Research Report

Journal of INTERNATIONAL MEDICAL RESEARCH

Journal of International Medical Research
2017, Vol. 45(3) 993–1006

DOI: 10.1177/0300060517701035
journals.sagepub.com/home/imr
SAGE

Short-term application of dexamethasone on stem cells derived from human gingiva reduces the expression of RUNX2 and β-catenin

Bo-Bae Kim[1], Minji Kim[1], Yun-Hee Park[2], Youngkyung Ko[1] and Jun-Beom Park[1]

Abstract

Objective: Next-generation sequencing was performed to evaluate the effects of short-term application of dexamethasone on human gingiva-derived mesenchymal stem cells.

Methods: Human gingiva-derived stem cells were treated with a final concentration of 10^{-7} M dexamethasone and the same concentration of vehicle control. This was followed by mRNA sequencing and data analysis, gene ontology and pathway analysis, quantitative real-time polymerase chain reaction of mRNA, and western blot analysis of RUNX2 and β-catenin.

Results: In total, 26,364 mRNAs were differentially expressed. Comparison of the results of dexamethasone versus control at 2 hours revealed that 7 mRNAs were upregulated and 25 mRNAs were downregulated. The application of dexamethasone reduced the expression of RUNX2 and β-catenin in human gingiva-derived mesenchymal stem cells.

Conclusion: The effects of dexamethasone on stem cells were evaluated with mRNA sequencing, and validation of the expression was performed with qualitative real-time polymerase chain reaction and western blot analysis. The results of this study can provide new insights into the role of mRNA sequencing in maxillofacial areas.

Keywords

Dexamethasone, gingival, messenger RNA, stem cells

Date received: 6 January 2017; accepted: 1 March 2017

Introduction

Mesenchymal stem cells have the capacity for self-renewal and multilineage differentiation. These cells include three lineages that exhibit osteogenic, chondrogenic, and

[1]Department of Periodontics, College of Medicine, The Catholic University of Korea, Seoul, Republic of Korea
[2]ebiogen, Seoul, Republic of Korea

Corresponding author:
Jun-Beom Park, Department of Periodontics, Seoul St Mary's Hospital, College of Medicine, The Catholic University of Korea, 222 Banpo-daero, Seocho-gu, Seoul 06591, Republic of Korea.
Email: jbassoonis@yahoo.co.kr

adipogenic differentiation, respectively[1,2] Mesenchymal stem cells play a crucial role in tissue engineering and regenerative medicine.[3] In recent years, stem cells have been derived from a variety of dental-related tissues such as the periodontal ligament, papilla, follicle, dental pulp of exfoliated deciduous and adult teeth, and maxillary sinus membrane, which represents a particularly rich source of mesenchymal stem cells.[4,5] Stem cells from dental-related tissues display multifactorial advantages including a high proliferation rate, high viability, and easy induction to distinct cell lineages.[4] Moreover, our group has reported that human gingiva-derived stem cells can be differentiated into osteoblasts under some conditions, including within osteogenic media, and that these cells may be applied to tissue engineering.[6,7]

Dexamethasone is a glucocorticoid drug that is broadly used to repress allergic reactions and treat autoimmune diseases.[8] One study revealed that dexamethasone treatment induced osteonecrosis of the femoral head via inhibition of osteogenic differentiation of human bone marrow stem cells.[9] Another study showed that osteoblastic MC3T3-E1 cell proliferation was directly inhibited by dexamethasone via deviant glucocorticoid receptor activation and subsequent P53 activation.[10] Moreover, dexamethasone induces bone loss by inhibiting bone formation through suppression of osteoblast proliferation and collagen synthesis and by stimulating mature osteoclasts.[11] As a result, high dosages of glucocorticoids can change bone remodeling and promote bone resorption, which can lead to osteoporosis.

In certain responses to a variety of physiological and pathological stimuli, mesenchymal stem cells can proliferate and differentiate into osteoblasts, chondrocytes, and adipocytes.[12] Differentiation of multipotent mesenchymal stem cells to the osteoblast lineage and maturation of osteoprogenitors are regulated and activated by transcription factors.[13] Among these transcription factors, runt-related transcription factor 2 (RUNX2) and the Wnt/β-catenin signaling pathway are crucial for osteoblast differentiation.[14,15] RUNX2 is a bone-specific factor and the master regulator of bone formation.[15] This transcription factor has been found to modulate a complex gene-regulatory network during osteoblastogenesis.[16] Additionally, ablation of RUNX2 results in the absence of a mineralized skeleton in mice and the development of cleidocranial dysplasia in humans.[17] The inactivation of β-catenin in mesenchymal progenitor cells exclusively blocks osteoblast differentiation.[14,18] However, whole-level gene changes have not been well described.[19] RNA sequencing can detect all coding and non-coding regions in the cell and determine their sequence and structure.[20] Massive parallel sequencing can be performed using next-generation sequencing or second-generation sequencing.[21] The present study was performed to evaluate the effects of short-term application of dexamethasone on human gingiva-derived mesenchymal stem cells with next-generation sequencing.

Materials and methods

Isolation and culture of human gingiva-derived stem cells

Gingival tissues were collected from healthy patients undergoing clinical crown-lengthening procedures. The design of the study was reviewed and approved by the Institutional Review Board of the Catholic University of Korea, College of Medicine (KC11SISI0348). Informed consent was obtained from all patients according to the Act on Legal Codes for Biomedical Ethics and Safety and the Declaration of Helsinki.

Human gingiva-derived stem cells were isolated and cultivated in accordance with

the procedure described in a previous study by one of the authors.[6] The gingival tissues were collected and contained in sterile phosphate-buffered saline (Welgene, Inc., Gyeongsan-si, Gyeongsangbuk-do, Korea) that included 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich Co., St. Louis, MO, USA) at 4°C. The tissues were de-epithelialized, separated into 1- to 2-mm^2 fragments, digested in 0.2-μm-pore filter, and modified in alpha minimum essential medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing dispase (1 mg/mL; Sigma-Aldrich Co.) and collagenase type IV (2 mg/mL; Sigma-Aldrich Co.) for 30 min at 37°C. The cell suspension was filtered with a 70-μm cell strainer (Falcon; BD Biosciences, Franklin Lakes, NJ, USA), and the cells were incubated at 37°C in a humidified incubator with 5% CO_2. After 24 hours, the nonadherent cells were washed with phosphate-buffered saline and replaced with fresh media every 3 to 4 days.

Cells were plated onto a 100-mm dish (passage 4) at a density of 1.0×10^6 cells/well and cultured in a growth medium including alpha minimum essential medium (Gibco), ascorbic acid (Sigma-Aldrich Co.), fetal bovine serum (Gibco), L-glutamine (Sigma-Aldrich Co.), penicillin, and streptomycin (Sigma-Aldrich Co.). Dexamethasone (Sigma-Aldrich Co.) was dissolved in 95% ethanol to a concentration of 2×10^{-3} M (stock solution). The cells were then treated with a final concentration of 10^{-7} M dexamethasone and the same concentration of vehicle control (95% ethanol) for 2 and 24 hours. The doses of dexamethasone and the time points used in the present study were based on those used in previously published studies.[22,23]

Total RNA extraction

The human gingiva-derived stem cells were harvested at 2 and 24 hours. Total RNA was isolated using Trizol reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). RNA quality was assessed with a bioanalyzer (Agilent 2100; Agilent Technologies, Amstelveen, The Netherlands) using the RNA 6000 Nano Chip (Agilent Technologies), and the quantity was determined by spectrophotometer (ND-2000; Thermo Fisher Scientific, Inc.) with a ratio of absorbance of >1.8 at 260 and 280 nm. To confirm high RNA quality for all samples, we checked whether the RNA integrity number was >7.0.

Sequencing of mRNA and analysis of data

A library of control and test RNAs was constructed using SENSE mRNA-Seq Library Prep Kit (Lexogen, Inc., Vienna, Austria) according to the manufacturer's instructions. Briefly, each 2 μg of total RNA was prepared and incubated with magnetic beads decorated with oligo-dT, and other RNAs (excluding mRNA) were then removed with a washing solution. Library production was initiated by the random hybridization of starter/stopper heterodimers to the poly(A) RNA still bound to the magnetic beads. These starter/stopper heterodimers contained linker sequences compatible with Illumina (San Diego, CA, USA). A single-tube reverse transcription and ligation reaction extends the starter to the next hybridized heterodimer, where the newly synthesized cDNA insert is ligated to the stopper. Second-strand synthesis is performed to release the library from the beads, and the library is then amplified. Barcodes were introduced when the library was amplified. High-throughput sequencing was performed as paired-end 100-base-pair sequencing using HiSeq 2500 (Illumina).

RNA-Seq reads were mapped using the Top Hat software tool (Toronto, ON, Canada) to obtain the alignment file. The alignment file was used to assemble transcripts, estimate their abundances, and

detect differential expressions of genes or isoforms using cufflinks. Gene classification was based on searches performed using the BioCarta (http://www.biocarta.com/), GenMAPP (http://www.genmapp.org/), DAVID (http://david.abcc.ncifcrf.gov/), and Medline databases (http://www.ncbi.nlm.nih.gov/).

Gene ontology and pathway analysis

Gene ontology analysis was performed and included osteoblast differentiation, bone remodeling, and Wnt signaling. *P* values were used to denote significance of gene ontology term enrichment ($P < 0.05$ was considered statistically significant). Pathway analysis was also performed for differentially expressed genes based on the PATHWAY database of the Kyoto Encyclopedia of Genes and Genomes. A fold change of 1.3 was applied in this study, and a log2 normalized read count of ≥ 4 was applied to minimize false counts. A *P* value of <0.05 was considered statistically significant.

Quantification by real-time polymerase chain reaction

Total RNA was isolated from cultured cells using GeneJET RNA Purification kit (Thermo Fisher Scientific, Inc.) and reverse-transcribed. The sense and antisense primers were designed based on GenBank. Primer sequences were as follows: RUNX2 Forward 5′-AAT GAT GGT GTT GAC GCT GA-3′, Reverse 5′-TTG ATA CGT GTG GGA TGT GG-3′; β-catenin Forward 5′-GAG GGG TGG GCT GGT ATC TC-3′, Reverse 5′-CTC GAC CAA AAA GGA CCA GA-3′; and β-actin Forward 5′-TGGCACCCAGCACAATGAA-3′, Reverse 5′-CTAAGTCATAGTCCGCCTA GAAGCA-3′. β-actin served as a housekeeping gene for normalization. The expression of mRNA was detected by real-time polymerase chain reaction (RT-PCR) using SYBR Green Real-Time PCR Master Mixes (Enzynomics, Daejeon, Korea) according to the manufacturer's protocol. Quantitative RT-PCR experiments were repeated three times.

Western blot analysis

Cells were lysed in ice-cold RIPA lysis and extraction buffer (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Whole cell lysates were quantified using the BCA assay (Thermo Fisher Scientific, Inc.). Protein samples were loaded and then transferred for immunoblotting. The membranes were incubated with the following primary antibodies overnight at 4°C: anti-RUNX2 antibody (Abcam, Cambridge, UK), anti-β-catenin (BD Biosciences, San Jose, CA, USA), or anti-GAPDH antibody (Abcam). After washing, membranes were incubated with secondary antibody (Abcam) for 1 hour at room temperature.

Data analysis

mRNA-Seq reads were mapped using Top Hat software to obtain the alignment file. Differentially expressed genes were determined based on counts from unique and multiple alignments using EdgeR within R version 3.2.2 (R development Core Team, 2011) with BIOCONDUCTOR version 3.0 (Gentleman et al., 2004). The alignment file was also used to assemble transcripts, estimate their abundances, and detect differential expression of genes or isoforms using cufflinks. We calculated the fragments per kilobase of exon per million fragments to determine the expression level of the gene regions. Global normalization was used for comparison between samples. Gene classification was based on searches done by DAVID (http://david.abcc.ncifcrf.gov/). The data are represented as mean ± standard deviation of the experiments. Either Student's t-test or a two-way analysis of

variance with a post-hoc test was performed to determine the differences between the groups using a commercially available program (SPSS 12 for Windows; SPSS Inc., Chicago, IL, USA). The level of significance was 0.05.

Results

Gene ontology

In total, 26,364 mRNAs were differentially expressed. Scatter plots, which differentially expressed the mRNAs, are shown in Figure 1. The gene ontology analysis results of the differentially expressed mRNAs are shown in Figure 2. We focused especially on osteoblast differentiation (fold change of 1.3, log2 normalized read count of $\geq$4 to minimize false counts, $P < 0.05$) (Table 1 and Figure 3). First, we assessed the effect of incubation time on the stem cells. Comparison of the controls at 24 versus 2 hours revealed that 16 mRNAs were upregulated and 26 were downregulated. Comparison of the dexamethasone group at 24 versus 2 hours revealed that 9 mRNAs were upregulated and 37 were downregulated. Comparison of the data in both groups at 24 versus 2 hours showed that 4 genes were upregulated and 17 genes were downregulated. The four genes that were upregulated were TWIST1, BMP4, BGLAP, and RDH14, and the 17 genes that were downregulated were FHL2, GTPBP4, SMAD3, SNAI2, PENK, LRP5, MEF2D, EPHA2, AMELX, JUND, MSX2, CHRD, SEMA7A, PTHLH, JUNB, LRRC17, and SOX8. However, two genes (ESR1 and ACHE) showed opposite trends.

Next, we assessed the effect of dexamethasone. Comparison of the dexamethasone and control groups at 2 hours showed that 7 mRNAs were upregulated and 25 mRNAs were downregulated. Comparison of the dexamethasone and control groups at 24 hours revealed that 6 mRNAs were upregulated and 31 mRNAs were downregulated. In both groups, 1 gene (BGLAP) was upregulated and 11 genes (WWTR1, CREB3L1, LRP5, IGFBP3, MSX2, SMAD3, RSPO2, CHRD, PTH1R, WWOX, and SPP1) were downregulated. However, four genes (ESR1, IGFBP5, LGR4, and ACHE) showed opposite trends. Expression of RUNX2 was decreased in the dexamethasone group at 24 hours. Decreased expression of CTNNB1 (for β-catenin expression) was also noted in the dexamethasone group at 24 hours (Figure 4).

Validation of mRNA expression

Quantitative RT-PCR revealed that the mRNA levels of RUNX2 and β-catenin were lower at 24 than at 2 hours (Figure 5). This change was more significant in the dexamethasone group at 24 hours.

Western blot analysis

Western blot analysis was performed to detect protein expression following treatment with dexamethasone at 2 and 24 hours compared with the untreated group at 2 and 24 hours. Normalization of the protein expressions revealed $34.3\% \pm 4.3\%$ expression of RUNX2 in the control group at 24 hours and $155.5\% \pm 29.8\%$ and $190.3\% \pm 71.3\%$ expression of RUNX2 in the dexamethasone group at 2 and 24 hours, respectively, when the control value at 2 hours was considered to be 100% ($P < 0.05$) (Figure 6). The expression of β-catenin in the control group was not significantly different between 2 and 24 hours. Normalization of the protein expression revealed $95.2\% \pm 14.5\%$ expression of β-catenin in the control group at 24 hours and $66.8\% \pm 13.5\%$ and $73.6\% \pm 43.9\%$ expression of β-catenin in the dexamethasone group at 2 and 24 hours, respectively, when the control value at 2 hours was considered to be 100% (Figure 6).

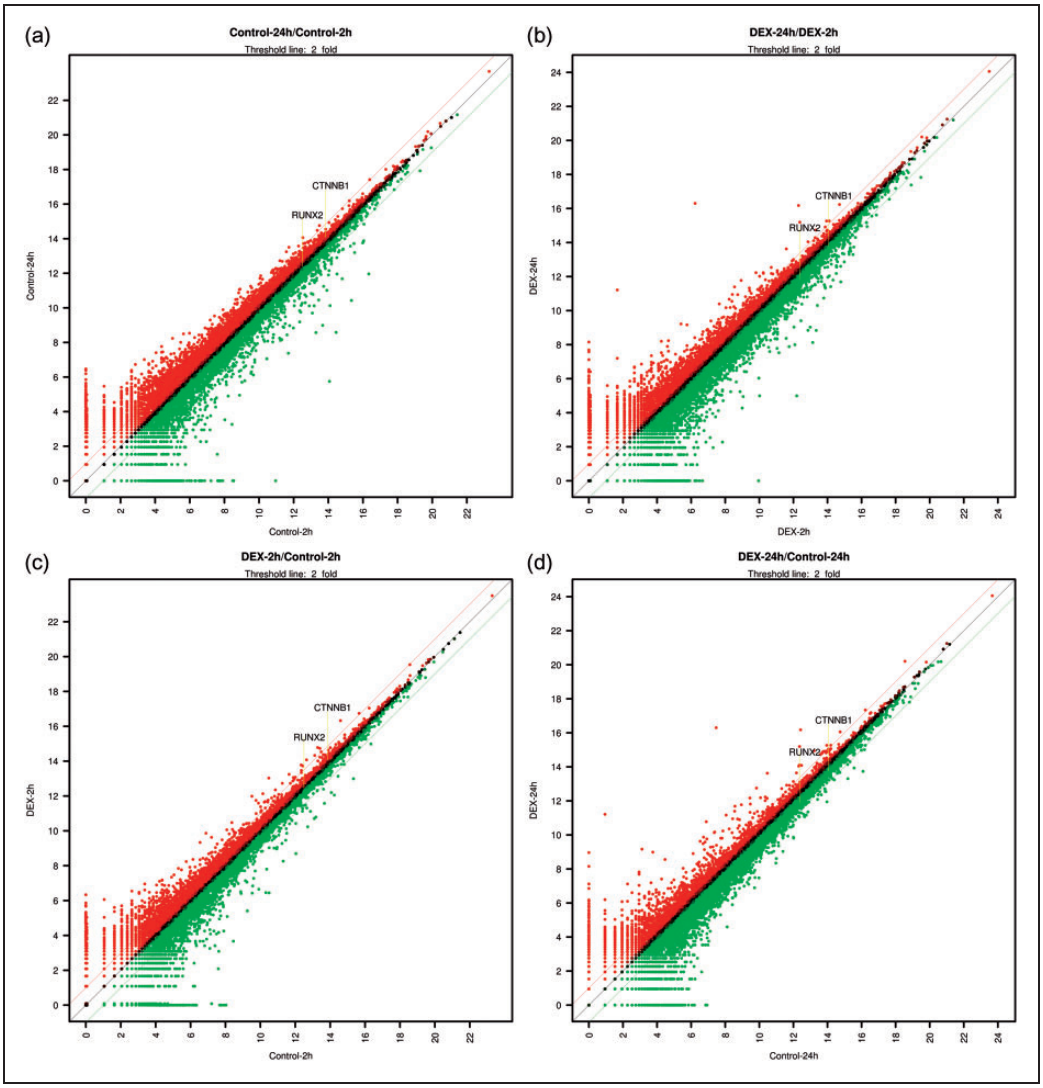

Figure 1. Scatter plots showing the expression in untreated controls at 2 and 24 hours and dexamethasone-treated cells at 2 and 24 hours (x, y-axis: relative expression levels; Red: Expression level of y-value is higher than expression level of x-value; Green: Expression level of y-value is lower than expression level of x-value). (a) Control at 24 hours/control at 2 hours, (b) Dexamethasone at 24 hours/dexamethasone at 2 hours, (c) Dexamethasone at 2 hours/control at 2 hours, (d) Dexamethasone at 24 hours/control at 24 hours.

Discussion

In this report, we examined the effects of predetermined concentrations of dexamethasone on stem cells at 2 and 24 hours. Sequencing of mRNA and validation of the expression was performed with qualitative RT-PCR and western blot analysis. The application of dexamethasone clearly resulted in reduced expression of RUNX2

Figure 2. Gene ontology analysis of mRNA expression. (a) Control at 24 hours/control at 2 hours. (b) Dexamethasone at 24 hours/dexamethasone at 2 hours. (c) Dexamethasone at 2 hours/control at 2 hours. (d) Dexamethasone at 24 hours/control at 24 hours. (e) Venn diagram analysis (fold change, 1.3; log2 normalized read counts of ≥ 4 were selected; $P < 0.05$).

and β-catenin in human gingiva-derived mesenchymal stem cells.

Stem cells derived from the gingiva were used in the present study; these cells have also been applied to tissue engineering.[24] Human gingiva-derived mesenchymal stem cells from the maxillofacial region can be considered a favorable source of mesenchymal stem cells because harvesting these cells from the mandible or maxilla can easily be performed under local anesthesia.[6,25] Moreover, one study revealed that human gingiva-derived mesenchymal stem cells are superior to bone marrow-derived mesenchymal stem cells for cell therapy in regenerative medicine.[26]

Two time points (2 and 24 hours) were selected to evaluate the effects of the dexamethasone incubation time. The immediate effects of dexamethasone were evaluated using the 2-hour results, and the more sustained effects were evaluated by using the 24-hour results. More pronounced effects were observed at 24 hours because of the cumulative effects of dexamethasone. Short-term, high-dose dexamethasone reportedly predisposes patients to complicated fracture union, especially patients with angle fractures.[27] However, long-term physiologic concentrations of dexamethasone resulted in more intense staining for alkaline phosphatase in human bone-derived cells.[28]

Table 1. Differentially expressed mRNA related to osteoblast differentiation (fold change, 1.3; log2 normalized read counts of ≥4 were selected; $P < 0.05$).

Gene Symbol	Control-24h/Control-2h	Gene Symbol	Dexamethasone-24h/dexamethasone-2h	Gene Symbol	Dexamethasone-2h/control-2h	Gene Symbol	Dexamethasone-24h/control-24h
TWIST1	2.334	ACHE	8.835	ESR1	2.811	ACHE	3.717
PTH1R	2.144	BMP4	2.478	ALPL	1.883	BMP6	2.424
SMO	1.858	BMP6	2.105	SMO	1.817	GLI1	1.597
LGR4	1.814	TWIST1	2.044	IGFBP5	1.782	ITGA11	1.517
BMP4	1.778	BGLAP	1.665	NR1I3	1.651	RPS15	1.461
BGLAP	1.638	MEN1	1.362	BGLAP	1.399	BGLAP	1.422
ESR1	1.626	RDH14	1.317	LGR4	1.375	SATB2	0.753
SHOX2	1.600	MYBBP1A	1.306	AMELX	0.767	GJA1	0.750
DLX5	1.599	ATP5B	1.301	WWTR1	0.760	GPNMB	0.743
ALPL	1.565	EPHA2	0.766	EPHA2	0.748	RBMX	0.740
NR1I3	1.511	GPNMB	0.765	TP53INP2	0.742	SYNCRIP	0.729
RBMX	1.452	RRAS2	0.755	SEMA7A	0.738	IGFBP5	0.716
RRAS2	1.391	FHL2	0.737	LEF1	0.734	LGR4	0.715
RDH14	1.388	GJA1	0.735	CREB3L1	0.734	COL1A1	0.713
UFL1	1.317	TPM4	0.724	MEF2C	0.723	FIGNL1	0.711
IFT80	1.306	IGFBP3	0.722	LRP5	0.715	RUNX2	0.708
HDAC4	0.745	FIGNL1	0.720	JUND	0.690	MSX2	0.706
FHL2	0.745	COL6A1	0.716	IGFBP3	0.689	SHOX2	0.696
GTPBP4	0.728	LRRC17	0.709	MSX2	0.686	RRAS2	0.692
SPP1	0.684	RUNX2	0.707	SOX8	0.682	IFT80	0.685
SMAD3	0.679	CREB3L1	0.706	SMAD3	0.680	COL6A1	0.683
TP53INP2	0.655	SYNCRIP	0.686	RSPO2	0.639	CREB3L1	0.671
SNAI2	0.650	WWTR1	0.679	BMP4	0.630	WWTR1	0.658
PENK	0.644	MEF2D	0.614	SNAI1	0.624	CHRD	0.637
LRP5	0.640	AMELX	0.608	JUNB	0.618	LRP5	0.630
LEF1	0.640	GTPBP4	0.605	CHRD	0.608	WWOX	0.608
MEF2D	0.535	DDX21	0.605	PTH1R	0.550	IGFBP3	0.561

(continued)

Table 1. Continued.

TMEM119	0.514	VCAN	0.604	WWOX	0.476	TMEM64	0.508
GLI1	0.512	RSPO2	0.593	NOG	0.357	SNAI2	0.505
EPHA2	0.466	CBFB	0.587	PTHLH	0.341	SMAD3	0.490
AMELX	0.465	SNAI1	0.575	ACHE	0.114	SFRP1	0.438
JUND	0.460	LRP5	0.564	SPP1	0.063	RSPO2	0.362
MSX2	0.436	JUND	0.545			TMEM119	0.360
SNAI1	0.406	SMAD3	0.489			ESR1	0.287
CHRD	0.395	SEMA7A	0.458			PTH1R	0.267
SEMA7A	0.367	MSX2	0.449			TNC	0.192
NOG	0.324	PENK	0.428			SPP1	0.009
ACHE	0.270	CHRD	0.414				
PTHLH	0.107	SFRP1	0.398				
JUNB	0.064	TMEM64	0.380				
LRRC17	0.046	IGFBP5	0.327				
SOX8	0.007	PTHLH	0.314				
		SNAI2	0.300				
		TMEM119	0.219				
		TNC	0.183				
		ESR1	0.166				
		JUNB	0.116				
		SOX8	0.010				

Next-generation sequencing can be performed in a high-throughput manner and is more cost-effective than Sanger sequencing.[21] This next-generation sequencing can be applied in several ways: gene expression profiling, chromatin immunoprecipitation sequencing, DNA methylation, de novo genome sequencing, metagenomics, noninvasive prenatal testing, identification of disease-related genes, assessment of human disease and health, and single-molecule and long-read sequencing.[29] The wide range of applications facilitated by automatic sequencing analyzers, the simple workflow, and a 100-fold decrease in cost have brought us significantly closer to understanding the links between genotype and phenotype and establishing the molecular basis of many

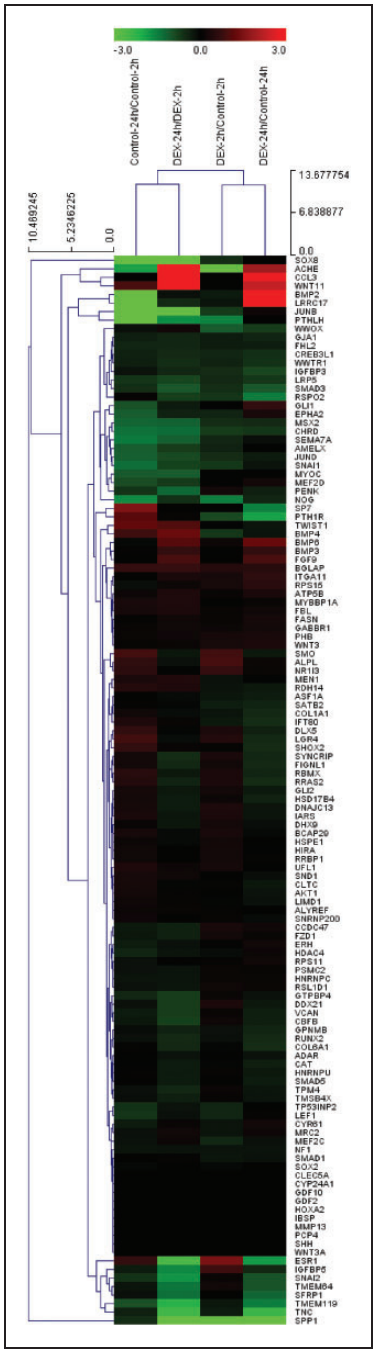

Figure 3. Clustering analysis of differentially expressed mRNA related to osteoblast differentiation (fold change, 1.3; log2 normalized read counts of ≥ 4 were selected; $P < 0.05$).

diseases.[30] HiSeq (Illumina), Roche 454 (Roche, Branford, CT, USA), and SOLiD (Life Technologies, Inc., Gaithersburg, MD, USA) systems are representative sequencers used for next-generation sequencing. Among them, the HiSeq is reportedly has particularly high-speed data quality and complete end-to-end sequencing solutions.[31]

The sequencing data showed the expression of a total of 26,364 mRNAs. Gene ontology analysis of mRNAs related to osteoblast differentiation was then performed. Previously, mRNA expression was measured by microarray methods or RT-PCR techniques.[32] These techniques are limited to the detection of known transcripts and have a limited capacity to differentiate between transcript variants.[33] Although RNA sequencing is still a technology under active development, it offers several advantages over existing technologies, including determination of all coding and non-coding regions; additionally, it is a popular method for genome analysis.[20,34] Notably, however, genes with low expression abundance might not be detected.[35]

Western blot analysis was performed to detect the protein expression of RUNX2 and β-catenin and provide information regarding possible mechanisms. Canonical Wnt/β-catenin signaling pathways are reportedly crucial for the osteoblastic lineage.[36] Wnt signaling suppresses mesenchymal stem cell commitment to the chondrogenic and adipogenic lineages and enhances the osteoblastic lineage, and both RUNX2 and β-catenin are major regulators of this signaling.[37] RUNX2 regulates a complex gene-regulatory network during osteoblastogenesis and upregulates various osteoblast lineage-specific genes.[38] One study revealed that RUNX2-targeting miRNAs might be essential to ensure that the RUNX2 level is attenuated at key stages of osteoblast lineage progression to accommodate the biological functions of RUNX2 in mesenchymal cell-fate

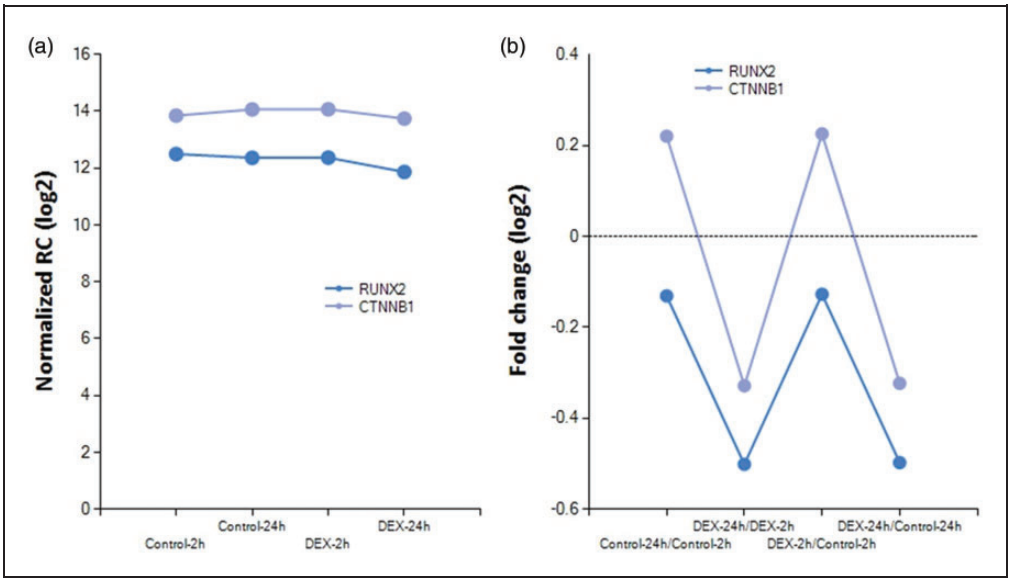

Figure 4. Change in expression of RUNX2 and CTNNB1 (for β-catenin expression) (fold change, 1.3; log2 normalized read counts of ≥ 4 were selected; $P < 0.05$).

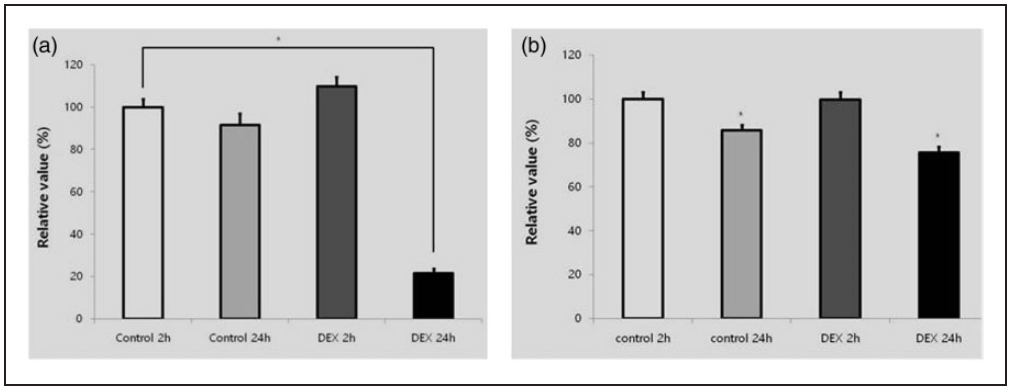

Figure 5. Validation of RUNX2 mRNA and β-catenin mRNA. (a) Expression of RUNX2. (b) Expression of β-catenin. *Statistically significant differences compared with the control at 2 hours.

determination and maturation of osteoblasts.[13] Inactivation of β-catenin in mesenchymal stem cells was shown to totally block osteoblast differentiation as well as mesenchymal cell differentiation into chondrocytes in the perichondrium and calvarium.[39] Dexamethasone has been shown to induce osteoporosis mainly by suppressing osteoblast-mediated osteogenesis; however, the precise mechanisms involved in these signaling pathways remain unclear.[40] For this reason, we examined the canonical Wnt/β-catenin signaling pathways in the present study and focused on changes

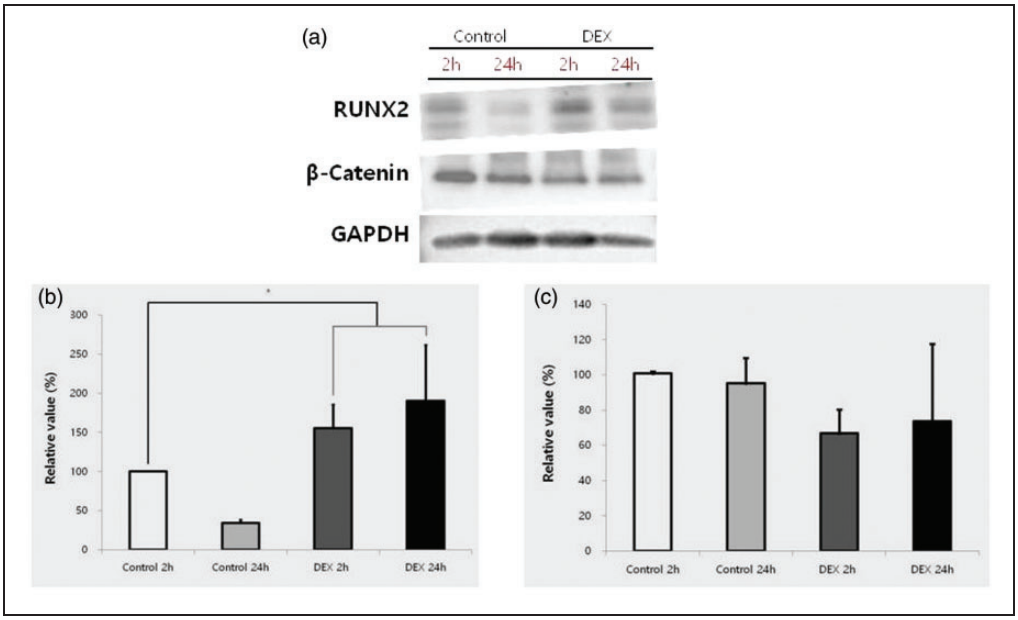

Figure 6. Western blot analysis for expression of RUNX2 and β-catenin. (a) Evaluation of protein expressions of RUNX2, β-catenin, and GAPDH. (b) Quantitative analysis of protein expressions of RUNX2 after normalization with GAPDH levels by densitometry. (c) Quantitative analysis of protein expressions of β-catenin after normalization with GAPDH levels by densitometry. *Statistically significant differences compared with the control at 2 hours.

in RUNX2 and β-catenin. The expression of RUNX2 was decreased by 70% after 24 hours of incubation with dexamethasone; the result for β-catenin was similar to this. We performed quantitative RT-PCR and western blot to validate this result by comparing mRNA and protein levels. Moderate consistency was found between the sequencing data and the RT-PCR results.

The effects of dexamethasone on stem cells were evaluated by mRNA sequencing, and the expression was validated by quantitative RT-PCR and western blot analysis. The results clearly showed that the application of dexamethasone reduced the expression of RUNX2 and β-catenin in human gingiva-derived mesenchymal stem cells. This study may provide new insights into the role of mRNA sequencing in maxillofacial areas.

Ethical approval statement

The design of the study was reviewed and approved by the Institutional Review Board of the Catholic University of Korea, College of Medicine (KC11SISI0348). Informed consent was obtained from all patients according to the Act on Legal Codes for Biomedical Ethics and Safety and the Declaration of Helsinki.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Declaration of conflicting interest

The Authors declare that there is no conflict of interest.

Funding

This research was partly supported by the Research Fund of Seoul St. Mary's Hospital, The Catholic University of Korea and partly supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, Information and Communication Technology & Future Planning (NRF-2014R1A1A1003106).

References

1. Huang GJ, Gronthos S and Shi S. Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. *J Dent Res* 2009; 88: 792–806.
2. Baksh D, Song L and Tuan R. Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. *J Cell Mol Med* 2004; 8: 301–316.
3. Seo BM, Miura M, Gronthos S, et al. Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* 2004; 364: 149–155.
4. Lei M, Li K, Li B, et al. Mesenchymal stem cell characteristics of dental pulp and periodontal ligament stem cells after in vivo transplantation. *Biomaterials* 2014; 35: 6332–6343.
5. Hakki SS, Kayis SA, Hakki EE, et al. Comparison of mesenchymal stem cells isolated from pulp and periodontal ligament. *J Periodontol* 2015; 86: 283–291.
6. Jin SH, Lee JE, Yun JH, et al. Isolation and characterization of human mesenchymal stem cells from gingival connective tissue. *J Periodontal Res* 2015; 50: 461–467.
7. Kim BB, Ko Y and Park JB. Effects of risedronate on the morphology and viability of gingiva-derived mesenchymal stem cells. *Biomed Rep* 2015; 3: 845–848.
8. Li T, Li H, Li T, et al. MicroRNA expression profile of dexamethasone-induced human bone marrow-derived mesenchymal stem cells during osteogenic differentiation. *J Cell Biochem* 2014; 115: 1683–1691.
9. Cárcamo-Orive I, Gaztelumendi A, Delgado J, et al. Regulation of human bone marrow stromal cell proliferation and differentiation capacity by glucocorticoid receptor and AP-1 crosstalk. *J Bone Miner Res* 2010; 25: 2115–2125.
10. Li H, Qian W, Weng X, et al. Glucocorticoid receptor and sequential P53 activation by dexamethasone mediates apoptosis and cell cycle arrest of osteoblastic MC3T3-E1 cells. *PLoS One* 2012; 7: e37030.
11. Laxman N, Rubin CJ, Mallmin H, et al. Second generation sequencing of microRNA in human bone cells treated with parathyroid hormone or dexamethasone. *Bone* 2016; 84: 181–188.
12. Zhou S. TGF-β regulates β-catenin signaling and osteoblast differentiation in human mesenchymal stem cells. *J Cell Biochem* 2011; 112: 1651–1660.
13. Zhang Y, Xie RL, Croce CM, et al. A program of microRNAs controls osteogenic lineage progression by targeting transcription factor Runx2. *Proc. Natl. Acad. Sci. U.S.A* 2011; 108: 9863–9868.
14. Haxaire C, Haÿ E and Geoffroy V. Runx2 controls bone resorption through the down-regulation of the Wnt pathway in osteoblasts. *Am J Pathol* 2016; 186: 1598–1609.
15. Kook SH, Heo JS and Lee JC. Crucial roles of canonical Runx2-dependent pathway on Wnt1-induced osteoblastic differentiation of human periodontal ligament fibroblasts. *Mol Cell Biochem* 2015; 402: 213–223.
16. Hecht J, Seitz V, Urban M, et al. Detection of novel skeletogenesis target genes by comprehensive analysis of a Runx2(−/−) mouse model. *Gene Expr Patterns* 2007; 7: 102–112.
17. Otto F, Kanegane H and Mundlos S. Mutations in the RUNX2 gene in patients with cleidocranial dysplasia. *Human mutat* 2002; 19: 209–216.
18. Day TF, Guo X, Garrett-Beal L, et al. Wnt/β-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. *Dev cell* 2005; 8: 739–750.
19. Laxman N, Rubin CJ, Mallmin H, et al. Second generation sequencing of microRNA in human bone cells treated with parathyroid

hormone or dexamethasone. *Bone* 2016; 84: 181–188.
20. t' Hoen PA, Friedländer MR, Almlöf J, et al. Reproducibility of high-throughput mRNA and small RNA sequencing across laboratories. *Nat Biotechnol* 2013; 31: 1015–1022.
21. Schuster SC. Next-generation sequencing transforms today's biology. *Nat Methods* 2008; 5: 16–18.
22. Park JB. The effects of dexamethasone, ascorbic acid, and beta-glycerophosphate on osteoblastic differentiation by regulating estrogen receptor and osteopontin expression. *J Surg Res* 2012; 173: 99–104.
23. Park JB. Effects of the combination of dexamethasone and fibroblast growth factor2 on differentiation of osteoprecursor cells. *Mol Med Rep* 2014; 9: 659–662.
24. Gentleman RC, Carey VJ, Bates DM, et al. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* 2004; 5: R80.
25. Jin SH, Kweon H, Park JB, et al. The effects of tetracycline-loaded silk fibroin membrane on proliferation and osteogenic potential of mesenchymal stem cells. *J Surg Res* 2014; 192: e1–e9.
26. Park JB, Kim YS, Lee G, et al. The effect of surface treatment of titanium with sand-blasting/acid-etching or hydroxyapatite-coating and application of bone morphogenetic protein-2 on attachment, proliferation, and differentiation of stem cells derived from buccal fat pad. *Tissue Eng Regen Med* 2013; 10: 115–121.
27. Tomar GB, Srivastava RK, Gupta N, et al. Human gingiva-derived mesenchymal stem cells are superior to bone marrow-derived mesenchymal stem cells for cell therapy in regenerative medicine. *Biochem Biophys Res Commun* 2010; 393: 377–383.
28. Snall J, Apajalahti S, Suominen AL, et al. Influence of perioperative dexamethasone on delayed union in mandibular fractures: a clinical and radiological study. *Med Oral Patol Oral Cir Bucal* 2015; 20: e621–e626.
29. Wong MM, Rao LG, Ly H, et al. Long-term effects of physiologic concentrations of dexamethasone on human bone-derived cells. *J Bone Miner Res* 1990; 5: 803–813.
30. Buermans HPJ and den Dunnen JT. Next generation sequencing technology: advances and applications. *BBA Mol Basis Dis* 2014; 1842: 1932–1941.
31. Janitz M. *Next-generation genome sequencing: towards personalized medicine*. Weinheim, Germany: John Wiley & Sons, 2011.
32. Levy SE and Myers RM. Advancements in next-generation sequencing. *Annu Rev Genomics Hum Genet* 2016; 17: 95–115.
33. Mutz KO, Heilkenbrinker A, Lönne M, et al. Transcriptome analysis using next-generation sequencing. *Curr Opin Biotechnol* 2013; 24: 22–30.
34. Zhou W, Calciano MA, Jordan H, et al. High resolution analysis of the human transcriptome: detection of extensive alternative splicing independent of transcriptional activity. *BMC Genet* 2009; 10: 63.
35. Wang Z, Gerstein M and Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* 2009; 10: 57–63.
36. Tarazona S, Garcia-Alcalde F, Dopazo J, et al. Differential expression in RNA-seq: a matter of depth. *Genome Res* 2011; 21: 2213–2223.
37. Regard JB, Zhong Z, Williams BO, et al. Wnt signaling in bone development and disease: making stronger bone with Wnts. *Cold Spring Harb Perspect Biol* 2012; 4: a007997.
38. Baron R and Kneissel M. WNT signaling in bone homeostasis and disease: from human mutations to treatments. *Nat Med* 2013; 19: 179–192.
39. Wu H, Whitfield TW, Gordon JA, et al. Genomic occupancy of Runx2 with global expression profiling identifies a novel dimension to control of osteoblastogenesis. *Genome Biol* 2014; 15: 1.
40. Hill TP, Später D, Taketo MM, et al. Canonical Wnt/β-catenin signaling prevents osteoblasts from differentiating into chondrocytes. *Dev Cell* 2005; 8: 727–738.
41. Hayashi K, Yamaguchi T, Yano S, et al. BMP/Wnt antagonists are upregulated by dexamethasone in osteoblasts and reversed by alendronate and PTH: potential therapeutic targets for glucocorticoid-induced osteoporosis. *Biochem Biophys Res Commun* 2009; 379: 261–266.