Spatial Controls of Ligamentous Tissue Orientations Using the Additively Manufactured Platforms in an In Vivo Model: A Pilot Study

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Abstract: The periodontal ligaments (PDLs) with specific orientations to tooth-root surfaces play a key role in generating biomechanical responses between the alveolar bone and cementum as a tooth-supporting tissue. However, control of angulations and regeneration of the ligamentous tissues within micron-scaled interfaces remains challenging. To overcome this limitation, this study investigated surface fabrications with microgroove patterns to control orientations of rat PDL cells in vitro and fibrous tissues in vivo. After being harvested, rat PDL cells were cultured and three different microgroove patterns (∠PDL groove = 0°, ∠PDL groove = 45°, and ∠PDL groove = 90°) were created by the digital slicing step in 3D printing. Cell-seeded scaffolds were subcutaneously transplanted at 3 and 6 weeks. In histology images, rat PDL cells were spatially controlled to angularly organize following the microgroove patterns and fibrous tissues were formed in scaffolds with specific angulations, which were reflected by additively manufactured microgroove topographies. Based on the results, specifically characterized surface topographies were significant to directly/indirectly organizing rat PDL cell alignments and fibrous tissue orientations. Therefore, interactions between surface topographies and tissue organizations could be one of the key moderators for the multiple tissue complex (bone-ligament-cementum) neogenesis in periodontal tissue engineering.

Keywords: periodontal ligament (PDL); biomaterials; regenerative medicine; tissue engineering; 3D printing; periodontal tissue

1. Introduction

The major component in periodontal tissues is alveolar bone, which is a mineralized tissue to support teeth and form geometrically tooth-adaptable sockets by the bone remodeling process [1,2]. In physiological alveolar bone remodeling, periodontal ligaments (PDLs) play a significant role in transmitting mechanical forces or distributing various physical stresses in masticatory or occlusal loading conditions [3,4] with tautly anchored fibrous connective tissue bundles between two different types of mineralized tissues, such as alveolar bone and cementum [3,5]. In particular, collagenous PDL bundles have specific orientations to tooth-root surfaces, such as perpendicular or oblique angulations [6,7] and they play significant roles in generating mechanical, physiological, or biological responses against external stimuli for natural tooth preservation [8–10].

Periodontitis is a widespread chronic inflammatory disease that induces tooth-supporting tissue destruction, which is generally initiated by cytokines or biochemicals from oral microorganisms [11,12]. In the case of severe periodontal disease, it is usually necessary to extract teeth and place dental prosthetics with tooth-extraction socket healing [13,14]. For the tooth replacement and stability of dental implants, various approaches have been concentrated on promoting bone regeneration in tooth-extraction sites or inducing osseointegration to the dental implant surfaces using biomaterials, bioactive molecules, or stem
cells [15–19]. However, advanced tissue engineering techniques have currently focused on the regeneration of tooth-supporting tissue complexes to save natural teeth by extending their lives, instead of using dental implants. For natural tooth preservation, the PDL formation is required to spatially secure PDL tissue interfaces within 250–300 µm thickness, with hierarchical compartmentalization between mineralized tissues (alveolar bone and cementum) including alveolar bone formation [3,20]. However, it has been challenging to fabricate micron-scaled 3D platforms to promote regeneration of engineered PDL structures and to control specific orientations of engineered PDL bundles for perpendicular or oblique tissue angulations to tooth surfaces [4,20]. The previous in vitro studies demonstrated that the specific micron-scaled topographies could moderate angular orientations of ligament cells after 7- and 21-day incubations [21]. In addition to angular pattern controls, the specific microgroove interval (25.40 µm) was a key factor for directional organizations of PDL cells with significant predictability; cell orientations on surfaces of PDL-guiding architectures at 7 and 21 days [22]. However, it was difficult to promote aligned fibrous tissue formations in scaffolds instead of cell organizations on the scaffold surfaces in the previous in vitro studies. In this pilot study using the mouse subcutaneous model, fibrous tissue formation and the fibrous bundle orientations were investigated using the PDL-guiding scaffolds with the 25.40 um microgroove interval, which was investigated in previous studies as the optimal topography [21,22]. This in vivo investigation demonstrated that the microgroove patterns can predictably control rat PDL cell alignments on surfaces of scaffolds and fibrous tissue bundle orientations in the scaffold interspaces, which were void regions between PDL-guiding architectures.

2. Materials and Methods

2.1. Harvesting and Primary-Culturing Rat PDL Cells

Primary rat PDL cells were provided as a kind gift from Dr. Joong-Hyun Kim (KBIO Health Osong Medical Innovation Foundation, Cheongju, Chungbuk, Korea). Briefly, rat PDL tissues were primarily obtained from the mandibles and maxillae of Sprague Dawley rats (Figure 1). After isolation of rat PDL cells from the surface of each incisor tooth, harvested tissues were digested in enzymatic solutions such as 0.2% collagenase type I (Worthington Biochem, Freehold, NJ, USA) and 0.1% dispase (Roche, Mannheim, Germany) for 3 h at 37 °C. The growth culture medium for the primary culture of rat PDL cells was prepared with minimum essential medium alpha (α-MEM; Hyclone, Cytiva, Marlborough, MA, USA), with 10% fetal bovine serum and antibiotics (100 units/mL penicillin). During the cell culture and incubation, growth media were changed every 4 days, and rat PDL cells were trypsinized and passaged (P1) at 80–90% confluency. For this study, rat PDL cells were cultured by passage 3.

![Figure 1](image-url)

**Figure 1.** Rat periodontal ligament (PDL) tissues are harvested from the incisors of mandibles, and primary cells growing from the tissue origin are cultured up to the passage number 3. A yellow arrow indicates PDL tissue on the incisor surface and a white arrow points the extracted incisor from the rat mandible.

2.2. Scaffold Manufacturing with the Creation of Angular Surface Topographies

The previous study demonstrated design and manufacturing methods of PDL-guiding scaffolds with three different angulated microgroove patterns [21]. Briefly, designed scaffold molds were manufactured using a 3D wax printer (Solid scape 3Z Studio; Solidscape®, Inc., Merrimack, NH, USA) with 0°, 45°, and 90° angulated pattern surfaces [21]. After
poly-ε-caprolactone (PCL; MW 43–50 kDa, Polysciences Inc., Warrington, PA, USA) was dissolved in 1,4-dioxane to make a 25% solution, the PCL solution was cased into the mold and scaffolds were created (Figure 2).

Figure 2. Experimental steps for designing, manufacturing, characterizing, and transplanting scaffolds. (a) Three different angulated microgroove patterns are designed and created on surfaces of periodontal ligament (PDL)-guiding architectures (∠PDL groove = 0°, ∠PDL groove = 45°, and ∠PDL groove = 90°). (b) Polymer casting wax molds are manufactured using 3D printing system and poly-ε-caprolactone (PCL) scaffolds are created by casting PCL to the casting mold, which could be obtained after removing blue wax material (build material) from the trimmed models. (c) The confocal microscope is utilized to characterize surface topographies with three different angles. The red boxes demonstrate that topography profiles can show specific groove intervals (25.40 µm). Moreover, a scanning electron microscope (SEM) can provide a high correlation of the microgroove patterns and 3D topographic images by the confocal microscope images. (d) After rat PDL cells are seeded into the scaffolds, cell-scaffold constructs are transplanted subcutaneously and harvested at 3- and 6-week timepoints. Yellow arrows indicate blood vessels, which are formed around scaffolds.

2.3. Rat PDL Cell Seeding to the Scaffold for In Vitro and In Vivo Studies

After passage 3 of the primary culture of rat PDL cells, 1.0 × 10^5 cells were seeded into all scaffolds (for the in vitro study, n_in_vitro = 3 per angular topography, which were ∠PDL groove = 0°, ∠PDL groove = 45°, and ∠PDL groove = 90°) and cell-scaffold constructs were incubated for 7 days. After a cell fixation step using 4% formaldehyde for 30 min at room temperature, fluorescence analyses were performed for the morphological qualifications, cell orientations or polarities following the angular microgroove patterns of PDL-guiding scaffolds by DAPI (4′,6-diamidino-2-phenylindole, Thermo Fisher Scientific, Waltham, MA, USA) and F-actin staining with phalloidin (Alexa Fluoro® 546 Phalloidin, Life Technologies, Carlsbad, CA, USA). The confocal microscope (ZEISS LSM700, Carl Zeiss Microscopy, LLC., Oberkochen, Germany) and the software (ZEISS ZEN, Carl Zeiss Microscopy, LLC.) created 2D projected images and z-stacked 3D images, which showed the morphological significance on surfaces of PDL-guiding architectures (Figure 3).

2.4. Quantification of Nuclear Deformation and Circularity

For the statistical analyses of cell shapes, nuclear deformation and circularity were calculated with the nuclear aspect ratio (NAR) and the nuclear shape index (NSI), respectively. The NAR was calculated with the long- and short axis of individual cell nuclei, and the NSI was analyzed with the perimeters and area of nuclei by ImageJ software (National Institute of Health; NIH, Bethesda, MD, USA).
Figure 3. The angulations and orientations of rat PDL cells were qualitatively analyzed using the fluorescence staining method with cell nuclei and actin filaments after 7-day cultures. Nuclear angulations and cytoskeleton polarities (or cell orientations) can be qualitatively determined to the reference, which was determined to be the parallel direction to the PDL-guiding architectures. White dash-lines represented PDL-guiding architecture border lines. Scale bars: 200 µm.

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2.5. In Vivo Experiments with the Cell-Scaffold Construct Transplantation

Two different surgical pockets on the dorsa of 6-week-old NIH III nude mice (approximately 24.51 ± 1.29 g; total n_{animal} = 10) were surgically created. Three different groups (n_{in vivo} = 3 per group with three different groups) were designed with 3- and 6-week timepoints. The experimental groups consisted of ∠PDL groove = 0°, ∠PDL groove = 45°, and ∠PDL groove = 90° after seeding 1.0 × 10^5 rat PDL cells in individual scaffolds and incubating them for 7 days. Under isofluorane anesthesia, incubated cell-scaffold constructs were implanted subcutaneously into surgically created pockets and the incisions were closed with surgical staples. All animals were euthanized with carbon dioxide (CO_2) and specimens were harvested. Animals were cared for and treated in accordance with guidelines established by the Kyungpook National University Institutional Animal Care and Use Committee (KNU-IACUC).

2.6. Histological Analysis for Fibrous Tissue Formation with Hematoxylin and Eosin (H&E) Staining

Scaffolds harvested after 3 and 6 weeks were fixed in 10% buffered formalin phosphate solution for 1 day, and then embedded in paraffin blocks for histological sectioning and
H&E staining. Using the light microscopy, images were captured and angular organized tissues were qualitatively analyzed (Figures 4 and 5).

![Image](image_url)

**Figure 4.** Hematoxylin and eosin (H&E) stained histology is evaluated for fibrous tissue formation and orientations between PDL-guiding architectures at 3 weeks. Black arrowed-dash lines represent the fibrous tissue directionality. Yellow arrows indicate blood vessels. The scale bars of low magnification and high magnification are 250 µm and 50 µm, respectively.

![Image](image_url)

**Figure 5.** H&E histology is evaluated for fibrous tissue formation and orientations between PDL-guiding architectures at 6 weeks. Black arrowed-dash lines represent the fibrous tissue directionality. Yellow arrows indicate blood vessels. The scale bars of low magnification and high magnification are 250 µm and 50 µm, respectively.

### 2.7. Statistical Analysis

After images from the in vitro study were assessed quantitatively, every group in the in vitro study was statistically analyzed for NASR and NSI using the one-way analysis of variance (one-way ANOVA) test with the α-value set at the 0.05 level of significance. Data were described using mean ± standard deviation.
3. Results

3.1. Morphological Analyses of Rat PDL Cell Alignments In Vitro

PDL-guiding scaffolds were designed and manufactured following the previous study, which created three different angulated microgroove patterns on surfaces of PDL-guiding architectures (\(\angle\)PDL groove = 0°, \(\angle\)PDL groove = 45°, and \(\angle\)PDL groove = 90°) [21]. After seeding 1.0 \(\times\) 10^5 rat PDL cells (primary-cultured) into the scaffolds, cell-scaffold complexes were cultured for seven days to analyze angular organizations of cells on surfaces of scaffolds, which had three different microgroove patterns. Using fluorescence-staining methods for nuclei (DAPI) and actin filaments (F-actin), angular organizations of rat PDL cells were analyzed qualitatively and morphologically as in previous in vitro studies described [21,22] (Figure 3). Similar to the previous in vitro study [21], angulations of rat PDL cells showed morphological similarity to angular microgroove patterns; nuclear angulation and nuclear deformation (Figure 3), which were parameters to assess cell shapes and orientations with NAR and NSI. The NAR for the nuclear deformation was quantified with the calculations with long axis/short axis using DAPI-stained nuclei; NAR (\(\angle\)PDL groove = 0°) = 2.42 ± 0.26, NAR (\(\angle\)PDL groove = 45°) = 2.61 ± 0.15, and NAR (\(\angle\)PDL groove = 90°) = 2.62 ± 0.14. The NSI for nuclear circularity was calculated with \((4\pi \times \text{area})/\text{perimeter}^2\) of a nucleus; NSI (\(\angle\)PDL groove = 0°) = 0.62 ± 0.038, NSI (\(\angle\)PDL groove = 45°) = 0.63 ± 0.013, and NSI (\(\angle\)PDL groove = 90°) = 0.62 ± 0.0069. Both the NAR and NSI showed no significant differences following the statistical analyses; \(p\)-value (NAR) = 0.390 and \(p\)-value (NSI) = 0.796. Therefore, the microgroove patterns could regulate morphological changes of nuclei in vitro, but cells in every group could provide similar shapes based on the statistical quantifications of deformation and circularity of cell nuclei.

In addition, although the seeded cell numbers (1.0 \(\times\) 10^5 rat PDL cells) were different from the previous studies (1.0 \(\times\) 10^3 human PDL cells), individual experimental groups (\(\angle\)PDL groove = 0°, \(\angle\)PDL groove = 45°, and \(\angle\)PDL groove = 90°) showed critically controlled cell angulations by the microgroove-patterned platforms of PDL-guiding scaffolds (Figure 3).

3.2. Histological Analyses of Rat PDL Tissue Orientations

Using the hematoxylin and eosin (H&E) staining method, three different fibrous tissue orientations were analyzed between PDL-guiding architectures at 3 and 6 weeks (Figures 4 and 5). Although the in vitro study demonstrated that seeded rat PDL cells were angularly organized following created surface topographies, the limitation was showing tissue alignments in scaffold interspaces, which were void regions between PDL-guiding struts. Interestingly, in the in vivo study, it was found that typical tissue morphologies were formed with critical alignments following created surface topographies (Figures 4 and 5). In particular, three topographical discrepancies led to significantly different tissue alignments at 3 and 6 weeks using parallel, oblique, and perpendicular angulations by angled microgroove patterns: \(\angle\)PDL groove = 0°, \(\angle\)PDL groove = 45°, and \(\angle\)PDL groove = 90°, respectively (Figures 4 and 5). Although the groups had low tissue densities between PDL architectures at the early timepoint (3 weeks), fibrous tissues with collagenous matrices had similar angulations to the surface patterns; the results could demonstrate that tissue alignments were possibly controlled by the surface patterns with angulated cells on the PDL-guiding architectures (Figure 4). Interestingly, fibrous tissue structures in all groups were significantly matured, and their angular organizations with invaded vascular structures into scaffolds could be controlled by specific surface patterns with predictability at 6 weeks (Figure 5).

4. Discussion

Various strategies have been developed to promote periodontal tissue regeneration using bioactive molecules, biomaterials, or their fabrication techniques [4,23,24]. In order to provide primary stability of dental implants or prosthetics after natural tooth extraction, the alveolar bone of periodontal tissues has been a major target tissue to regenerate or repair [25]. However, the tissue engineering paradigm has shifted to tissue regeneration...
and functioning restoration of tissue complexes for natural tooth preservation rather than tissue replacements with single bone formation [26–28]. In particular, digital design and manufacturing techniques of scaffolds have been rapidly developed in order to provide appropriate bioactive microenvironments and specific localization of tissue formation in sites defected by diseases or traumatic injuries [29–31]. Moreover, the investigations for local tissue regeneration could provide significant approaches to overcome limitations of systemic drug delivery systems; (1) the availability to affect other organs, (2) requirement of high concentrations and quantitative optimization of biologics, and (3) uncontrolled activities of factors with less effectiveness at distant wound sites [10,32,33].

Recently, periodontal tissues such as PDL or cementum have been studied as multiple tissue neogenesis, with spatial compartmentalization of hierarchical structures and functioning restoration of regenerated tissues to support natural tooth constructs [20,34]. Of periodontia, fibrous connective tissues (PDL) have a specific orientation and are anchored to two different surfaces of mineralized tissues (alveolar bone and cementum) with Sharpey’s fibers [3,35]. The natural PDLs with perpendicular or oblique orientations to tooth-root surfaces play a significant role in spatially distributing masticatory/occlusal stress [2,6] or contributing to mineralized tissue remodeling [36].

Although some techniques have been developed to spatially provide the small dimension of PDL interspaces (approximately 250–300 µm), it is still limited to predictably controlled specific angulations of PDL cells and fibrous tissue bundles using 3D engineering platforms [4,37]. Park et al. demonstrated that surface artifacts (or stair-stepping errors) by the additive manufacturing technique could be reinterpreted as microgroove patterns on the scaffold surfaces, and it could be highly predictable and reproducible with specific angulations. Using angulated topographies, cell orientations and cytoskeletal polarities could be modulated for 7 and 21 days in vitro [21] and the follow-up study recently reported the optimization of microgroove intervals to regulate cell angulations [22].

Based on the achievement of organizing highly populated PDL cells following angular topographies, which were created during the slicing process of 3D printing [21,22], this study investigated fibrous tissue formations and the tissue orientations in the interspace regions between PDL-guiding architectures in the scaffold, as well as cell alignments. After rat PDL cells were harvested from the incisors and primarily cultured by passage number three, rat PDL cells were seeded into scaffolds with three different topographies, and cell-scaffold constructs were subcutaneously transplanted into the immunodeficient nude mouse model (Figure 1). After 3 and 6 weeks, vasculature formations around scaffolds could be found clearly without any clinical signs such as inflammation or infection (Figure 2); histology images also showed that blood vessels infiltrated into the scaffolds (Figures 4 and 5). As vasculatures can promote and accelerate tissue differentiation or morphogenesis by supplying oxygen and nutrients to tissues [38–40], angiogenesis or vasculogenesis could be essential to regulating physiological tissue regeneration processes and facilitating pathological tissue maturition [41]. In particular, global pore architectures of a scaffold lead to blood vessel infiltration and neo-vascular formation from pre-existing vasculature in tissues; thus, vasculature can affect the physiological microenvironments of the ligament tissue development and regeneration in this study (Figures 4 and 5).

In addition to the vascular formation and infiltration of scaffolds, an interesting finding was that fibrous tissues were formed along with specific alignments in the interspace regions, which was between PDL-guiding architectures in the scaffold, as well as cell alignments. After rat PDL cells were harvested from the incisors and primarily cultured by passage number three, rat PDL cells were seeded into scaffolds with three different topographies, and cell-scaffold constructs were subcutaneously transplanted into the immunodeficient nude mouse model (Figure 1). After 3 and 6 weeks, vasculature formations around scaffolds could be found clearly without any clinical signs such as inflammation or infection (Figure 2); histology images also showed that blood vessels infiltrated into the scaffolds (Figures 4 and 5). As vasculatures can promote and accelerate tissue differentiation or morphogenesis by supplying oxygen and nutrients to tissues [38–40], angiogenesis or vasculogenesis could be essential to regulating physiological tissue regeneration processes and facilitating pathological tissue maturition [41]. In particular, global pore architectures of a scaffold lead to blood vessel infiltration and neo-vascular formation from pre-existing vasculature in tissues; thus, vasculature can affect the physiological microenvironments of the ligament tissue development and regeneration in this study (Figures 4 and 5).

In addition to the vascular formation and infiltration of scaffolds, an interesting finding was that fibrous tissues were formed along with specific alignments in the interspace regions, which was between PDL-guiding architectures (Figures 4 and 5). Although the short-term timepoint (3 weeks) had a low density of newly formed fibers, orientations of the limited tissues were significantly correlated with engineered angular topographies on the surfaces of the PDL-guiding architectures (Figure 4). In 6-week transplantation groups, fibrous tissue bundles were newly formed with high density and angularly organized with the specific orientations in interspaces of scaffolds following three different surface topographies: ∠PDL groove = 0°, ∠PDL groove = 45°, and ∠PDL groove = 90° (Figure 5). Due to the multipotency of PDL cells for various cell differentiation such as osteogenesis, adipogenesis, chondrogenesis, angiogenesis, or neurogenesis [42–44], it has
guiding architectures.

Figure 6. The schematic illustration shows the rat PDL cell orientations on PDL-guiding structure surfaces with angulated microgroove patterns and fibrous tissue alignments in the interface of PDL-guiding architectures.

5. Conclusions

Of various strategies, specific surface topographies and 3D scaffolding systems have been one of the major approaches to spatiotemporally controlling angular morphogenesis of cells or tissues. Previous preliminary studies and this pilot study have concentrated on the 3D-printing system (or additive manufacturing system), which can rapidly and easily manufacture various 3D microarchitectures with micron-scaled patterns. Using topographical cues of microgrooves based on preliminary studies, rat PDL cells can be spatiotemporally regulated to form and organize angular fibrous tissue bundles in vivo at 3 and 6 weeks, and cell orientations on the PDL-guiding architecture surfaces in vitro. In conclusion, specific surface topographies with the optimized microgroove interval (25.40 μm) and three different microgroove angles (∠PDL groove = 0°, ∠PDL groove = 45°, and ∠PDL groove = 90°) can show angular patterns of rat PDL cells on scaffold surfaces (in vitro) and significantly concern tissue orientations (in vivo). Although this investigation was of in vivo tissue orientations using the subcutaneous model and immunodeficient mice, this study significantly demonstrated that specifically characterized topographies can directly/indirectly organize fibrous tissue orientations with structural similarities to...
natural PDL bundles and provide microstructural cues to form bone-ligament complexes in periodontal tissue engineering.

Author Contributions: M.G.K. cultured rat periodontal ligament (PDL) cells and analyzed histology images; C.H.P. designed/manufactured the scaffolds, performed in vitro experiments, and edited figures; M.G.K. and C.H.P. performed surgeries to transplant cell-scaffold constructs for in vivo study and wrote the manuscript as well as revised the manuscript. Both authors have read and agreed to the published version of the manuscript.

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