Appendix

Periodontal Tissue Engineering Using a Multiphasic Construct with Cell Sheets

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1. Materials and methods:

1.1 Bone compartment

The bone compartment was electrospun using an in-house melt electrospinning device. The polymer pellets were loaded into a 2 mL syringe and electrospun at a temperature of 80°C at a feed rate of 20 µL/h, at 7 kV and at a 4 cm tip to collector distance for 8 hours. This resulted in the formation of a 5 mm thick melt electrospun scaffold and prior to use the melt electropsun scaffolds were sectioned with a sharp scalpel blade into 5x5x5 mm^3 specimens.

2.1 Periodontal compartment

Medical grade polycaprolactone (mPCL, Corbion) membranes were prepared using an in-house solution electrospinner. The polymer was first dissolved in a mixture of chloroform and dimethylformamide (9/1 vol/vol) at a concentration of 25% wt/vol. The polymer solution was loaded into a 10 mL syringe and electrospun at a feed rate of 2 mL/hr, at 10 kV and at a 20 cm tip to collector distance for 30 min.

3.1 Assembly of the biphasic scaffold

The 3D melt electrospun scaffold component was placed at a 1 cm distance from a hot plate heated to 300°C for 4 seconds and then quickly press-fitted for 10 seconds onto the PCL solution-electrospun membrane. This heat treatment partially melted the first layers of the melt electrospun scaffold and enabled strong adhesion to the solution electrospun membrane. The biphasic scaffolds were surface etched using sodium hydroxide in order to improve their hydrophilicity according to the following protocol: the scaffolds were immersed in 100% ethanol for 15 minutes under vacuum and thereafter immersed in sodium hydroxide 2 M for 30 min at 37ºC, multiple rinse-immersions in ultrapure water were performed until a water pH of 7 was reached. The scaffolds were dried and kept in a dessicator until further use.

4.1 Scanning electron microscopy (SEM)

The scaffolds were immersed in liquid nitrogen for 5-10 min and a sharp razor blade was used to section the structures. The samples were gold coated for 3 min and observed with a FEI Quanta 200 Environmental SEM operating at 10 kV. The average fibre diameter was calculated using ImageJ from 3 micrographs taken at a magnification of 1000 from 3 different scaffolds (tallying over 50 fibres). Similarly, the average pore area in mm^2 and average pore size in microns was calculated in ImageJ as previously reported (Vaquette et al. 2018).

5.1 Cell sources

The cells were obtained from the ten Merino sheep undergoing the experimental surgery (Queensland University of Technology, Animal Ethics Committee approval number: 0900001332). The cells were sourced from autologous tissue (extracted incisors, explanted gingival tissue, and bone marrow aspirate) obtained with the animals under general anaesthesia The cells were isolated according to established protocols (Costa et al. 2014; Haase et al. 2003; Vaquette et al. 2012; Vaquette et al. 2010) and used at P3. The PDLCs were sourced from two extracted incisors per animal and the cells were isolated using the explant culture method as we previously reported (Costa et al. 2014; Vaquette et al. 2012) Similarly, GC were extracted from two small pieces of gingiva, the epithelium was removed and the gingival tissues were further sectioned and the cells were extracted using the same explant culture method. Bm-MSc

were obtained from the iliac crest, an 18G needle was utilised to aspirate 20 mL of bone marrow into 5 mL of PBS with 5,000 UI of heparin. The aspirate was subsequently diluted at a one to two ratio in DMEM prior to centrifugation at 2,500 rpm. The nucleated layer was then collected and plated in a 175cm^2 culture flask. The media was initially changed after 4 days and thrice a week thereafter. All cells were passaged using 0.25% trypsin, replated at 2,500 cell/cm^2 and further expanded until P3.

6.1 Mineralisation potential of the various cell sources

Each of the primary cell cultures were seeded in triplicate at a density of 10,000 cell/cm^2 in 24-well plates and cultured for 7 days in either expansion media (DMEM containing 10% FBS, 1% penicillin/streptomycin) or in osteogenic induction media (expansion media supplemented with 50 µg/mL ascorbate-2-phosphate, 10 mM β-glycerophosphate and 0.1 µM dexamethasone). At the end of the 7 days culture, the wells were rinsed twice in PBS and then fixed for 10 min in cold methanol. After rinsing in Millipore H_2O, 500 µL of a 1% alizarin Red S solution (Sigma Aldrich) were added and incubated for 10 min at room temperature. The unfixed dye was removed by gently rinsing the stained structures with Millipore H_2O until a clear solution was obtained (generally after 5 rinses). The samples were air dried overnight and photographed. Thereafter, the alizarin red was solubilised using 400µL of a 10% acetic for 10 min under gentle stirring prior to adding 100µL of 10% ammonium hydroxide in order to bring the pH to 4.1. The solutions were transferred in triplicates of 100 µL into 96 well plates and the absorbance at 405 nm was read using a plate reader (Benchmark Plus, microplate spectrophotometer, BIO RAD).

7.1 Cell sheet culture and harvesting

Each primary autologous cell type was seeded in 6 well plates at a density of 10,000 cells/cm^2 and cultured in osteogenic media for 7 days. At this time point, the cells had become fully confluent and formed a mature cell sheet which could be mechanically harvested using tweezers. Prior to harvesting, the biphasic scaffold was sterilised in ethanol for 30 min followed by a 30 min UV-irradiation. The cell sheets were folded four times and placed on the solution electrospun membrane (periodontal compartment) of the scaffold, thus creating a multiphasic tissue engineered construct. The cell sheets were allowed to attach for 4 hours prior to implantation. Autologous cells were utilised in all cases.

8.1 Multiphasic construct implantation:

The defects were created using an extra-oral approach involving a full skin incision in the lower jaw and elevation of a full thickness mucoperiosteal flap adjacent to the second pre-molar and first molar of the mandible (Figure 1B). In each animal, four defects were created by bilaterally removing the alveolar bone and cementum covering the roots of the mandibular P2 and M1 using round burs with copious saline irrigation, with the assistance of 2.5X magnification surgical loupes and headmounted illumination. Four experimental groups were thus tested as follows: (1) non cellularised biphasic scaffold (Control), (2) Gingival cell sheet on the biphasic scaffold (GC), (3) Bone marrow mesenchymal stem cell sheet on the biphasic scaffold (Bm-MSC), (4) Periodontal cell sheet on the biphasic scaffold (PDLC). Each group was randomly assigned to a defect (Figure 1C) and a cell occlusive solution electrospun PCL membrane was placed over the defect once the highly porous scaffolds were inserted to preclude soft tissue infiltration, according to the principles of GTR. The sites were sutured and primary closure was achieved. Following 5 or 10 weeks of healing (5 animals at each time points; 10 animals in total), the animals were euthanised by an injection of Lethabarb (pentobarbiton sodium, 100mg/kg) and the animal head was perfused with 1L of a 4% paraformaldehyde solution in

PBS at pH 7.4. Thereafter the mandible was harvested and further fixed in 4% paraformaldehyde for 7 days and subsequently rinsed and stored in PBS. Thereafter, the specimens were further trimmed in order to isolate the various regions of interest.

9.1 Micro-Computed Tomography (µCT)

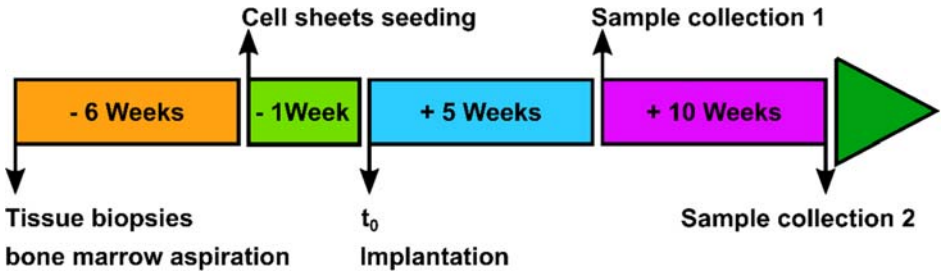
Micro-CT scanning (µCT40, SCANCO Medical AG, Bruttisellen, Switzerland) was performed at a resolution of 30µm, voltage of 70kVP, current of 114 µA, power of 8W, and an integration time of 200ms.

10.1 Histology and histomorphometry

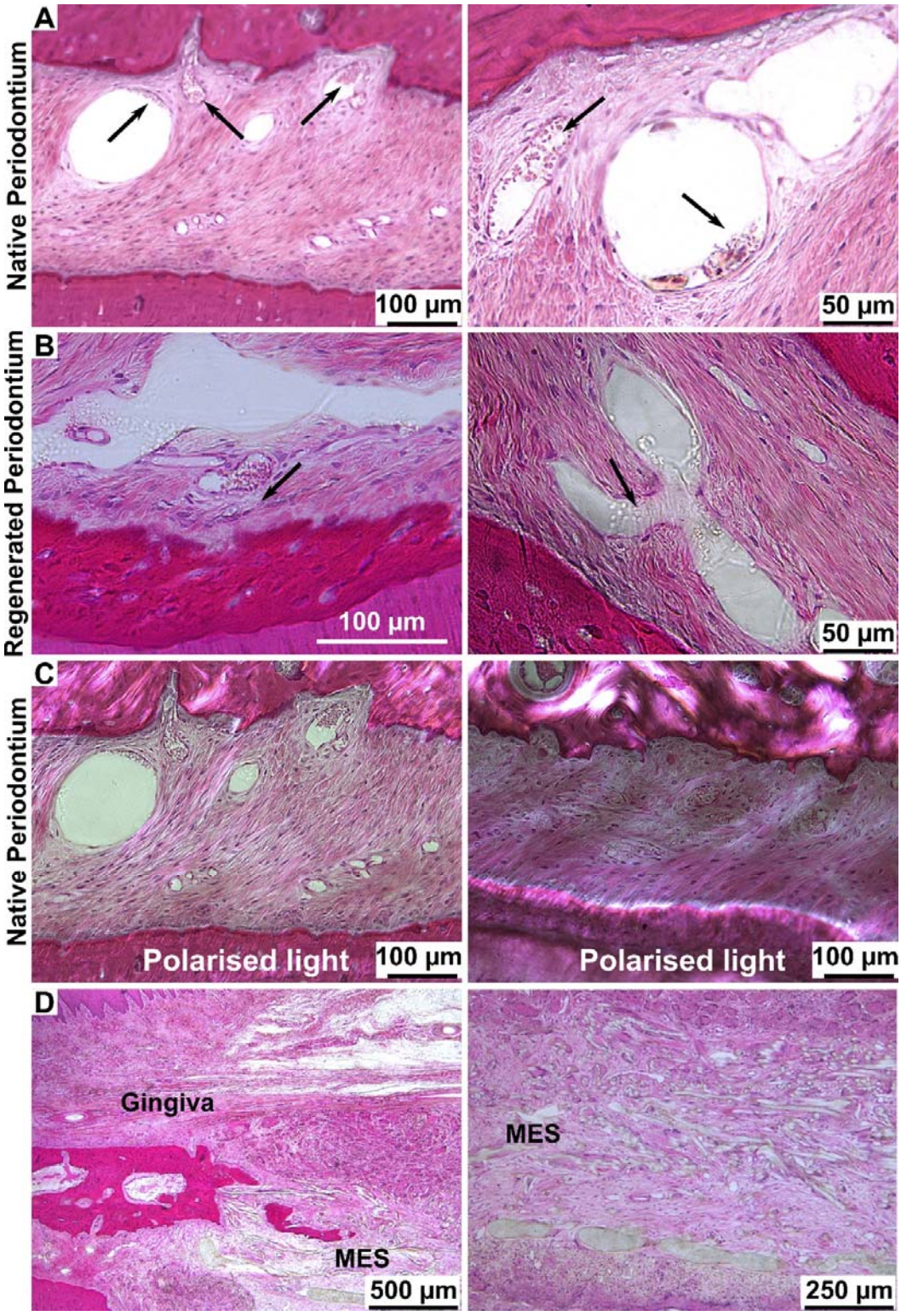
The samples were thereafter dehydrated in a graded series of ethanol and infiltrated with resin (Methyl Methacrylate / Glycol Methacrylate, Tecknovit 7200, Heraeus Kulzer, Germany) for hard tissue sectioning. Once cured, the resin blocks were ground using an EXAKT 400 CS micro-grinding system to expose the samples, glued onto a glass slide and subsequently sectioned to a thickness of 150µm using an EXAKT 300 cutting system (Exact Apparatebau, GmbH Norderstedt Germany). The slides were polished down to a thickness of 20 µm using the EXAKT 400 CS micro grinding system. The thin specimens were then stained with H&E for light microscopy. Histomorphometrical analysis using Osteomeasure was performed in order to quantify the percentage of bone fill, periodontal attachment and periodontal regeneration. The percentage bone fill was determined by measuring the area covered by newly formed bone normalised by the total area of the defect (Figure 1 E). Percentage of cementum coverage was calculated as the length of newly formed cementum divided by total length of the root within the defect. Cementum thickness was calculated for each specimen at 5 random locations along the root surface. 'Periodontal attachment' was defined as oblique fibres attachment into cementum and was calculated by measuring the length where this feature was present divided by the total length of the root within the defect. 'Periodontal regeneration' was defined as periodontal ligament attachment with the addition of the presence of new bone in the direct vicinity (Figure 1 F), and was determined as a percentage of the total length of the root within the defect.

11.1 Statistical analysis

For the in vitro data, the statistical analysis was performed using one-way ANOVA followed by a Tukey HSD post hoc test using IBM SPSS Statistics for Windows version 21.0 (IBM Corporation 2012©, Armonk, NY, USA). For all other quantitative data, Generalized Estimating Equations were applied using individual animals as the clustering variable in order to take into account that 4 different groups were implanted in each animal. This enabled testing for the potential influence of individual animal characteristics that may influence the regenerative outcome, ie. test whether any differences can be attributed to differences between animals rather than other treatment variables as we have previously published (Alayan et al. 2016; 2017). To this end, time and treatment were used as explanatory variables along with all two-way interactions. A backward elimination process was used for arriving at the most parsimonious model. A $p \leq 0.05$ was considered to represent statistically significant differences.



Appendix Figure 1: Project surgical timeline.



Appendix Figure 2. A: Vascularisation within the native ovine periodontal ligament indicating the presence of large blood vessels (in the hundredth of micron scale). B: vascularization of the regenerated periodontal ligament at 10 weeks post-implantation, the arrows indicate the presence of red blood cells, C: Polarised light images of the ovine native periodontium indicating the insertion of Sharpey's fibres in the cementum and showing the attachment of the

periodontal ligament in the alveolar bone. D: presence of connective tissue in the bone compartment (melt electrospun scaffold, MES), and tissue integration of the multiphasic scaffold with the surrounding gingival tissues.

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