Abstract: Periodontitis leads to destruction of periodontal ligament, cementum and alveolar bone. Regeneration of periodontal tissue is dependent on mesenchymal stem cells (MSC) present in the periodontal ligament, and transcription factors determine the direction of MSC differentiation. The present study was conducted to investigate the transcription factors that are crucial for maintaining the characteristics of the periodontal ligament. The mRNA levels of several transcription factors were measured in cultured human periodontal ligament (HPDL) cells, human gingival fibroblasts and osteoblast-like Saos2 cells. HPDL cells were transfected for 72 h with siTwist2, siKlf12, or siMix (siTwist2, siPax9, and siKlf12). The cells were then harvested and subjected to real-time PCR and Western blotting. siTwist2 suppressed the levels of Twist2, Sox2 and Colla1 mRNAs, and increased those of Sox5 and aggrecan mRNAs. siKlf12 decreased the mRNA levels of Klf12, Runx3, Zfp521, and Stab2, and increased those of Sox2, Klf4, and the MSC markers CD90 and CD105. These results suggest that transfection with siMix and siTwist2 induced chondrogenesis, and that siKlf12 induced the differentiation of MSC in HPDL cells. Thus, inhibition of Twist2 or Klf12 induced the differentiation of chondrogenic or mesenchymal stem cells in this setting, suggesting that the characteristics of HPDL cells may be altered by inhibition of specific transcription factors.

Keywords: gingival fibroblasts; periodontal ligament cells; osteoblasts; transcription factor; differentiation.

Introduction

Periodontal tissue essentially consists of four components: gingiva, periodontal ligament, cementum and alveolar bone. Periodontal ligament is a non-mineralized connective tissue located between two mineralized tissues, i.e. cementum and alveolar bone (1,2). Periodontal tissue that has been damaged by periodontitis can be regenerated using barrier membranes, enamel matrix derivative (EMD), platelet-derived growth factor BB and fibroblast growth factor 2 (FGF2) (3-7). The success of periodontal tissue regeneration is dependent on mesenchymal stem cells (MSC) present in the periodontal ligament. A recent study has suggested that FGF2 is an effective and safe therapeutic agent for periodontal tissue regeneration, with an efficacy superior to that of EMD (7). FGF2 increases transcription of runt-related transcription factor 2 (Runx2), thus inducing osteoblast differentiation and chondrocyte maturation (8). Osteoblasts, chondrocytes, myoblasts, and adipocytes are all derived from common mesenchymal stem cells. Several transcription factors are crucial for differentiation of these cells: Runx2 and Osterix for differentiation of osteoblasts (9,10), and Sox5,
Sox6 and Sox9 for chondrocyte differentiation (11). Myogenic regulatory factors (MyoD, Myf5, myogenin, and Mrf4) are necessary for myoblast differentiation (12), and the C/EBP family and PPARγ2 play a fundamental role in adipocyte differentiation (13). Overexpression of Runx2 in osteoblasts inhibits osteoblast maturation and causes osteopenia (14). Osterix induces the expression of Col1a2 and osteocalcin mRNAs in osteoblasts (15). Sox9 overexpression promotes glioma metastasis via Wnt/β-catenin signaling (16). MyoD induces myogenic differentiation in immortalized bovine embryonic fibroblasts (17). PPARγ overexpression in vascular smooth muscle cells rescues smooth muscle contractile protein expression and attenuates the proliferative smooth muscle cell phenotype (18). Pluripotent stem cells have been induced from adult human fibroblasts by transfection with Oct3/4, Sox2, c-Myc, and Klf4 (19). Two reprogramming factors (c-Myc and Klf4) and one chondrogenic factor (Sox9) induce the differentiation of chondrogenic cells from mouse dermal fibroblasts without going through a pluripotent state (20,21). RNA interference (RNAi), discovered in 1998 (22), has been widely used for treating genetic diseases and cancers (23,24). Delivery of siRNA via cationic sterosomes is a powerful method for efficient gene knockdown (25). Transfection of 3T3-L1 cells with siRNA targeting the transcriptional coactivator with PDZ-binding motif (TAZ) increases lipid deposition and adipogenic gene expression (26).

The purpose of this study was to compare the expression of transcription factors in human gingival fibroblasts (HGF), human periodontal ligament (HPDL) cells and osteoblast-like cells, and determine which are crucial for maintaining the characteristics of the periodontal ligament. Induction of chondrogenic and mesenchymal stem cells from HPDL cells through inhibition of specific transcription factors was also investigated.

Materials and Methods

Materials

Dulbecco’s Modified Eagle Medium (DMEM), α-Minimum Essential Medium (αMEM), and alcian blue were purchased from Wako (Tokyo, Japan). Fetal calf serum (FCS), TRIzol, penicillin, and streptomycin, TrypLE Express, Oligofectamine, and Opti-MEM were purchased from Invitrogen (Carlsbad, CA, USA). Nonsilencing oligos (on-target plus siControl non-targeting pool; D-001810-10), on-target plus siRNA human Pax9 (J-012242-05), Twist2 (J-012862-01) and Klf12 (J-013353-06) were from GE healthcare Dharmaco Inc. (Lafayette, CO, USA). A PrimeScript RT reagent kit and a SYBR Premix Ex Taq II were obtained from Takara Bio (Tokyo, Japan). Anti-rabbit IgG (whole molecule)-horseradish peroxidase goat serum was purchased from Sigma-Aldrich Japan (Tokyo, Japan). ELC plus Western Blotting Detection Reagent was purchased from GE Healthcare UK Ltd. (Amersham, UK).

Cell culture

DMEM or αMEM supplemented with 10% FCS and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) were used for cell culture. Primary human gingival fibroblasts (HGF) were derived from explants of gingival connective tissues. Human periodontal ligament (HPDL) cells were derived from explants of periodontal ligament tissues obtained from fully erupted third molars (27). HGF and HPDL were cultured in DMEM at 37°C under 5% CO₂/95% air, and human osteoblast-like Saos2 cells were cultured in αMEM until confluence. The Institutional Internal Review and Ethics Board at Nihon University School of Dentistry at Matsudo approved the study (EC03-016, EC03-041, EC10-040). Written informed consent was obtained from each study subject after all the procedures had been fully explained.

RNA interference

Twenty-four hours after plating in 6-well plates, HPDL cells at 30-40% confluence were transfected with siTwist2, siKlf12, or siMix (siTwist2, siPax9, and siKlf12) oligonucleotides using Oligofectamine in 0.5 mL of Opti-MEM in accordance with the manufacturer’s instructions. After 4 h, 0.5 mL of fresh culture medium containing 2× concentrated FCS and 0.5 mL of Opti-MEM was added. Cells were harvested 72 h after transfection. Control cells were treated with non-silencing oligos.

Real-time PCR

Total RNA was isolated from cultured cells using TRIzol reagent in accordance with the manufacturer’s protocol. Total RNA (1 μg) was used as a template for cDNA synthesis. cDNA was prepared using the EXScript RT reagent Kit. Quantitative real-time PCR was performed using the SYBR Premix Ex Taq in a TP800 thermal cycler dice real-time system (Takara Bio, Tokyo, Japan). Relative mRNA expression levels were determined by the ΔΔCt method. All transcript levels were normalized to the transcription level of GAPDH. Sequences of primers are presented in Table 1. Experiments were performed in triplicate and results are displayed as mean values ±SD.
Western blotting
Total proteins from HPDL cells were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto membranes. The membranes were then incubated for 3 h with anti-Twist2 (ab57997; Abcam), anti-Klf12 (ab68347; Abcam), anti-Pax9 (ab84830; Abcam), anti-Sox5 (ab157003; Abcam), anti-Sox2 (Y-17; Santa Cruz Biotechnology), anti-klf4 (GKLF, H-180; Santa Cruz Biotechnology) and anti-actin (I-19; Santa Cruz Biotechnology) antibodies. Anti-rabbit IgG, anti-mouse IgG, or anti-goat IgG conjugated with horseradish peroxidase was used as the secondary antibody. Immunoreactivities were detected using ELC plus Western blotting detection reagents. Band densities were quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

Alcian blue staining
Twenty-one days after transfection with siControl, siMix, siTwist2, or siKlf12, HPDL cells were fixed with 100% ethanol at −20°C for 10 min, then incubated with 0.1% alcian blue in 0.1N HCl for 10 min, then incubated with 0.1% alcian blue in 0.1N HCl for 10 min.

Statistical analysis
Triplicate samples were analyzed for each experiment, and experiments were replicated to ensure the consistency of expression in the cell lines. Significance of differences was determined using one-way ANOVA and Tukey-Kramer test.

Results
Detection of mRNAs for transcription factors in HGF, HPDL and Saos2 cells
Real-time PCR was performed to confirm the expression of mRNAs for alkaline phosphatase (Alp) and several transcription factors in HGF, HPDL and osteoblast-like Saos2 cells. Levels of Alp mRNA in Saos2 cells were characteristically higher than those in HGF and HPDL cells (Fig. 1U). Levels of mRNAs for Runx2, Runx3, Sox4 and Sox5 were higher in Saos2 cells than in HGF and HPDL cells (Fig. 1B, C, E, F). These transcription factors may play roles in osteogenesis (Alp, Runx2, and Sox4), chondrogenesis (Sox5) and tumor suppression (Runx3). Levels of Pax3, Klf3 and c-Myc mRNAs were almost the same in HGF, HPDL and Saos2 cells (Fig. 1K, M, Q). Levels of mRNAs for Runx1, Twist1, Twist2, Pax9, Klf4, Klf10, Klf12, Zfp521, and Prrx1 were higher in HGF and HPDL cells than in Saos2 cells (Fig. 1A, I, J, L, N, O, P, R, S). Twist2 may inhibit osteoblast maturation and maintain cells in a preosteoblast phenotype during osteoblast development. Pax9 is required for...
normal development of the thymus, parathyroid glands, ultimobranchial bodies, teeth, skeletal elements of the skull and larynx, as well as the distal limbs. Klf12 transcriptionally represses the transcription factor AP-2 alpha (Tfap2a) gene. Tfap2a-knockout mice die perinatally due to cranio-abdominoschisis and severe dysmorphogenesis of the face, skull, sensory organs, and cranial ganglia. These previous findings suggest that three transcription factors (Twist2, Pax9, and Klf12) could be important for maintaining the characteristics of HGF and HPDL cells (Fig. 1J, L, P).
siMix (siTwist2, siPax9 and siKlf12), siTwist2, and siKlf12 induce differentiation of HPDL cells to different phenotypes

Transfection of HPDL cells with siMix (siTwist2, siPax9, and siKlf12) suppressed the levels of mRNAs for Twist2, Pax9 and Klf12 (Fig. 2J, L, P). Levels of mRNAs for Sox4, Sox5, c-Myc, Zfp521 and aggrecan were increased (Fig. 2E, F, Q, R, U), whereas that of Col1a1 was suppressed (Fig. 2V). Levels of mRNAs for other transcription factors were not changed after siMix transfection (Fig. 2A-D, G-I, K, M-O, S, T). siMix suppressed the protein levels of Twist2 and Klf12 (Fig. 3A-C), but had no effect on the level of Pax9 protein (Fig. 3A, D). Moreover, siMix increased the protein level of Sox5 (Fig. 3A, E). These results suggest that siMix increased the expression of mRNAs for chondrogenic markers, and decreased that of mRNAs for fibroblast markers.

Transfection of HPDL cells with siTwist2 suppressed the mRNA and protein levels of Twist2 (Figs. 4J, 6A, B), suppressed the level of Sox2 mRNA (Fig. 4D), and increased the mRNA and protein levels of Sox5 (Figs. 4F, 6A, B). Furthermore, siTwist2 increased the level of aggrecan mRNA (Fig. 5A), and decreased that of Col1a1 mRNA (Fig. 5B). However, there were no effects on other transcription factors (Fig. 4A-C, E, G-I, K-T). Transfection of HPDL cells with siKlf12 suppressed the mRNA and protein levels of Klf12 (Figs. 4P, 7A, C), suppressed the mRNA levels of Runx3, Zfp521 and Satb2 (Fig. 4C, R, T), and increased the mRNA levels of Sox2, Klf4, and the MSC markers CD90 and CD105 (Figs. 4D, N, 5C, D). Moreover, siKlf12 increased the protein level of Klf4, but not that of Sox2 (Fig. 7A, F, G).

To examine whether siMix, siTwist2, or siKlf12 can induce differentiation of HPDL cells, alcian blue staining was performed 21 days after transfection. Alcian blue staining of HPDL cells was evident after siMix and siTwist2 transfection (Fig. 8).
Discussion

This study has demonstrated that inhibition of Twist2, which is highly expressed in HGF and HPDL cells, induces chondrogenesis of HPDL cells (Fig. 8). The results indicated that Twist2 and Klf12 are important transcription factors in HPDL cells. Twist has recently been identified as a negative regulator of osteoblast differentiation. Twist2 has a conserved C-terminal domain known as the Twist box that interacts with Runx2 and inhibits the expression of downstream targets of Runx2, including osteoblast-specific genes (28). Overexpression of Twist protein impacts proteins in various metabolic pathways and the levels of mRNAs for inflammatory cytokines (29). Inhibition of Twist1 or Twist2 has been reported to reduce bone formation and osteoblast differentiation in Twist1- and Twist2-haploinsufficient mice (30). The Klf12 gene plays an important role in progression of poorly differentiated gastric cancer and is a potential therapeutic target (31). HPDL cells express transcription factors involved in inhibition of osteoblast differentiation and fibroblast growth. Here, inhibition of Twist2 in HPDL cells increased the levels of Sox5 mRNA and protein, and decreased the level of Sox2 mRNA (Fig. 4). Sox5 plays important roles in the commitment of mesenchymal cells to the chondrogenic lineage, and activates the transcription of chondrocyte-marker genes by binding to their enhancers (32). Furthermore, inhibition of Twist2 in HPDL cells increased the level of aggrecan mRNA (Fig. 5A). Sox5 drives expression of the aggrecan gene in cartilage (33). However, it has been reported that levels of mRNAs for chondrogenic and hypertrophic/osteoarthritic markers were markedly decreased or delayed in ATDC5 cells overexpressing Sox5 (34). These results suggest that Sox5 induces differentiation of mesenchymal cells into chondrocytes, whereas Sox5 overexpression suppresses chondrocyte differentiation. In the present study, inhibition of Twist2 in HPDL cells induced differentiation of HPDL cells into chondrocytes (Fig. 8). When Klf12 was inhibited in HPDL cells, levels
of mRNAs for Sox2, Klf4, and the MSC markers CD90 and CD105 were increased, whereas those of mRNAs for Runx3, Zfp521 and Satb2 were decreased (Figs. 4, 5). Although inhibition of Klf12 increased the level of Klf4 protein, the level of Sox2 protein did not change. Previous reports have indicated that overexpression of Klf4 promotes self-renewal of embryonic stem cells (35). Also, an *in vitro* study has demonstrated that the HMG-box transcription factor Sox2 (sex-determining region Y-box 2) gene is expressed in stem/progenitor cells (36). Runx3 deficiency affects bone mineralization and constitutes a risk factor for human osteopenia (37). Satb2 acts as a potent transcription factor to enhance osteoblastogenesis and promote bone regeneration (38). *In vitro* overexpression of Zfp521 antagonizes differentiation and bone nodule formation, whereas Zfp521 knockdown promotes these processes (39). Here, down-regulation of Klf12 by siRNA increased the expression of stem cell-related transcription factors, and suppressed that of osteogenic transcription factors. However, siMix (Twist2, Klf12 and Pax9) induced differentiation of HPDL cells into the chondrogenic phenotype. Although Twist2, Klf12, and Pax9 are highly expressed in HGF and HPDL cells (Fig. 1), cross-talk between them has yet to be defined.

Inhibition of Twist2 or Klf12 in HPLD cells induced chondrogenesis or MSC (Fig. 9). Further study is required to validate the role of these transcription factors *in vivo*, with a view to their future therapeutic application for periodontal regeneration.

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**Conflict of interest**

None declared.

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