Journal of Dentistry and Oral Biology

Research Article
Published: 22 Jun, 2023

HIF-1α Overexpression Using a Protein Transduction Domain to Increase the Osteogenic Potential of SHED

*Kim IH[1#], Jeon M[2#], Lee YH[2], Shin JS[3], Lee T[2], Lee SK[3], Cheon K[4] and Song JS[1,2]**

[1]Department of Pediatric Dentistry, College of Dentistry, Yonsei University, Korea

[2]Oral Science Research Center, College of Dentistry, Yonsei University, Korea

[3]Department of Biotechnology, College of Life Science and Biotechnology, Yonsei University, Korea

[4]Department of Pediatric Dentistry, University of Alabama at Birmingham, USA

[#]These authors contributed equally to this work

Abstract

Background: Hypoxia-Inducible Factor 1 (HIF-1) alpha (HIF-1α) is known to be expressed in hypoxia and affects stemness and bone differentiation. Protein Transduction Domains (PTDs) safely and effectively deliver proteins into cells. This study combined HIF-1α delta Oxygen-Dependent Degradation Domain–Transcription Modulation Domain (ntHIF-1α-ΔODD-TMD) with the PTD and then used it to treat Stem cells from Human Exfoliated Deciduous teeth (SHED) to determine its effect on stemness and bone differentiation.

Methods: The SHED were treated using PDT-conjugated ntHIF-1α-ΔODD-TMD, and its effects on STRO-1 expression, cytotoxicity, stemness-related gene expression, and osteogenic differentiation were confirmed. These were also transferred to 5-week-old mice in order to confirm the effects *in vivo* after recellularization of SHED treated using ntHIF-1α-ΔODD-TMD on decellularized tooth specimens. After 9 weeks, they were sacrificed to confirm the expression of genes related to bone differentiation and angiogenesis.

Results: ntHIF-1α-ΔODD-TMD increased the expression levels of STRO-1, HIF-1α-related genes, and stemness-related genes in SHED. Osteogenic differentiation was also increased both *in vitro* and *in vivo*.

Conclusion: These results suggest that ntHIF-1α-ΔODD-TMD maintains or increases the stemness of SHED and also increases the osteogenic potential. SHED have previously been found to have excellent proliferative but low differentiation capacities compared with other cells. However, properly using ntHIF-1α-ΔODD-TMD increases the osteogenic potential of SHED, indicating the potential of using SHED as a useful source for hard-tissue regeneration.

Keywords: Hypoxia-inducible factor 1 alpha; Stem cells from human exfoliated deciduous teeth; Protein transduction domain; Stemness; Osteogenic differentiation

OPEN ACCESS

Correspondence:
Je Seon Song, Department of Pediatric Dentistry, College of Dentistry, Yonsei University, Seoul, Korea, Tel: +82-2-22283170; Fax: +82-2-3927420; E-mail: songjs@yuhs.ac

Received Date: *01 Jun 2023*
Accepted Date: *16 Jun 2023*
Published Date: *22 Jun 2023*

Citation:
Kim IH, Jeon M, Lee YH, Shin JS, Lee T, Lee SK, et al. HIF-1α Overexpression Using a Protein Transduction Domain to Increase the Osteogenic Potential of SHED. J Dent Oral Biol. 2023; 8(3): 1215.

ISSN: 2475-5680

Introduction

Stem Cells from Human Exfoliated Deciduous Teeth (SHED), which can be obtained from the pulp of primary teeth, have the advantage of being obtainable noninvasively, unlike other stem cells that can be obtained from teeth. In particular, they represent an attractive source of stem cells due to its excellent proliferative capacity [1,2]. It can be considered that excellent stem cell function means that there is potential for them to differentiate into various cell types [1]. However, although SHED have excellent stemness, their bone regeneration ability is not as good as that of other cells that can be obtained from teeth [3,4]. If SHED can increase the bone regeneration ability, they can be a useful stem cell source for both tooth and hard-tissue regeneration.

Hypoxia-Inducible Factor 1 (HIF-1) alpha (HIF-1α) affects stemness and osteogenesis, and is known to be a master regulator of the hypoxic response that regulates the expression of important genes related to hypoxia [5]. HIF-1α is composed of multiple domains, and Oxygen-Dependent Degradation Domain (ODD) is involved in HIF-1α degradation. This domain induces HIF-1α degradation under normoxia [6], and it may therefore be effective to use HIF-1α delta ODD with the ODD removed to obtain a sustained effect of HIF-1α in normoxic conditions.

Gene delivery using cell-penetrating proteins is a recently recognized method of gene delivery into the cell nucleus. Using viral or nonviral vectors or lipofectamine were previously common methods for regulating transcription factor expression. However, these had problems such as viral infection and immunological response, low delivery efficiency, and increased cell death [7-11]. A method for directly delivering transcription factors into the nucleus using a protein with a short amino acid sequence of less than 30 bp called the Protein Transduction Domain (PTD) was recently identified [12-15]. This method has been found to have higher gene transfer efficiency than using viral vectors. Among various PTDs, PTD (ARVRRRGPRR) discovered in human transcription factor hph-1 has strong *in vitro* and *in vivo* delivery abilities [16].

HIF-1α was used to increase the bone differentiation ability of SHED in the present study. We identified any changes in the stemness and bone differentiation potential of SHED by treating HIF-1α with the ODD removed. hph-1-PTD was used in this process to effectively deliver HIF-1α from which ODD was removed into cells. This process can confirm the effect of HIF-1α on the stem cell function and bone differentiation ability of SHED.

Materials and Methods

Production of ntHIF-1α-ΔODD-TMD

To generate the HIF-1α-ΔODD, single-site mutagenesis reaction was carried out in Gly27 using Arg in p(HA)HIF-1α(401delta603) R27G plasmid (#52215, Addgene, Watertown, MA, USA). The primer sequence used for mutagenesis was as follows: forward primer 5' - A A A A G T C T C G A G A T G C A G C C A G A T C T C G G C G A A G T A A A G A A - 3' and reverse primer 5' - T T C T T T A C T T C G C C G A G A T C T G G C T G C A T C T C G A G A C T T T T - 3'. The HIF-1α(401delta603) plasmid generated through mutagenesis was amplified by the Polymerase Chain Reaction (PCR) using the following primers: Forward primer 5' - C T A G C T A G C A T G G A G G G C G C C G G C - 3' and reverse primer 5' - G G G G T A C C G T G T T A A C T T G A T C C A A A G - 3'. The PCR product was inserted into the pEGFPN1 plasmid expression vector (Invitrogen, Carlsbad, CA, USA) using restriction enzyme NheI (Takara Bio, Otsu, Japan) at 5' termini and KpnI (Takara Bio) at 3' termini of the PCR fragment.

To generate the DNA construct of ntHIF-1α-ΔODD-TMD (nucleus transducible HIF-1α-ΔODD–transcription modulation domain), DNA sequences that encode the N-terminus of HIF-1α-ΔODD and hph-1-PTD were amplified using PCR and inserted into the pET-28a (+) vector (Novagen Merck Millipore, Billerica, MA, USA). The DNA construct was expressed in *Escherichia coli* [Rosetta2 (DE3), Sigma-Aldrich, St Louis, MO, USA], and affinity chromatography was used to purify the proteins. The supernatant of the cells that expressed ntHIF-1α-ΔODD-PTD were harvested through sonication in a native lysis buffer (10 mM imidazole, 50 mM NaH_2PO_4, and 300 mM NaCl). The filtered supernatant was mixed with an Ni-NTA agarose (QIAGEN, Hilden, Germany), and non-specifically bound proteins were washed using a native wash buffer composed of 30 mM imidazole, 50 mM NaH_2PO_4, and 300 mM NaCl. The proteins were eluted using a native elution buffer composed of 500 mM imidazole, 50 mM NaH_2PO_4, and 300 mM NaCl, and diluted using an SP binding buffer (50 mM NaH_2PO_4 and 300 mM NaCl). Ion-exchange chromatography was performed using SP Sepharose (GE Healthcare Life Sciences, Marlborough, MA, USA), and the purified proteins were concentrated using an Amicon filter (Merck Millipore, Carrigtwohill, Ireland). The final product was desalted in a PD-10 Sephadex G-25 column (GE Healthcare Life Sciences) filled with 10% glycerol Phosphate Buffered Saline (PBS, Invitrogen).

Cell culture

The SHED was obtained from the deciduous teeth of six children (aged 2 to 7 years; two males and four females) according to the approved guidelines set by the Institutional Review Board of the Yonsei University Dental Hospital (approval no. #IRB 2-2020-0033). The deciduous anterior teeth used in this study were almost at the natural exfoliation stage, had less than one-third of the root remaining, and had no deep caries, restorations, periapical lesions, or internal resorption. The deciduous teeth were isolated using enzymatic disaggregation after extraction. In brief, the pulp tissue was treated using collagenase type I (3 mg/ml, Invitrogen) and dispase (4 mg/ml, Invitrogen) for 30 min at 37°C and were then filtered through a 70-μm cell strainer. The SHED was cultured in a cell culture medium comprising alpha-minimum essential medium (α-MEM; Invitrogen) that contained 10% Fetal Bovine Serum (FBS; Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen), and 2 mM L-glutamine (Invitrogen) at 37°C in 5% CO_2. The isolated stem cells that originated from at least four different donors from each group were blended at passage 2, and cells at passages 3 to 5 were used for further experiments.

Western blot

After 24 h of ntHIF-1α-ΔODD-TMD treatment, SHED lysate was electrophoresed and transferred onto polyvinylidene difluoride (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked to prevent nonspecific antibody binding using blocking buffer [5% skim milk (LPS solution, Daejeon, South Korea) in diluted 10X Tris-buffered saline (Bio-Rad Laboratories) with 0.05% Tween-20 (TBST; Bio Basic, Markham, ON, Canada] for 30 min. The blocked membrane was incubated at 4°C overnight using diluted primary antibodies [beta-actin (β-actin) rabbit monoclonal antibody (1:2000; Cell Signaling Technology, Danvers, MA, USA) and DYKDDDDK (FLAG) Tag mouse monoclonal antibody (1:2000, Cell Signaling Technology)]. After washing with TBST, anti-mouse IgG (1:2000, Cell Signaling Technology) or antirabbit IgG (1:2000, Cell Signaling Technology) were used to detect each primary antibody. ECL reagent (Bio-Rad Laboratories) was added into the membrane for the chemiluminescence reaction and the signal was observed using ChemiDoc (Bio-Rad Laboratories).

Flow cytometry analysis

The flow cytometry analysis method used in this study has been described previously [17]. In brief, SHED was treated for 24 h using 1 μM ntHIF-1α-ΔODD-TMD. The SHED was detached after 24 h using 0.2% ethylenediaminetetraacetic acid (Fisher Scientific, Houston, TX, USA) in PBS (Invitrogen), and then the cells were resuspended in PBS (Invitrogen). The cells (1×10^6) were then incubated using 5 μg of antihuman STRO-1 antibody (Alexa Fluro 388 conjugated, R&D Systems, Minneapolis, MN, USA) diluted at a concentration of 1:10. The control conditions involved omitting the fluorescent-dye-conjugated antibodies. All procedures were performed in the dark at 4°C. The expression profiles were observed using a flow cytometer (LSRII Flow Cytometer, BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using FCS Express 7 Research Edition (De Novo Software, Los Angeles, CA, USA). Data were obtained from three independent experiments.

Cell cytotoxicity

Cell cytotoxicity was determined using the Cell Counting Kit (CCK)-8 assay (Dojindo Laboratories, Kumamoto, Japan). In brief, the SHED was plated into 24-well culture plates (BD Falcon, Lincoln Park, NJ, USA) at a density of 2×10^4 cells per well. After 24 h, the cells were treated using ntHIF-1α-ΔODD-TMD for another 24 h. After 48 h, the quantity of water-soluble colored formazan from the CCK-8 assay formed by dehydrogenase activity in living cells was measured using a spectrophotometer (Benchmark Plus Microplate Spectrophotometer, Bio-Rad Laboratories) at 450 nm. Cell cytotoxicity data were obtained from three independent experiments, with all samples used in triplicate.

Quantitative real-time PCR

The SHED was treated using ntHIF-1α-ΔODD-TMD for 24 h. After 24 h, total RNA was isolated using the RNeasy Mini Kit (QIAGEN) according to the instructions of the manufacturer. The integrity and concentration of the extracted RNA were evaluated using a spectrophotometer (NanoDrop ND-2000, Thermo Scientific, Waltham, MA, USA). cDNA was synthesized by reverse transcribing 500 μg of RNA using a Maxime RT PreMix kit (oligo dT15 primer, iNtRON Biotechnology, Gyeonggi, South Korea) according to the instructions of the manufacturer. A quantitative real-time PCR (qPCR) assay was performed using TB Green Premix Ex Taq (Tli RNase H Plus, Takara Bio) and a real-time PCR system (ABI 7300, Applied Biosystems, Carlsbad, CA, USA) according to the instructions of the manufacturer. The qPCR condition comprised 95°C for 10 sec followed by 40 cycles at 95°C for 5 sec and 60°C for 31 sec, with a final 5-min extension at 72°C. The sequences and sizes of the primers are listed in Table 1. The values for each gene were normalized to the expression levels of the gene encoding β-actin, and the relative expression levels of the studied genes were calculated using the 2-ΔΔCt method [18]. Data were obtained from three independent experiments, with all samples used in duplicates.

Osteogenic differentiation

A modified version of the differentiation methods described by Song et al. [19] was used. In brief, the cells were prepared in 12-well culture plates and treated using 1 μM ntHIF-1α-ΔODD-TMD for 24 h after reaching 80% confluence. After 24 h, the culture medium was swapped with α-MEM that contained 10% FBS, 1% antibiotics, 0.1 μM dexamethasone, 2 mM β-glycerophosphate (Sigma-Aldrich), and 50 μM ascorbic acid 2-phosphatasee. Relative changes in the expression levels of osteogenesis-related genes encoding Alkaline Phosphatase (ALP) and Osteocalcin (OC) and runt-related transcription factor 2 (RUNX2) were evaluated using qPCR at days 3 and 7. Data were obtained from three independent experiments, with all samples used in duplicates. Mineralization nodules were visualized using alizarin red S stain at day 14. In brief, cells were fixed and then stained using 2% alizarin red S (pH 4.2; Sigma-Aldrich) for 20 min at room temperature. Data were obtained from three independent experiments.

Decellularization and recellularization

Tooth slices were incubated in 1% Triton X-100 (Bio Basic) for 24 h and then with 1% sodium dodecyl sulfate (Tech & Innovation, Gangwon, South Korea) for 24 h to decellularize the samples. This cycle was repeated three times. These procedures were performed at room temperature with constant gentle agitation of the samples in an SH30 Orbital Shaker (FinePCR, Gyeonggi, South Korea) in the presence of protease inhibitor cocktail (EMD Millipore, Darmstadt, Germany). Samples were rinsed three times for 10 min each using PBS (Invitrogen) at the end of each cycle. The tooth slices were recellularized by pipetting rat tail collagen I (Corning, Corning, NY, USA) 1×10^7 SHED/ml in 12-well culture plates (Corning). After 30 min, 1 ml of basal culture media was added to each well, which was changed every 3 days. Cells were cultured at 37°C in 5% CO_2 for 2 weeks.

In vivo transplantation

In vivo procedures were performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee of Yonsei University (approval no. #2022-0016). Samples were prepared using the recellularization experiments. Decellularized teeth were used as the control samples. Samples were subcutaneously transplanted into the dorsal surfaces of 5-week-old male immunocompromised BALB/c nude mice (SLC, Shizuoka, Japan). Two pockets were made in each mouse (n=12) and either recellularized SHED or SHED treated using recellularized ntHIF-1α-ΔODD-TMD were inserted into each. All transplants were retrieved after 9 weeks. Of the 24 pockets, 4 from decellularized tooth plants were used as controls. The remaining 20 pockets were recellularized SHED and or SHED treated using recellularized ntHIF-1α-ΔODD-TMD.

Gene expression analysis using qPCR

Gene expression levels in samples were evaluated using qPCR. Total RNA was extracted from the samples using a RNeasy Mini Kit (QIAGEN) according to the instructions of the manufacturer. The integrity and concentration of the extracted RNA were measured using a NanoDrop ND-2000 spectrophotometer (Thermo Scientific). cDNA was synthesized from 500 ng of RNA using the oligo dT15 primer Maxime RT PreMix Kit (iNtRON Biotechnology) according to the instructions of the manufacturer. A qPCR assay was performed using the TB Green Premix EX Taq II (Takara Bio) and the ABI 7300 Real-time PCR system (Applied Biosystems) according to the instructions of the manufacturer. Information about the primers is provided in Table 1. The expression level of each gene was normalized to that of the gene encoding β-actin, and the relative gene expression levels were calculated using the 2-ΔΔCt method [18]. Gene expression levels were calculated relative to their expression levels in the decellularized tooth control samples.

Statistical analyses

All experiments were performed in triplicate at least. Statistical analyses were performed using SPSS (version 26.0, SPSS, Chicago, IL, USA). Data normality was evaluated using the Shapiro-Wilk test ($p<0.05$). The Mann-Whitney U test ($p<0.05$) was also performed for all experiments.

Results

Generation of ntHIF-1α-ΔODD-TMD and transduction efficiency of ntHIF-1α-ΔODD-TMD

HIF-1α-ΔODD was produced by deleting ODD, an oxygen-dependent domain, from wild-type HIF-1α. The N-terminus of HIF-1α-ΔODD has a TMD comprising DNA-binding amino acid residues and isotype-specific sequences that may play key roles in the functional specificity of HIF-1α-ΔODD. We generated a transducible fusion protein (HIF-1α-ΔODD-TMD) of HIF-1α-ΔODD with hph-1-PTD (YARVRRRGPRR) to modulate the functions of HIF-1α.

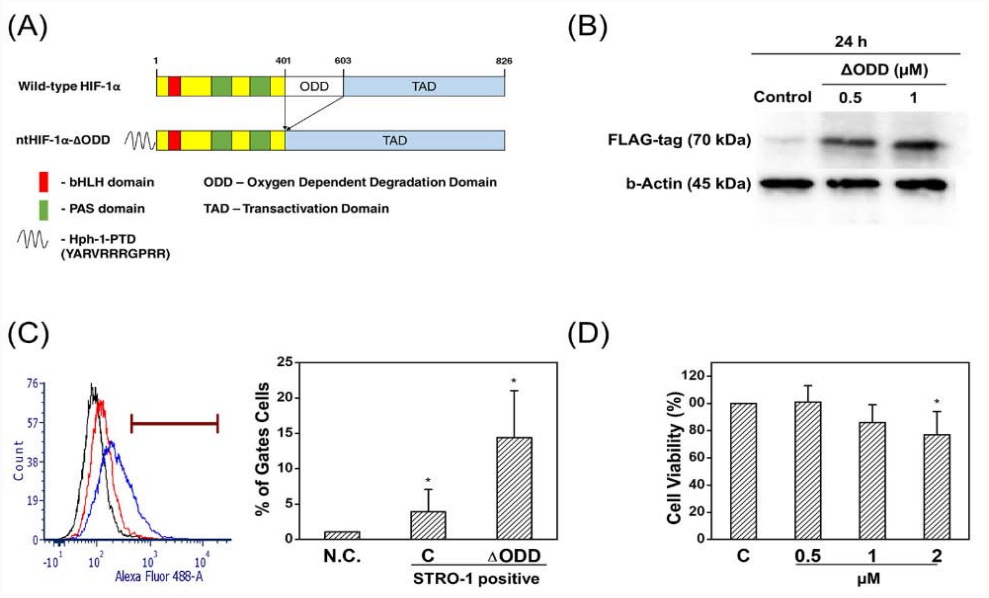

Figure 1: (A) Structure of ntHIF-1α-ΔODD-TMD. (B) Transduction efficiency of ntHIF-1α-ΔODD-TMD. Dose-dependent intracellular transduction efficiency of ntHIF-1α-ΔODD-TMD into SHED. ntp65-TMD was detected using immunoblot analysis with anti-FLAG antibody, with β-actin as a loading control. (C) STRO-1 expression of ntHIF-1α-ΔODD-TMD in SHED (black lines, negative controls; red lines, SHED controls; blue lines, SHED treated using ntHIF-1α-ΔODD-TMD). Horizontal bars indicate 1% of the control samples. Flow cytometry analysis was performed in three independent experiments. (D) Cell viability of ntHIF-1α-ΔODD-TMD in SHED. Cells were treated using ntHIF-1α-ΔODD-TMD at different concentrations for 24 h, and cell viability was assayed using the CCK-8 assay kit. No treatment group was used for the positive controls. The data are mean and standard-deviation values. Cell viability differed significantly in the treatment group with 2 μM ntHIF-1α-ΔODD-TMD. Values are the means and standard deviations of three independent experiments. *p<0.05 indicates a significant difference from the control value (100%).

Table 1: qPCR forward and reverse primer sequences and sizes. The annealing procedures were performed at 60ºC for all primers.

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Size (bp)
ALP	GGACCATTCCCACGTCTCAC	CCTTGTAGCCAGGCCCATTG	137
CD146	CCGTCTCGTAAGAGCGAACT	CAGGGAAGGGAGCTGAAGTG	166
GAPDH	TCCTGCACCACCAACTGCTT	TGGCAGTGATGGCATGGAC	100
HIF1A	AGCTTGCTCATCAGTTGCCA	CCAGAAGTTTCCTCACACGC	105
OC	CAAAGGTGCAGCCTTTGTGTC	TCACAGTCCGGATTGAGCTCA	150
REX1	ACCATCGCTGAGCTGAAACAAA	ATGTTTTCCTGCCTGTGCCC	191
RUNX2	CACTGGCGCTGCAACAAGA	CATTCCGGAGCTCAGCAGAATAA	127
SOX2	ACCAGCGCATGGACAGTTAC	CCGTTCATGTAGGTCTGCGA	196
VEGF	TCCTCACACCATTGAAACCA	GATCCTGCCCTGTCTCTCTG	131
Beta actin	TCACCATGGATGATGATATCGC	GGAATCCTTCTGACCCATGC	161

Abbreviations: *ALP*: Gene Encoding Alkaline Phosphatase; *CD146*: Gene Encoding Cell Differentiation 146; *GAPDH*: Gene Encoding Glyceraldehyde-3-Phosphate Dehydrogenase; *HIF1A*: Gene Encoding Hypoxia Inducible Factor 1 Alpha; *OC*: Gene Encoding Osteocalcin; *RUNX2*: Gene Encoding Runt-Related Protein 2; *SOX2*: Gene Encoding SRY (Sex-Determining Region of Y)-box 2; *VEGF*: Gene Encoding Vascular Endothelial Growth Factor

ntHIF-1α-ΔODD was designed as a novel therapeutic to efficiently deliver HIF-1α-ΔODD-TMD into the cell nuclei *in vitro* and *in vivo* (Figure 1A). We added treated ntHIF-1α-ΔODD-TMD into SHED to examine the effects on intracellular transduction efficiency. FLAG-tagged ntHIF-1α-ΔODD-TMD was confirmed using Western blotting. ntHIF-1α-ΔODD-TMD was effectively transduced into the cells after 24 h of treatment (Figure 1B).

STRO-1 expression and cell cytotoxicity of SHED treated using ntHIF-1α-ΔODD-TMD

STRO-1 expression was examined using flow cytometry analysis. STRO-1 expression differed significantly between the ntHIF-1α-ΔODD-TMD treatment group and the control group (Figure 1C). Cell cytotoxicity was determined using the CCK-8 assay kit and SHED grown in α-MEM were used as the controls. Cell cytotoxicity differed significantly at 2 μM in the ntHIF-1α-ΔODD-TMD treatment group (Figure 1D).

Identification of markers related to HIF-1α and stemness of SHED treated using ntHIF-1α-ΔODD-TMD

SHED treated using ntHIF-1α-ΔODD-TMD increased HIF-1α expression compared with the control SHED (not treated) (Figure 2A). VEGF and GAPDH expression levels, which are affected by HIF-1α expression, also increased. Measuring the expression of stemness-related genes revealed that the expression levels of the REX1, CD146, and SOX2 genes increased. The expression level of each gene increased with the ntHIF-1α-ΔODD-TMD concentration.

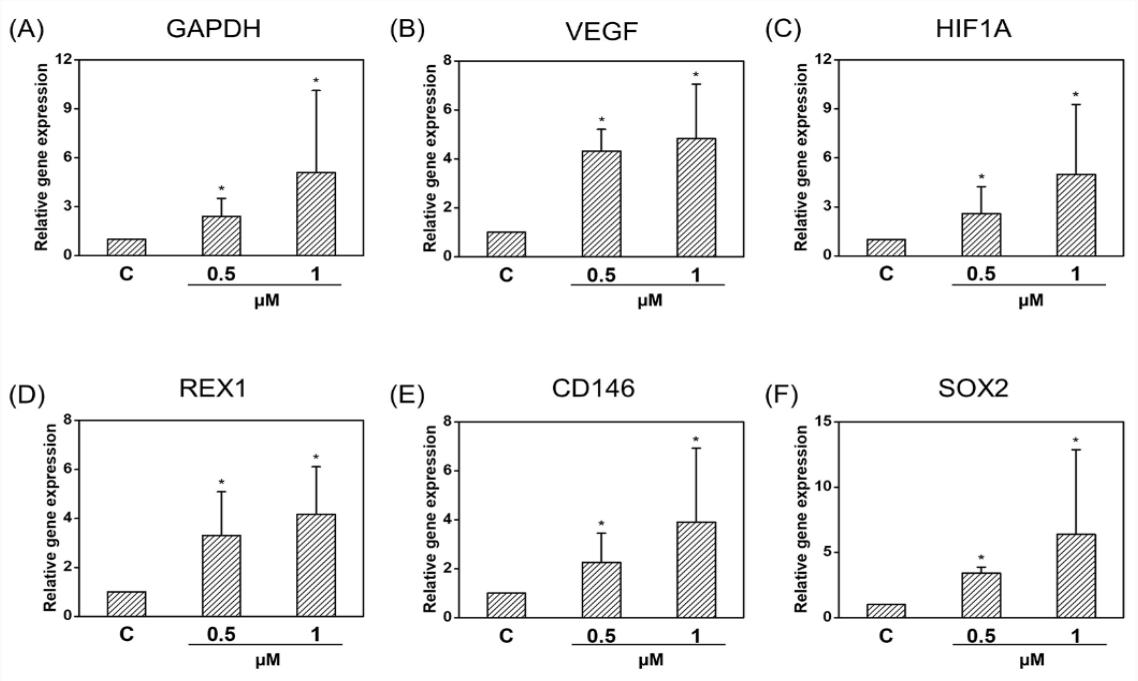

Figure 2: Gene expression of ntHIF-1α-ΔODD-TMD in SHED. SHED without ntHIF-1α-ΔODD-TMD were used as the controls. Values are the means and standard deviations of three independent experiments. *p<0.05 indicates a significant difference from the control value.

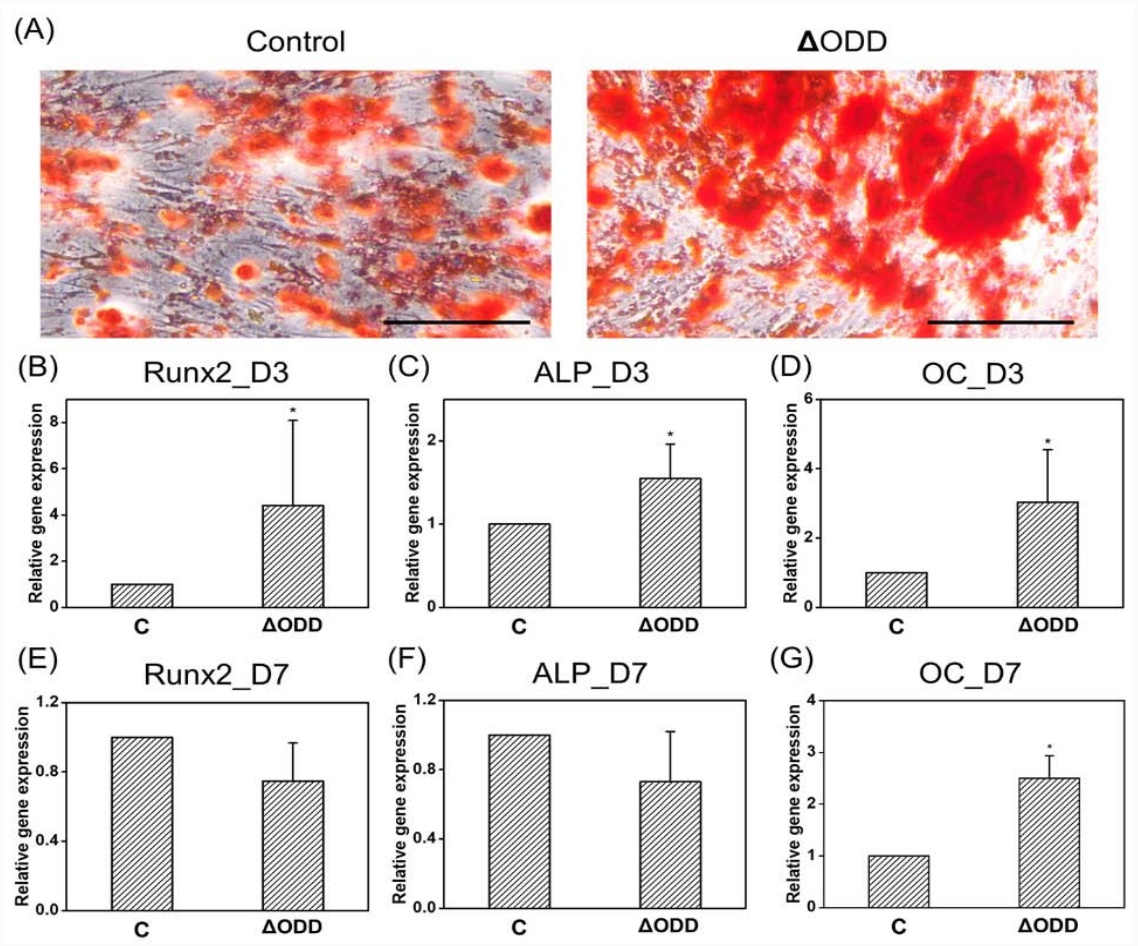

Figure 3: Osteogenic differentiation of ntHIF-1α-ΔODD-TMD in SHED. (A) Alizarin red S staining. Data were obtained from three independent experiments. Scale bars: 200 μm. (B–G) Gene expression of ntHIF-1α-ΔODD-TMD in SHED during osteogenic differentiation. Data were obtained from three independent experiments, with all samples used in duplicate. The data are mean and standard-deviation values.

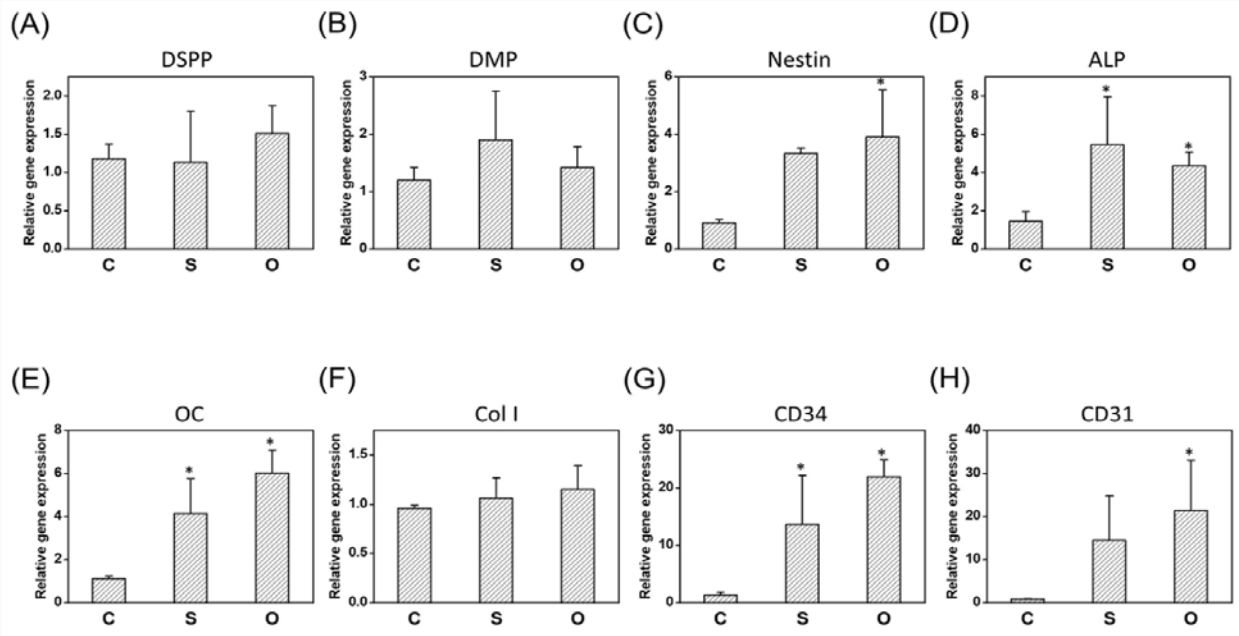

Figure 4: Gene expression of markers of osteogenic differentiation for *in vivo* transplantation. The gene expression levels of decellularized teeth transplants were set as the control (normalized to 1). Data were obtained from four transplants for the controls, ten for the recellularized SHED, and ten for the SHED treated using recellularized ntHIF-1α-ΔODD-TMD. Data are mean and standard-deviation values. *p<0.05 indicates a significant difference from the control value.
C: Control; S: SHED; O: SHED treated using ntHIF-1α-ΔODD-TMD

In vitro osteogenic differentiation of ntHIF-1α-ΔODD-TMD treated SHED

Both SHED and SHED treated using ntHIF-1α-ΔODD-TMD had the ability to differentiate into cells that produce mineralized nodules following treatment using an osteogenic induction medium. The degree of osteogenic differentiation was confirmed through alizarin red S staining, which indicated more differentiation in SHED treated using ntHIF-1α-ΔODD-TMD than in nontreated SHED (Figure 3A).

SHED treated using ntHIF-1α-ΔODD-TMD increased osteogenic marker expression compared with the control SHED at day 3. RUNX2 and ALP expression levels were significantly higher in SHED treated using ntHIF-1α-ΔODD-TMD than in the control SHED at day 3 (Figure 3B, 3C; Mann-Whitney U test, $p<0.05$). OC expression was higher in SHED treated using ntHIF-1α-ΔODD-TMD than in the control SHED at days 3 and 7 (Figure 3D, 3G), but the difference was not significant. The expression levels of RUNX2 and ALP, which are early markers of osteogenic differentiation, also decreased on day 7 (Figure 3E, 3F).

In vivo gene expression levels of SHED treated using recellularized ntHIF-1α-ΔODD-TMD

The expression levels of genes related to osteogenesis and angiogenesis were confirmed using qPCR (Figure 4). Compared with the decellularized teeth in the control group, the expression levels of all genes were increased in recellularized SHED and SHED treated using recellularized ntHIF-1α-ΔODD-TMD. Gene expression levels were all higher in SHED treated using recellularized ntHIF-1α-ΔODD-TMD than in recellularized SHED, except for ALP.

Discussion

This study confirmed that the stemness of SHED increased when overexpression of ntHIF-1α-ΔODD-TMD was induced. In particular, it was confirmed that the osteogenic differentiation ability of SHED increased.

HIF-1α is known to increase the stem cell generation ability of dental stem cells. HIF-1α is known to increase expression during hypoxia, and is involved in increased angiogenesis. The ntHIF-1α-ΔODD-TMD used in this study removes the ODD involved in HIF-1α degradation during hypoxia, and then adds the PTD hph-1-PTD to deliver it into the cell nuclei [19]. The delivery effect and safety of the PTD used in the study have been demonstrated previously [16,20-23]. Treating SHED using ntHIF-1α-ΔODD-TMD affected the increase in stemness and increased angiogenesis-related gene expression (Figure 2), which was consistent with the findings of previous studies. Hypoxia plays an essential role in maintaining stem cell properties such as self-renewal, survival, and pluripotency. Culturing stem cells under a low oxygen concentration has been found to increase the expression levels of proliferation- and stem-cell-related markers [24]. STRO-1 expression also ranges from 0.02% to 9.56% in human pulp stem cell culture, but it has been found to increase under hypoxic conditions [25,26]. HIF-1α expression is activated in hypoxic conditions. In this study, treating SHED using ntHIF-1α-ΔODD-TMD increased HIF-1α expression, and the SHED recognized this condition as hypoxia. As a result, the expression levels of STRO-1 and stem-cell-related genes increased in SHED treated using ntHIF-1α-ΔODD-TMD.

HIF-1α also plays an important role in bone formation [27,28]. Osteogenesis and angiogenesis are very closely related. HIF-1α is an adaptive protein regulated by biomechanical and proinflammatory signals during bone regeneration. Increased bone mass and rich skeletal vasculature were observed in mice in which osteoblasts were induced to overexpress HIF-1α, and enhanced osteogenesis was found in Bone marrow stem cells transfected with HIF-1α [29,30]. A study also found that the role of HIF-1α changes according to age in relation to osteogenesis. HIF-1α expression is known to increase in young mice, which affects the increase in osteogenesis. However, in

aged mice, HIF-1α expression increased p53 expression, suppressed osteogenesis-related gene expression, and decreased VEGF expression [31]. The SHED used in the current experiment are cells that can be obtained at a lower age than other dental stem cells. It is therefore thought that HIF-1α overexpression helped to increase osteogenesis.

Among the various stem cells that can be obtained from human teeth, there are two types that can be obtained from dental pulp tissue: Primary and permanent dental pulp stem cells. These two cell types are a type of mesenchymal stem cell, have proliferative ability, and can differentiate into various cells. Both cell types have the characteristics of being able to differentiate into odontoblasts, osteoblasts, adipocytes, and neural cells, [1,2,4], which can also differentiate into dental pulp and dentine as well as periodontal tissue, so they can be used as stem cell sources to regenerate various dental tissues. SHED are cells that have the advantage of being obtained in a noninvasive way among the various stem cells that can be obtained from teeth. Since they can be obtained from decidual primary teeth, they have the advantage of being easier to obtain than Dental Pulp Stem Cell (DPSC) and have been found to have excellent cell proliferation ability. However, the osteogenic differentiation potential is lower for SHED than for DPSC [3,4,32]. We also confirmed that there is a difference in the stem cell generation ability of SHED depending on the method used to isolate stem cells. SHED isolated by enzymatic disaggregation were confirmed to have lower osteogenic potential than cells isolated using the outgrowth method [17]. This appeared to vary depending on whether or not the cells were isolated from the stem cell niche. However, it has recently been common to isolate stem cells using the enzymatic disaggregation method rather than outgrowth. Therefore, if SHED isolated using the enzymatic disaggregation method retain stemness and increase bone differentiation ability, they can be used as a good source for hard-tissue regeneration. Since ntHIF-1α-ΔODD-TMD increases the osteogenic potential while maintaining the stemness of SHED, it is thought that appropriate treatment with ntHIF-1α-ΔODD-TMD can further increase the applicability of SHED.

This study had several limitations. Further studies should be conducted to confirm the characteristics of hard tissues generated through *in vivo* experiments. It also needs to be confirmed whether the increase in stemness and osteogenic potential when using ntHIF-1α-ΔODD-TMD treatment also occurs in other dental stem cells. It is further necessary to conduct an experiment to confirm that there is no change in the ability of SHED treated using ntHIF-1α-ΔODD-TMD to differentiate into other cells regardless of the osteogenic potential. Nevertheless, we believe that processing ntHIF-1α-ΔODD-TMD into SHED increases their potential for use as a better source for hard-tissue regeneration.

Conclusion

SHED, which can be obtained from primary teeth, are stem cells that are easier to obtain than those from other dental tissues, and have excellent proliferative ability. However, it was found that its ability to differentiate into hard tissue was lower than that of other cells. Treatment using ntHIF-1α-ΔODD-TMD increases the ability of SHED to differentiate into hard tissue, so they can be used as a more useful source for tooth tissue regeneration.

Funding

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2018R1D1A1B07041657) and the Yonsei University College of Dentistry Fund (6-2021-0013).

Ethics Approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Yonsei University Dental Hospital (July 09, 2020 / approval no. #IRB 2-2020-0033).

In vivo procedures were performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee of Yonsei University (March 07, 2022 /approval no. #2022-0016).

References

1. Zhai Q, Dong Z, Wang W, Li B, Jin Y. Dental stem cell and dental tissue regeneration. Front Med. 2019(2):152-9.
2. Rodríguez-Lozano FJ, Bueno C, Insausti CL, Meseguer L, Ramírez MC, Blanquer M, et al. Mesenchymal stem cells derived from dental tissues. Int Endod J 2011;44:800-6.
3. Sabbagh J, Ghassibe-Sabbagh M, Fayyad-Kazan M, Al-Nemer F, Fahed JC, Berberi A, et al. Differences in osteogenic and odontogenic differentiation potential of DPSCs and SHED. J Dent 2020;101:103413.
4. Wang X, Sha XJ, Li GH, Yang FS, Ji K, Wen LY, et al. Comparative characterization of stem cells from human exfoliated deciduous teeth and dental pulp stem cells. Arch Oral Biol. 2012;57:1231-40.
5. Semenza GL, Wang GL. A nuclear factor induced by hypoxia *via de novo* protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol 1992;12:5447-54.
6. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielber J, Gaskell SJ, et al. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science. 2001;292(5516):468-72.
7. Gao X, Huang L. Cationic liposome-mediated gene transfer. Gene Ther. 1995;2(10):710-22.
8. Torchilin VP, Levchenko TS, Rammohan R, Volodina N, Papahadjopoulos-Sternberg B, D'Souza GG. Cell transfection *in vitro* and *in vivo* with nontoxic TAT peptide-liposome-DNA complexes. Proc Natl Acad Sci U S A. 2003;100(4):1972-7.
9. Lundstrom K. Latest development in viral vectors for gene therapy. Trends Biotechnol. 2003;21(3):117-22.
10. Liu F, Yang J, Huang L, Liu D. New cationic lipid formulations for gene transfer. Pharm Res. 1996;13(12):1856-60.
11. Stone D. Novel viral vector systems for gene therapy. Viruses. 2010;2(4):1002-7.
12. Harreither E, Rydberg HA, Amand HL, Jadhav V, Fliedl L, Benda C, et al. Characterization of a novel cell penetrating peptide derived from human Oct4. Cell Regen. 2014;3(1):2.
13. De Coupade C, Fittipaldi A, Chagnas V, Michel M, Carlier S, Tasciotti E, et al. Novel human-derived cell-penetrating peptides for specific subcellular delivery of therapeutic biomolecules. Biochem J. 2005;390:407-18.
14. Nagahara H, Vocero-Akbani AM, Snyder EL, Ho A, Latham DG, Lissy NA, et al. Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27Kip1 induces cell migration. Nat Med. 1998;4(12):1449-52.
15. Veldhoen M, Magee AI, Penha-Goncalves MN, Stockinger B. Transduction of naive CD4 T cells with kinase-deficient Lck-HIV-Tat fusion protein dampens T cell activation and provokes a switch to regulatory function. Eur J Immunol. 2005;35(1):207-16.
16. Choi JM, Ahn MH, Chae WJ, Jung YG, Park JC, Song HM, et al. Intranasal delivery of the cytoplasmic domain of CTLA-4 using a novel protein transduction domain prevents allergic inflammation. Nat Med.

2006;12(5):574-9.

17. Jeon M, Song JS, Choi BJ, Choi HJ, Shin DM, Jung HS, et al. *In vitro* and *in vivo* characteristics of stem cells from human exfoliated deciduous teeth obtained by enzymatic disaggregation and outgrowth. Arch Oral Biol. 2014;59(10):1013-23.

18. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8.

19. Song JS, Kim SO, Kim SH, Choi HJ, Son HK, Jung HS, et al. *In vitro* and *in vivo* characteristics of stem cells derived from the periodontal ligament of human deciduous and permanent teeth. Tissue Eng Part A. 2012;18(19-20):2040-51.

20. Cheon SY, Kim JM, Kam EH, Ho CC, Kim EJ, Chung S, et al. Cell-penetrating interactomic inhibition of nuclear factor-kappa B in a mouse model of postoperative cognitive dysfunction. Sci Rep. 2017;7(1):13482.

21. Kang CM, Mo S, Jeon M, Jung UW, Shin Y, Shin JS, et al. Intranuclear delivery of nuclear factor-kappa B p65 in a rat model of tooth replantation. Int J Mol Sci. 2021;22.

22. Cheon SY, Kim JM, Kim EJ, Kim SY, Kam EH, Ho CC, et al. Intranuclear delivery of synthetic nuclear factor-kappa B p65 reduces inflammasomes after surgery. Biochem Pharmacol 2018;158:141-52.

23. Park SD, Cheon SY, Park TY, Shin BY, Oh H, Ghosh S, et al. Intranuclear interactomic inhibition of NF-κB suppresses LPS-induced severe sepsis. Biochem Biophys Res Commun. 2015;464(3):711-7.

24. Ahmed NE, Murakami M, Kaneko S, Nakashima M. The effects of hypoxia on the stemness properties of human Dental Pulp Stem Cells (DPSCs). Sci Rep. 2016;6:35476.

25. Werle SB, Chagastelles P, Pranke P, Casagrande L. The effects of hypoxia on in vitro culture of dental-derived stem cells. Arch Oral Biol. 2016;68:13-20.

26. Sakdee JB, White RR, Pagonis TC, Hauschka PV. Hypoxia-amplified proliferation of human dental pulp cells. J Endod. 2009;35(6):818-23.

27. Jahangir S, Hosseini S, Mostafaei F, Sayahpour FA, Baghaban Eslaminejad M. 3D-porous β-tricalcium phosphate-alginate-gelatin scaffold with DMOG delivery promotes angiogenesis and bone formation in rat calvarial defects. J Mater Sci Mater Med. 2018;30(1):1.

28. Tan L, Zhang Y, Huang Y, Luo Y, Liu Y. Preservation of alveolar ridge after tooth extraction with hypoxia-inducible factor-1α protein in a dog model. Exp Ther Med. 2019;17(4):2913-20.

29. Wang Y, Wan C, Deng L, Liu X, Cao X, Gilbert SR, et al. The hypoxia-inducible factor alpha pathway couples angiogenesis to osteogenesis during skeletal development. J Clin Invest. 2007;117(16):1616-26.

30. Zou D, Han W, You S, Ye D, Wang L, Wang S, et al. *In vitro* study of enhanced osteogenesis induced by HIF-1α-transduced bone marrow stem cells. Cell Prolif. 2011;44(3):234-43.

31. Shao J, Liu S, Zhang M, Chen S, Gan S, Chen C, et al. A dual role of HIF1α in regulating osteogenesis-angiogenesis coupling. Stem Cell Res Ther. 2022;13:59.

32. Nakamura S, Yamada Y, Katagiri W, Sugito T, Ito K, Ueda M. Stem cell proliferation pathways comparison between human exfoliated deciduous teeth and dental pulp stem cells by gene expression profile from promising dental pulp. J Endod. 2009;35:1536-42.