Mechanical Stretch Increases the Proliferation While Inhibiting the Osteogenic Differentiation in Dental Pulp Stem Cells

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Dental pulp stem cells (DPSCs), which can differentiate into several types of cells, are subjected to mechanical stress by jaw movement and occlusal forces. In this study, we evaluated how the uniaxial mechanical stretch influences proliferation and differentiation of DPSCs. DPSCs were isolated and cultured from male Sprague-Dawley rats. Cultured DPSCs were identified by surface markers and the differentiation capabilities as adipocytes or osteoblasts. To examine the response to mechanical stress, uniaxial stretch was exposed to cultured DPSCs. We evaluated the impact of stretch on the intracellular signaling, proliferation, osteogenic differentiation, and gene expressions of DPSCs. Stretch increased the phosphorylation of Akt, ERK1/2, and p38 MAP kinase as well as the proliferation of DPSCs. The stretch-induced proliferation of DPSCs was abolished by the inhibition of the ERK pathway. On the other hand, stretch significantly decreased the osteogenic differentiation of DPSCs, but did not affect the adipogenic differentiation. We also confirmed mRNA expressions of osteocalcin and osteopontin were significantly suppressed by stretch. In conclusion, uniaxial stretch increased the proliferation of DPSCs, while suppressing osteogenic differentiation. These results suggest a crucial role of mechanical stretch in the preservation of DPSCs in dentin. Furthermore, mechanical stretch may be a useful tool for increasing the quantity of DPSCs *in vitro* for regenerative medicine.

Introduction

REGENERATIVE MEDICINE is expected to become useful for the replacement of damaged organs. Somatic stem cells, which predominantly exist in bone marrow and adipose tissue as well as dental pulp are suitable for tissue generation because of their particular characteristics of proliferation, differentiation, and plasticity.^{1–4} Bone marrow-derived mesenchymal stem cells differentiate into mesenchymal cell types, such as osteoblasts, chondrocytes, and adipocytes.⁵ Dental pulp stem cells (DPSCs), which are a kind of mesenchymal stem cells have the ability to differentiate into adipocytes, osteoblasts, and odontoblasts,^{6–8} which might enable the regeneration of oral tissues and bone.⁹ DPSCs are easy to obtain by the extraction of the third molar or the premolar for orthodontic reasons, and can be cryopreserved for long periods and retain their differentiation capability.^{10,11} Furthermore, DPSCs have an immuno-

suppressive activity that inhibits T cell responses.¹² These results suggest that DPSCs could be a promising cell source for regenerative medicine.

DPSCs are subjected to mechanical stress by jaw movement and occlusal forces.¹³ Mechanical stress is an essential biological stimulation, and mechanical loading plays an important role in regulating the function of mesenchymal stem cells.¹⁴ The type of mechanical stress are varied, such as fluid-shear stress, compression, hydrostatic pressure, and uniaxially vertical and horizontal stretch.¹⁵ Dental pulp is stretched vertically during tooth eruption and orthodontic forces transfer horizontal stretch to DPSCs as well as to periodontal ligament tissues. Mechanical stress activates several intracellular signaling through mechanoreceptors.^{16,17} However, the effects of mechanical stress on the differentiation of DPSCs are controversial. Some demonstrated the promotion of odontoblastic differentiation by mechanical stress in human DPSCs,^{18,19} whereas others reported that mechanical stress

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had no significant effect on the differentiation of human dental pulp.²⁰ These results suggest that the response to mechanical stress differs by the type and the duration of mechanical force. Furthermore, the intracellular signaling by mechanical force was poorly investigated in DPSCs.

The aim of this study is to assess specific characteristics and intracellular signaling of DPSCs focusing on the response to uniaxial stretch. We examined the effects of uniaxially stretch on the proliferation of DPSCs. We revealed that stretch increased the phosphorylation of Akt, extracellular signal-related kinase (ERK), and p38 mitogen-activated protein kinase (p38 MAPK). Stretch increased the proliferation of DPSCs via ERK accompanied with the suppression of osteogenic differentiation. These results suggest not only that stretch increases the number of DPSCs without osteogenic differentiation *in vivo*, but also that stretch is a useful tool for increasing the quantity of DPSCs.

Materials and Methods

Isolation and cell culture of DPSCs

DPSCs were isolated from 6-week-old male Sprague-Dawley rats and cultured. Rats were killed with an overdose of pentobarbital. Immediately after the incisors were surgically extracted, the dental pulp was retrieved and minced pulp was digested with phosphate-buffered saline (PBS) containing 0.1% collagenase and 0.25% trypsin.²¹ DPSCs were cultured with alpha modification of the Eagle's medium (α -MEM) (GIBCO) supplemented with 20% fetal bovine serum (GIBCO). DPSCs from passage 3 to 6 were used for all experiments. Experimental protocols were conducted according to the Regulations for Animal Experiments in the Aichi Gakuin University, and were approved by the Institutional Animal Care and Use Committees of the Aichi Gakuin University.

Characterization of DPSCs

DPSCs at passage 3 were characterized by fluorescence-activated cell sorting (FACS) (FACS Calibur; Becton Dickinson). Cells were incubated with the FITC-conjugated mouse monoclonal antibody against rat CD90, the FITC-conjugated hamster antibody against rat CD29, and R-PE-conjugated mouse monoclonal antibodies against rat CD34, CD49d, and CD45 (Becton Dickinson). Isotype-identical antibodies served as controls. Data were analyzed with CELLQUEST software (Becton Dickinson).

The inductions and detections of adipogenic or osteogenic differentiation

The adipogenic or osteogenic differentiation was conducted according to the manufacturer's instructions (R&D Systems). For adipogenic differentiation, we used the adipogenic induction medium containing with 1% h-insulin, 2% L-glutamine, 10% mesenchymal cell growth supplement, 0.5% dexamethasone, 0.2% indomethacin, 0.1% 3-isobutylmethyl-xanthine, and the adipogenic maintenance medium containing with 1% h-insulin, 2% L-glutamine, and 10% mesenchymal cell growth supplement (all are supplied in the adipogenic differentiation kit by R&D Systems). Three cycles of induction/maintenance stimulated optimal adipogenic differentiation. Each cycle consists of 3-day culture with a supplemented adipogenesis induction medium followed by

3-day culture with a supplemented adipogenic maintenance medium. After three complete cycles of induction/maintenance, cells were cultured for more 4 days in the adipogenic maintenance medium before the analysis of adipogenic differentiation. For the detection of adipogenic differentiation, the cells were stained with oil red O (Polysciences) and the fatty acid-binding protein-4 (FABP-4) (R&D Systems).

For osteogenic differentiation, we used the osteogenic induction medium with 0.5% dexamethasone, 2% L-glutamine, 0.5% ascorbate, and 10% mesenchymal cell growth supplement and 1% β -glycerophosphate (all are supplied in osteogenic differentiation kit by R&D Systems). Osteogenic differentiation was started by replacing by the osteogenic induction medium and maintained culture with the medium replacement every 3–4 days for 2–3 weeks. For the detection of osteogenic differentiation, the cells were stained with alkaline phosphatase (ALP) (Millipore) and osteocalcin (R&D Systems). To examine matrix mineralization, cells were stained with 1% Alizarin Red S (Merck) in distilled water and 28% ammonia solution (pH 6.3) for 5 min at room temperature.²²

Uniaxial stretch

Uniaxial stretch is one of mechanical forces by jaw movement as well as pressure and shear stress.^{23,24} To examine the response to uniaxial stretch, DPSCs were seeded onto a stretch chamber (size 2.0×2.0 cm). After cells were allowed to attach to the chamber bottom for 72 h, uniaxial stretch (110% peak to peak, at 0.167 Hz), which means that the maximum uniaxial stretched length is 2.2 cm (ST-140; STREX, Inc.), was applied for the indicated periods (Supplementary Video SV1; Supplementary Data are available online at www.liebertpub.com/tea).

Western blotting

After stretching for the indicated periods, cells were lysed with the lysis buffer as described previously.²⁵ Cells were washed three times with ice-cold PBS and lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/mL aprotinin, 1 μ g/mL leupeptin, 1 mM Na₃VO₄, and 1 mM NaF at 4°C. Samples containing the same amount of protein were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was incubated overnight at 4°C with each first antibody to phospho-Akt (Ser473), Akt, phospho-ERK 1/2, ERK1/2, phospho-p38 MAPK, and p38 MAPK (Cell signaling), followed by incubation with an HRP-conjugated anti-rabbit polyclonal IgG antibody. The proteins were visualized using ECL plus (GE healthcare) chemiluminescence detection kits. Images were scanned and densities were determined by ImageJ (NIH). The phosphorylation of Akt, ERK 1/2, and p38 MAPK were analyzed by Western blot analyses using antibodies to phospho-Akt (Ser473), Akt, phospho-ERK 1/2, ERK1/2, phospho-p38 MAPK, and p38 MAPK (Cell signaling).

Proliferation analyses of DPSCs under the mechanical stretch

DPSCs at 3 passages were seeded onto a stretch chamber (4 cm²) at 2×10^4 cells and cultured for 3 days. The

proliferation ability of DPSCs was investigated by MTT assay according to the manufacturer's procedure. After stretching for 1 h, which we decided to resemble the chewing time, cells were seeded in a 96-well plate at a density of 3×10^3 and cultured for an additional 6 h. Then, the MTT Reagent (Cayman Chemical Company) was added to each well and the plates were incubated for 3 h. Formazan crystals were dissolved and the absorbance was measured at 570 nm using a multilabel luminescence counter (1420 ARVO MX/Light, PerkinElmer, Inc.).

The number of DPSCs was counted with a hemocytometer 1 day after 1-h stretch. Control cells were cultured on the chamber without stretch. To examine which intracellular signaling is involved in the stretch-stimulated proliferation of DPSCs, the cells were pretreated with or without a PI 3-kinase/Akt inhibitor (LY294002; 20 μ M) (Enzo Life Sciences), a MEK/ERK inhibitor (PD98059; 20 μ M) (Sigma), or a p38 MAP kinase inhibitor (SB203580; 5 μ M) (Merck) 30 min before stretch. Subsequently, cells were uni-axially stretched for 1 h and the numbers of cells were counted 1 day after the stretch.

Osteogenic and adipogenic differentiation of DPSCs under the mechanical stretch

The osteogenic and adipogenic differentiation were conducted as described above with or without 1-h stretch every day for 2–3 weeks. The osteogenic differentiation was detected by the staining with osteocalcin (R&D Systems). The adipogenic differentiation was detected by the staining with FABP-4 (R&D Systems). Total cell count was calculated by the nuclei staining with 4'-6-Diamidino-2-phenylindole (DAPI). We randomly counted the number of the osteocalcin or FABP-

4-positive cells and the cell nuclei stained by DAPI at 10 different areas and averaged the counting number in each stretch chamber. The ratios of the osteogenic or adipogenic differentiation were calculated as the percentage of osteocalcin and FABP-4-positive cells in total cells, respectively.

mRNA expressions of DPSCs under the mechanical stretch

Next, we examined the effects of stretch on osteogenic differentiation-related mRNA expressions of DPSCs. DPSCs were pretreated with LY294002 (20 μ M), PD98059 (20 μ M), or SB203580 (5 μ M) 30 min before stretch, and then cells were uniaxial stretched for 1 h. Twenty four hour after stretch, total RNA was extracted using TRIzol (Invitrogen) and purified using the purification column (Qiagen) and was reverse transcribed using RevaTra Ace (TOYOBO) according to the manufacturers' protocols. Real-time PCR was performed with TaqMan probes (Applied Biosystems) to detect osteocalcin and osteopontin, and monitored by the ABI PRISM[®]7000 Sequence Detection System (Applied Biosystems). The values were referenced to 18S rRNA (Applied Biosystems) expression using fluorescently labeled 18S rRNA

FIG. 1. Culture and identification of dental pulp stem cells (DPSCs). **(A)** Cultured DPSCs. **(B)** Flow cytometric analysis revealed the surface markers of DPSCs. Cells were incubated with fluorescence-conjugated antibodies against CD90, CD29, CD34, CD49d, and CD45. Isotype-identical antibodies served as controls (Line filled in black). Color images available online at www.liebertpub.com/tea

FIG. 2. Differentiation capability of DPSCs. **(A)** Adipogenesis was assessed by oil red-O and FABP-4 staining. Bar=200 μ m. **(B)** Osteogenesis was assessed by ALP and osteocalcin staining. Matrix mineralization was assessed by Alizarin Red S staining. Bar=200 μ m.

with VIC and MGB (Applied Biosystems). The relative quantity was calculated by the $\Delta\Delta C^t$ method.²⁶

Statistical analysis

All group values are expressed as means $\pm$ standard error of the mean (SEM). Statistical analyses were made by the Student *t* test for a single comparison or one-way analysis of variance followed by the Bonferroni correction for multiple comparisons using SPSS version 14.0.1 for Windows (SPSS). Differences were considered significant at the $p < 0.05$ level.

Results

Characteristics of DPSCs

Cultured DPSCs (Fig. 1A) were characterized by their surface markers using FACS (Fig. 1B). DPSCs showed that high expressions of CD29 and CD90, which were common stem cell markers in mesenchymal stem cells and DPSCs.^{27,28} DPSCs also showed relatively high expressions of CD49d, while lacked the expressions of CD34 and CD45.²⁹

Differentiation into adipocytes and osteoblasts

After 21 days of culture with the adipogenic and the osteogenic induction medium, adipogenesis was assessed by oil red O staining and FABP-4, and osteogenesis was assessed by ALP-activity staining and osteocalcin. DPSCs de-

veloped into lipid-laden fat cells stained with oil red O and FABP (Fig. 2A). DPSCs were also differentiated into osteoblasts stained with ALP and osteocalcin (Fig. 2B). To assess the matrix mineralization, we performed Alizarin Red S staining after 21 days of culture with the osteogenic induction medium. DPSCs showed marked mineralization (Fig. 2B).

Stretch increased the phosphorylation of Akt, ERK, and p38 MAPK in DPSCs

To elucidate intracellular signaling during 1-h stretch, we investigated the phosphorylation of Akt, p38 MAPK, and ERK1/2 in the indicated periods of stretch (Fig. 3A–C). The phosphorylation of Akt was increased in a time-dependent manner up to 60-min stretch (Fig. 3A). The peak phosphorylation of Akt was observed at 60-min and subsequently decreased (Supplementary Fig. S1). On the other hand, the peak phosphorylations were observed at 5-min stretch in p38 MAPK and at 10 to 30-min stretch in ERK 1/2. The phosphorylation of p38 MAPK and ERK were decreased after 10-min stretch and 60-min stretch, respectively (Fig. 3B, C).

Stretch stimulates the proliferation of DPSCs via ERK

The effect of stretch on the proliferation of DPSCs was evaluated by MTT assay and cell count. MTT assay revealed that stretch increased the proliferation activity of DPSCs by 1.8-fold compared with nonstretch control ($p < 0.05$) (Fig. 4A).

FIG. 3. Phosphorylation in response to uniaxial stretch for the indicated periods in DPSCs. (A) Stretch-stimulated phosphorylation of Akt ($n=4$). (B) Stretch-stimulated phosphorylation of p38 MAPK ($n=4$). (C) Stretch-stimulated phosphorylation of ERK 1/2. Results are expressed as mean $\pm$ standard error of the mean (SEM) ($n=4$). * $p < 0.05$.

FIG. 4. Effect of uniaxial stretch on the proliferation of DPSCs. (A) The proliferation activity of DPSCs with or without 1-h stretch was assessed by the MTT assay. Results are expressed as mean $\pm$ SEM ($n=4$). * $p<0.05$ versus DPSCs without the stretch (Stretch (-)). (B) The numbers of DPSCs were calculated 24-h after 1-h stretch stimulation. To examine the effects of inhibitors on the stretch-stimulated proliferation of DPSCs, cells were pretreated with or without a PI3K/Akt inhibitor (LY; LY294002), a MEK/ERK inhibitor (PD; PD98059), or a p38 MAPK inhibitor (SB; SB203580) 30 min before the stretch. Results are expressed as mean $\pm$ SEM ($n=4$). * $p<0.05$, ** $p<0.01$.

The cell numbers of DPSCs were calculated 1 day after 1-h stretch. The stretch significantly increased the numbers of DPSCs (control; $7.2 \pm 2.9 \times 10^4$ cells, stretch; $13.2 \pm 6.0 \times 10^4$ cells per chamber, $p<0.05$).

To investigate the involvement of the intracellular signaling pathways in the stretch-induced proliferation of DPSCs, we assessed the effects of inhibitors of Akt, ERK, and p38 MAPK on the number of DPSCs 1 day after 1-h stretch (Fig. 4B). Stretch-stimulated proliferation was completely inhibited by the inhibition of ERK using PD98059, suggesting that stretch stimulated the proliferation of DPSCs via the ERK pathway. The proliferation was not inhibited by the inhibition of Akt nor p38 MAPK.

Stretch suppressed the osteogenic differentiation, but not the adipogenic differentiation

To examine the effect of stretch on the osteogenic and adipogenic differentiation in DPSCs, osteogenic or adipogenic induction was applied to DPSCs with or without 1-h uniaxial stretch every day for 2–3 weeks. The osteogenic and adipogenic differentiation were evaluated by the immunohistological staining of osteocalcin and FABP-4, respectively. The rate of osteocalcin-positive cells to whole cells were significantly decreased by the mechanical stretch compared with nonstretched cells (nonstretched cells; $63.0\% \pm 4.2\%$, stretched cells; $35.1\% \pm 1.1\%$, $p<0.001$) (Fig. 5A, B). These results indicate that 1-h uniaxial stretch for 2 weeks significantly suppressed the osteogenic differentiation by 44% compared with nonstretched cells.

On the other hand, the rate of FABP-4-positive cells to whole cells was low and was not changed between nonstretched cells and stretched cells (nonstretch cells; $10.9\% \pm 1.1\%$, stretched cells; $14.1\% \pm 1.2\%$), indicating that mechanical stretch did not affect the adipogenic differentiation (Fig. 5C, D).

Stretch suppressed the mRNA expressions of osteocalcin and osteopontin

To determine whether stretch affected osteogenic gene expressions in DPSCs, we examined mRNA expression of osteocalcin and osteopontin, which was expressed in osteoblasts. Stretch significantly suppressed the mRNA level of osteocalcin by $76.5\% \pm 3.4\%$ ($p<0.01$) (Fig. 6A). The inhibition of the Akt, ERK, or p38MAPK pathway did not restore the stretch-induced suppression of osteocalcin expression (Fig. 6B). Stretch also suppressed the mRNA level of osteopontin by $39.4\% \pm 9.9\%$ ($p<0.01$), which was abolished by the inhibition of the Akt pathway and the ERK pathway by LY294002 and PD98059, respectively (Fig. 6C, D), suggesting that the stretch suppressed mRNA expression of osteopontin via Akt and ERK.

Discussion

DPSCs not only play an essential role in dentinogenesis, but also are expected to be a promising source of regenerative medicine.^{6,7,30} We have revealed that uniaxial mechanical stretch stimulated the phosphorylation of ERK and Akt as well as p38 MAPK in DPSCs, suggesting that stretch affects multiple cell functions of DPSCs. In addition, we have first demonstrated that stretch increased the proliferation of DPSCs via ERK activation, while inhibiting the osteogenic differentiation. Since DPSCs physiologically receive mechanical stress by mastication and swallowing, our results suggest that mechanical stretch may have an essential role in the maintenance of DPSCs through increasing the proliferation, while suppressing osteogenic differentiation.

We have revealed that stretch increased the proliferation of DPSCs via the ERK pathway, which is consistent with other type of cells.³¹ ERK is reported to be the key molecule in the proliferation and the production of cytokines. The basic fibroblast growth factor induced the proliferation of DPSCs via the phosphorylation of ERK,³² and lipopolysaccharide increased the production of the vascular endothelial growth factor via the phosphorylation of ERK.³³ Our results

FIG. 5. Effect of uniaxial stretch on the osteogenic differentiation and the adipogenic differentiation of DPSCs. The osteogenic differentiation was induced using the osteogenic induction medium with or without 1-h stretch every day for 2 weeks. The osteogenic differentiation was detected by the staining with osteocalcin. The adipogenic differentiation was induced using the adipogenic induction medium with or without 1-h stretch every day for 3 weeks. The adipogenic differentiation was detected by the staining with FABP-4. The cell nuclei were stained with 4'-6-Diamidino-2-phenylindole (DAPI). We randomly counted the number of the osteocalcin or FABP-4-positive cells and the cell nuclei stained by DAPI at 10 different areas in each stretch chamber and the positive cells/the cell nuclei were calculated. **(A)** Representative photographs of immunological staining of osteocalcin and DAPI in nonstretched and stretched cells. **(B)** The ratio of osteocalcin-positive cells to total cells in nonstretched and stretched cells. Results are expressed as mean $\pm$ SEM ($n=5$). *** $p<0.001$. **(C)** Representative photographs of immunological staining of FABP-4 and DAPI in nonstretched and stretched cells. **(D)** The ratio of FABP-4-positive cells to total cells in nonstretched and stretched cells. Results are expressed as mean $\pm$ SEM ($n=5$).

indicate that the ERK pathway is the major pathway, which is involved with the stretch-induced proliferation of DPSCs.

Dental pulp and periodontal ligaments are considered to have unique mechanisms involved in bone remodeling and dentin formation in response to mechanical stress.³⁴ Previous studies demonstrated the influence of mechanical stress in the differentiation of DPSCs, although the results are controversial.^{18–20} Han *et al.* revealed that continuous cyclic mechanical tension for 4–10 days increased proliferation and collagen/osteopontin expression in DPSCs.³⁵ Yu *et al.* showed DPSCs increased odontogenic differentiation, early *in vitro* mineralization, with slightly disrupted survival by hydrostatic pressure for 2 h.¹⁵ Cai *et al.* indicated that a 6-h uni-axially cyclic tensile stretch inhibited osteogenic differentiation of DPSCs.³⁶ We have demonstrated that 1-h uni-axially stretch inhibited the gene expressions of osteocalcin and osteopontin, which are expressed in osteoblasts. We also confirmed that 1-h uniaxial stretch inhibited osteogenic differentiation induced by the osteogenic induction medium. These findings suggest that the responses to mechanical stress are varied depending on the type of mechanical

stresses and the uniaxial stretch is at least partially involved in suppressing the osteogenic differentiation of DPSCs. The inhibition of differentiation by mechanical stress makes sense for a physiological role of DPSCs. In considering the *in vivo* condition, it is suggested that DPSCs located near the roots of teeth are exposed to higher levels of oral mechanical stress by jaw movement and occlusal forces, whereas those located near the crown are exposed to lower levels of mechanical stress. Tissue-specific localization of the stem cell population was reported in periodontal tissues.^{37,38} The difference of mechanical stress might affect such distribution of stem cells.

To our knowledge, this is the first report that the uniaxial stretch stimulates Akt, p38 MAPK, and ERK in DPSCs. We also demonstrated that the mechanical stretch increased the proliferation via ERK and suppressed the mRNA expression of osteopontin via ERK and Akt in DPSCs. Previous studies demonstrated that mechanical stress increased these intracellular signaling and affected the proliferation and differentiation through these signaling in other type of cells. 10-day cyclic mechanical stretch increased cardiomyocyte proliferation via p38 MAPK phosphorylation.³⁹ Shear stress

FIG. 6. Effect of stretch on mRNA expressions of osteocalcin and osteopontin. DPSCs were pretreated with a PI3K/Akt inhibitor (LY; LY294002), MEK/ERK inhibitor (PD; PD98059), or p38 MAPK inhibitor (SB; SB203580) 30 min before stretch, and then stretched for 1-h. Total RNA was extracted 24-h after stretch and mRNA expression was evaluated by real-time PCR method. **(A)** Comparison between nonstretched control and stretched cells in the mRNA expression of osteocalcin. $^{**}p < 0.01$ **(B)** comparison between the stretched cells with or without inhibitors in mRNA expression of osteocalcin. **(C)** Comparison between nonstretched control and stretched cells in mRNA expression of osteopontin. $^{**}p < 0.01$ **(D)** Comparison between the stretched cells with or without inhibitors and the three inhibitors in mRNA expression of osteopontin. $^{**}p < 0.01$. Results are expressed as mean $\pm$ SEM ($n = 5$).

induced proliferation of rat mesenchymal stem cells through pim-1 via ERK and p38 MAPK.⁴⁰ Oscillatory flow induces human osteoblast-like MG63 cell proliferation through activation of $\beta 3$ and $\beta 1$ integrins leading to the modulation of downstream ERK and Akt pathways.⁴¹ These results suggest that mechanical stress affect cell functions via these intracellular signaling, which differs by cell type and the way/duration of mechanical stress. The expressions of some integrins are changed by uniaxial stretch in DPSCs in our preliminary study (Data not shown). Integrins may be the candidates to mediate mechanotransduction in DPSCs. On the other hand, we demonstrated that stretch suppressed mRNA expression of osteopontin via the Akt and ERK pathway; however, the stretch-suppressed mRNA expression of osteocalcin did not restore by the inhibition of Akt, ERK, nor p38MAPK, suggesting the involvements of other pathways. Further study is required to clarify these issues in response to mechanical stress in DPSCs.

In conclusion, stretch increases the quantity of DPSCs via the ERK pathway, while suppressing osteogenic differentiation in which PI3K/Akt and ERK pathways are partly involved. These results suggest that uniaxial mechanical stretch not only plays an important role to keep the number of DPSCs without differentiation *in vivo*, but also may be a useful tool for increasing the quantity of DPSCs *in vitro* for regenerative medicine.

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Disclosure Statement

Keiji Naruse has served on the STREX Advisory Board. He has a patent pending with STREX for a stretch machine, and owns stock. None of the other authors have a financial interest in the subject of this manuscript.

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