ORIGINAL ARTICLE

Effect of substrate stiffness on proliferation and differentiation of periodontal ligament stem cells

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Abstract

Objectives: The aim of this study was to understand the effect of substrate stiffness (a mechanical factor of the extracellular matrix) on periodontal ligament stem cells (PDLSCs) and its underlying mechanism.

Materials and methods: Elastic substrates were fabricated by mixing 2 components, a base and curing agent in proportions of 10:1, 20:1, 30:1 or 40:1. PDLSC morphology was observed using scanning electron microscopy (SEM). Cell proliferation and differentiation were assessed after PDLSCs was cultured on various elastic substrates. Data were analysed using one-way ANOVA.

Results: SEM revealed variations in the morphology of PDLSCs cultured on elastic substrates. PDLSC proliferation increased with substrate stiffness ($P < .05$). Osteogenic differentiation of PDLSCs was higher on stiff substrates. Notch pathway markers were up-regulated in PDLSCs cultured on stiff substrates.

Conclusions: Results suggested that the osteogenic differentiation of PDLSCs might be promoted by culturing them in a stiffness-dependent manner, which regulates the Notch pathway. This might provide a new method of enhancing osteogenesis in PDLSCs.

1 | INTRODUCTION

Maxillofacial surgery, tooth extraction, traumatic jaw cyst and oral carcinoma cause bone defects, which affect facial aesthetics and physical oral function. In addition to extensive bone defects that occur in the maxilla and mandible, alveolar bone loss is a common disease associated with periodontal lesions.¹ Alveolar bone deficiency poses a major challenge during dental implantation.² Therefore, the improvements in bone regeneration and repair strategies in maxillofacial surgery, dental implantation and periodontology are

important. To promote bone defect repair, various biological materials and methods such as guided bone regeneration, autogenous bone graft, allograft and distraction osteogenesis were utilized.³⁻⁷ However, these methods are of limited efficiency. Tissue engineering might be a promising alternative for developing advanced bone regeneration methods⁸ as it can utilize the three essential elements of tissue engineering, namely, seed cells, scaffolds and growth factors.⁹⁻¹¹

Periodontal ligament stem cells (PDLSCs) are adult stem cells that possess characteristics similar to those of other stem cells, such

as self-renewal and clonogenicity.^{12,13} PDLSCs are abundant and can be easily obtained from the periodontal ligament. They have the ability of multi-directional differentiation including osteogenic, chondrogenic, neural and adipogenic lineages under certain conditions.¹⁴ Osteogenic differentiation of PDLSCs is being extensively studied owing to its applicability in bone regenerative therapy, particularly in a large scale.¹⁵⁻¹⁸ Therefore, investigating factors that affect osteogenic differentiation of PDLSCs is necessary for developing novel therapeutic strategies for bone defects.

The extracellular matrix (ECM) exhibits specific biophysical and biochemical characteristics with respect to cell types and locations. ECM regulates the fate of stem cells that bind to or are in contact with its components.¹⁹ Recently, it was proposed that the mechanical properties of the substrate influence stem cell differentiation.^{20,21} Mesenchymal stem cells (MSCs) underwent osteogenesis when cultured on stiff substrates, whereas they tended to undergo adipogenic differentiation on soft substrates.²² Additionally, alteration of substrate stiffness affects biological features such as proliferation; for example, MSCs cultured on stiff substrates exhibit increase in proliferative activity compared to MSCs grown on soft surfaces.²³ Therefore, we aimed to detect the influence of substrate stiffness on the osteogenic potential of PDLSCs, which has not been adequately studied so far.

The Notch signalling pathway mainly involves in the determination of cell behaviours like differentiation and proliferation.²⁴ The impact of Notch signalling pathway during osteogenic differentiation has drawn a lot of attention in many researches. Osteogenic differentiation and mineralization were induced by Notch pathway activation in vascular smooth muscle cells.²⁵ Osteogenesis in human primary bone marrow stromal cells was enhanced, whereas adipogenic differentiation of these cells was inhibited by the Notch pathway.^{26,27} Moreover, a study indicated that the Notch pathway plays a crucial role during osteogenesis in PDLSCs isolated from osteoporotic rats.²⁸ Therefore, we attempted to obtain evidence regarding the participation of Notch pathway during the induction of osteogenesis in PDLSCs cultured on substrates with varied stiffness.

2 | MATERIALS AND METHODS

2.1 | Fabrication of polydimethylsiloxane (PDMS) substrates

Initially, a liquid oligomeric base and curing agent (Sylgard 184, Corning, NY, USA) were vigorously mixed in varying proportions of 10:1, 20:1, 30:1 and 40:1 (PDLSCs cultured on substrate obtained from each of these proportions were considered as experimental groups for subsequent experiments) with constant stirring. This mixture was spread on the bottom of Petri dishes (Corning, New York, NY, USA). The PDMS substrates were obtained after the mixture was allowed to cross-link in an oven at 60°C for 24 h. Subsequently, PDMS substrates were immersed twice in dopamine solution for 24 h to improve the hydrophilicity of the PDMS surface, which increases cell adhesion. Prior to use with PDMS

substrates, the Petri dishes were air-dried and sterilized using ultraviolet irradiation for 1 h. In our previous study, the Young's modulus of PDMS substrates has been measured.

2.2 | Cell culture

The third molars and premolars of patients (adults and children) were collected after acquiring consent from their guardians who were informed regarding our experimental purposes and procedures. Teeth were obtained from donors who were ≤18-20 years old at the West China Hospital of Stomatology. Our study was approved by the Board of Inspection and Survey. Periodontal ligament pieces acquired by gentle scraping of exclusively the middle of tooth root surfaces were cut into small fragments, which were treated with 0.5% type I collagenase for 40 min. Subsequently, periodontal ligament debris were obtained by centrifuging at 10 000 rpm for 5 min. Sediments were resuspended in growth medium containing α -minimal essential medium (Invitrogen, Carlsbad, CA, USA), 10% foetal bovine serum, and 1% penicillin-streptomycin solution.

2.3 | Scanning electron microscope

PDLSCs cultured on PDMS substrates for 2 days were fixed overnight using 2.5%-3% glutaraldehyde. Gradient dehydration was performed using alcohol (30, 50, 75, 85, 95 and 100%) for 15 min in each dilution. Specimens were cut into small pieces, dried in an exhaust hood, coated with a thin layer of gold and observed using SEM.

2.4 | Analysis of cell proliferation

PDLSCs were seeded onto the surface of PDMS substrates with varied stiffness, at a density of approximately 3500 cells per well and cultured in growth medium for 7 days. The cell counting kit-8 (Sigma, St. Louis, MO, USA) assay was performed to evaluate cell proliferation on day 1, 3, 5 and 7, post-seeding. The assay was conducted according to the manufacturer's instructions. The BioTek ELX800 microplate reader (Bio-Tek, VT, USA) was taken to measure the optical density at a wavelength of 450 nm.

2.5 | Osteogenic differentiation assay

Osteogenic differentiation was induced by transferring PDLSCs into osteogenic medium, which contained the growth medium, 10 mM β -glycerophosphate (Sigma), 50 μ g/mL ascorbic acid 2-phosphate (Sigma) and 10^{-7} M dexamethasone (Sigma). PDLSCs were treated with BCIP/NBT alkaline phosphatase colour development kit (Beyotime, China) for alkaline phosphatase staining after culturing for 7 days. Additionally, after incubating PDLSCs in osteogenic medium for 14 days, alizarin red staining assay was performed to evaluate osteogenesis in PDLSCs. PDLSCs were fixed in 4% paraformaldehyde for 25 min and then immersed in 2% alizarin red dye for 10 min.

2.6 | Polymerase chain reaction

PDLSCs were treated with TRIzol reagent to extract total RNA after induction by osteogenic medium. RNA samples were isolated and purified with RNeasy plus mini kit (Qiagen, CA, USA). Reverse-transcription reactions were performed using dissolved RNA samples and a reverse transcriptase kit (TAKARA, Osaka, Japan). Primers are tabulated in the Table 1. Semi-quantitative PCR (SQ-PCR) and quantitative reverse transcription PCR (qRT-PCR) were conducted to evaluate the transcriptional levels of osteogenic markers. Agarose gel electrophoresis was performed in SQ-PCR and the DNA bands were visualized using Quantity One software (Bio-Rad, Hercules, CA, USA). And RT-qPCR was proceed with ABI 7300 (Applied Biosystems, Shanghai, China).

2.7 | Immunofluorescent staining

PDLSCs seeded on PDMS substrates were slightly rinsed thrice using PBS, and immobilized using 4% paraformaldehyde for 20 min. PDLSCs were incubated in 5% goat serum for 1 h at 37°C for blocking and then in primary antibodies (overnight) at 4°C. On the next day, the samples were rewarmed at 37°C for 1 h, incubated in secondary antibodies for 1 h, and in DAPI to stain nuclei for 10 min. The primary and secondary antibodies are mentioned in Table 2. Images were captured using fluorescent microscope.

2.8 | Western blot analysis

Total protein of PDLSCs was obtained through lysing in lysis buffer containing protease inhibitors. The supernatants were collected from the lysates by centrifuging at 10 310 × g for 5 min. After protein samples were solubilized and boiled in sodium dodecyl sulphate (SDS) sample buffer for 5 min, bicinchoninic acid assay was performed to measure their concentrations. Sodium dodecyl sulphate (SDS) -polyacrylamide gel electrophoresis was performed at 100 V

TABLE 1 Sequences of forward and reverse primers of selected genes designed for qPCR

Target gene (Human)	Primer pairs
GAPDH	Forward: CAGGGCTGCTTTAACTCTGG Reverse: TGGGTGGAATCATATTGGAACA
ALP	Forward: ACTGGTACTCAGACAACGAGAT Reverse: ACGTCAATGTCCTGATGTTATG
Runx2	Forward: TGGTTACTGTCATGGCGGGTA Reverse: TCTCAGATCGTTGAACCTTGCTA
OCN	Forward: AGCCCATAGTGCTTGTAAGG Reverse: CCCTCTGCTTGACACAAAG
OPN	Forward: GAA GTT TCG CAG ACC TGA CAT Reverse: GTA TGC ACC ATT CAA CTC CTC G
BMP-2	Forward: ACT ACC AGA AAC GAG TGG GAA Reverse: GCA TCT GTT CTC GGA AAA CCT

TABLE 2 Primary antibody and secondary antibody used for immunofluorescence staining

	Product number	Company
Primary antibody		
GAPDH	ab181602	Abcam, Cambridge, MA, USA
OPN	ab91655	Abcam, Cambridge, MA, USA
Runx-2	ab92336	Abcam, Cambridge, MA, USA
Notch-1	ab52627	Abcam, Cambridge, MA, USA
Hey-1	ab22614	Abcam, Cambridge, MA, USA
Hes-1	ab108937	Abcam, Cambridge, MA, USA
Secondary antibody		
Goat anti-Rabbit IgG H&L (Alexa Fluor® 594)	ab150080	Abcam, Cambridge, MA, USA

for 90 min and the resolved proteins were electrophoretically transferred onto a polyvinylidene fluoride (PVDF) membrane. After being soaked in 5% bovine serum albumin for 45 min, these PVDF membranes were immersed overnight into specific primary antibody at 4°C. On the second day, the membranes were rinsed thrice by Tris-buffered saline Tween (TBST), incubated in an appropriate secondary antibody for 1 h at 37°C, and visualized using enhanced chemiluminescence reagent. The grey value of each protein band was quantified using ImageJ software and normalized to that of the 10:1 group.

2.9 | Statistical analysis

The results from independent experiments conducted in triplicates were analysed using one-way analysis of variance (ANOVA) of SPSS 21.0 (IBM, Armonk, NY, USA) and represented as mean ± SD. The variations among the mean values of groups were considered statistically at $P < .05$ in the two-tailed test.

3 | RESULTS

3.1 | Elastic PDMS substrates influenced the cellular morphology of PDLSCs

In our previous study, the stiffness values of PDMS substrates prepared by mixing the base and curing agent at the proportions of 10:1, 20:1, 30:1 and 40:1 were approximately 135, 54, 16 and 6 kPa, respectively²⁹ (Figure 1A). Distinct cell morphologies of PDLSCs were

FIGURE 1 A, Schematic showing periodontal ligament stem cells (PDLSCs) exposed to PDMS substrates. B, SEM images of PDLSCs cultured on varying elastic PDMS material. Scale bar: 100 μ m. C, Proliferation of PDLSCs plated on PDMS substrates with varying stiffness. The results of the CCK-8 assay were normalized to that of group 10:1 and represented as histograms. The results shown are representative of 5 different samples ($n = 5$). Data are presented as mean $\pm$ SD, * $P < .05$, ** $P < .01$

observed in SEM images. The morphologies of PDLSCs cultured on various PDMS substrates were as follows: (i) 10:1 group: cells adhered onto the substrate surface and were widely stretched out, exhibiting a polygonal shape; (ii) 20:1 group: cells vaguely displayed similar shape but were smaller in size than those in the 10:1 group; (iii) 30:1 group: the majority of cells cultured on this soft substrate underwent reshaping, assumed small size, and were trapped in the substrate; and (iv) 40:1 group: cells prominently shrank into extremely small size and were embedded in the soft substrate (Figure 1B).

3.2 | Stiff PDMS substrates enhanced PDLSC proliferation

Cell proliferative rates determine the availability and abundance of stem cells that exhibit regenerative properties. We statistically analysed the measured absorbance value and observed that the proliferation rate of PDLSCs cultured on stiff substrates was higher than that of PDLSCs cultured on soft substrates throughout the experimental period (Figure 1C). This indicated that soft substrates lead to loss of proliferative potential of PDLSCs.

3.3 | Stiff substrates promoted osteoblastic differentiation of PDLSCs

To investigate the expression of osteogenesis-related genes in PDLSCs, SQ-PCR and RT-qPCR were performed after they were incubated in osteogenic medium for 3 and 7 days. Results demonstrated that the expression levels of alkaline phosphatase (ALP), osteopontin (OPN), runt-related transcription factor-2 (RUNX2),

bone morphogenetic protein-2 (BMP2) and osteocalcin (OCN) in PDLSCs decreased with reduction in substrate stiffness. Significant variations were observed between the 20:1, 30:1 or 40:1, and 10:1 groups ($P < .05$) in greyscale value and RT-qPCR analysis (Figures 2A,B and 3A). Western blot analysis demonstrated that the expression level of RUNX2 was up-regulated in PDLSCs cultured on stiff substrates for 7 and 14 days after the induction to osteogenic differentiation (Figure 3B-D). After the PDLSCs were incubated in osteogenic medium for 3 days, immunofluorescence staining was performed with OPN and RUNX2. Results demonstrated that the osteogenesis of PDLSCs increased on stiff substrates (Figure 4). ALP and alizarin red staining indicated that the staining intensities in the 10:1 and 40:1 groups were the strongest and weakest, respectively (Figures 2C and 3E). Our results indicated that the osteogenic differentiation of PDLSCs on elastic PDMS substrates occurred in a stiffness-dependent manner.

3.4 | Notch pathway was up-regulated during osteogenic differentiation of PDLSCs cultured on stiff PDMS substrates

We explored the role of the Notch pathway during osteogenesis to understand the mechanism of action of substrate stiffness during osteogenesis of PDLSCs. After the PDLSCs were induced to osteogenic differentiation for 7 and 14 days, the protein levels of NOTCH1 and HES1 were determined using western blotting. Results demonstrated that protein expression was higher in PDLSCs cultured on stiff substrates (10:1 and 20:1 groups)

FIGURE 2 A, Transcriptional levels of ALP, OPN, OCN, BMP2 and RUNX2 in PDLSCs at 3-d post-seeding. Grayscale images were calculated and represented as histograms. The results were first normalized to GAPDH levels, and then normalized to that of group 10:1. The results shown are representative of three different samples ($n = 3$). Data presented are mean $\pm$ SD, * $P < .05$, ** $P < .01$, *** $P < .001$. B, The gene expression levels of ALP, OPN, OCN, BMP2 and RUNX2 in PDLSCs at 7-d post-seeding. C, ALP staining of PDLSCs at 7-d post-seeding

than in those cultured on soft substrates (30:1 and 40:1 groups) (Figure 5B-D). The immunofluorescence intensities of NOTCH1, HES1 and HEY1 increased with PDMS substrate stiffness in PDLSCs incubated in osteogenic medium for 3 days (Figure 5A). These results indicated that the Notch pathway potentially enhanced osteogenic differentiation of PDLSCs on stiff substrates.

4 | DISCUSSION

This study indicated that the enhanced osteogenic potential of PDLSCs in response to increased substrate stiffness might probably involve Notch pathway activation. To investigate how substrate stiffness regulates cellular functions, we used elastic PDMS material produced by mixing a liquid oligomeric base and curing agent in the proportions of 10:1, 20:1, 30:1 and 40:1, which acted as a biomechanical signal similar to the ECM signal. Previous studies have shown stiffness-mediated stem cell responses, such as changes in cell proliferation rate as well as osteogenic, adipogenic or neuronal differentiation. For example, MSCs preferably undergo neuronal differentiation on soft matrix with stiffness of 0.1–1 kPa, whereas

they tend to undergo osteogenic differentiation on stiff matrix with stiffness of 25–40 kPa.²² The osteogenic differentiation of osteoblasts was enhanced with increase in substrate stiffness.³⁰ In agreement with the aforementioned results, certain cellular functions of PDLSCs were increased in a stiffness-dependent manner in our study. Therefore, our results demonstrated that substrate stiffness is an important physical factor that regulates the cellular functions of PDLSCs.

The periodontal ligament surrounding the tooth is a thin non-mineralized connective tissue, which exhibits osteoblast-like characteristics. PDLSCs exist in the periodontal ligaments of teeth and participate in the regeneration of periodontal tissues in adults. Since PDLSCs was firstly reported by Seo et al. (2004),¹³ it has been extensively studied in recent years. However, till date, the properties of PDLSCs as well as the effect of the microenvironment and the mechanisms that regulate their function remain unclear. The rapid development of stem cell applications in regenerative therapies has promoted research on adult stem cells, which can continuously replenish dead or damaged cells and are easily obtained as an essential component in regenerating tissue. Owing to the innate ability of PDLSCs to undergo osteogenic differentiation, the study of specific

FIGURE 3 A, Transcriptional levels of ALP, OPN, OCN, BMP2 and RUNX2 in PDLSCs at 7-d post-seeding measured by q-PCR. The results were first normalized to GAPDH levels, and then normalized to that of group 10:1. The results shown are representative of three different samples ($n = 3$). Data presented are mean $\pm$ SD, * $P < .05$, ** $P < .01$, *** $P < .001$. B, RUNX2 protein levels in PDLSCs at 7-d and 14-d post-seeding. (C, D), Grayscale analysis of the bands obtained by western blotting. E, Osteogenic differentiation was analysed by alizarin red staining assay on day 14

factors that regulate this process might promote PDLSC application for clinical therapy and biological research.³¹

Various factors that affect osteogenic differentiation of PDLSCs have been studied; however, the effect of extracellular physical factors on PDLSCs has been rarely investigated. Substrate stiffness is an important mechanical factor that might influence cell fate. Three-dimensional (3D) materials^{32,33} are being widely used for encapsulating cells as they simulate the *in vivo* environment of the cells. However, the range of stiffness of most of these 3D materials is lower than those of two-dimensional (2D) materials,³⁴ which is not favourable for investigating the effect of substrate stiffness on cells and their underlying mechanisms. In our study, we selected the PDMS substrate because it is 2D with variable stiffness and can support cell growth. However, further studies on how the stiffness of 3D materials influences cell behaviour are required.

In our study, PDLSCs exhibited variations in cell morphology when cultured on substrates of varied stiffness. This might be because of interactions between the contractile force of the cells and the constraint tension of the substrate during the course of mechanical sensing. This indicates that the biological functions of cells are influenced by substrate stiffness as cell morphology is generally closely associated with cell function.³⁵ Cell proliferation and differentiation are two critical processes regulating stem cell regeneration.³⁶ A reduction in cell proliferation affects the quantity of stem cells that can be isolated (*in vivo*) from the human body as well as those obtained by *in vitro* culture methods, which are ultimately used in regenerative therapies. Our results demonstrated that the proliferation rate of PDLSCs increased with substrate stiffness (range: 6–135 kPa). Therefore, the enhanced proliferation of PDLSCs on stiff substrates increases the quantity of available

FIGURE 4 Immunofluorescence of RUNX2 and OPN was observed in PDLCs cultured on substrates of varying elasticities. Scale bar: 50 μm

FIGURE 5 A, Protein levels of NOTCH1, HEY1 and HES1 in PDLCs after 3 d of culture by immunofluorescence staining. B, HES1 and NOTCH1 expression levels were assessed using western blotting on day 7 and 14. (C, D), The protein levels were first normalized to GAPDH levels, followed by normalization to that of group 10:1. The results shown are representative of three different samples ($n = 3$). Data presented are mean $\pm$ SD, * $P < .05$, ** $P < .01$, *** $P < .001$

PDLSCs. Repair at damaged sites during the reconstruction phase is partially because of stem cell differentiation into tissue-specific cells.^{37,38} The ability of PDLSCs to differentiate into osteoblasts is the fundamental principle involved in bone regeneration, and hence, PDLSCs can be used as seed cells for this process. ALP and alizarin red staining, as well as the expression levels of osteogenic markers, concordantly suggested that substrate stiffness regulates osteogenic differentiation in PDLSCs and that increase in substrate stiffness (range: 6–135 kPa) enhances osteogenesis in PDLSCs. We demonstrated that substrate stiffness influences proliferation as well as differentiation of PDLSCs. This provides a new decisive element for scaffold designing and improving therapeutic applications, such as periodontal tissue and alveolar bone regeneration.^{39–42}

Various signalling pathways determine cell behaviour.⁴³ Among them, the Notch pathway regulates cell differentiation.^{44,45} For dental pulp stem cells, Notch1 signalling enhanced proliferation and regulated cell differentiation.^{46–48} Osteogenesis of PDLSCs isolated from ovariectomized rats was induced by the Notch pathway.²⁰ In our study, NOTCH, HEY1 and HES1 expression levels were increased in a stiffness-dependent manner in PDLSCs cultured (in vitro) on substrates of varying stiffness in the presence of osteogenic medium, confirming that the Notch pathway participates during osteogenic differentiation in PDLSCs. Although this phenomenon was observed in human PDLSCs, the underlying mechanism remained undefined. Our study indicated that substrate stiffness is an important factor that influences osteogenic differentiation via the Notch pathway. Further studies regarding (i) the mechanism of regulation of PDLSC differentiation by substrate stiffness (studied using broad range of stiffness and in vivo models); and (ii) the involvement of Notch signalling in impairing alveolar bone loss, are necessary for applying PDLSCs in regenerative medicine.

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