



# Age-related decline in the matrix contents and functional properties of human periodontal ligament stem cell sheets



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## ABSTRACT

In this study, periodontal ligament (PDL) stem cells (PDLSCs) derived from different-aged donors were used to evaluate the effect of aging on cell sheet formation. The activity of PDLSCs was first determined based on their colony-forming ability, surface markers, proliferative/differentiative potentials, senescence-associated  $\beta$ -galactosidase (SA- $\beta$ G) staining, and expression of pluripotency-associated transcription factors. The ability of these cells to form sheets, based on their extracellular matrix (ECM) contents and their functional properties necessary for osteogenic differentiation, was evaluated to predict the age-related changes in the regenerative capacity of the cell sheets in their further application. It was found that human PDLSCs could be isolated from the PDL tissue of different-aged subjects. However, the ability of the PDLSCs to proliferate and to undergo osteogenic differentiation and their expression of pluripotency-associated transcription factors displayed age-related decreases. In addition, these cells exhibited an age-related increase in SA- $\beta$ G expression. Aged cells showed an impaired ability to form functional cell sheets, as determined by morphological observations and Ki-67 immunohistochemistry staining. Based on the production of ECM proteins, such as fibronectin, integrin  $\beta$ 1, and collagen type I; alkaline phosphatase (ALP) activity; and the expression of osteogenic genes, such as *ALP*, *Runt-related transcription factor 2*, and *osteocalcin*, cell sheets formed by PDLSCs derived from older donors demonstrated a less potent osteogenic capacity compared to those formed by PDLSCs from younger donors. Our data suggest that the age-associated decline in the matrix contents and osteogenic properties of PDLSC sheets should be taken into account in cell sheet engineering research and clinical periodontal regenerative therapy.

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## 1. Introduction

Periodontitis is a ubiquitous disease induced by bacterial infection that causes pathological alterations in the periodontium, and if untreated, it leads to the continual destruction of the tooth-supporting tissues until the tooth is finally lost [1]. Periodontitis is the primary cause of tooth loss in adults, and the development of effective therapies to treat periodontitis and restore damaged periodontal structures has long been an important goal of dental medicine [2,3]. Current clinically available therapies, such as tooth scaling, root planing and open flap debridement, are generally effective in removing pathogenetic agents, eliminate active disease, and promote tissue repair. Other techniques, such as bone grafting and protein product use, have largely been applied to

guide regeneration of the damaged periodontium; where the reestablishment of lost supporting structures of the tooth is the ultimate purpose [4]. Although concerted efforts have been and still are being made in this field and although satisfactory bone repair has been achieved in several ideal clinical scenarios, the regeneration of lost periodontal structure to its original form in a predictable way remains unrealized. Human periodontal tissue exhibits very restricted regeneration, and little is known about factors that might modulate its healing potential, rendering various strategies and materials to regenerate the periodontium pursued over the past decades relatively fruitless [3]. Recently, stem cell therapy, a nascent but rapidly expanding field, has been introduced into the periodontal arena to potentially overcome the limitations of conventional regenerative procedures, leading to more effective therapeutics for predictable periodontal regeneration [5–8]. In particular, *in vitro*-expanded cell populations derived from autologous PDL tissue have already been utilized in the clinic for the treatment of human periodontitis [9–12].

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Although stem cell therapies have been progressing rapidly in the field of periodontal regenerative medicine and although several clinical trials have already been started or are in preparation, the standard protocol in terms of the best cell populations and their delivery mode remains to be optimized [9,12–15]. In animal models, local administration of dissociated cells was found to exert anti-inflammatory and immunomodulatory effects in addition to repairing defects caused by periodontitis [16]. Unfortunately, poor survival of the transplanted cells with regard to traditional delivery models (e.g. cell suspension injection) might impede the expected therapeutic effects in humans [9].

In recent years, cell sheet engineering has rapidly progressed to allow the development of confluent cell sheets for delivering therapeutic cells along with their *in vitro*-secreted extracellular matrix (ECM) in the field of regenerative medicine [17,18]. In using this unique, scaffold-free method of cell processing, the preservation of cell-growing microenvironments in terms of various mechanical, chemical, and biological properties as well as cell–matrix and cell–cell interactions may minimize cell loss and increase cell survival rate and engraftment after cell sheet transplantation [17]. In this regard, cell sheet technology is an innovative method to regenerate defective tissues during periodontal regenerative procedures, where cell sheets have been frequently used in combination with clinically available filler materials. The overall procedure was based on guided tissue regeneration techniques [8,19]. Indeed, in the field of periodontal medicine, this technology has already been safely and effectively used in a wide variety of preclinical studies [20–22] as well as in cytotераpy trials in human patients [8]; most, if not all, clinical periodontal cell therapies have been performed based on this cell delivery strategy [9–12].

The therapeutic benefits of periodontal cell therapy need to be confirmed by randomized controlled clinical trials in a relatively large number of periodontitis patients. If cell sheet technology is used for cell delivery, the functionality of the cell sheets used in the trials, which may be influenced by the cell populations employed [21] as well as by the methods used for cell sheet production [23–25], must be predicted. Previously published data have demonstrated that the multipotent capacity and regenerative potential of human PDLSCs are severely compromised with increasing donor age [26,27]. In the present study, we clarified the potential age-related impairment of the matrix production and functional properties of cell sheets derived from aging cells. The identification of age-related changes in stem cell sheets is crucial for autologous cell therapy development in older subjects, the population that periodontitis typically afflicts. The elucidation of the degree of such alterations may be helpful in analyzing data obtained from forthcoming clinical trials, particularly those using autologous tissue-derived stem cells, because the involved patients may be distributed across different ages.

## 2. Materials and methods

### 2.1. Study design

The human third molars used for cell isolation in the present study were obtained from the Dental Clinic of School of Stomatology, the Fourth Military Medical University, Xi'an, China.

These teeth were extracted due to impaction or non-functional reasons, without the presence of endodontic or periodontal disease. All of the donors were systemically healthy and lacked moderate/severe periodontitis in the teeth neighboring the teeth selected for cell isolation. An informed consent form was signed by each donor before the contribution of his or her teeth to this research project. The Institutional Review Board for Human Subjects Research of the Fourth Military Medical University approved the experimental protocols. The obtained teeth were divided into Groups A, B, and C according to the donors' age as shown in Table 1. If PDLSCs were not successfully obtained from the PDL tissue of a tooth or if the isolated PDLSCs suffered from contamination/damage for technical reasons, the matched teeth were automatically excluded from the current study.

### 2.2. Isolation and purification of PDLSCs

PDLSCs were isolated and cultured from fresh PDL tissues using a tissue-block-based enzymolytic method, as described previously [27]. Briefly, the extracted teeth were rinsed with sterile phosphate-buffered saline (PBS) repeatedly within 2 h of extraction, and the PDL tissue was scraped from the middle part of the root surface of each tooth using sterile surgical blades. The PDL tissue was then cut into tiny blocks (approximately 1 mm × 1 mm × 1 mm) and digested in a mixed solution of 3 mg/mL collagenase type I and 4 mg/mL dispase (both from Sigma–Aldrich, St. Louis, MO, USA) at a ratio of 1:1 for 1 h. After discarding the digestive solution, the digested tissues were suspended with complete medium, and then the digested tissue suspension was transferred into 3.5-cm-diameter culture dishes (Corning, Lowell, MA, USA) containing complete medium, namely,  $\alpha$ -minimum essential medium ( $\alpha$ -MEM; Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Zhejiang, China), 0.292 mg/mL glutamine (Invitrogen Life Technologies, Carlsbad, CA, USA), 100 U/mL penicillin (Gibco BRL) and 100 mg/mL streptomycin (Gibco BRL). Finally, the dishes were incubated at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>, and the medium was freshly changed every 3 days until the primary cells migrated from the tissue blocks and reached confluence. As we have reported previously [23], the limiting dilution technique was used to purify stem cells from the primary cells, and the resultant cells at passages 1–3 (P1–P3) were used in subsequent studies to avoid cell behavioral changes due to long-term *in vitro* culture.

### 2.3. Colony-forming assay

PDLSCs (P3) were plated into 10-cm-diameter culture dishes (Costar, Cambridge, MA, USA) at a density of  $1 \times 10^3$  cells per dish and cultured in complete medium prior to a colony-forming unit-fibroblast (CFU-F) assay. The medium was changed every 2 days, and after 14 days, the cells were fixed in 4% paraformaldehyde for 30 min and then stained with 0.1% crystal violet (Sigma–Aldrich) for 10–15 min. A cell colony containing 50 or more cells when observed under an inverted microscopy was viewed as a colony and included in the final statistical analysis; otherwise, the colony was excluded.

**Table 1**  
Group design and clinical information.

Group	Age range (years)	Average age (years)	Donors (n)	Sex	Teeth (n)
A	18–30	24.8 ± 5.7	14	M = 8; F = 6	20
B	31–45	37.6 ± 6.8	16	M = 7; F = 9	24
C	46–62	53.4 ± 4.9	12	M = 5; F = 7	18

M, male; F, female.

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