formation were observed throughout all constructs at all timepoints. The percentage of adipose tissue compared to overall tissue area increased from 37.17% to 62.30% between week 5 and week 15 ($p < 0.01$), and increased to 81.2% at week 24 ($p < 0.01$), while the seeded endothelial cells self-organised to form a functional capillary network. The presented approach of fabricating customised scaffolds using 3D scans represents a facile approach towards engineering clinically relevant volumes of adipose tissue for breast reconstruction.

1. Introduction

Breast cancer is a global problem and a major cause of morbidity, with an estimated number of 300,000 new cases diagnosed in 2013 [1]. While treatment concepts have moved from radical mastectomy to lumpectomy and adjuvant therapies such as chemotherapy, radiation or hormone therapy, there is still a significant number of patients suffering from the consequences of surgical removal of breast tissue. Such procedures often have negative psychological effects on the well-being of the patients. Earlier studies by Renneker et al. [2] showed that mastectomy is directly related to a psychological syndrome "marked by anxiety, insomnia, depressive attitudes, occasional ideas of suicide, and feelings of shame and worthlessness" [2]. Although several new approaches exist today in order to anatomically reconstruct the breast, some women report the feeling of an altered body image [3]. This might partly be due to the fact that reconstructive methods available today replace, yet do not regenerate native adipose tissue, which is necessary for restoring the natural shape and feel of a breast. Prosthetic silicone-based implants are associated with a high occurrence of a mid and long-term foreign body response in form of a fibrous implant encapsulation altering the shape of the breast and more importantly leading to pain and discomfort [4–6]. Autologous fat tissue transplantation and free tissue flap transfers are also often used by plastic surgeons. However they are associated with a high risk of tissue shrinkage, fat necrosis and/or oil cyst formation [7,8].

To overcome the limitations of currently used breast reconstructive techniques, impetus has been steadily growing towards cell-based regeneration of adipose tissue. Tissue Engineering and Regenerative Medicine (TE&RM) approaches aim to move the field

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away from methods to replace damaged tissue with permanent implants to more biological solutions that are able to restore structure and function of autologous tissue [9]. Since the publication of the pioneering research paper in 1999 by Patrick et al. [10] who used preadipocyte-seeded polyglycolic acid (PLGA) scaffolds for regenerating small volumes of adipose tissue, research groups from around the world [11–19] have achieved breakthroughs which moved the field forward. However, formation of sufficient volumes of mature adipose tissue remains a major problem and so far it has not been possible to maintain the structural entity of tissue engineered adipose constructs for more than 8–16 weeks [20,21]. Current developmental efforts are therefore directed towards resolving problems that prevent the upscale of adipose tissue engineering concepts towards clinically relevant volumes that can be maintained throughout the life of the patient.

The shape and size of the breast of each patient is different, hence the scaffold of each breast reconstruction should be customised [9,22,23]. Previous studies undertaken in our laboratory have established a clinically viable methodology to design scaffolds for breast tissue engineering applications from patient scan data sets [24]. In this project, our main focus was on the design and fabrication of anatomically-shaped and patient-specific scaffolds using a biopolymer that degrades over the time scale of 1–2 years. Previous reports have indicated that a scaffold made out of such a slow-degrading polymer provides a long-term stable platform for adipose tissue to regenerate and to mature, remodel its environment and stabilise [25]. It is well known that not only large but fully interconnected pore architectures facilitate cell invasion and blood vessel ingrowth, which is crucial for maintaining long-term viability of adipose tissue [26,27]. Since the diameters of a great number of the larger rat blood vessels infiltrating into a tissue-engineered construct lie in the 500–1500 μm range [28], we hypothesised that a slow-degrading biopolymer with pore sizes in the range of 1000–2000 microns allows for efficient vascular ingrowth and long-term sustained regeneration of high volume adipose tissue within a rat subcutaneous model.

2. Methods and materials

2.1. Image acquisition and computer aided design (CAD) model generation

A 3D CAD file was generated based on a 3D laser scan performed on a 46 year old female patient, after informed consent, who suffered from an invasive ductal carcinoma from a data set previously derived [24]. Briefly, a Vivid 910 dot-laser scanner with built-in high precision camera (Konica Minolta, Marinouchi, Japan) was used to perform a 3D scan of the patient from three angles (0° frontal, and −30° and +30° oblique anterior). The images were imported in Rapidform2006 (Inus Technology, Seoul, South Korea) and merged into a single-shell object [29]. A custom surfacing algorithm was generated to model a virtual chest wall which was merged with the breast surface shell to obtain a watertight model of the solid breast. This watertight model was then meshed and exported as a Standard-Tesselation-Language (STL) file.

2.2. Design & fabrication of scaffolds

The volume of the original CAD model (194 cm^3) was scaled down to 3 cm^3. Skeinforge-50, open-source slicing software, was used to generate machining computer-numerical-control (CNC) code from the CAD file. Custom software was designed to selectively remove the perimeter shells of each layer. The resultant CNC code was then used to fabricate the scaffolds using the Replicator 3D printer (Makerbot Industries, New York, USA) and Poly(D,L)-Lactide polymer (PDLLA). During extrusion of the scaffolds, the layer thickness was set to 0.37 mm, porosity value was set to 90%, operating flow rate and feed rate were matched to 41 mm/s and extrusion temperature was set to 220 °C.

2.3. MicroCT (μCT) evaluation of scaffolds

The specimens were scanned on a Scanco μCT40 (Scanco AG, Brüttisellen, Switzerland) at 8 μm resolution, employing 55 kV and 145 μA with 250 ms exposure time. Porosity, pore-size and filament-size distributions were obtained by employing a modified trabecular bone histomorphometry algorithm.

2.4. Isolation and culturing of primary human umbilical cord perivascular cells (HUCPVCs)

HUCPVCs represent a rich source of human mesenchymal cells found in the perivascular region of the human umbilical cord [30] and have recently gained attention in the tissue engineering field due to their short doubling time, and high occurrence of colony-forming-unit-fibroblast (CFU-F). HUCPVCs isolated from consenting full-term caesarean section patients were obtained from Tissue Regeneration Therapeutics Inc. (Toronto, Canada). The HUCPVCs were isolated as reported previously [30] and were received in passage 2. HUCPVCs were maintained in Dulbecco's Modified Eagle Medium (containing 4600 mg/L glucose) (Invitrogen, Carlsbad, USA) growth media supplemented with 10% (v/v) foetal bovine serum (Lonza, Basel, Switzerland) and 1% (v/v) Penicillin (10,000 U/ml) – Streptomycin (10,000 μg/ml) (Invitrogen, Carlsbad, USA) in a humidified incubator at 37 °C and 5% (v/v) CO_2.

2.5. Culturing of primary human umbilical vein endothelial cells (HUVECs)

HUVECs were obtained from American Type Culture Collection (Rockville, MD, USA) and maintained in F12K medium (Invitrogen, Carlsbad, USA) supplemented with 0.1 mg/ml heparin (Sigma, St Louis, USA), 0.05 mg/ml Endothelial Cell Growth Supplement (Millipore, Billerica, USA), 10% (v/v) foetal bovine serum (Lonza) and 1% (v/v) and Penicillin (10,000 U/ml) – Streptomycin (10,000 μg/ml) (Invitrogen, Carlsbad, USA), in a humidified incubator at 37 °C and 5% (v/v) CO_2. HUVECs were used between passages 2 and 8.

2.6. Production of GFP lentiviruses and transduction of HUVECs

HUVECs were labelled with enhanced green fluorescent protein (eGFP) using a pLenti CMV GFP Puro (658-5) plasmid (Addgene 17448) and packaged with plasmids pRSV-Rev, pMDLg/pRRE and pMD2.G (contains VSV.G gene) (Addgene 12253, 12251 and 12259). The pLenti CMV GFP Puro plasmid were amplified in *Escherichia coli* and purified using W/Endo-free Qiagen Maxi-Prep Kit (Promega) according to the manufacturer's instructions and packaging plasmids were amplified in *E. coli* and purified using Maxi-Prep Kits (Qiagen). Packing cell line 293T cells (GenHunter Corp.) in a T75 flask were transfected with 6.6 mg pLenti CMV GFP Puro (empty) and 3.3 mg of each packaging plasmids pRSVRev, pMDLgpRRE and pMD.G in 133 μl 1.25 M $CaCl_2$, 0.5 ml H_2O and 0.66 ml 2 × HEPES buffered saline and the cells were incubated at 37 °C/5% CO_2. Four hours later, the medium was removed, cells were washed twice with warm phosphate-buffered saline (PBS) and replaced with 5 ml of complete DMEM. The cell culture supernatant was harvested 48 h later by centrifugation at 850 g for 7 min at 4 °C, followed by filtration of the supernatant through a 0.45 μm filter. The viral supernatant was concentrated 80–100 times using Amicon Ultra 15 ml centrifugal filter units (Millipore). The lentiviral stocks were stored in small aliquot at −80 °C for titration and cell transfection. Titre of the concentrated lentiviral was determined by plating 4×10^5 293T cells in 6-well plate and infecting the cells with serial (10-fold) dilutions of concentrated lentivirus. After 48 h, cells were trypsinised, washed three times with cold PBS and fixed with 1% paraformaldehyde (PFA) for 30 min. The fixed cells were analysed using FACSCalibur for eGFP expression and for a typical preparation, the titre was approximately 10^7–10^8 infectious units per ml. GFP.

HUVECs (2×10^6) in T175 flask were infected with 10 infectious units per cell in 5 ml of complete DMEM with polybrene (8 μg/ml) (Santa Cruz biotechnology) for 1 h incubation at 37 °C/5% CO_2 before the addition of 15 ml of complete DMEM/polybrene. Incubation was continued for 24 h before DMEM/polybrene media was replaced by complete DMEM. 48 h post-transduction, the media was replaced with DMEM complete with 0.5 μg/ml of puromycin (Invitrogen), as determined previously by a puromycin kill curve (0.2–4 μg/ml) with HUVECs, in order to select for eGFP positive cells.

2.7. Preparation of cell-seeded TECs

Breast-shaped PDLLA scaffolds were first washed with sterile PBS and for sterilisation immersed in 70% ethanol overnight. Once the ethanol evaporated, the scaffolds were subjected to UV-irradiation for 30 min on each surface. Sterilised scaffolds were later placed in PBS overnight to pre-wet them. HUCPVC were detached using 0.25% trypsin, washed with PBS and resuspended in culture media. 20×10^6 HUCPVC were seeded onto each scaffold within a fibrin matrix (Baxter, Toongabbie, Australia) to improve cell attachment. After trypsinisation, the cell suspension was diluted with thrombin solution at a ratio of 1:1. 600 μL of cell/thrombin suspension was mixed with 600 μL fibrinogen solution and seeded on each scaffold. Cell-seeded scaffolds were cultured at 37 °C and 5% CO_2 in complete DMEM.

After 4 weeks of static culture, all scaffolds were subjected to a dynamic culture environment for 2 weeks utilising the TisXell biaxially rotating bioreactor (QuinXell Technologies, Singapore) for 8 h per day at 5 rpm. The bioreactor setup has been previously described elsewhere [31,32].