치의과학박사 학위논문

# Enhanced osteogenic potential by inhibiting *LHX8* overexpression-associated *ID1* in human dental pulp stem cells

# *LHX8* 과발현 관련 *ID1*의 억제를 통한 인간 치수줄기세포의 골분화 유도 효과

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서울대학교 대학원

치의과학과 구강악안면외과학 전공

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Abstract

# Enhanced osteogenic potential by inhibiting *LHX8* overexpression-associated *ID1* in human dental pulp stem cells

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*LIM homeobox 8 (LHX8)* encodes a transcription factor protein that is necessary for embryonic development of craniofacial tissues including bone and teeth. Previous animal studies suggest that *LHX8* may have a role in osteogenesis, but the finding requires further supporting evidence in human dental pulp cells which possess the osteogenic ability. A lentiviral system was utilized in this study to overexpress *LHX8* in human dental pulp stem cells (DPSCs) and the effect of overexpressed *LHX8* on osteogenesis was analyzed.

*LHX8* overexpression did not modulate proliferation, migration, nor the angiogenic property of DPSCs. On the other hand, the osteogenic differentiation ability of DPSCs was attenuated, demonstrated by decreased alkaline phosphatase activity and calcium accumulation. cDNA microarray analysis showed that genes associated with protein binding were differentially expressed in osteodifferentiated DPSCs. There were 38 differentially expressed genes (DEGs) which were common both in response to osteodifferentiation and *LHX8* overexpression, and there were grouped into

two clusters. Each cluster responded in an opposite way, i.e. upregulated during osteodifferentiation but downregulated by *LHX8* overexpression or vice versa, supporting an anti-osteogenic role of *LHX8*. In order to develop a molecular approach to promote osteogenesis of DPSCs, genes downregulated in control cells but upregulated in *LHX8* cells during osteodifferentiation were screened. Chemical inhibitors were treated to their protein products to see if osteodifferentiation of DPSCs were enhanced. ML323, which target DNA-binding protein inhibitor ID-1, promoted osteodifferentiation of DPSCs.

In summary, an anti-osteogenic effect of *LHX8* was confirmed in this study by a *LHX8* overexpressing DPSCs model. An anti-osteogenic gene *ID1* was discovered. Finally, it was demonstrated that by inhibiting DNA-binding protein inhibitor ID-1, osteogenesis was promoted in human DPSCs.

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**Keywords: dental pulp stem cell, osteogenesis, *LHX8*, *ID1,* ML323**
**Student number: 2010-31184**

# Table of Contents

# LIST OF TABLES

## Part 1



## Part 2

# LIST OF FIGURES

## Part 1



## Part 2

# GENERAL INTRODUCTION

LIM homeodomain proteins are a family of transcription factors [1]. Among them, *LHX8* may play a role in osteogenesis [2-5], but the finding requires further supporting evidence in human cells. Mesenchymal stem cells (MSCs) reside in various parts of adult tissues and organs [6]. Among the subtypes of MSCs, dental pulp stem cells (DPSCs) possess osteogenic characteristics [7], and less invasive to isolate [8, 9, 10]. Therefore, DPSCs are good sources of studying the role of *LHX8* in osteodifferentiation.

Craniofacial bone defects caused by osteomyelitis, malignancy or traumatic conditions require bone reconstruction [11, 12]. Autologous bone grafting is the traditional approach for reconstruction due to the craniofacial bone defects [11-13]. However, there are limitations for wide-spread use [14, 15]. Bone morphogenic proteins (BMPs), are often used in clinics to aid bone reconstruction, but there are also drawbacks [16, 17]. There is an urgent need for the development of osteogenic agents which may aid bone regeneration.

In this study, *LHX8* overexpression on DPSCs has revealed the anti-osteogenic properties of the gene. Using the *LHX8* DPSCs model, druggable *LHX8*-associated genes were screened and functional inhibitors were treated to find compounds with the osteogenic property. These findings may lead to the discovery of osteogenic agents which might provide clinical benefit in bone regeneration.

# PART 1

## Anti-osteogenic *LHX8* in human dental pulp stem cells

# INTRODUCTION

LIM homeodomain proteins are a family of transcription factors which in mammals 12 subtypes have been discovered [1]. *LHX8*, or formerly L3 and *LHX7*, is a member of the LIM homeodomain proteins and is expressed in the craniofacial tissues during the development process [2]. The molecular function of *LHX8*, often working in combination with its paralogous gene *LHX6*, has been proposed mainly by studies using mouse models. Homozygous deletion or mutation of *LHX8* leads to cleft palate development in mice [3]. Lhx6/8 double knockout mice have defects of the cranial skeleton and die shortly after birth [4]. Failure of dental mesenchyme differentiation and tooth formation was observed in the Lhx6/8 double knockout mouse [5]. These studies suggest that *LHX8* may play a role in osteogenesis, but the finding requires further supporting evidence in human cells.

MSCs reside in various parts of adult tissues and organs, can be isolated and expanded easily, and possess the ability to differentiate into various tissue types including the bone [6]. Among the subtypes of MSCs, DPSCs PBScomparable osteogenic characteristics to bone marrow stem cells (BMSCs), the most widely studied MSCs [7]. DPSCs can be isolated and expanded from dental pulp either from permanent teeth removed for orthodontic reasons or third molar teeth for prophylactic reasons [8], whereas an invasive surgical intervention is necessary to obtain BMSCs [9]. Furthermore, the osteogenic potential of DPSCs was superior to other stromal

cells of dental origin, the gingival fibroblasts [10]. Therefore, DPSCs are good sources of studying the role of *LHX8* in osteodifferentiation.

In this study, DPSCs were isolated and expanded from human third molar teeth. *LHX8* was overexpressed in DPSCs using a lentiviral system. The functional changes that occurred in *LHX8* overexpressing DPSCs were observed using various functional assay skills, including quantitative PCR, alkaline phosphate activity measurement, and calcium staining with Alizarin Red S.

# MATERIALS AND METHODS

## 1. Human dental pulp stem cells

Human third molars were collected from three healthy young males and a female (18–25 years old) under the protocol approved by the Institutional Review Board of the Seoul National University Dental Hospital, Seoul, South Korea (IRB No. 05004). Dental pulps were gently separated from extracted teeth and the separated tissues were digested in a solution of 3 mg/mL collagenase type I (Worthington Biochem, Freehold, NJ, USA) and 4 mg/mL dispase (Boehringer, Mannheim, Germany) for 1 hour at 37°C. Single-cell suspensions were obtained by passing the cells through a 70 μm strainer (Falcon BD Labware, Franklin Lakes, NJ, USA) and were cultured in the alpha-modification of Eagle's medium (aMEM, Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA), 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen) and incubated at 37°C in 5% $CO_2$. The medium was changed after the first 24 hours and then every 3-4 days. All primary cells used in this study were in passage 2-5. Cells were imaged using an Olympus CKX53 microscope.

## 2. Flow cytometric analysis

To characterize the immunophenotype of DPSCs, the expression of mesenchymal stem cell (MSC)-associated surface markers at passage 4 was

analyzed by flow cytometry. Briefly, cells in their third passage (1.0 x10e$^6$ cells) were fixed with 3.7% paraformaldehyde for 10 minutes and resuspended in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA, ICN Biomedicals, Aurora, OH, USA) for 30 minutes for blocking. Cells were then incubated with specific antibodies for CD34, CD13, CD90, or CD146 at 4 °C for 1 hour, followed by incubation with fluorescence secondary antibodies at room temperature for 1 hour. All antibodies were purchased from BD Biosciences (San Jose, CA, USA). The percentage of CD13-, CD90-, and CD146-positive and CD34-negative cells was measured with a FACSVerse flow cytometer (Becton Dickinson, San Jose, CA, USA).

**3. Lentivirus transduction**

The full-length coding sequences of *LHX8* were amplified by PCR from DPSCs. PCR amplification products were cloned into the pCDH-CMV-MCS-EF1-copGFP lentiviral vector (System Biosciences, Mountain View, CA, USA) and packaged by co-transfection with psPAX2 and pMD2.G plasmids with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in HEK293FT (Invitrogen, Carlsbad, CA, USA) cells. The virus was harvested and concentrated by ultracentrifugation 48 hours later. For *LHX8* overexpression, passage 2 DPSCs were treated with different multiplicities of infection (MOIs) of lentivirus for 24 hours and examined for green fluorescent protein (GFP) expression after 3 days. MOIs that generated at least 95% of GFP-positive cells were chosen for further culture (MOI 30 for control, MOI 100 for *LHX8*). These cells were at least twice passaged before used for experiments.

HEK293FT cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Welgene, Seoul, Korea) media with 10% FBS.

**4. Western blotting**

*LHX8* overexpressing DPSCs and control DPSCs were cultured in 100 mm dishes until approximately 80% confluent. Cells were washed twice with PBS and detached from the culture plate using a cell scraper. Cells were lysed on ice for 15 minutes with radio-immuno-precipitation assay (RIPA) buffer (Thermo Scientific, Rockford, IL, USA), which contains 1% proteinase inhibitors. The samples were loaded onto a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and subjected to electrophoresis on ice. The proteins resolved were transferred onto nitrocellulose membrane for 1 hour and blocked for 1 hour at room temperature with 5% skim milk. The membranes were incubated overnight at 4°C with the primary antibodies. Anti-beta actin and anti-*LHX8* were obtained from Abcam (Abcam, Cambridge, MA, USA). The membranes were washed in Tris-buffered saline-Tween 20 (TBST) and incubated with the secondary antibody. Detection was done in the dark using enhanced chemiluminescence solution (GE healthcare, Chicago, IL, USA).

**5. Cell proliferation assay**

Cell proliferation was measured using the tetrazolium salt WST-1 assay kit (Clonetech, Palo Alto, CA, USA). Briefly, DPSCs ($1.0\times10e^3$ cells/well) were seeded in 96-well plates and were incubated in 5% $CO_2$ at 37°C for 5 days.

Every day, the WST-1 dye solution was added and cells were incubated in 5% $CO_2$ at 37°C for 2 hours until the stop solution was added to the cultures to stabilize the formazan product. Each condition was prepared in triplicate, and reactions were assessed using an enzyme-linked immunosorbent assay (ELISA) reader at optical density (OD) 450nm.

**6. Cell migration assay**

Migration of DPSCs was observed using the Transwell® culture insert with 8 μm pores (Corning, Grand Island, NY, USA). After one-day serum starvation (0.1%), DPSCs were loaded onto the insert with low serum medium (0.1%). The lower chamber was filled with complete growth medium with 10% FBS to facilitate migration through pores. Following 24 hours incubation, remaining cells in the upper chamber were removed using a cotton swab and cells in the bottom layer of the Transwell® were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet solution. After microscopic images were obtained, crystal violet stained cells were quantified by solubilizing them using 0.1% SDS.

**7. Real-time cell migration assay**

The real-time analysis of cell migration assay was performed using the xCELLigence DP Real Time Cell Analyzer and CIM-16 plates with 8 μm pore membranes. The bottom electrodes of the CIM-16 plates were coated with 0.2% gelatin and incubated in a laminar airflow chamber for 30 minutes. DPSCs were loaded onto the upper compartments with low serum medium (0.1%).

The lower side was filled with complete growth medium with 10% FBS to facilitate migration. The impedance data, reported as cell index and proportional to the area of the bottom electrodes covered by migrated cells, were collected every 15 minutes. For easy visualization, 10-time points during 24 hours of observation were depicted.

**8. Angiogenesis assay**

To measure the angiogenic property of DPSCs, a tube formation assay was performed. The inner wells of a μ-slide (Ibidi, Planegg, Germany) were coated with Matrigel® basement membrane matrix (BD Biosciences, Franklin Lakes, NJ). Human umbilical vein endothelial cells (HUVECs) were seeded on top of the Matrigel® with 48 hours conditioned medium of DPSCs. The cultures were incubated for 16 hours before microscopic images of tubular structures were taken. The numbers of tubes were manually counted for quantification. HUVECs were cultured in M200 media supplemented with LVES (Thermo, A1460801)

**9. Osteodifferentiation**

For osteogenesis, the DPSCs were grown with complete culture media until confluence. Then the medium was changed to an osteogenic differentiation medium with 50 μg/mL ascorbic acid, 10 mM β-glycerophosphate, and 100 nM dexamethasone (all from Sigma-Aldrich) for up to 4 weeks.

**10. Real-time reverse transcription–polymerase chain reaction (real-time RT-PCR)**

To evaluate gene expression levels in osteodifferentiated DPSCs, *LHX8* overexpressed cells or control cells were cultured under an osteogenic differentiation condition. Total RNA was prepared using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA using reverse transcriptase (Superscript II Preamplification System; Invitrogen, Carlsbad, CA, USA). RT-PCR was conducted as on an ABI7500 thermal cycler. The expression levels of genes were calculated using the relative quantification method. The specific primer-probe sets used for RT-PCR are listed in Table 1.

**Table 1. Primer-probe information**

| Gene | Accession | Company | Product no. |
|---|---|---|---|
| *GAPDH* | NM_002046 | IDT | Hs.PT.39a.22214836 |
| *ALPL* | NM_000478.6 | Thermo | Hs01029143_m1 |
| *COL1A1* | NM_000088.3 | Thermo | Hs00164004_m1 |
| *IBSP* | NM_004967.3 | Thermo | Hs00913377_m1 |
| *RUNX2* | NM_001015051.3 | Thermo | Hs01047975_m1 |

**11. Alkaline phosphatase activity measurement**

Osteodifferentiated DPSCs were fixed with 4% paraformaldehyde and stained for alkaline phosphatase (ALP) activity with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) color development substrate (Promega, Madison, WI, USA) on differentiation day 7. After the acquisition of microscopic images, stained ALP substrate was solubilized with SDS solution with 0.1M hydochloride for quantification.

**12. Calcium accumulation measurement**

Accumulation of mineral nodules was detected by staining with 2% Alizarin red S staining at pH 4.2 (Sigma-Aldrich, St Louis, MO, USA). For the destaining procedure to measure the calcium content, 3 mL of 10 mM sodium phosphate in 10% acetylpyrimidium (pH 7.0, Sigma-Aldrich) solution was added to each stained well and incubated at room temperature for 15 minutes. The destained sample was transferred to a 96-well plate and the absorbance was measured at 562 nm.

**13. Statistical analysis**

Statistical analysis was performed using Prism software (GraphPad Software, San Diego, CA, USA). Comparison between two groups was made with Student's t-test. Significance was defined as $P \leq 0.05$. Values in each graph represent mean±standard deviation. All assays were performed at least thrice and representative data are presented.

# RESULTS

**DPSCs were successfully isolated and expanded from surgically removed human third molar teeth.**

To explore roles of *LHX8* in craniofacial calcifying tissues, human dental pulp stem cells were isolated and expanded in vitro. These cells are of a mesenchymal origin, showing spindle morphology when adhered on tissue culture plastic surfaces (Figure 1). Typical mesenchymal stem cell markers CD13, CD90, and CD146 were positive in these cells and were negative for hematopoietic stem cell marker CD34 (Figure 2).

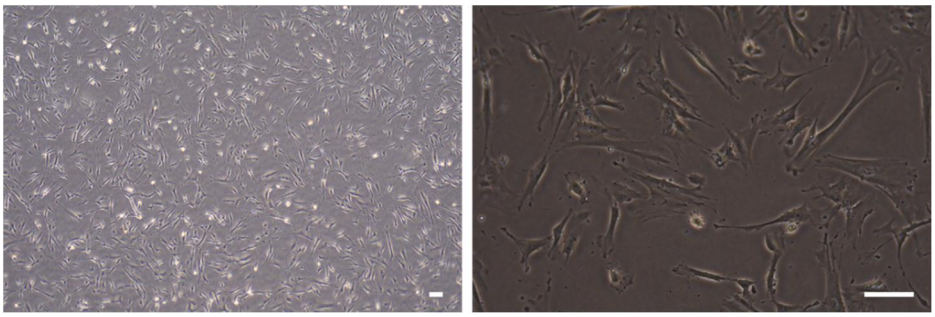

**Figure 1. Microscopic images of passage 4 human DPSCs isolated from molar teeth**. Human dental pulp stem cells were isolated and expanded from surgically removed third molar teeth. Their spindle shape morphology was confirmed via microscopic observation. Scale bars, 100 μm.

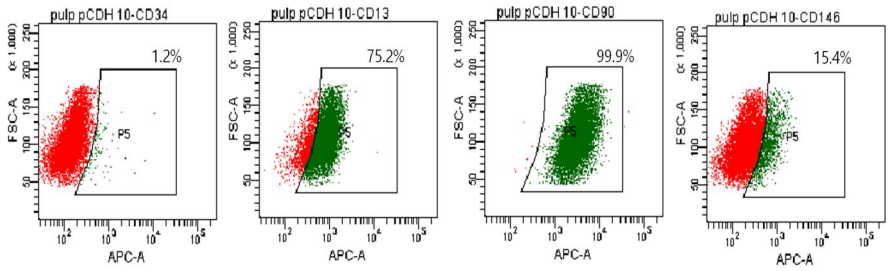


**Figure 2. Expression levels of mesenchymal cell surface markers.** Expression of typical mesenchymal stem cell markers CD13, CD90, and CD146 was confirmed via flow cytometry analyses. These cells were negative for lymphocytic marker CD34.

***LHX8* is overexpressed using a lentiviral system**

Since osteodifferentiation is a long process often take up to 4 weeks in culture, I assumed a stable expression of the target gene was necessary for osteodifferentiation studies. Therefore, lentiviral vector system was chosen for *LHX8* gene overexpression in DPSCs (Figure 3). Using the lentiviruses, LHX8 protein was overexpressed in DPSCs as confirmed by flow cytometry analysis of GFP marker protein (Figure 4) and by antibody detection of LHX8 protein (Figure 5).

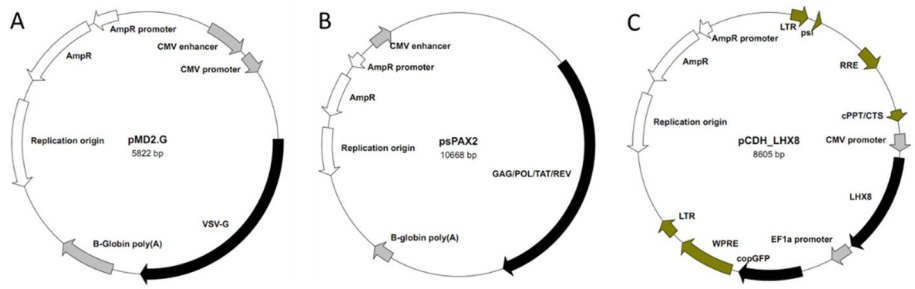


**Figure 3. Plasmid vectors used in this study.** (A) pMD2.G plasmid encodes vesicular stomatitis virus glycoprotein which facilitates binding to target cells. (B) psPAX2 plasmid encodes a portion of human immunodeficiency virus proteins to enable genomic integration of desired sequences. (C) pCDH_*LHX8* plasmid encodes sequences required for genomic integration and expression of LHX8 protein and green fluorescent protein.

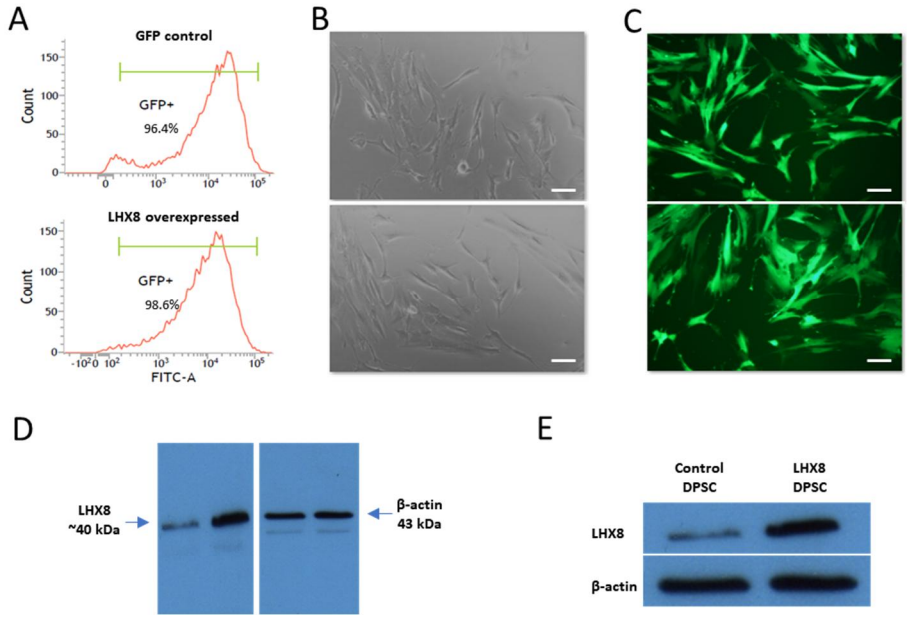


**Figure 4. Lentivirus-mediated overexpression of LHX8 in human DPSCs.** (A) Overexpression of human LHX8 protein was confirmed via flow cytometry analyses. Vector-only control cells expressed similar amounts of GFP marker proteins. (B) Similar morphology of *LHX8* DPSCs and GFP control cells. (C) GFP expression levels were observed via fluorescence microscopy. (D) LHX8 protein was detected with a specific antibody by western blotting. (E) An enlarged version of (D). Scale bars, 100 μm.

***LHX8* overexpression does not affect proliferation of DPSCs**

To determine the consequences of *LHX8* overexpression, the proliferation rate of DPSCs were observed. *LHX8* overexpressing cells and control cells did not show any significant difference in proliferation by WST-1 assay (Figure 5)

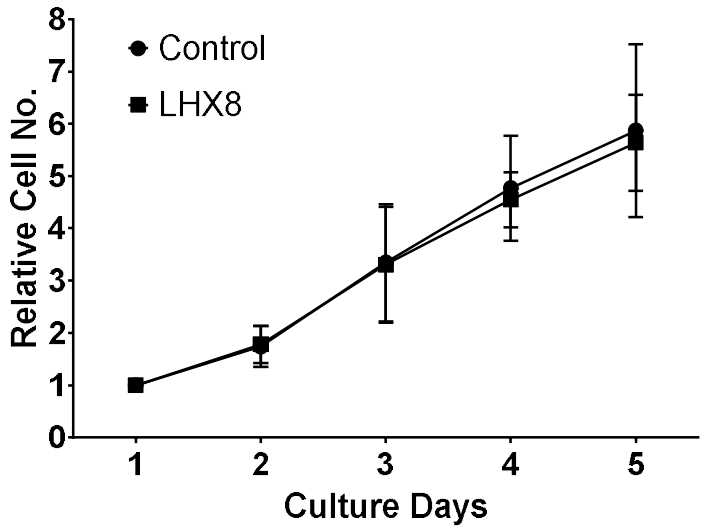


**Figure 5. Effect of *LHX8* overexpression on cell growth**. The proliferation of DPSCs with and without *LHX8* overexpression was compared using WST-1 assay. Control, empty vector-incorporated control DPSCs. *LHX8*, *LHX8* overexpressed DPSCs.

***LHX8* overexpression does not affect the migration of DPSCs**

The migrating ability of DPSCs was analyzed by allowing cells to migrate through small pores toward chemoattractant. DPSCs with or without *LHX8* overexpression did not show any difference in migration when Boyden chamber culture system was used (Figure 6A-C). When migration was monitored real-time using the Real Time Cell Analyzer (RTCA), *LHX8* DPSCs showed marginally attenuated migration but there was no statistical significance (Figure 6D).

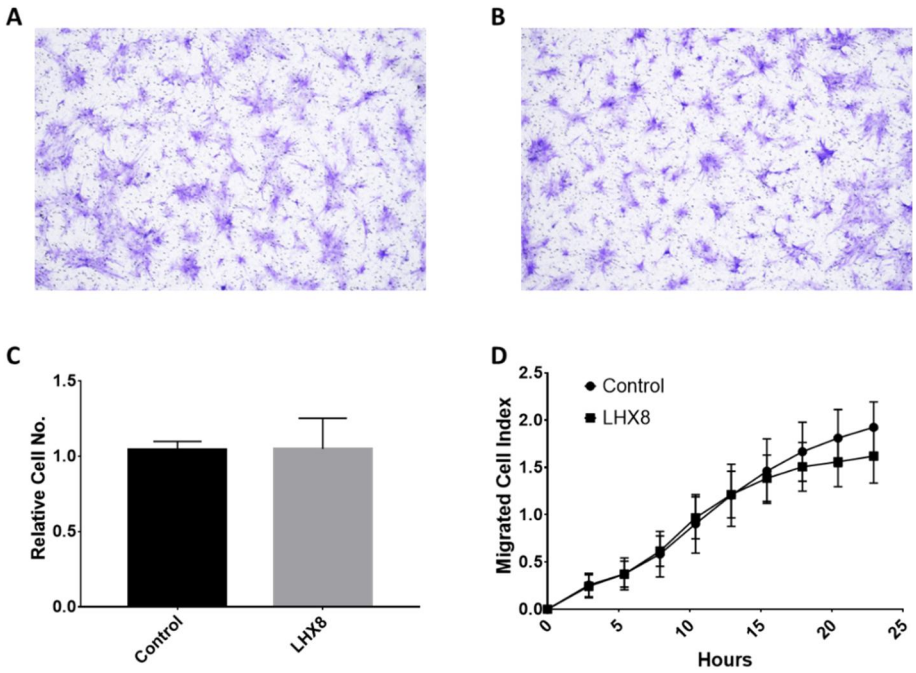


**Figure 6. Effect of *LHX8* overexpression on migration.** Migration of DPSCs with and without *LHX8* overexpression was compared. Crystal violet stained migrated (A) control cells and (B) *LHX8* cells in the Boyden chamber assay. Cells were allowed to migrate through 8 μm-sized pores for 24hours.

(C) The relative ratio of migrated cells was quantified by solubilizing crystal violet crystals. (D) Migration of DPSCs was recorded real-time using RTCA device. Control, empty vector-incorporated control DPSCs. *LHX8*, *LHX8* overexpressed DPSCs.

***LHX8* overexpression does not affect the angiogenic ability of DPSCs**

DPSCs are known to possess angiogenic abilities. I sought to examine if *LHX8* overexpression dysregulates angiogenic potential of DPSCs. The results of tube assay using HUVEC cells showed slightly decreased angiogenic tube formation in *LHX8* overexpressed cells, but without statistical significance (Figure 7).

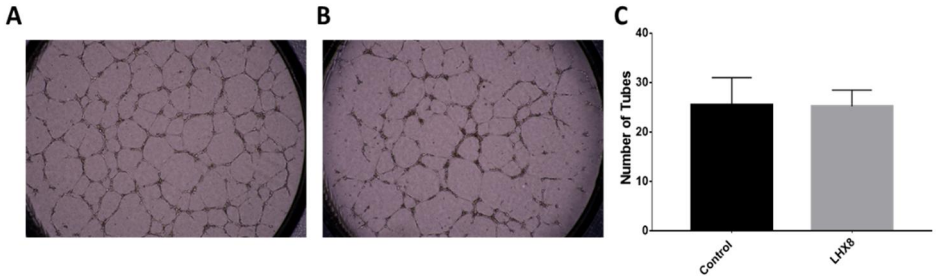


**Figure 7. Effect of *LHX8* overexpression on the angiogenic property of DPSCs.** Human umbilical cord endothelial cell tube formation assay was performed. Two-day conditioned media from DPSCs with and without *LHX8* overexpression was treated on human umbilical cord endothelial cells layered on Matrigel® matrix. Endothelial cell tube formation was observed microscopically after 24 hours. The number of tubes was counted from four randomly selected images. Control, empty vector-incorporated control DPSCs. *LHX8*, *LHX8* overexpressed DPSCs.

***LHX8* overexpression attenuates osteogenic differentiation of DPSCs**

The effect of *LHX8* overexpression on osteodifferentiation was analyzed with various methods. Osteogenic genes were upregulated on osteogenic day 14, and the upregulation was attenuated in *LHX8* cells (Figure 8). The activity of alkaline phosphatase, an enzyme which plays important roles in skeletal development, was diminished in *LHX8* overexpressing DPSCs (Figure 9). Additionally, the amount of calcium accumulation was reduced in *LHX8* cells (Figure 10).

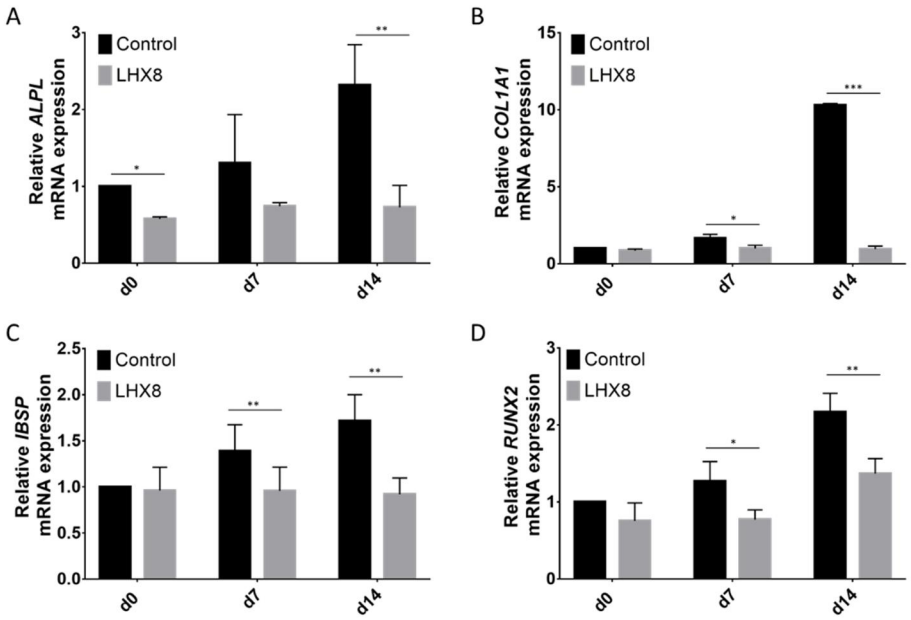


**Figure 8. Effect of *LHX8* overexpression on osteogenic gene expression signature.** Control DPSCs and *LHX8* overexpressing DPSCs were osteodifferentiated and RNA was isolated on indicated days. Expression levels of osteogenic genes were analyzed by real-time PCR. Control, empty vector-incorporated control DPSCs. *LHX8*, *LHX8* overexpressed DPSCs. *, $P<0.05$, **, $P<0.01$

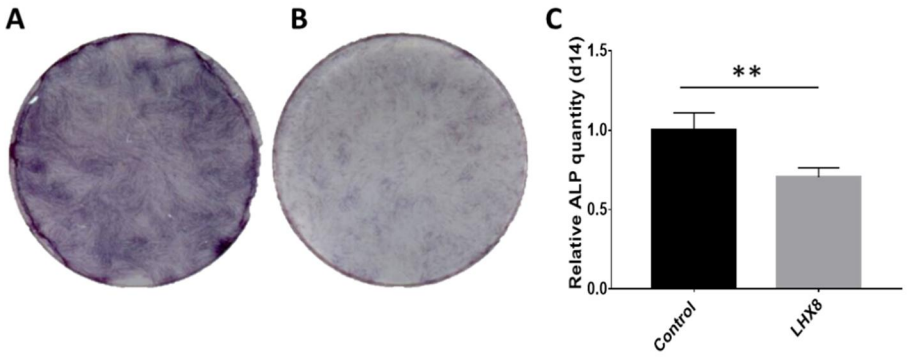


**Figure 9. Effect of *LHX8* overexpression on alkaline phosphatase (ALP) activity of DPSCs.** Control DPSCs and *LHX8* overexpressing DPSCs were osteodifferentiated for 14 days. ALP activity was measured using BCIP/NBT method in control (A) and *LHX8* (B) cells. (C) Chromogens were solubilized with acidic sodium dodecyl sulfate solution for quantification of ALP levels. Control, empty vector-incorporated control DPSCs. *LHX8*, *LHX8* overexpressed DPSCs. **, $P<0.01$

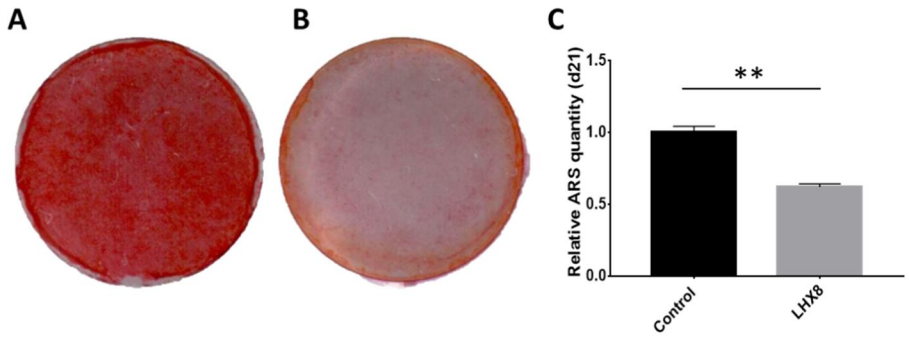


**Figure 10. Effect of *LHX8* overexpression on calcification of DPSCs.** Control DPSCs and *LHX8* overexpressing DPSCs were osteodifferentiated for 21 days. Accumulated calcium nodules were stained with alizarin red S (ARS) in control (A) and *LHX8* (B) cells. (C) Calcium levels were quantified by solubilizing calcium nodules with acidic sodium dodecyl sulfate solution. Control, empty vector-incorporated control DPSCs. *LHX8*, *LHX8* overexpressed DPSCs. **, $P<0.01$

# DISCUSSION

DPSCs have been successfully isolated from human third molars and were expanded in vitro. These cells showed typical MSC morphology and expressed MSC surface marker proteins. *LHX8* was overexpressed in these cells using the lentiviral vector system. Expression of *LHX8* was confirmed by flow cytometric analysis of co-expression of the fluorescent marker protein and direct detection of *LHX8* by the western blot. The consequences of *LHX8* overexpression was analyzed regarding cell proliferation, migration, angiogenic property, and osteodifferentiation property. Among these portraits tested, osteodifferentiation ability was attenuated in *LHX8* DPSCs and the results were visualized by real-time PCR analyses of relevant genes, ALP activity measurement, and calcium accumulation measurement.

Previous reports of *LHX8*, mostly animal studies, suggested a role of the gene in osteogenesis, and my results provided that the role of the gene is inhibitory in a human cell model. A study demonstrated that siRNA-mediated knockdown of LHX8 lead to increased ALP activities and calcium nodule formation [11].

There are chances that suppression of osteodifferentiation by *LHX8* may lead to enhancement of other cellular functions. I have searched for consequences of *LHX8* overexpression on cells, and unfortunately, no difference was observed regarding proliferation, migration, angiogenic abilities of DPSCs. Angiogenic properties are known to be strengthened

during the process of osteodifferentiation [12]. Since *LHX8* attenuates osteodifferentiation, the gene may also reduce the angiogenic potential in cells. The angiogenic tube assay results showed that the reduction in tube formation was marginally observed in *LHX8* cells, but was not with a statistical significance. A stronger and longer stimulation with the osteogenic condition may clarify this issue.

In summary, a *LHX8* overexpressing DPSC model has been successfully constructed. The cell model showed that *LHX8* plays an inhibitory role in osteogenesis. Other portraits, such as proliferation, migration, and angiogenic property, were not significantly changed.

# PART 2

Inhibition of *LHX8* overexpression–associated *ID1* in human dental pulp stem cells

# INTRODUCTION

Craniofacial bone defects caused by osteomyelitis, malignancy or traumatic conditions require bone reconstruction [13, 14]. Autologous bone grafting is the traditional approach for reconstruction [15]. However, increased surgical costs, pain due to invasive procedures, graft infection, and graft resorption are shortcomings of present bone grafting procedures [16]. Most importantly, available autologous bone is inherently limited by nature [17]. Recent advances in technology lead to the development of bone graft substitutes, stem cell therapies, osteogenic agents, or a combination of all, which may be beneficial in addressing the problem [17]. Among osteogenic agents, bone morphogenic proteins (BMPs), are often used in clinics to aid bone reconstruction. However, adverse side-effects associated with its use has been well-described and being a recombinant protein its high cost blocks BMPs to be widely used [18, 19]. There are attempts to develop chemical agents as alternatives to BMPs, but results are still preliminary. There is an urgent need for the development of osteogenic agents which may aid bone regeneration.

In this study, cDNA microarray was performed on control DPSCs and *LHX8* overexpressed DPSCs. The characteristics of differentially expressed genes were analyzed. The osteogenic genes which were also associated with *LHX8* overexpression were screened, and chemical inhibitors or neutralizing antibodies for their protein products were treated to see if these genes are indeed functional in osteogenesis.

## MATERIALS AND METHODS

### 1. Cell culture

The generations of *LHX8* overexpressing DPSCs and control DPSCs have been described in Part 1. These cells were cultured in the aMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin and incubated at 37°C in 5% $CO_2$. The medium was changed every 3-4 days. All primary cells used in this study were in passages 2-5.

### 2. cDNA microarray

DPSCs were osteodifferentiated for 4 days and total RNA was extracted as described above. cDNA microarray was performed with GeneChip® Human Gene 2.0 ST Array. cDNA was synthesized using the GeneChip Whole Transcript (WT) Amplification kit as described by the manufacturer. The sense cDNA was then fragmented and biotin-labeled with terminal deoxynucleotidyl transferase (TdT) using the GeneChip WT Terminal Labeling Kit. Approximately 5.5 μg of labeled DNA target was hybridized to the Affymetrix GeneChip Array at 45°C for 16 hours. Hybridized arrays were washed and stained on a GeneChip Fluidics Station 450 and scanned on a GCS3000 Scanner (all from Affymetrix, Santa Clara, CA, USA). Array data export processing and analysis were performed using Affymetrix® GeneChip Command Console® Software, Affymetrix Power Tools and R 3.1.2 (https://www.r-project.org/).

**3. Transcriptomic data analyses**

Gene ontology analysis was performed by the Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Resources version 6.8, which is developed by National Institutes of Health (Bethesda, MD, USA).

**4. Statistical analysis**

Statistical analysis was performed using Prism software (GraphPad Software, San Diego, CA, USA). Comparison between two groups was made with Student's t-test. Significance was defined as $P \leq 0.05$. Values in each graph represent mean±standard deviation. All assays were performed at least thrice and representative data are presented.

# RESULTS

### *LHX8* overexpression regulated expression levels of diverse genes

To study transcriptional aberrations due to *LHX8* overexpression, cDNA microarray was performed on *LHX8* and control DPSCs. *LHX8* overexpression lead to 716 differentially expressed genes (DEGs) and top 20 upregulated and top 20 downregulated DEGs are listed in Tables 1 and 2, respectively. DEGs are defined as genes whose absolute fold changes were above 1.5 with a statistical significance ($P<0.05$).

### Osteodifferentiation of DPSCs regulated expression levels of diverse genes

Osteodifferentiation modulates the expression of a multitude of genes, and the osteogenic stimulus resulted in 345 DEGs in the control DPSCs versus undifferentiated. Top 20 upregulated and top 20 downregulated DEGs are listed in Tables 3 and 4, respectively. cDNA microarray was performed on osteodifferentiation day 4.

**Table 1. Top 20 upregulated genes in *LHX8* overexpressing DPSCs compared to control DPSCs**

| Gene | Accession | Fold Change |
|---|---|---|
| *GPC3* | NM_001164617 | 6.73 |
| *LHX8* | NM_001001933 | 6.36 |
| *SULT1B1* | NM_014465 | 5.52 |
| *CEMIP* | NM_001293298 | 3.91 |
| *CHRNA1* | NM_000079 | 3.84 |
| *AQP11* | NM_173039 | 3.83 |
| *PDZRN4* | NM_001164595 | 3.78 |
| *ACTA2* | NM_001141945 | 3.70 |
| *B3GALT2* | NM_003783 | 3.62 |
| *PTX3* | NM_002852 | 3.55 |
| *PLCXD3* | NM_001005473 | 3.46 |
| *LRRN3* | NM_001099658 | 3.42 |
| *EML5* | NM_183387 | 3.38 |
| *RARB* | NM_000965 | 3.35 |
| *P3H2* | NM_001134418 | 3.31 |
| *PLCE1-AS1* | NR_033969 | 3.29 |
| *CLDN1* | NM_021101 | 3.10 |
| *GREM2* | NM_022469 | 3.07 |
| *A2M* | NM_000014 | 3.03 |
| *F2RL2* | NM_001256566 | 2.85 |

**Table 2. Top 20 downregulated genes in *LHX8* overexpressing DPSCs compared to control DPSCs**

| Gene | Accession | Fold Change |
|---|---|---|
| *HIST1H2BB* | NM_021062 | -2.46 |
| *CPA4* | NM_001163446 | -2.49 |
| *SLC14A1* | NM_001128588 | -2.50 |
| *FNDC1* | NM_032532 | -2.53 |
| *ASPM* | NM_001206846 | -2.54 |
| *CCNB1* | NM_031966 | -2.55 |
| *NR4A2* | NM_006186 | -2.61 |
| *PLXDC2* | NM_001282736 | -2.67 |
| *KCNJ15* | NM_001276435 | -2.73 |
| *MAP2K6* | NM_002758 | -2.73 |
| *IL11* | NM_000641 | -2.79 |
| *RAB27B* | NM_004163 | -2.81 |
| *KIF14* | NM_001305792 | -2.83 |
| *IGFBP5* | NM_000599 | -2.87 |
| *FAM72D* | NM_207418 | -2.88 |
| *EGR1* | NM_001964 | -3.15 |
| *SPTLC3* | NM_018327 | -3.18 |
| *GALNT15* | NM_054110 | -3.75 |
| *WNT2* | NM_003391 | -3.77 |
| *STC1* | NM_003155 | -4.03 |

**Table 3. Top 20 upregulated genes in day 4 osteodifferentiated DPSCs compared to undifferentiated DPSCs**

| Gene | Accession | Fold Change |
|---|---|---|
| *NABP1* | ENST00000410026 | 2.69 |
| *SLC2A12* | NM_145176 | 2.61 |
| *MIR1245A* | NR_031647 | 2.60 |
| *CEMIP* | NM_001293298 | 2.57 |
| *LOC105375734* | NR_131202 | 2.34 |
| *ENPP2* | NM_001040092 | 2.33 |
| *POM121L9P* | NR_003714 | 2.28 |
| *NPR3* | NM_000908 | 2.17 |
| *CYP39A1* | NM_001278738 | 2.16 |
| *HAS2* | NM_005328 | 2.15 |
| *SNORD114-6* | NR_003198 | 2.14 |
| *CFH* | NM_000186 | 2.11 |
| *LOC105370777* | XR_916226 | 2.00 |
| *NAV2-IT1* | BC018444 | 1.96 |
| *ANXA8L1* | OTTHUMT00000047846 | 1.95 |
| *MAOA* | NM_000240 | 1.94 |
| *PTX3* | NM_002852 | 1.93 |
| *ZEB2* | ENST00000628473 | 1.92 |
| *PIP* | NM_002652 | 1.90 |
| *ABCA6* | NM_080284 | 1.89 |

**Table 4. Top 20 downregulated genes in day 4 osteodifferentiated DPSCs compared to undifferentiated DPSCs**

| Gene | Accession | Fold Change |
|---|---|---|
| *DCLK1* | NM_001195415 | -2.01 |
| *CDCP1* | NM_022842 | -2.04 |
| *TRIB1* | NM_001282985 | -2.04 |
| *HIST1H2AI* | NM_003509 | -2.05 |
| *FST* | NM_006350 | -2.06 |
| *HIST1H1B* | NM_005322 | -2.09 |
| *KRTAP2-3* | NM_001165252 | -2.09 |
| *APCDD1L* | NM_001304787 | -2.10 |
| *CXCL14* | NM_004887 | -2.20 |
| *MAP2K6* | NM_002758 | -2.21 |
| *HIST1H3G* | NM_003534 | -2.24 |
| *IER3* | NM_003897 | -2.29 |
| *ITGA2* | NM_002203 | -2.30 |
| *FAM72D* | NM_207418 | -2.34 |
| *IL11* | NM_000641 | -2.44 |
| *MMP3* | NM_002422 | -2.52 |
| *ID1* | NM_002165 | -2.64 |
| *CLSTN2* | NM_022131 | -2.66 |
| *LRRC15* | NM_001135057 | -2.76 |
| *EGR1* | NM_001964 | -2.80 |

To achieve a more comprehensive genome-wide understanding, gene ontology terms associated with DEGs were analyzed. The gene ontology analysis showed that osteogenic stimulus of control DPSCs lead to dysregulation of DEGs related to the binding function of the cell, especially protein binding (Figure 1). Interestingly, *LHX8*-associated DEGs were also related to the binding function of the cell, implying possible involvement of *LHX8* in osteodifferentiation (Figure 2).

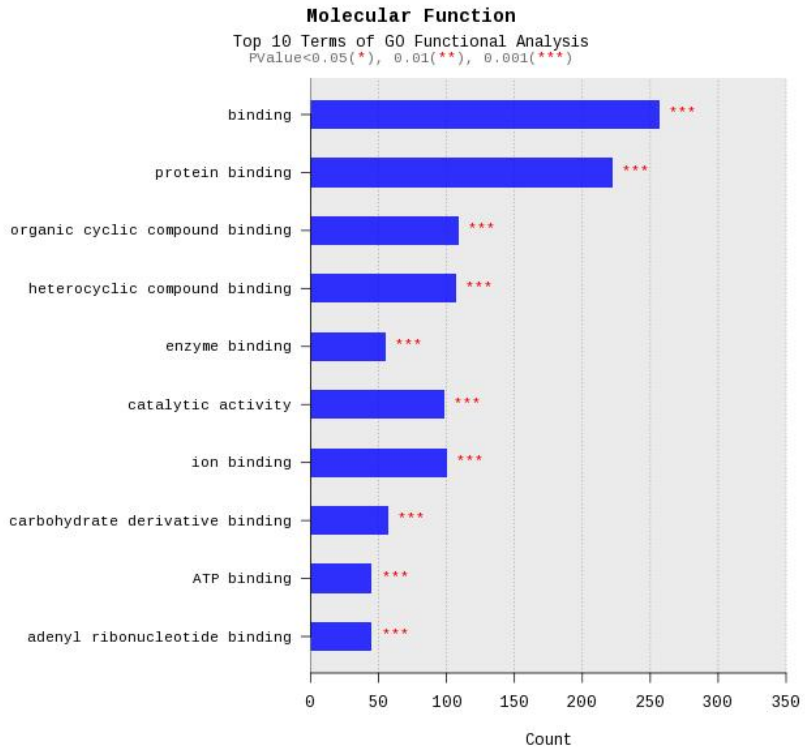


**Figure 1. Gene ontology terms associated with osteodifferentiation of DPSCs.** Top ten gene ontology terms in the molecular function category which were dysregulated during osteodifferentiation in control DPSCs were listed. ***. P<001.

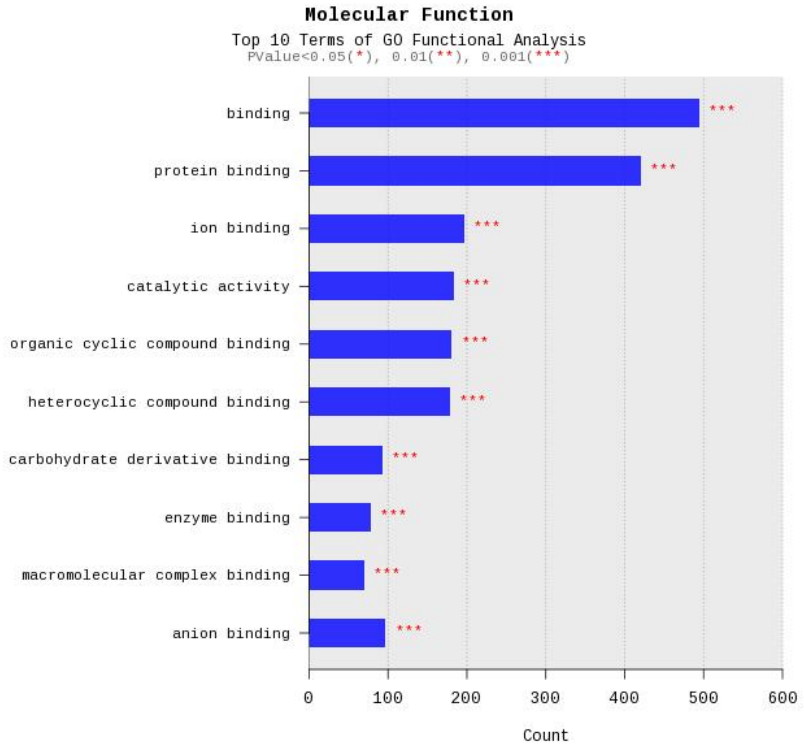


**Figure 2. Gene ontology terms associated with *LHX8* overexpression of DPSCs.** Top ten gene ontology terms in the molecular function category which were dysregulated by *LHX8* overexpression in DPSCs were listed. ***. P<001.

***LHX8* regulates osteogenic genes in an opposite way when compared with control DPSCs.**

When a comparison was made with undifferentiated DPSCs, the number of osteodifferentiation associated DEGs were 345 in control DPSCs and 53 in *LHX8* DPSCs, showing *LHX8* DPSCs were less responsive to osteodifferentiation stimulus. The majority (38/53, 71.7%) of DEGs in *LHX8*

DPSCs that were responsive to osteodifferentiation were also DEGs of control DPSCs (Figure 3). When the 38 common DEGs which were associated with osteodifferentiation both in control and *LHX8* DPSCs were depicted as a heatmap, the genes were grouped into two separate clusters (Figure 4). One cluster (Cluster A) were genes with decreased mRNA expression during osteodifferentiation in control DPSCs and with increased mRNA expression in *LHX8* cells, and the other cluster the opposite (Cluster B). Therefore, it can be concluded that the role of *LHX8* during the osteodifferentiation process of DPSCs may be inhibitory, supporting findings in previous functional studies.

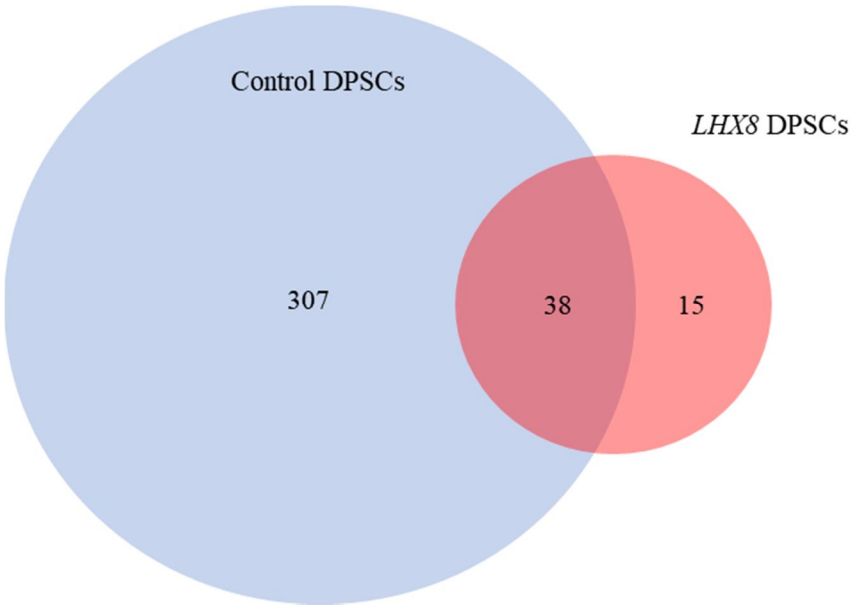


**Figure 3. Osteodifferentiation associated DEGs**. The number of DEGs in response to osteodifferentiation stimulus was counted in control and *LHX8* DPSCs. DEG, differentially expressed genes.

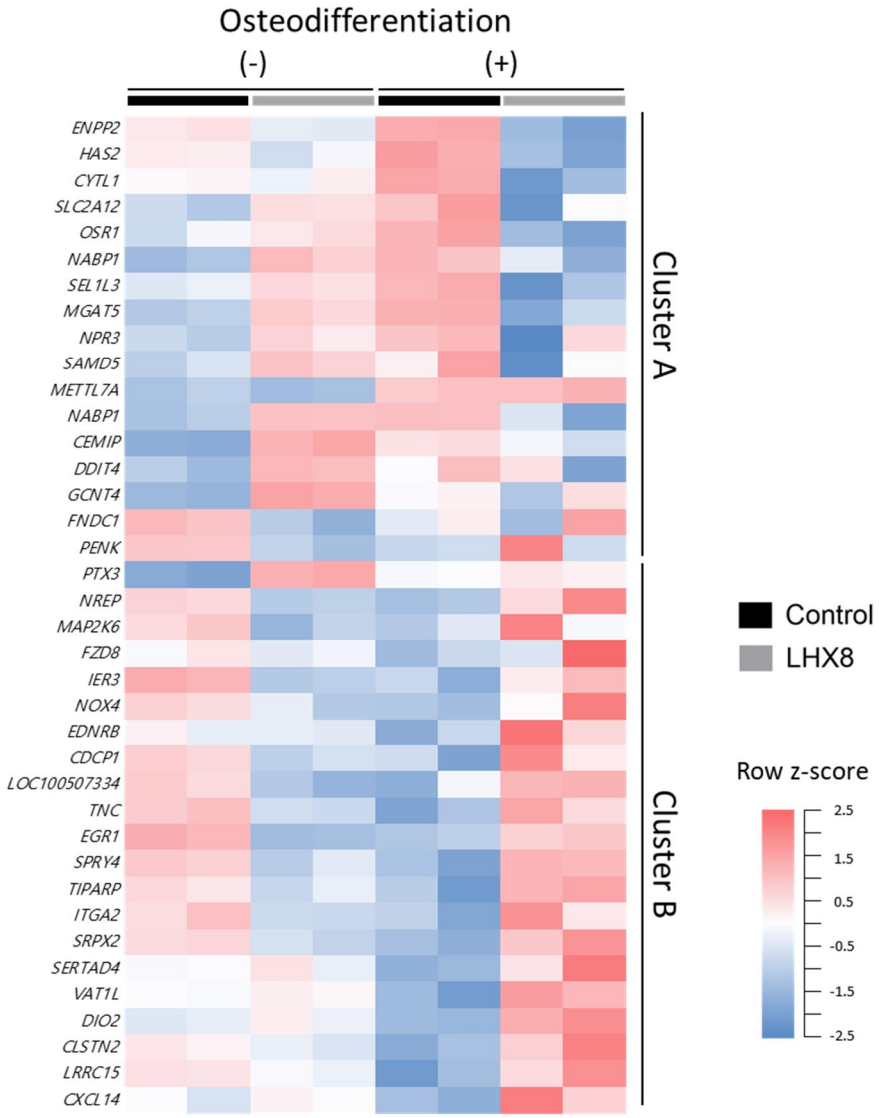


**Figure 4. Heatmap of osteodifferentiation DEGs common in control and *LHX8* DPSCs.** The 38 genes which were common osteogenic DEGs both in control and *LHX8* DPSCs were clustered and the expression levels were depicted as a heatmap. The Row z-score is calculated as the following: (expression – mean expression) / standard deviation.

### *LHX8*-associated DEGs upon osteodifferentiation

Upon osteodifferentiation, *LHX8* overexpression resulted in 427 DEGs. These are also associated with gene ontology term of protein binding (data not shown). Top 20 upregulated and top 20 downregulated DEGs are listed in Tables 5 and 6, respectively.

**Table 5. Top 20 upregulated genes in *LHX8* DPSCs compared to control DPSCs when osteodifferentiated**

| Gene | Accession | Fold Change |
|---|---|---|
| *CXCL14* | NM_004887 | 8.23 |
| *DIO2* | NM_000793 | 7.63 |
| *LOC105369463* | XR_913470 | 6.44 |
| *CLSTN2* | NM_022131 | 5.56 |
| *CHRNA1* | NM_000079 | 5.33 |
| *PDZRN4* | NM_001164595 | 5.02 |
| *AQP11* | NM_173039 | 4.63 |
| *LRRC15* | NM_001135057 | 4.17 |
| *DCLK1* | NM_001195415 | 3.88 |
| *USP9Y* | NM_004654 | 3.83 |
| *CLDN1* | NM_021101 | 3.82 |
| *B3GALT2* | NM_003783 | 3.81 |
| *SPRY1* | NM_001258038 | 3.72 |
| *LHX8* | NM_001001933 | 3.64 |
| *PSG5* | NM_001130014 | 3.57 |
| *PLCXD3* | NM_001005473 | 3.46 |
| *IGFBP3* | NM_000598 | 3.39 |
| *EDNRB* | NM_000115 | 3.38 |
| *KRTAP1-5* | NM_031957 | 3.21 |
| *NFE2L3* | NM_004289 | 3.21 |

**Table 6. Top 20 downregulated genes in *LHX8* DPSCs compared to control DPSCs when osteodifferentiated**

| Gene | Accession | Fold Change |
|---|---|---|
| *NFIL3* | NM_001289999 | -3.02 |
| *OLFML1* | NM_198474 | -3.09 |
| *IGFBP5* | NM_000599 | -3.11 |
| *WNT2* | NM_003391 | -3.13 |
| *STC1* | NM_003155 | -3.18 |
| *ACKR3* | NM_020311 | -3.22 |
| *RGS18* | NM_130782 | -3.22 |
| *CYTL1* | NM_018659 | -3.24 |
| *TIMP4* | NM_003256 | -3.32 |
| *JAM2* | NM_001270407 | -3.40 |
| *CYP39A1* | NM_001278738 | -3.73 |
| *EGFL6* | NM_001167890 | -3.77 |
| *NR4A3* | NM_006981 | -3.84 |
| *GALNT15* | NM_054110 | -3.87 |
| *MIR1245A* | NR_031647 | -4.01 |
| *STEAP1* | NM_012449 | -4.07 |
| *PAPPA* | NM_002581 | -4.58 |
| *LOC105375734* | NR_131202 | -5.51 |
| *HAS2* | NM_005328 | -6.02 |
| *ENPP2* | NM_001040092 | -11.07 |

**Inhibition of *LHX8*-associated protein ID1 by a small molecule results in increased osteogenesis.**

The anti-osteogenic role of *LHX8* has been partly demonstrated in Part 1 of this study, by in vitro functional experiments. Confirmation of these results by functional blockage of *LHX8* with a small molecule is unfortunately impossible since no chemical inhibitor targeting LHX8 protein has been developed to my knowledge. I assumed that inhibition of *LHX8*-associated proteins may result in altered osteodifferentiation of DPSCs. From those top 20 genes whose expression has been downregulated during osteodifferentiation in control DPSCs (Table 4), target genes were selected with these following criteria: i) decreased expression in control DPSCs during osteodifferentiation, ii) increased expression in *LHX8* DPSCs during osteodifferentiation, iii) availability of chemical inhibitor or a neutralizing antibody. Five potential target genes were chosen: *DCLK1, CXCL14, MAP2K6, ID1, and EGR1*. The neutralizing antibodies or chemical inhibitors for *IL11* and *MMP3* have been developed, but were not tested in this study due to commercial availability at the time of experiments (Table 7). Inhibitor information of these target genes is summarized in Table 8. Among the inhibitors tested, ML323, targeting ID1, resulted in an increased osteodifferentiation of DPSCs shown by increased ALP activity and increased calcium accumulation (Figure 4).

**Table 7. *LHX8*-associated genes with potential role in osteogenesis**

| Gene Symbol | mRNA Accession | CD/CO Fold Change | LD/CD Fold Change | Inhibitor Availability |
|---|---|---|---|---|
| *EGR1* | NM_001964 | -2.8 | 2.28 | Yes |
| *LRRC15* | NM_001135057 | -2.76 | 4.17 | |
| *CLSTN2* | NM_022131 | -2.66 | 5.56 | |
| *ID1* | NM_002165 | -2.64 | 1.78 | Yes |
| *MMP3* | NM_002422 | -2.52 | 1.49 | Yes [a] |
| *IL11* | NM_000641 | -2.44 | 1.32 | Yes [a] |
| *FAM72D* | NM_207418 | -2.34 | 1.19 | |
| *ITGA2* | NM_002203 | -2.3 | 2.64 | |
| *IER3* | NM_003897 | -2.29 | 1.86 | |
| *HIST1H3G* | NM_003534 | -2.24 | 1.08 | |
| *MAP2K6* | NM_002758 | -2.21 | 2.56 | Yes |
| *CXCL14* | NM_004887 | -2.2 | 8.23 | Yes |
| *APCDD1L* | NM_001304787 | -2.1 | 1.46 | |
| *KRTAP2-3* | NM_001165252 | -2.09 | 1.34 | |
| *HIST1H1B* | NM_005322 | -2.09 | 1.14 | |
| *FST* | NM_006350 | -2.06 | 1.97 | |
| *HIST1H2AI* | NM_003509 | -2.05 | 1.03 | |
| *TRIB1* | NM_001282985 | -2.04 | 1.47 | |
| *CDCP1* | NM_022842 | -2.04 | 2.41 | |
| *DCLK1* | NM_001195415 | -2.01 | 3.88 | Yes |

[a] not included in the study due to commercial availability limitations
Abbreviations: CD, control DPSCs osteodifferentiated; CO, control DPSCs undifferentiated; LD, *LHX8* DPSCs osteodifferentiated

**Table 8. Chemical inhibitors and neutralizing antibodies tested.**

| Compound Name | Protein Target | Gene Target | Concentration | Cat. No. | References |
|---|---|---|---|---|---|
| LRRK2-IN-1 | Doublecortin like kinase 1 | *DCLK1* | 10 μM | TOCRIS 4273 | [20] |
| CXCL14 neutralizing antibody | CXC motif chemokine ligand 14 | *CXCL14* | 200 μg/mL | RnD AF866 | [21] |
| Ethacrynic acid | MAPK/ERK kinase 6 | *MAP2K6* | 10 μM | Sigma SML1083 | [22] |
| ML323 | Inhibitor of DNA binding 1 | *ID1* | 10 μM | Sigma 531131 | [23, 24] |
| Mithramycin A | Early growth response 1 | *EGR1* | 10 nM | Sigma M6891 | [25] |

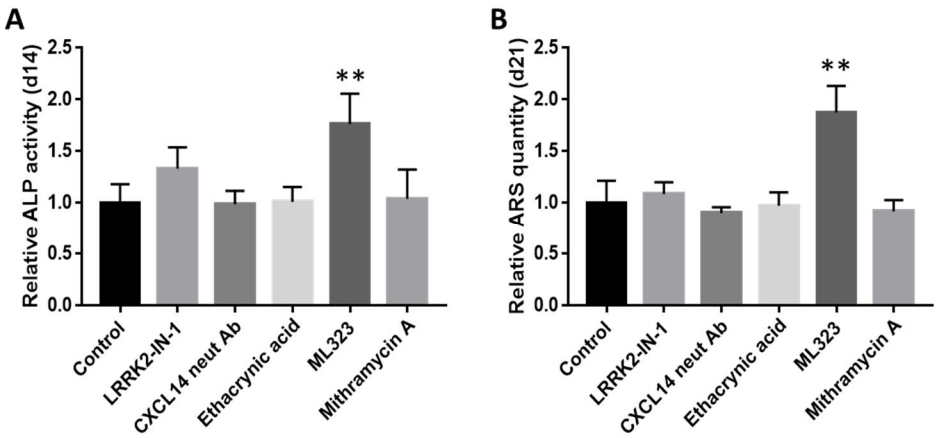


**Figure 5. ML323 enhances the osteogenic function of DPSCs**. (A) Alkaline phosphatase (ALP) activity of DPSCs on day 14 of osteodifferentiation with various compounds. (B) Accumulated calcium staining by alizarin red S (ARS) on day 21. Control, empty vector-incorporated control DPSCs. *LHX8*, *LHX8* overexpressed DPSCs. neut ab, neutralizing antibody. **, $P<0.01$

# DISCUSSION

Transcriptomic analyses were performed on *LHX8* overexpressing DPSCs and control DPSCs. cDNA microarray data supported the finding in Part 1 that *LHX8* plays an osteo-inhibitory role. For the genes which were associated with both osteodifferentiation and *LHX8* overexpression, chemical inhibitors or neutralizing antibodies were treated to their protein products to confirm their functional role in osteogenesis.

The microarray results showed differential expression of 716 genes in *LHX8* overexpressed DPSCs compared to control DPSCs, and these genes were mostly associated with the binding function of the cell. When DPSCs were osteodifferentiated, 345 genes were differentially expressed compared to control DPSCs. These genes were also associated with binding, implying the involvement of *LHX8* in osteodifferentiation. Interestingly, a smaller number of genes (n=53) were differentially expressed during the osteodifferentiation process in *LHX8* DPSCs, partially supporting the attenuated response of *LHX8* overexpressed DPSCs to osteogenic stimulus. The common osteodifferentiation-associated DEGs of control DPSCs and *LHX8* DPSCs were clearly clustered into two groups: when a cluster of genes was downregulated by osteodifferentiation signal, the same genes were upregulated by *LHX8* and vice versa. This also supports the anti-osteogenic role of *LHX8*.

Dental mesenchyme, where *LHX8* is strongly expressed during the course of embryonic development, goes through the condensation process

[26]. Gene products regulating protein binding may play important roles during mesenchymal condensation. Therefore, the role of binding function genes which were dysregulated by *LHX8* needs to be elucidated. It is a pity that there is no mesenchyme condensation cell model available to my knowledge.

Microarray analyses were performed on the fourth day of osteodifferentiation, which is a relatively early time point taking into an account that osteodifferentiation is a long process often taking weeks [27]. I assumed that *LHX8* may play a role at the early stage of craniofacial tissue development considering the precedent reports that *LHX8* is expressed from embryonic day 9.5 [28]. Therefore cDNA array comparisons were made before full osteogenic signals were activated. My real-time PCR results showed some osteogenic genes were even not fully activated on osteodifferentiation day 7, suggesting that DEGs in my microarray results are indeed early response genes. A thorough comparison of early response genes and late response genes during the course of osteodifferentiation is also an interesting topic to commence further studies.

There is no chemical inhibitor for LHX8 protein, however, and this limits the clinical utility of the finding. I have used transcriptomic analysis to discover *LHX8*-associated genes which are functional during the process of osteogenesis. Among the *LHX8*-regulated osteogenic genes, *ID1* showed an anti-osteogenic role which was proven by the small molecule inhibitor treatment. ID1 is an oncogenic protein that is normally poly-ubiquitinated and rapidly degraded in the normal condition [29]. An ubiquitin-specific protease

USP1 deubiquitinates ID1 and rescues it from proteasome degradation [24]. Blockage of USP1 function leads to subsequent degradation of ID1 [24]. My results show that ML323, an inhibitor of USP1, enhances osteodifferentiation of DPSCs possibly by suppressing USP1-mediated deubiquitination of ID1 [30]. A more in-depth study has to be performed to discover clinically adaptable small molecule osteogenic agents, possibly by using chemical libraries of FDA-approved drugs [31].

In summary, *LHX8* overexpression showed anti-osteogenic effects on DPSCs. Chemical inhibitor studies for *LHX8* downstream genes confirmed that treatment of ML323 during osteodifferentiation lead to enhanced osteogenesis. These results may provide clinical insights which may lead to the development of osteogenic agents.

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# 국문 초록

전사인자 단백질을 발현하는 *LIM homeobox 8 (LHX8)* 유전자는 두개부의 치아 및 골조직 배아발달 단계에서 중요한 역할을 한다. 그러나 골 분화능을 가진 인간 치수줄기세포의 골분화에 있어서 *LHX8*의 역할은 명확하지 않다. 본 연구는 렌티바이러스 시스템을 활용하여 인간치수줄기세포에서 *LHX8*를 과발현시키고, 과발현된 *LHX8*이 골분화에 미치는 영향을 연구하였다. *LHX8* 과발현은 세포의 성장속도, 이동속도, 혈관형성능에 영향이 없었다. 반면 골분화는 *LHX8* 과발현 모델에서 억제되었는데 염기성인산분해효소 활성의 감소 및 칼슘 축적량 감소를 통해 확인되었다. cDNA 마이크로어레이 분석을 시행한 결과 골분화 유도시 치수줄기세포에서 단백질결합에 관계된 유전자군에 유의한 변화가 있었다. 골분화 유도와 골분화를 억제하는 *LHX8* 과발현에 의해 공통적으로 발현양이 변화한 유전자는 총 38개였는데 2개의 유전자군으로 구별되었다. 각 군의 유전자는 골분화 유도에 의해 발현이 증가할 때 *LHX8* 과발현에 의해 감소하거나 혹은 그 반대의 경우로서 *LHX8*의 골분화 억제능을 재확인할 수 있는 결과였다. 다음은 치수줄기세포의 골분화를 촉진할 수 있는 분자적 방법을 찾기 위해서 *LHX8* 과발현 없는 골분화 조건에서는 발현이 감소하지만 골분화를 억제하는 *LHX8* 과발현 조건에서 골분화 유도시에는 발현이 증가하는 유전자를 도출하였다. 도출된 유전

자는 각 유전자가 발현하는 단백질에 대한 화학적 저해제를 처리한 후 골분화가 촉진되는지 살펴보았다. *ID1* 발현 단백질인 DNA-binding protein inhibitor ID-1 활성을 억제하는 화학적 저해제 ML323에 의해 치수줄기세포의 골분화가 촉진되었다. 이로서 본 연구는 *LHX8* 과발현 인간 치수유래 줄기세포 모델을 활용하여 *LHX8*가 인간 치수유래 줄기세포의 골분화를 억제함을 밝혔으며 골분화 억제 유전자 *ID1*을 발굴하였고 DNA-binding protein inhibitor ID-1을 억제함으로써 인간 치수줄기세포의 골분화능을 촉진할 수 있음을 보여주었다.

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**주요어 : 치아줄기세포, 골분화, *LHX8*, *ID1*, *ML323***

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