Original Article

Long noncoding RNA expression profile of mouse cementoblasts under compressive force

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ABSTRACT

Objectives: To investigate the long noncoding RNA (lncRNA) expression profile of cementoblasts under compressive force.

Materials and Methods: Mouse cementoblasts were exposed to compression (1.5 g/cm^2) for 8 hours. RNA sequencing (RNA-seq) was performed to compare the transcriptomes of the compressed and control cells. Quantitative real-time polymerase chain reaction **(**qRT-PCR) was used to validate five of the differentially expressed lncRNAs of interest. Gene Ontology (GO) functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were also performed.

Results: A total of 70 lncRNAs and 521 mRNAs were differentially regulated in cementoblasts subjected to compressive loading. Among the differentially expressed lncRNAs, 57 were upregulated and 13 downregulated. The expression levels of the five selected lncRNAs (Prkcz2, Hklos, Trp53cor1, Gdap10, and Ak312-ps) were validated by qRT-PCR and consistent with the RNA-seq results. GO functional annotation demonstrated upregulation of genes associated with cellular response to hypoxia and apoptotic processes during compressive loading. KEGG analysis identified the crucial pathways involving the hypoxia-inducing factor-1α, forkhead box O, and mammalian target of rapamycin signaling pathways.

Conclusions: Mechanical compression changes the lncRNA expression profile of cementoblasts, providing important references for further investigation into the role and regulation of lncRNAs in compressed cementoblasts and root resorption during orthodontic treatment. (*Angle Orthod.* 2019;89:455–463.)

KEY WORDS: Long noncoding RNA; Cementoblasts; Compressive stress; RNA-seq; Root resorption

INTRODUCTION

External apical root resorption is an unavoidable side effect of orthodontic treatment. This undesirable effect can be attributed to cementum remodeling during treatment. Cementum is a thin layer of avascular mineralized tissue generated by cementoblasts. Because of its slow mineral-remodeling pattern, cementum is regarded as a protective barrier against mechanical stimuli during treatment.[1] Cementoblasts repair the resorption lacunae by secreting acellular and cellular cementum.[1] Irreversible severe root resorption can occur due to the apoptosis of cementoblasts and decreased cementogenesis on the pressure side of the tooth root.[2] Thus, exploring the performance of cementoblasts under compressive stress may provide

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novel strategies for reducing the incidence of orthodontically induced root resorption.

Previous studies have demonstrated the mechanosensitivity of cementoblasts.[3] Under mechanical stress, cementoblastic activity is changed, and various molecules are involved such as bone sialoprotein (BSP), alkaline phosphatase (ALP), osteoprotegerin (OPG), and receptor activator for nuclear factor κB ligand (RANKL). However, the exact mechanism underlying the mechanical transduction process remains unclear. A study found that mechanical compression leads to the reduction of BSP expression.[3] However, another study claimed that compression alone upregulates BSP level, while compression accompanied by interleukin-1β reduces BSP expression.[4] Similarly, ALP, OPG, and RANKL are expressed inconsistently in cementoblasts under mechanical force.[5–8] The process of transduction of mechanical compression in cementoblasts may be affected epigenetically, but this has rarely been reported.

In the transcribed mammalian genome, only 2% to 3% of RNAs are messenger RNAs (mRNAs), and noncoding RNAs compose the rest. Most noncoding RNAs are >200 nucleotides, and these are defined as long noncoding RNAs (lncRNAs).[9] LncRNAs regulate a variety of biological responses via different mechanisms.[9] Recent studies have shown that lncRNAs respond to mechanical stress in health and disease. The lncRNA expression profiles in mechanically stretched human aortic smooth muscle cells have been identified,[10] and a mechanoresponsive lncRNA (XR007793) modulates cell proliferation and migration and participates in vascular remodeling during hypertension.[11] The lncRNA H19 mediates the osteogenesis induced by mechanical tension in bone marrow mesenchymal stem cells.[12] However, the role and function of lncRNAs in cementoblasts under compressive stress remain unclear. Understanding what lncRNAs act in compressed cementoblasts may provide essential information for understanding external apical root resorption during orthodontic treatment.

The present study was designed to identify the differentially expressed lncRNAs and mRNAs in compressed cementoblasts and thus provide clues for possible mechanisms underlying orthodontically induced root resorption.

MATERIALS AND METHODS

Cell Culture and Pressure Application

Immortalized mouse cementoblast-like cells (OCCM-30) were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, Calif). Mechanical stress was applied as described previously.[13] In brief, a glass cover with additional metal weights on top was placed over an 80% confluent cell layer in a dish (Figure 1A). The compressive force was adjusted to 1.5 g/cm^2 by adding lead granules and maintained for 8 hours.

Cell Proliferation Assay

Cell proliferation was measured with a Cell Counting Kit (CCK-8, Dojindo, Kumamoto, Japan). Briefly, after exposure to compressive stress for 8 hours, the cells were seeded into each well of a 96-well plate. Cell viability was analyzed at 0, 12, 24, and 48 hours. CCK-8 (10 μL) was added to each well and incubated at 37°C for 3 hours. Absorbance values at 450 nm were measured using a microplate spectrophotometer (Bio-Tek Instruments Inc., Winosski, Vt).

DAPI Staining

Cells grown on glass coverslips were subjected to compressive force for 8 hours. The cells were washed, fixed in 4% paraformaldehyde, and stained with DAPI. Images were captured with a confocal imaging system (Carl Zeiss, Jena, Germany).

Caspase-3/7 Activity

The activity of caspase-3/7 was determined using a colorimetric assay kit (Caspase-Glo 3/7 Assay Systems, Promega, Madison, Wis). Briefly, after exposure to compressive force, cells were lysed and incubated with a luminogenic substrate, which is cleaved by activated caspase-3/7 in apoptotic cells. After incubation for 1 hour, luminescence was quantified using a luminometer (Berthold, Bad Wildbad, Germany).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's procedure. A total of 1 μg RNA was reverse transcribed using a cDNA Reverse Transcription Kit (Takara, Tokyo, Japan). qRT-PCR was performed using SYBR Green Master Mix on the ABI Prism 7500 real-time PCR system (Applied Biosystems, Foster City, Calif). All expression data were normalized to the mean of glyceraldehyde-3-phosphate dehydrogenase. The data were analyzed using the $2^{-\Delta\Delta Ct}$ relative expression method as described previously.[14]

ALP Assay

After the mechanical pressure, ALP staining was performed using the NBT/BCIP staining kit (CoWin

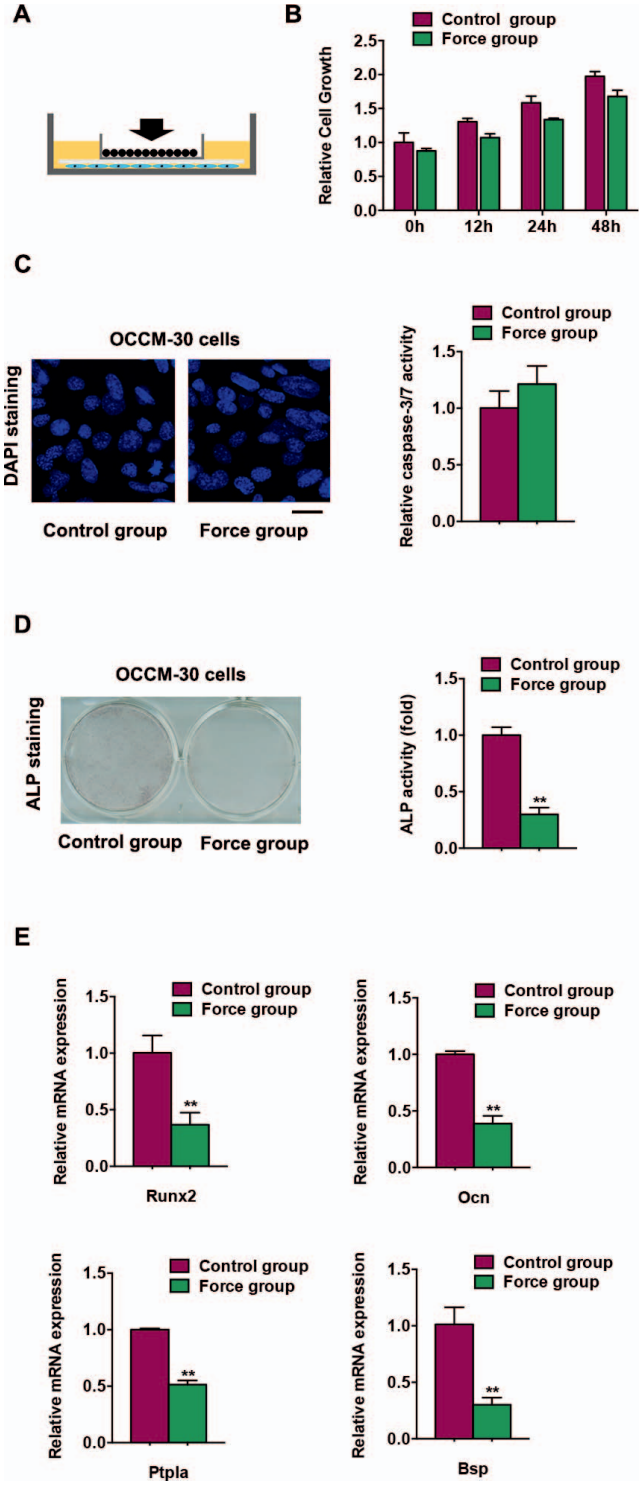

Figure 1. Cell viability and cementoblast differentiation of OCCM-30 under mechanical pressure. (A) Diagram showing the application of compressive force. (B) Relative cell growth with or without compression. (C) DAPI staining (left) and relative capase-3/7 activity (right) of OCCM-30 with or without compression. Scale bar, 50 μm. (D) ALP staining images (left) and ALP activity (right) of these cells. (E) Relative mRNA expression of Runx2, Ocn, Ptpla, and Bsp in these cells. Results are presented as mean ± SD (**$P < .01$).

Biotech, Beijing, China). The cells were fixed in 4% paraformaldehyde and stained with ALP substrate. ALP activity was analyzed using a colorimetric assay kit (Biovision, Milpitas, Calif) according to the manufacturer's protocol. The activity was normalized to the protein content as determined by a BCA protein assay reagent kit (Thermo Fisher Scientific, Waltham, Mass).

cDNA Library Construction and High-Throughput Sequencing

A total of 4 μg RNA was extracted and treated with DNase (Qiagen, Hilden, Germany) as described previously.[14] A Ribo-Zero Magnetic Kit (Illumina, San Diego, Calif) was used to deplete the ribosomal RNA. An RNA library was prepared, and paired-end sequencing was performed on a Hiseq 2000 system (Illumina). Whole transcriptome sequencing data were filtered using domestic java code and then mapped to the mouse genome using HISAT2.

Bioinformatics Analysis

Differential gene expression was analyzed using the EBSeq package in the Bioconductor R program as previously described.[14] The false discovery rate (FDR) was applied using the Benjamini-Hochberg algorithm[15] based on the *P* value. The differentially expressed genes were defined under the following criteria: (1) fold change >1.5 or <0.6667 and (2) FDR <0.05. The Database for Annotation, Visualization and Integrated Discovery was used to analyze the potential functions of mRNAs. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were performed.

Statistics

The quantitative data are presented as mean ± SD for each group. Student's *t*-test was used to determine the differences between groups using SPSS 16.0 software (IBM, Armonk, NY). A *P* value $<.05$ was considered statistically significant.

RESULTS

Cell Growth and Cementoblast Differentiation Under Compressive Force

There were no significant differences in growth between the force group and the control group, although a trend toward a decrease was noted in the loaded group (three samples, three technical replicates per sample; Figure 1B). DAPI staining and caspase-3/7 activity assay showed that apoptosis in the force group did not change significantly (three samples, three technical replicates per sample; Figure 1C).

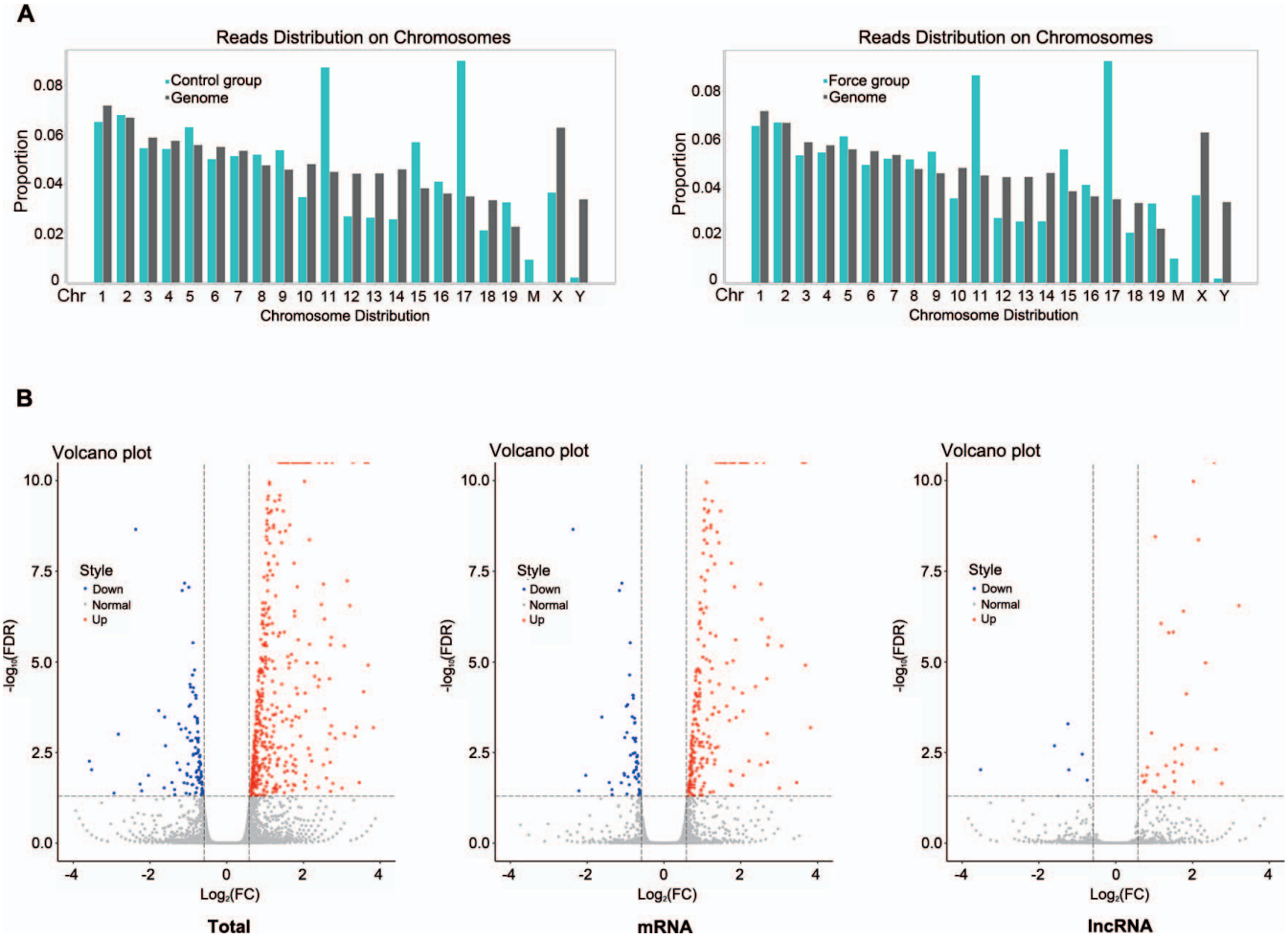

Figure 2. The differentially expressed lncRNAs and mRNAs under compressive stress. (A) Chromosomal read distributions of control cells (left) and compression loaded cells (right). (B) Volcano plot of differentially expressed transcripts (lncRNAs and mRNAs) in the control and force group. Red points: upregulated mRNAs or lncRNAs; blue points: downregulated mRNAs or lncRNAs.

The influence of compression force on cementoblast differentiation was also evaluated. ALP staining demonstrated a significant decrease in the staining area in the force group (three samples, three technical replicates per sample; Figure 1D). Consistently, ALP activity was also significantly decreased after compressive stress. The expression of the cementoblast-related genes, runt-related transcription factor 2 (Runx2), osteocalcin (Ocn), protein tyrosine phosphatase-like member A (Ptpla), and Bsp, were significantly downregulated in the force group (three samples, three technical replicates per sample; Figure 1E).

Overview of RNA Sequencing

The number of raw reads generated from either sample was >130 million, 136,560,044 for the force group (one sample) and 138,098,342 for the control group (one sample). After filtering the low-quality reads, a total of 237,111,920 cleaned reads (86.3%) were obtained. More than 91.2% of clean reads were perfectly mapped to the reference genome. The uniquely mapped reads comprised 95.4% of the total mapped reads in the force group and 95.5% of those in the control group. After a stringent screening procedure, 3714 lncRNAs and 13,013 mRNAs were identified from the two samples. Among these RNAs, 1137 lncRNAs and 205 mRNAs were uncharacterized. Both lncRNAs and mRNAs were distributed across chromosomes 1–19, M, X, and Y (Figure 2A).

Differential Expression of lncRNAs and mRNAs Induced by Compressive Force

According to the criteria, a total of 70 lncRNAs and 521 mRNAs were expressed differentially, including 27 lncRNAs and 9 mRNAs that were uncharacterized. Among these, 57 upregulated and 13 downregulated lncRNAs as well as 427 upregulated and 94 downreg-

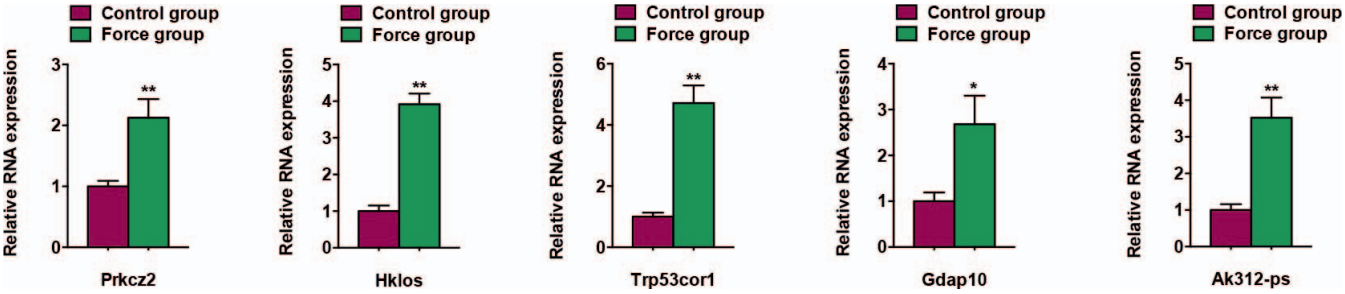

Figure 3. Differentially expressed lncRNAs (Prkcz2, Hklos, Trp53cor1, Gdap10, and Ak312-ps) validated by qRT-PCR. Results are presented as mean ± SD (*$P < .05$; **$P < .01$).

ulated mRNAs were identified in the force group compared with the control group (Figure 2B).

Differentially Expressed lncRNAs Validated by qRT-PCR

Considering the results of the bioinformatics analysis, five lncRNAs (Prkcz2, Hklos, Trp53cor1, Gdap 10, and Ak312-ps) were selected for validation. The results showed that all five lncRNAs were significantly increased under mechanical pressure compared with the control group, consistent with the results of RNA-seq (five samples, three technical replicates per sample; Figure 3).

Several Important Pathways Represented by Differentially Expressed Genes

GO term analyses showed that, when subjected to compressive force, OCCM-30 cells showed notable upregulation of genes associated with cellular response to hypoxia, apoptotic process, DNA-templated transcription, regulation of cell cycle, and downregulation associated with regulation of the G1/S transition of the mitotic cell cycle (Figure 4A).

KEGG pathway analyses showed that notable pathway enrichment of the differentially expressed genes occurred in the force group (Figure 4B). Among the top 20 overrepresented KEGG pathways, five were associated with signal transduction: hypoxia-inducing factor-1 (HIF-1), forkhead box O (FoxO), mammalian target of rapamycin (mTOR), Notch, and Ras-proximate-1 (Rap1) signaling pathways. The differentially expressed genes in three top 5 signaling pathways (the HIF-1, FoxO, and mTOR signaling pathways) under compressive stress are shown in Figure 5.

DISCUSSION

Cementoblasts play an essential role in cementum repair.[9] To better understand the response of cementoblasts to compression, the differentially expressed lncRNAs and mRNAs in compressed cementoblasts were studied.

The mechanical loading approach has been widely used. The compressive forces applied ranged from 0.25 g/cm^2 to 3 g/cm^2 in previous studies.[5,13] Since root resorption occurs in orthodontic treatment with light force as well, the response of cementoblasts under appropriate compressive force instead of heavy force was investigated. To avoid pathological damage of cementoblasts, the force magnitude was set at 1.5 g/cm^2 for 8 hours, and the cell viability and apoptosis assays confirmed the absence of significant increased apoptosis. Cementogenesis was inhibited under compression as indicated by ALP staining and cementoblast-related gene expression, consistent with previous studies.[3,5] External root resorption results from abnormal cementum remodeling. Thus, compression decreases cementoblastic function and subsequently induces root resorption.

A total of 70 differentially expressed lncRNAs were detected in this study, suggesting that they contribute to the mechanosensitivity of cementoblasts. However, their functional roles are poorly elaborated, especially in cementoblasts. Among them, 27 lncRNAs were uncharacterized, and only a small percentage have been studied. Five lncRNAs that possessed significant fold change were chosen for qRT-PCR validation. Trp53cor1, a transcriptional target of p53, was increased significantly in the compressive condition. It has been reported to promote p53[16] and HIF-1α[17] activity. These two pathways were also upregulated under compression. In addition, Trp53cor1 inhibits the Wnt pathway via sponging miR-17-5p,[18] and reduction of endogenous Wnt signaling results in idiopathic root resorption in mice.[19] Thus, Trp53cor1 may be involved in the biological function of compressed cementoblasts via p53, HIF-1α, and Wnt pathways. However, further studies, including in vitro and in vivo experiments, are needed to investigate the specific functions and mechanisms of these lncRNAs. A previous study used a Cre-LoxP system to conditionally delete Wntless and found that the genetic model of reduced Wnt signaling displays thinner cementum and extensive spontaneous root resorption.[19] Further study using transgenic

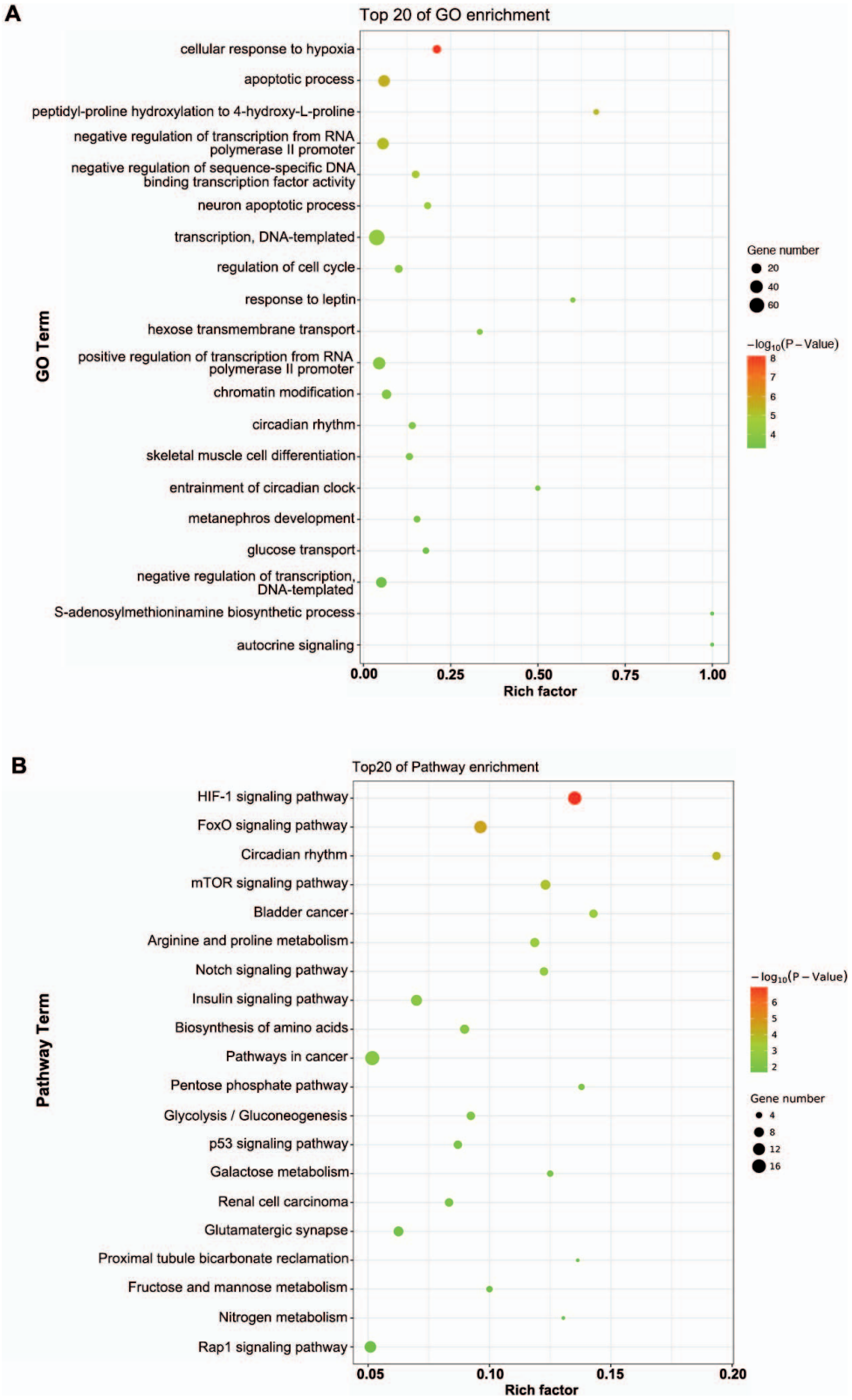

Figure 4. The top 20 enrichment in the GO terms (A) and the KEGG pathway analysis (B) of differentially expressed genes.

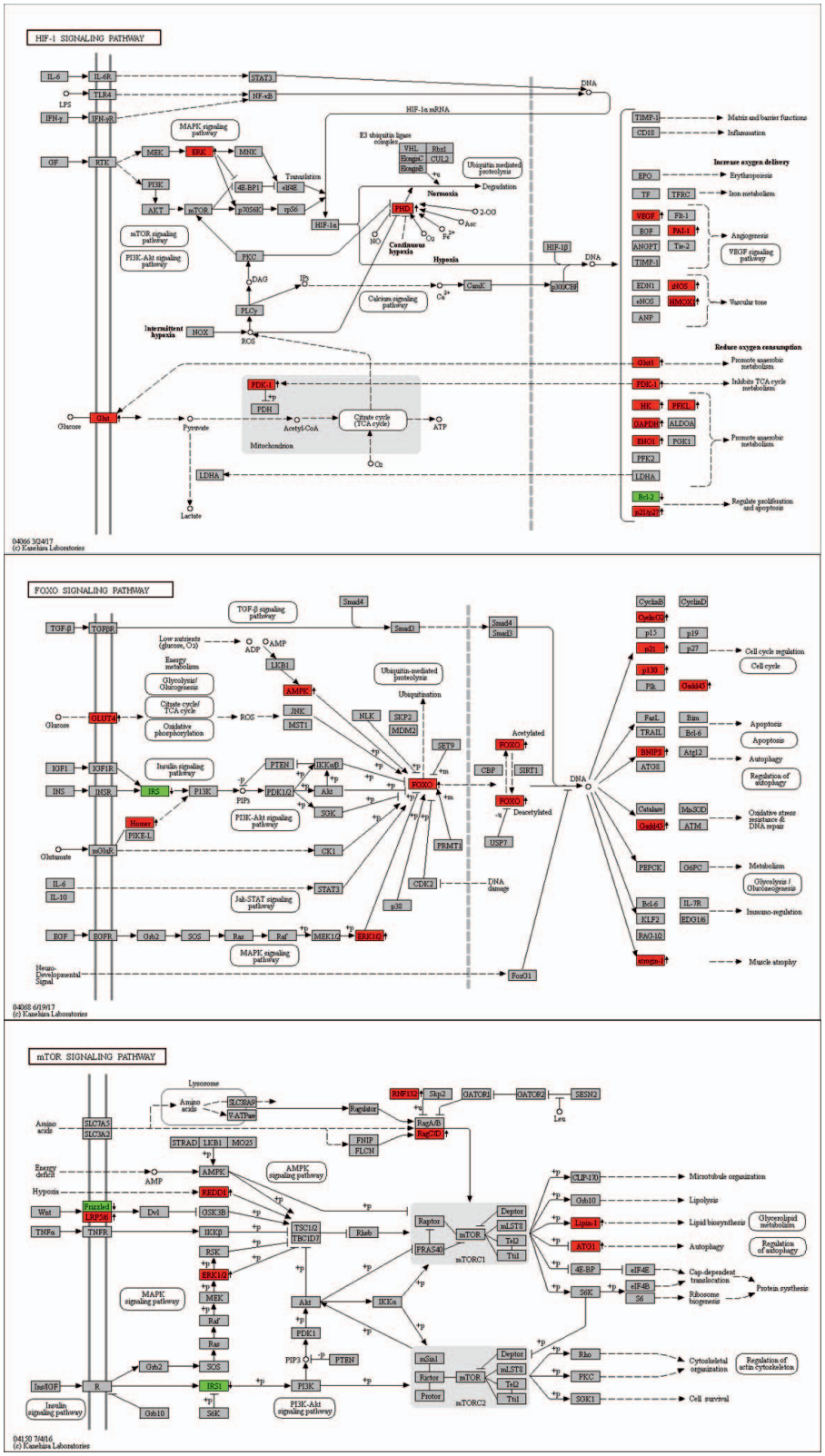

Figure 5. KEGG maps of differentially expressed genes in HIF-1, FoxO, and mTOR signaling pathways. Red boxes: upregulated genes; green boxes: downregulated genes.

models would provide more evidence of the role of lncRNAs in the biological process during root resorption or cementum repair.

Functional analyses showed that the upregulated expression in compressed cementoblasts mostly involved cell response to hypoxia and apoptotic processes, both of which occur in response to orthodontic tooth movement. The loading approach in vitro induces hypoxia, especially in the central area of the glasses. This is the inherent limitation of this method. To reduce the influence of hypoxia, light force was used. However, in vivo, mechanical compression decreases vascular activity on the pressure side of the periodontal ligament and results in local hypoxia in the compressed area during orthodontic treatment.[20] Hypoxia increases HIF-1α activity and decreases cementoblastic function, which then may result in root cementum resorption.[21] In addition, even though light force was used and the absence of pathological change of cementoblasts was confirmed, the apoptotic process was still highly enriched. Mechanical compression induces apoptosis of cementoblasts and decreased cementogenesis,[2] resulting in failed root resorption repair.

The FoxO signaling pathway was also affected under compressive loading. It regulates cellular physiological events including apoptosis, cell-cycle control, glucose metabolism, and oxidative stress resistance. FoxO1 is also involved in bone mineralization via Wnt/β-catenin signaling.[22] The mTOR signaling pathway ranked fourth in pathway enrichment. mTOR is viewed as a central regulator that adapts metabolism to changing environments.[23] It participates in the regulation of cell proliferation and apoptosis.[23] In addition, this pathway is associated with other pathways, such as the HIF-1α and p53 signaling pathways,[23] listed in the top 20 of KEGG analysis in the current study. These pathways respond to compressive stress in cementoblasts and may participate in root resorption and repair. Further study regarding the association between lncRNAs and these enriched biological processes would help elucidate the function and mechanism of lncRNAs under compression.

CONCLUSIONS

- Fifty-seven upregulated and 13 downregulated lncRNAs as well as 427 upregulated and 94 downregulated mRNAs were identified in cementoblasts under compressive force.
- Via GO and KEGG analyses, biological processes involving cellular response to hypoxia and apoptosis, as well as related signaling pathways, were identified. The lncRNA Trp53cor1 may play a role in the response to compression.

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