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The influence of cellular source on periodontal regeneration using calcium phosphate coated polycaprolactone scaffold supported cell sheets

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Abstract

Cell-based therapy is considered a promising approach to achieving predictable periodontal regeneration. In this study, the regenerative potential of cell sheets derived from different parts of the periodontium (gingival connective tissue, alveolar bone and periodontal ligament) were investigated in an athymic rat periodontal defect model. Periodontal ligament (PDLC), alveolar bone (ABC) and gingival margin-derived cells (GMC) were obtained from human donors. The osteogenic potential of the primary cultures was demonstrated *in vitro*. Cell sheets supported by a calcium phosphate coated melt electrospun polycaprolactone (CaP-PCL) scaffold were transplanted to denuded root surfaces in surgically created periodontal defects, and allowed to heal for 1 and 4 weeks. The CaP-PCL scaffold alone was able to promote alveolar bone formation within the defect after 4 weeks. The addition of ABC and PDLC sheets resulted in significant periodontal attachment formation. The GMC sheets did not promote periodontal regeneration on the root surface and inhibited bone formation within the CaP-PCL scaffold. In conclusion, the combination of either PDLC or ABC sheets with a CaP-PCL scaffold could promote periodontal regeneration, but ABC sheets were not as effective as PDLC sheets in promoting new attachment formation.

Keywords: cell sheet; periodontal ligament cell; alveolar bone-derived cell; gingival margin-derived cell; PCL scaffold; periodontal regeneration

1. Introduction

Periodontitis is a common inflammatory disease that results in irreversible destruction of the tooth supporting structures and eventually leads to tooth loss [1]. The ultimate goal of periodontal treatment is to restore the structure and function of the damaged periodontium. This is challenging as it requires the restoration of both hard and soft tissues, with the formation of functionally oriented periodontal ligament fibers which insert into newly formed cementum and alveolar bone. In the past few decades, various procedures, including root conditioning, bone grafting, guided tissue regeneration (GTR) and the application of biological factors have been utilized in order to promote periodontal regeneration. Unfortunately, currently regenerative procedures have had limited success in achieving this goal [2, 3].

Recent advances in progenitor cell biology and tissue engineering have enabled the development of cell-based therapeutics that are aimed at achieving periodontal regeneration with greater efficacy and predictability [2, 4]. A variety of cell types, including bone marrow mesenchymal stem cells, adipose-derived stem cells and dental follicle cells, have been shown to promote periodontal regeneration to various degrees in animal models [5-7].

The progenitor capacity of periodontal tissue itself has also been extensively studied during the past few decades [8]. Although studies utilizing the progenitor capacity of cells derived from periodontal tissues have mainly focused on periodontal ligament-derived cells (PDLC), recent studies have demonstrated that alveolar bone and gingival tissue may also contain progenitor cells that can be used for periodontal regeneration [9-12]. These cell sources are important given the highly specialized nature of the various

periodontal components, and their ready availability from redundant tissues obtained from periodontal and oral surgery. However, the periodontal regenerative capacity of cells derived from different periodontal tissues has not as yet been compared in an *in vivo* model.

The delivery of intact cell sheets onto a diseased tooth root is an attractive periodontal regeneration approach as it simulates the anatomical features of the periodontal ligament, whose presence is necessary for reforming the periodontal attachment between alveolar bone and root surface cementum. Intact cell sheets for transplantation can be prepared using thermo-responsive culture flasks [13]. Using this technique, cells are detached from the culture flask by lowering the temperature, without the need for enzymatic treatment. This results in cell sheets with an intact extracellular matrix. The cell sheet method has been used to promote periodontal regeneration in various periodontal defect models, and the safety and efficacy of PDLC sheets has been evaluated both *in vitro* and *in vivo* in pre-clinical studies [14-18]. Although the results of these studies suggest that therapy based on cell sheet engineering can be effective and practical for clinical periodontal regeneration, an inherent challenge of the cell sheet transplantation approach is the difficulty in stabilizing and securing the cell sheet within the periodontal defect. Therefore, there is a need to identify effective strategies for the delivery of cell sheets into periodontal defects. To this end, the ideal characteristics of a cell sheet 'carrier' scaffold to be used for periodontal regeneration would include the ability to stabilize the cell sheets on the root surface, as well as facilitate bone formation within the periodontal defect.

Our hypothesis was that the combination of an osteoconductive scaffold and cell sheets prepared from primary periodontal tissue derived cell cultures would result in a construct that facilitates periodontal regeneration by not only recreating the complex hierarchical structure of periodontal tissues, but also ensuring that the cells are secured to the denuded root surface within the periodontal defect. The first aim of this study was to compare the regenerative potential of cell sheets produced by human primary cell cultures derived from different periodontal tissues in an athymic rat periodontal defect model. The second aim was to assess the suitability of a previously characterized electrospun calcium phosphate coated polycaprolactone (CaP-PCL) scaffold [19] to support and stabilize a cell sheet at the periodontal defect site.

2. Materials and Methods

The experimental design of the study is summarized in **Fig.** 1A. Briefly, cell sheets obtained from well characterized primary cell cultures were harvested intact using thermoresponsive culture plates and delivered into rat periodontal defects using CaP-PCL scaffolds. The periodontal defects were subsequently assessed at 1 and 4 weeks using micro-CT, histomorphometry and immunohistochemistry.

2.1 Cell isolation and culture

Human periodontal ligament cells (PDLC), gingival margin-derived cells (GMC) and alveolar bone-derived cells (ABC) were isolated and cultured as previously described [20, 21]. Briefly, gingival margin, periodontal ligament and alveolar bone were collected from four healthy female subjects (age range 20-25 years old) who required third molar extraction. The selected teeth had no periodontal or endodontic disease. Ethical approval for the use of this redundant tissue was attained through the Griffith University Human Research Ethics Committee (DOH/17/7/HREC).

For establishment of the PDLC and GMC cultures, the extracted tooth and marginal gingival tissue were collected into Dulbecco's Modification of Eagle's Medium (DMEM) (Gibco) supplemented with 10% Fetal Calf Serum (FCS), 2.5 μg/ml fungizone, 100 units/ml penicillin, 100 μg/ml streptomycin and 1% non-

essential amino acids. Periodontal ligament, curetted from the middle third of the root, and gingival tissues were then cut into small pieces (less than $3mm^3$) and placed in tissue-culture dishes to allow the establishment of explant cultures. Subsequent subcultures were maintained in DMEM containing 10% FCS, 50 units/ml penicillin, 50 μg/ml streptomycin and 1% non-essential amino acids at 37°C in a 5% CO_2 incubator.

For generation of ABC cells, alveolar bone collected during third molar extraction was treated with 0.05% trypsin (37°C, 30 min) followed by 0.2% collagenase (37°C, 30 min), then washed three times with phosphate-buffered saline before the bone was chipped into small segments, covered with explant media and placed into a 5% CO_2 incubator at 37°C [21]. The explant cultures were subsequently subcultured as described above for the gingival and periodontal ligament cells.

2.2 *In vitro* characterization of periodontal tissue derived primary cell cultures

GMC, ABC and PDLC at passage 5 were used in all the *in vitro* studies. In the control group, cells were cultured in DMEM containing 10% FCS, 50 units/ml penicillin, 50 μg/ml streptomycin and 1% non-essential amino acids (control media, CM). In the experimental group, cells were cultured in CM supplemented with 50 μg/ml ascorbate-2-phosphate, 10mM β-glycerophosphate and 0.1μM dexamethasone (osteogenic media, OM) [22]. The cells were cultured in either CM or OM and then submitted to assays described below. The osteogenic potential of the three cell types derived from the four patients was initially assessed by measuring both mineralization (Von Kossa staining) and hydroxyapatite formation (Osteoimage Assay, Lonza, Waverly, NSW, Australia). A dermal cell (DC) line (Lonza, Waverly, Australia) was used as a negative control for the osteogenic differentiation studies. Subsequently, one set of representative matched cell cultures derived from a 25 year old female was chosen for the *in vivo* experiment and the osteogenic differentiation potential of these cells was further characterized by Alazarin Red S staining and assessment of BSP mRNA expression.

2.2.1 Von Kossa staining

Von Kossa staining was used to detect the phosphate content of the cell cultures. DC, GMC, ABC and PDLC were seeded at the density of 1×10^4 cells/well in a 48-well plate and cultured in OM at 37°C for 2 and 4 weeks. Cell monolayers were fixed with 10% formalin for 10 minutes. The cells were then washed

with phosphate buffered saline (PBS) and 500µl of 2% silver nitrate solution was added and subjected to ultra-violet light for 1 hour. Subsequently, the wells were washed with three changes of distilled water before 500uL of 5% sodium thiosulfate was added for 2min. The cells were again washed in three changes of distilled water and assessed visually.

2.2.2 Hydroxyapatite Formation

Cells were seeded at a density of 1×10^4 cells/well in a 48-well plate and cultured in CM and OM at 37°C for 4 weeks. Hydroxyapatite formation was assessed using the OsteoImage™ assay according to the manufacturer's instructions. The excitation and emission wavelengths used were 492nm and 520nm respectively.

2.2.3 Alizarin Red S staining

GMC, ABC and PDLC were seeded at a density of 2×10^4 cells/well in a 24-well plate and cultured in CM and OM at 37°C for 3 and 6 weeks. Alizarin Red S staining was carried out using a modification of the method described by Reinholz et al.[23]. Briefly, the cell monolayer was first washed in PBS and then fixed in 10% formalin for 10 minutes. The cells were then washed with PBS and 1ml of 2% Alizarin Red S (pH 4.3) was added for 20 minutes. The cells were then washed four times with PBS to remove excess dye before being allowed to dry and photographed.

2.2.4 Bone sialoprotein (BSP) gene expression

Cells were seeded at a density of 1×10^5 cells/well in a 48-well plate and cultured in CM or OM for 3 weeks. Total RNA was harvested from cultured cells using TRIzol® reagent (Invitrogen). The quality and quantity of RNA was assessed using a spectrophotometer (NanoDrop ND-1000) before cDNA was prepared by reverse transcription using the iScript cDNA synthesis kit (Bio-Rad, NSW). Real time PCR was performed in an ABI PRISM 7900 (Applied Biosystems) thermocycler using TaqMan Universal PCR Master Mix and Taqman 'Assay-on-Demand' primer/probe oligonucleotides for BSP (Applied Biosystems). The cycling conditions comprised an initial 10 min at 95ºC to activate the DNA polymerase, followed by 45 cycles of denaturation at 95°C for 15 sec and primer/probe annealing and extension at 60ºC for 1 min.

2.2.5 CaP-PCL carrier scaffold

A porous melt electrospun, CaP coated scaffold (**Fig.** 1B, D) with osteoconductive potential was used as a carrier for the periodontal cell sheets. The fabrication and characterization of this scaffold was described in a previous study [19]. Briefly, polycaprolactone (PCL, medical grade 80 kDa) was obtained from Osteopore (Singapore). PCL was electrospun using an in-house device. For scaffold preparation, the polymer pellets were loaded into a 2 mL syringe and melted at 80°C. The polymer was melt electrospun through a 21G needle at the same temperature using the following conditions: feed rate of 20 μL/hr, 7 kV, a tip-collector distance of 4 cm and for 5 min. Following this, the scaffold was coated with calcium phosphate and rinsed in NaOH. The scaffold had a thickness of 500 μm and was sectioned into small pieces (approximately 3mm ×1.5mm × 0.5mm in size) before use.

2.2.6 Covering membrane

A solution electrospun PCL membrane (**Fig.** 1C, E) was used to cover the periodontal defect following the insertion of the construct, in order to prevent the infiltration of overlying soft tissues. The fabrication of this membrane has been previously described [24]. Briefly, the polymer was first dissolved in a mixture of chloroform and dimethylformamide (9/1 vol/vol) at a concentration of 15% wt/vol. The polymer solution was loaded into a 10 mL syringe and electrospun at a feed rate of 2 mL/h, at 10 kV and at a 20 cm tip to collector distance for 30 min. The electrospinning process was performed onto a flat collector surface, which resulted in the formation of randomly orientated fibers. The PCL electrospun membranes are highly flexible and resilient when folded. This membrane was shown to have a fiber diameter of 3 μm, a small pore size from 5 to10 μm and therefore is partially occlusive. The physical and biomechanical integrity of the membrane was not compromised when wet and it retained its handling properties. The membranes demonstrated excellent attachment and stability once implanted. The PCL membrane had a thickness of approximately 300~400 μm and was sectioned into small pieces (approximately 8mm ×5mm × 0.3mm in size) prior to implantation

2.3 *In vivo* study

2.3.1 Cell sheet preparation

Cells at passage 5 were seeded in 35mm diameter temperature-responsive culture dishes (Thermo Scientific Nunc UpCell^{TM} surface, U.S.A.) at a concentration of 1×10^5 cells per well and cultured in DMEM media

supplemented with 50 μg/ml ascorbate-2-phosphate (Sigma) at 37°C for 4 days. Then the cells were cultured in osteogenic media at 37°C for another 4 days. The cell sheets were subsequently harvested by lowering the culture temperature to 20°C for one hour and then removed as a continuous sheet. The cell sheet was folded 4 times using sterile tweezers and a CaP-PCL scaffold, as described in section 2.1, was used for harvesting and supporting the cell sheet during the subsequent transplantation process.

2.3.2 Rat periodontal defect

This defect model was modified from a periodontal fenestration defect model described by Yang *et al* [5]. The protocols of this study were approved by the Animal Ethics Committee of Griffith University (DOH/02/11/AEC). In brief (**Fig.** 1A), twelve-week-old athymic rats (Rattus norvegicus, Strain-CBH-rnu/Arc, Animal Resources Centre, Murdoch, Western Australia) were anaesthetized via the inhalation method, using Isoflurane (AttaneTM, Bomac Animal Health Pty Ltd, Australia). The surgical site was shaved and disinfected using Povidone-Iodine on gauze swabs. A full thickness skin incision was made through the skin along the inferior border of the mandible, the masseter muscle and periosteum covering the buccal surface of the mandible was elevated as a flap. The alveolar bone and cementum covering the roots of the mandibular first molar were removed using round burs with copious saline irrigation, with the assistance of 2.5 × magnification surgical loupes and head-mounted illumination.. The surgical defect was approximately 3mm × 1.5 mm. The defect was then washed with abundant saline. The cell sheet carried by the CaP-PCL scaffold was implanted into the defect, with the cell sheet side of the construct facing the denuded root surface. Defects alone (Blank group) or defects with the CaP-PCL scaffold alone (Scaffold group) were used as controls. All defects were then covered by a piece of PCL membrane (**Fig.** 1C, E). The masseter muscle and skin were repositioned and sutured to ensure healing by primary intention. Temgesic® (0.02mg/kg) was used to control post-surgical pain. Prophylactic antibiotics were given (200μl/day of Enrofloxacin (Baytril®25) antibacterial oral solution, active ingredient 25mg/ml Enrofloxacin, Bayer Australia Ltd) on a daily basis post-surgically. Rats were sacrificed at 1and 4 weeks after surgery and the mandible samples (n=3 for each group at each time point) were harvested and fixed in 4% paraformaldehyde solution overnight at room temperature and then washed in PBS.

2.3.3 Micro CT

Scans were performed in a microcomputed tomography (micro-CT) scanner (mCT40, SCANCO Medical AG, Brüttisellen, Switzerland) at a resolution of 12 μm and a voltage of 45kVp and a current of 177 mA. Three-dimensional (3D) images of the defects were reconstructed from the scans by the micro-CT system software.

2.3.4 Histomorphometric analysis

After micro-CT scanning, the mandible samples were decalcified in 10% EDTA solution for 4 weeks and subsequently embedded in paraffin. The samples were sectioned horizontally every 5 μm with every 10th slide stained using hematoxylin and eosin to locate the defect. The stained slides were scanned using the Scanscope Digital Slide Scanner (Aperio, Vista, CA, USA).

The defect was demarcated by the histologically visible cut edge of cortical bone and exposed root surface. New bone formation was defined as the bone island observed within the defect. Percentage of new bone formation was calculated by dividing the area of new bone with the area of the defect (**Fig.** 2A).

New cementum was defined as the mineralized tissue formed on the denuded root surface with collagen fibers inserted (**Fig.** 2B, C). The percentage of new cementum covering the denuded root surface was calculated by dividing the length of root surface with new cementum coverage with the length of the whole denuded root surface.

The orientation of the fibers covering the denuded root surface was measured as the angle between the long axis of the fibers and the new cementum. Fibers with an angle greater than 60 degrees were considered as functional new periodontal ligament attachment (**Fig.** 2D, E). The percentage of new periodontal ligament was calculated by dividing the length of root surface with functional new periodontal ligament attachment with the length of the whole denuded root surface.

2.3.5 Immunohistochemistry

Immunohistochemistry was performed on slides using antibodies to bone sialoprotein (BSP, LF100, provided by Dr. Larry Fisher, dilution 1/1000), osteopontin (OPN, LF166, provided by Dr. Larry Fisher, dilution 1/1000) and human-specific nuclei (MAB 1281 clone 235-1,Millipore, dilution 1/50). In brief, slides were de-waxed, rehydrated and the antigen retrieved using citrate acid buffer (pH 6.0). Slides were

permeabilized with 0.2% Triton X-100 for 10 minutes, peroxidase blocked with 3% hydrogen peroxide solution for another 10 minutes and then blocked using 10% swine serum in 5% bovine serum albumin (Sigma) for 30 minutes. The samples were subsequently incubated with primary antibodies (diluted in 5% bovine serum albumin) for 30 minutes and the staining was visualized with Dako LSAB® 2 System-HRP kit (DAKO, Noble park, VIC, Australia) according to the manufacturer's instructions.

2.4 Statistical analysis

Statistical Package for Social Science (SPSS) version 20.0 for windows was used to analyze the data. One-way analysis of variance (ANOVA) was used to compare differences in the percentage of new bone formation, cementum formation and new periodontal ligament formation between groups. Statistical significance was assumed for *p* values less than 0. 05.

3 Results

3.1 *In vitro* characterization

3.1.1 Von Kossa staining

All three primary cell types (GMC, ABC, PDLC) exhibited staining at both time points with more intense staining at week 4 compared to week 2 (**Fig.** 3A). ABC displayed more intensive staining than GMC and PDLC after 2 weeks, while both ABC and PDLC displayed more staining than the GMC after 4 weeks. The dermal fibroblasts displayed minimal staining at both time points

3.1.2 Hydroxyapatite Formation

All three primary cell types showed a significant increase in hydroxyapatite formation when cultured in osteogenic media compared with the control media (**Fig**. 3B). There were no differences between the three cell types. The dermal fibroblasts did not exhibit any hydroxyapatite formation.

3.1.3 Alizarin Red S staining

In general, all three cell types cultured in the osteogenic media (+) demonstrated more intensive staining than those in the control media (-). GMC displayed the strongest staining at both time points, followed by ABC, and then PDLC (**Fig.** 3C).

3.1.4 BSP gene expression

All three cell types showed a significant increase of BSP gene expression when cultured in osteogenic media (**Fig.** 3D). In addition, ABC showed higher BSP gene expression than GMC and PDLC ($p<0.05$).

3.2 *In vivo* assessment

3.2.1 MicroCT analysis

Primary healing with no adverse post-operative sequelae was observed in all animals. 3D micro CT reconstruction was used to evaluate the healing of the defect at different time points. As shown in **Fig.** 4A, mineralized tissue covering the defect could be observed in the Scaffold, ABC and PDLC groups at 4 weeks, while in the other groups, most of the root surfaces remained exposed.

3.2.2 Histological and immunohistochemical analysis

3.2.2.1 New bone formation

In all groups the original margin of the defect was still identifiable, and this made it possible to distinguish new bone from the old bone. One week after surgery (**Fig.** 4B and **Fig.** 5B), new bone formation could be observed in the defect of the Scaffold and ABC groups, but the difference was not significant compared to the other groups. None of the other groups had observable new bone formation at this time point.

Four weeks after surgery, new bone formation could sbe observed in all groups (**Fig.** 4B and **Fig.** 5B). The Scaffold, ABC and PDLC groups had a significantly higher percentage of bone coverage compared with the Blank group ($p<0.05$). No significant difference was found between the GMC group and the Blank group. Also, it was observed that in the GMC and Blank groups, new bone was mainly formed at the edge of the defects, with none of the exposed root surfaces being covered by new bone.

3.2.2.2 New cementum formation

The Blank and GMC groups did not generate new cementum at any time point (**Fig.** 5A and C). In the other groups, new cementum could not be observed 1 week after the surgery. However, after 4 weeks, the ABC and PDLC groups generated significantly more cementum than the other groups ($p<0.05$). In the Scaffold group, although some new cementum formation could be detected, the difference was not significant compared with the blank group ($p<0.05$).

3.2.2.3 New periodontal ligament formation

The rats in the Blank and GMC groups did not generate any meaningful amount of oriented ($>60^{o}$) periodontal ligament fibers at any time point (**Fig.** 5A, D). PDLC sheets had significantly more appropriately oriented periodontal ligament fibers than all of the other groups at 4 weeks following cell sheet transplantation ($p<0.05$). The ABC group displayed significantly more functionally oriented periodontal ligament fibers than the blank group ($p<0.05$). A few oriented periodontal ligament fibers could also been observed in the Scaffold group at 4 weeks, however, the difference was not significant compared with the Blank group.

3.2.2.4 Immunohistochemistry of bone matrix proteins bone sialoprotein (BSP) and osteopontin (OPN)

BSP and OPN were strongly expressed in the native periodontium in bone and cementum, especially acellular cementum (**Fig.** 6A, D). Positive staining of BSP and OPN was also detected in newly formed bone and in the cementum formed on the denuded root surface (**Fig.** 6B, E). In those samples without new attachment formation, no expression of BSP or OPN was detected on the denuded root surface (**Fig.** 6D, F).

3.2.2.5 Immunohistochemistry of human nuclei

Human cells from the transplanted cell sheets were tracked by immunostaining of the donor cells with a primary antibody against human nuclei. All three cell types could be detected in great quantity in the defect 1 week after the surgery, and were mainly distributed within the soft tissues of the defect (**Fig.** 6G-I). Four weeks after surgery, a few cells of human origin were found scattered in the defect area, and there seemed to be more human cells present in the PDLC group compared with the GMC and ABC (**Fig.** 6J-L) groups at this time point. In the GMC and ABC groups, human cells were found in the soft tissues within the defects, while in the PDLC group, human cells were found surrounding the new bone, as well as on the root surface (**Fig.** 6I and L).

4 Discussion

Cell-based therapy represents a promising approach to achieving periodontal regeneration [2, 4]. This study aimed to assess the relative effectiveness of human primary cell sheets derived from different periodontal tissues (gingiva, periodontal ligament and alveolar bone) in promoting periodontal regeneration. The rationale for using primary periodontal

tissue derived cells is the ready availability of these tissues, especially gingiva and alveolar bone, to dental surgeons.

In the current study, the cell sheets were combined with a well characterized, osteoconductive carrier scaffold made from medical grade PCL [19]. The cell sheets were placed directly in contact with the denuded root in order to increase the number of potential progenitor cells in this area. The rationale of this approach was to recapitulate the composition of the periodontal tissue, as the cell sheets have a mature extracellular matrix, which allows them to remain intact during transplantation and early wound healing. As the healing progresses, these cells may exit this matrix and contribute to the regeneration of adjacent periodontal structures, such as bone and cementum. The CaP-PCL scaffold was used for supporting the cell sheets, maintaining the space in the defect and physically allowing the migration and infiltration of local progenitor cells into the bone healing compartment.

The regenerative potential of the combined CaP-PCL scaffold/cell sheet construct was evaluated in terms of bone formation in the defect area, cementum coverage and functional periodontal ligament formation on the exposed root surface. The results showed that although all three cell types exhibited mineralization potential when cultured in osteogenic media in vitro, their ability to promote periodontal regeneration varied greatly in vivo. When transplanted into rat periodontal defects, the ABC and PDLC sheets resulted in the formation of a periodontal ligament-like complex after 4 weeks, while the GMC sheets did not promote any significant periodontal regeneration. The new bone and cementum tissue formation was confirmed using immunohistochemical staining

for bone sialoprotein and osteopontin, which are recognized markers of osteogenesis and cenmentogenesis [25, 26].

It is well established that the periodontal ligament (PDL) contains cells capable of forming new cementum on the tooth root surface and re-establishing new attachment between cementum and bone [27-29]. The presence of progenitor cells in the PDL is supported by in vitro studies showing that PDL-derived cells have multipotent differentiation potential [30, 31]; and further confirmed by the isolation and characterization of stem cells from PDL cell cultures [32, 33]. The potential of PDL-derived cells in promoting periodontal regeneration has been demonstrated in several animal models [14, 16, 34-37], as well as some preclinical trials [18, 38]. However, one major drawback of using PDL-derived cells in periodontal regeneration is that the tooth has to be extracted in order to obtain these cells, and the quantity of cells obtained is relatively small.

Compared to the PDL, gingival tissue and alveolar bone can be readily obtained during oral surgery. Cells isolated from the gingival connective tissue are usually termed gingival fibroblasts (GF) [39], and they have been utilized as a cell source for tissue engineering of non-dental soft tissues [40]. However, GF were originally considered not to have any mineralization potential [22, 41], and hence be of limited use in promoting periodontal regeneration where the formation of mineralized tissues (bone and cementum) is essential. However, more recently, cells with mesenchymal stem cell characteristics have been isolated from gingival tissue [11, 12, 42], indicating that GF are in fact a heterogeneous population of cells and may also be a potential cell source for periodontal regeneration. In particular, gingival margin cells (GMC) have been shown to have

mesenchymal stem cell properties [43], which is not surprising given that this tissue incorporates periodontal attachment fibers between gingival connective tissue and root cementum. Further, it has been reported that non-stem cell enriched primary GF could differentiate into osteogenic and chondrogenic phenotype under certain inductive conditions [44, 45], which is in agreement with our observation of *in vivo* osteogenic potential of primary GMC cells.

Alveolar bone is one of the most active bones in the human body and accordingly considered as a useful site for harvesting bone cells for tissue-engineering purposes in the oral cavity [46]. Recently, a progenitor cell population with multipotent differentiation potential has also been isolated from the alveolar bone proper [47]. The ability of alveolar bone-derived cells to induce bone regeneration has been demonstrated *in vivo* [9], but these cells have not been tested for the purpose of promoting periodontal regeneration.

Among the three cell types, PDLC and ABC sheets had a significantly higher potential to promote bone formation than the GMC sheets. PDLC sheets exhibited the highest capacity to promote new periodontal attachment formation, which was not surprising, as the effect of PDLC on cementum and periodontal ligament regeneration are well recognized [14, 16, 34-37]. The ABC sheets also promoted more cementum and periodontal ligament formation than the control group, suggesting that alveolar bone can be considered as a potential cell source in tissue engineering therapies aimed at achieving periodontal regeneration.

A notable finding of this study was that the GMC sheet had an inhibitory effect on bone formation in the periodontal defect. These results are consistent with Nakajima et.al's findings in a similar periodontal defect model, in which defects that received GMC sheets

generated less bone than empty defects [48]. However, a recent study by Fawzy El-Sayed et al [43] reported significant periodontal regenerative potential of gingival margin-derived stem/progenitor cells. In their study, progenitor-enriched cultures were used instead of primary cells. As the progenitor cells only constitute a small proportion of the gingival cell population [49, 50], it is possible that the characteristics of the entire primary GMC population may inhibit the regenerative potential of the limited number of cell with progenitors characteristics. Indeed, this is the rationale behind the use of the Guided Tissue Regeneration (GTR) surgical technique, which aims to exclude gingival tissues from periodontal defects, giving preference to periodontal ligament cells and osteoblast [3]. The concept of GTR was also used in this current study whereby an occlusive membrane was placed on top of the scaffold, which prevented soft connective tissues of the mucosa from infiltrating the scaffold during the crucial early healing period.

In order to determine whether the transplanted cell sheets contributed directly to periodontal regeneration in this rat model, we tracked the cells using a human-specific anti-nuclei antibody. Cells of human origin could be detected in all of the groups transplanted with cell sheets at week 1, although the numbers greatly decreased by week 4. In the PDLC group, cells of human origin could be detected within the newly formed bone and cementum, indicating that the human cells directly contributed to the newly formed periodontal tissues. In the ABC group, human cells were observed directly next to new bone at the earlier time point (1 week), but were found scattered in the soft tissues in the defect at the later time point (4 weeks). These results suggest that the ABC sheets may indirectly induce periodontal regeneration, without necessarily differentiating into mineralized tissue forming cells themselves.

One of the challenges of the cells sheet approach is the difficulty in delivering and securing the sheet onto the tooth root surface. To this end, we used a well characterized [19], melt electrospun, CaP coated, porous PCL scaffold to harvest, carry, deliver and secure the cell sheet to the root surface. The PCL scaffold was fabricated into a highly porous and interconnected structure and cut to the defect size, which physically allowed the migration and infiltration of local progenitor cells. The CaP-PCL scaffold has been shown to accelerate the osteogenic process and promote ectopic bone formation [20]. The scaffold was shown to have the dual favorable characteristics of securing the cell sheet on the tooth root surface and promoting bone formation within the periodontal defect. In the histological analysis, the ABC and PDLC groups were found to have significantly higher percentage of new bone formation compared with the Blank group at 4 weeks; however, there were no statistically significant differences between these two groups and the Scaffold group, showing that the scaffold alone could support alveolar bone regeneration. Although the CaP-PCL scaffold had similar potential to promote bone formation as the ABC and PDLC sheets, the effect of the scaffold on periodontal attachment formation was limited. These findings indicate that the CaP-PCL scaffolds could promote bone formation in periodontal defects, but needs to be combined with the cell sheets to achieve periodontal attachment formation and cementum regeneration.

5 Conclusion

The CaP-PCL scaffold promoted bone formation within the periodontal defect, but had limited effect on the formation of a functional periodontal attachment. Combined with the CaP-PCL scaffold, the PDLC sheets performed the best in terms of promoting new periodontal attachment formation. The ABC sheets facilitated the formation of new

attachment to a moderate extent, suggesting that ABCs may be an alternative to PDLCs for periodontal tissue engineering therapies. The GMC cell sheet did not promote either bone, cementum or new attachment formation; on the contrary, it had an inhibitory effect on bone formation, suggesting that these cells are poor candidates for utilization in periodontal tissue engineering.

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Figure legends:

Fig. 1: A: Flow chart of cell culture, cell sheet harvesting and transplantation. B and D: morphology of the CaP-PCL scaffold; C and E: morphology of the covering membrane; D and E display the morphology of the structures under Scanning Electron Microscopy (SEM).

Fig. 2: Methods used to calculate the formation of new bone, cementum and periodontal ligament. A: the percentage of new bone formation was calculated by dividing the area of new bone (white outline) by the area of the defect (black outline) using ImageScope software (Aperio). B-C: the percentage of new cementum (black arrow) formation was calculated by the length of the exposed root surface covered by mineralized tissue with inserted fibers divided by length of the root surface. D-E: the percentage of new periodontal ligament formation was calculated by the length of the exposed root surface covered by fibers with an inserting angle greater than 60 degrees divided by the length of the exposed root surface.

Fig. 3: Osteogenic differentiation potential of periodontal tissue derived primary cell cultures. A: Von Kossa staining; B: hydroxyapatite formation was assessed in gingival margin (GMC), alveolar bone (ABC) and periodontal ligament (PDLC) primary cell sheets from four subjects following culture in osteogenic media for 4 weeks. A commercial dermal fibroblast cell line was used as a negative control. C: Calcium content using Alizarin Red S staining and D: bone sialoprotein (BSP) relative gene expression following culture in osteogenic media were assessed in one representative set of matched primary cell lines that were subsequently used for the in vivo periodontal defect model.

Fig. 4: A: representative microCT reconstruction images showing new bone formation in the different groups at 1 and 4 weeks. New bone formation (white arrow) was observed in the Scaffold, ABC and PDLC groups at 4 weeks. B: representative hematoxylin and eosin stained sections of the defects at 1 and 4 weeks. At 1 week, new bone was observed in the Scaffold and OB groups. b: bone; nb: new bone; c: cementum; d: dentin; black arrow: scaffold. At 4 weeks, new bone formation was observed in all groups. b: bone; nb: new bone; c: cementum; d: dentin; s: scaffold.

Fig. 5: Histomorphometry of bone, new cementum and periodontal ligament formation. A: hematoxylin and eosin and Azan staining indicating new cementum and periodontal ligament formation at 4 weeks (white arrow: new cementum; black arrow: functionally oriented periodontal ligament). B: percentage of the defect covered by new bone at different time points. *: $P<0.05$ compared to Blank group; ▼: $P<0.05$ compared to Scaffold group. C: percentage of exposed root surface covered by newly formed mineralized tissue with inserted collagen fibers. D: percentage of the exposed root surface that had inserted fibers with an angle greater than 60 degrees to the root surface. *: $P<0.05$ compared to Blank group; ▼: $P<0.05$ compared to Scaffold group.

Fig. 6: A-E: immunostaining of BSP and OPN. A and D: Positive staining of BSP and OPN (black arrow) was detected in bone and cementum of the native periodontium. B and E: positive staining of BSP and OPN (black arrow) in the newly formed bone and cementum for the samples with implanted cell sheets. C and F: in the samples without new attachment formation, no expression of BSP or OPN was detected on the denuded root surface. G-L: tracking of transplanted cells. Sections from periodontal defects transplanted with GMC, ABC and PDLC cells at different time points were stained with

human-specific anti-nuclei antibody. Black arrows show the positive staining exemplifying the presence of transplanted human cells within the regenerated area; nb: new bone; s: scaffold.

Figure 1

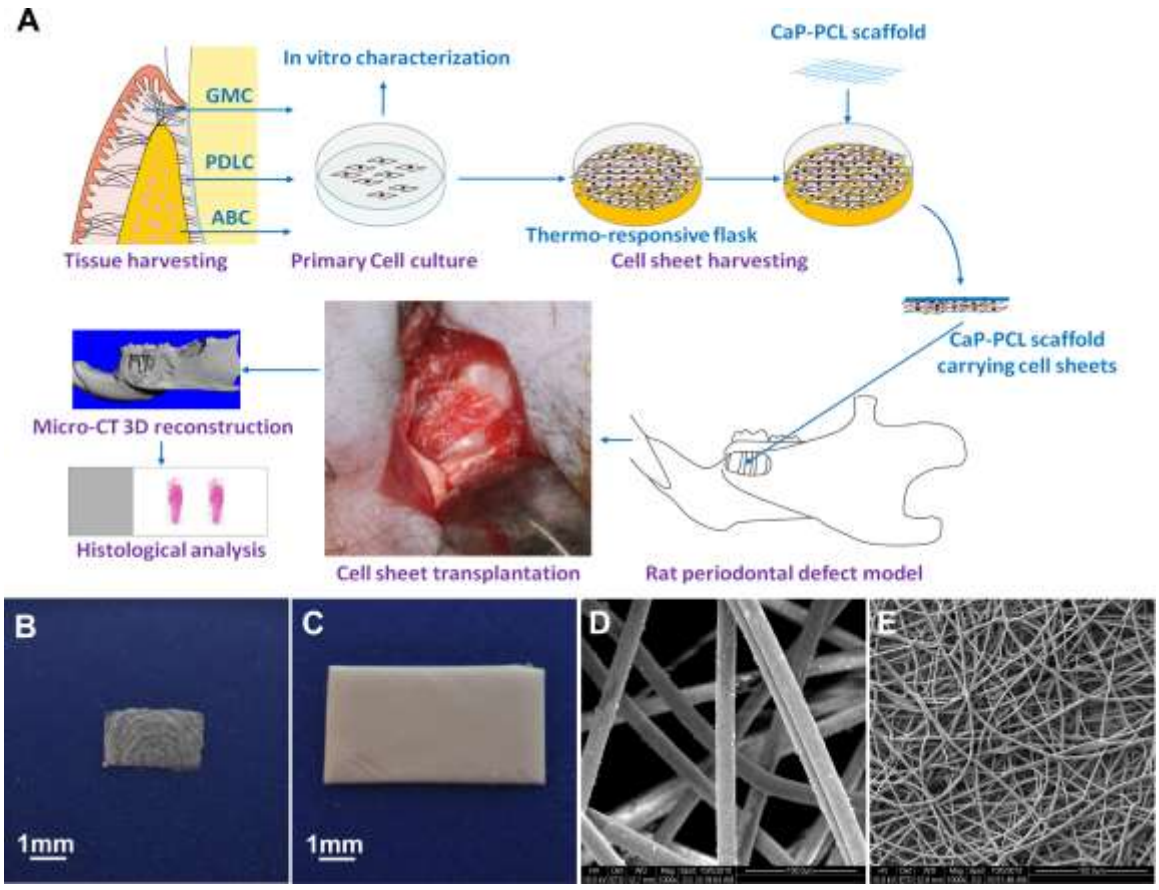

A
In vitro characterization
CaP-PCL scaffold
GMC
PDLC
ABC
Tissue harvesting
Primary Cell culture
Thermo-responsive flask
Cell sheet harvesting
CaP-PCL scaffold
carrying cell sheets
Micro-CT 3D reconstruction
Histological analysis
Cell sheet transplantation
Rat periodontal defect model
B
1mm
C
1mm
D
E

Figure 2

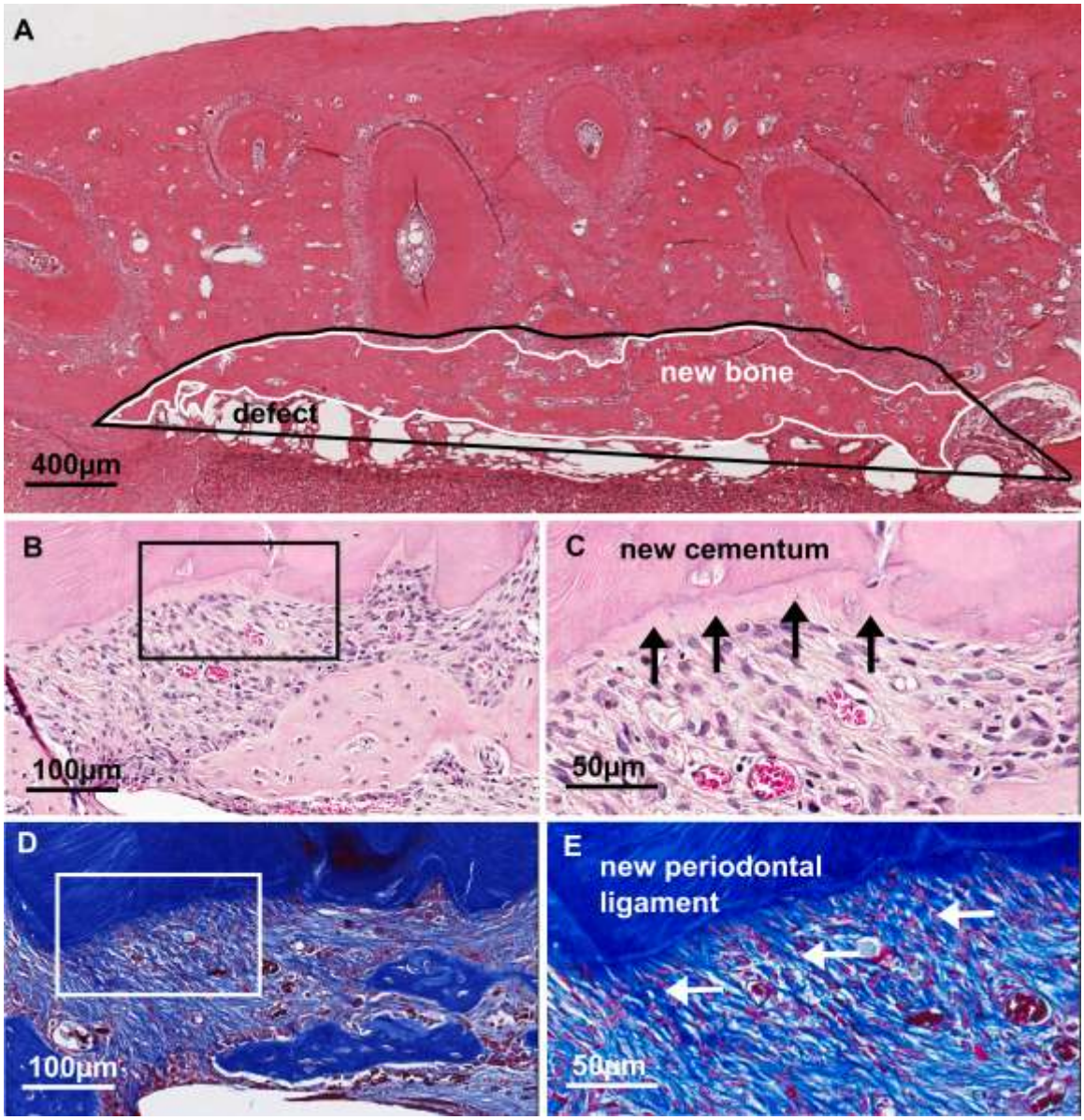

A
new bone
defect
400μm
B
100μm
C
new cementum
50μm
D
100μm
E
new periodontal
ligament
50μm

Figure 3

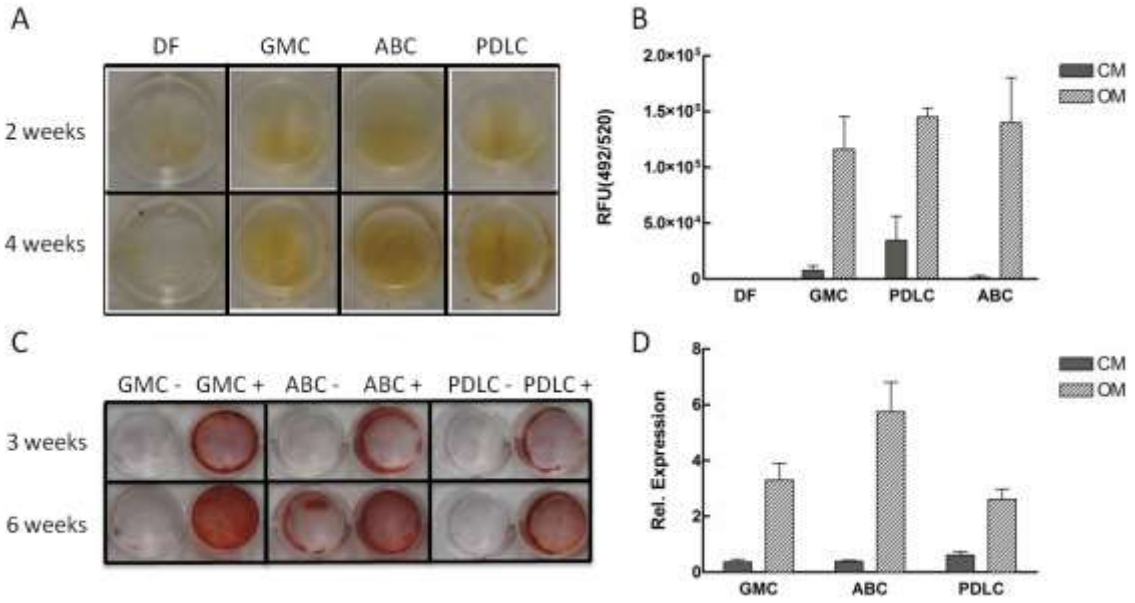

A
DF
GMC
ABC
PDLC
2 weeks
4 weeks
B
RFU(492/520)
2.0×10⁵
1.5×10⁵
1.0×10⁵
5.0×10⁴
0
DF
GMC
PDLC
ABC
CM
OM
C
GMC -
GMC +
ABC -
ABC +
PDLC -
PDLC +
3 weeks
6 weeks
D
Rel. Expression
8
6
4
2
0
GMC
ABC
PDLC
CM
OM

Figure 4

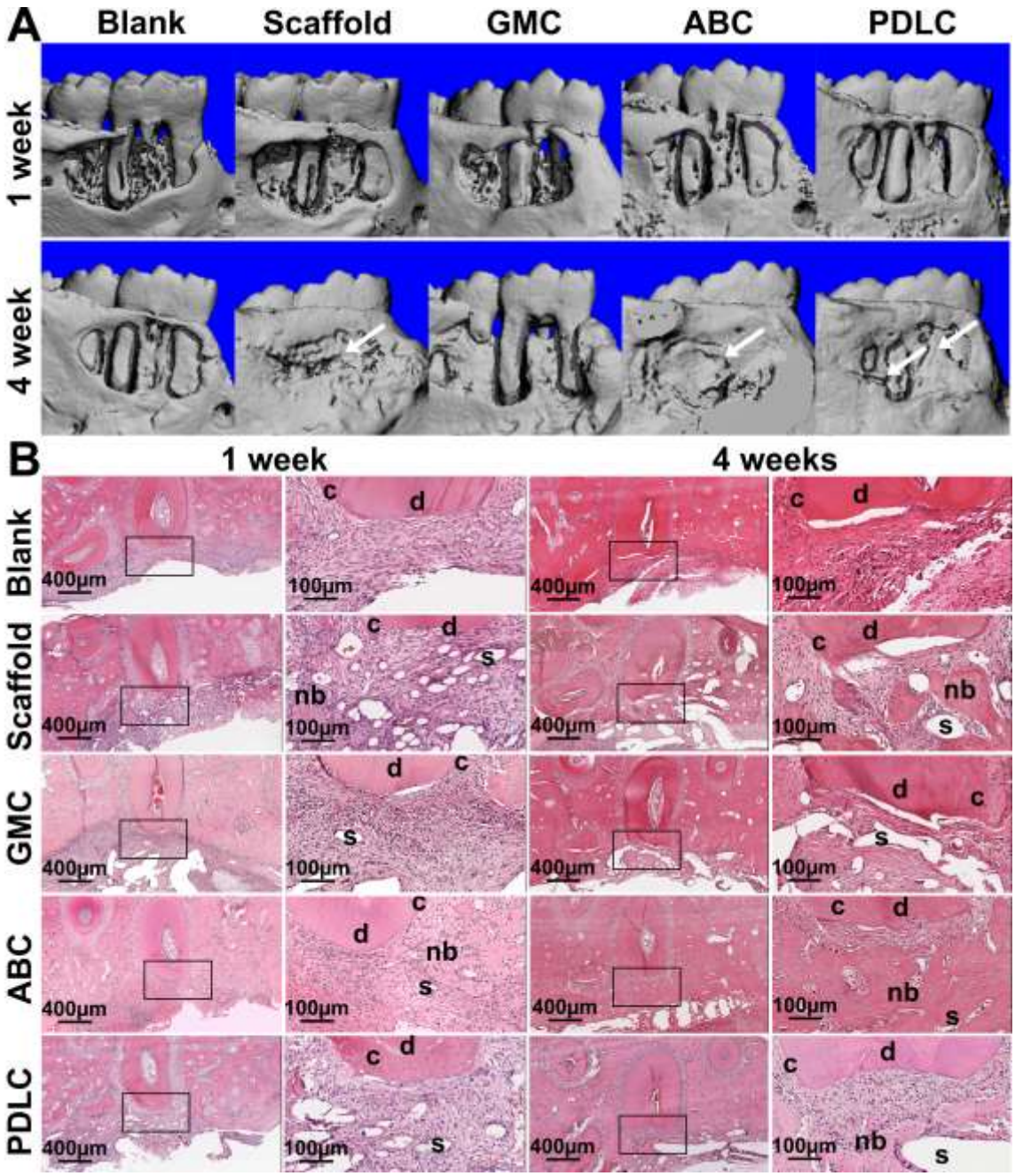

A
Blank
Scaffold
GMC
ABC
PDLC
1 week
4 week
B
1 week
4 weeks
Blank
Scaffold
GMC
ABC
PDLC
400μm
100μm
c
d
s
nb

Figure 5

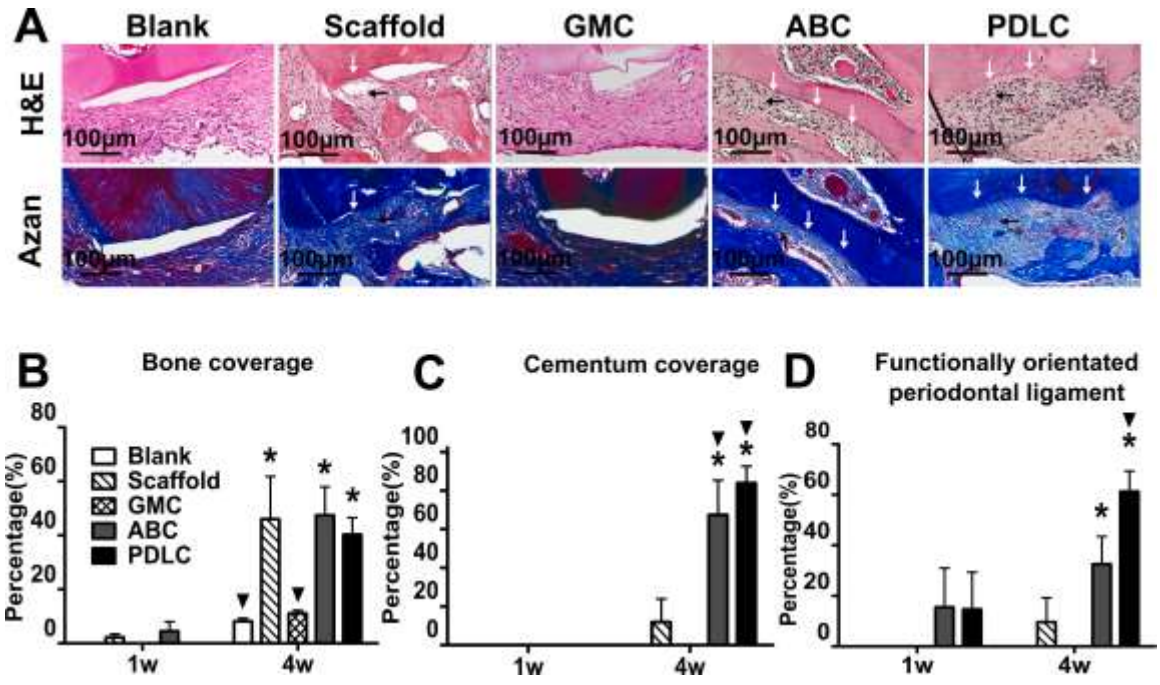
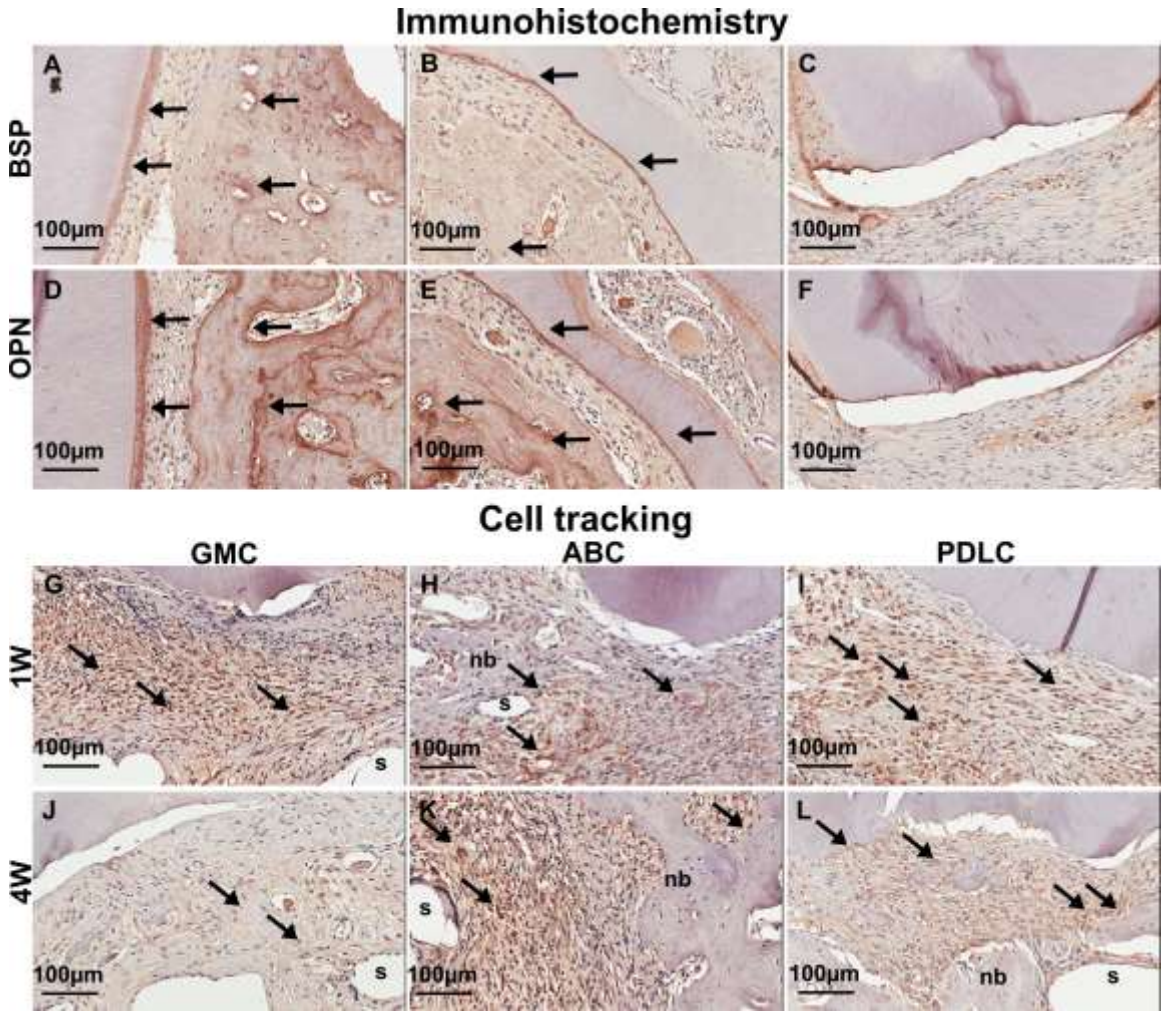

A
Blank
Scaffold
GMC
ABC
PDLC
H&E
Azan
100μm
B
Bone coverage
Percentage(%)
Blank
Scaffold
GMC
ABC
PDLC
1w
4w
C
Cementum coverage
D
Functionally orientated periodontal ligament

Figure 6


Immunohistochemistry
BSP
OPN
A
B
C
D
E
F
100μm
Cell tracking
GMC
ABC
PDLC
1W
4W
G
H
I
J
K
L
nb
s