ORIGINAL ARTICLE

# Possible Involvement of X-Box Binding Protein-1 in the Onset of Pulpitis

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## ABSTRACT

**Objective:** Endoplasmic reticulum (ER) stress plays important roles not only in stress avoidance, but also in cell differentiation and maturation, cell proliferation, and promotion of bone formation. This study aimed to investigate the involvement of ER stress in the onset of pulpitis.

**Methods:** Immunohistochemical analysis was conducted on human teeth extracted for orthodontic reasons. The effects of tunicamycin (TM), an inducer of ER stress, lipopolysaccharide (LPS), and 4µ8c, an inhibitor of inositol-requiring enzyme 1 (IRE1) on cultured human dental pulp cells (hDPCs) were also examined.

**Results:** The expressions of two ER stress markers, X-box binding protein (XBP)-1 and binding immunoglobulin protein (BiP)/78 kDa glucose-regulated protein (GRP78), were found in the human pulp tissues of a decayed tooth that had not developed irreversible acute pulpitis, but not in an impacted tooth without inflammation in pulp tissue. Both TM and LPS increased the mRNA levels of XBP-1, interleukin (IL)-6, and IL-8, whereas TM, but not LPS, enhanced the mRNA expression of BiP/GRP78 in hDPCs. 4µ8c significantly suppressed the increased level of XBP-1 by LPS.

**Conclusion:** This study demonstrated that XBP-1, in addition to inflammatory cytokines, may participate in the onset of pulpitis through IRE1. These findings provide a more comprehensive understanding of pulpitis pathogenesis through the cooperation of ER stress and inflammatory cytokines.

**Keywords:** ER stress, IRE1, pulpitis, XBP1

**HIGHLIGHTS**

- ER stress may be involved in the onset of pulpitis.
- The present findings provide a more comprehensive understanding of pulpitis pathogenesis through the cooperation of ER stress and inflammatory cytokines.
- This study may be useful to identify new therapeutic targets for pulpitis.

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## INTRODUCTION

Endoplasmic reticulum (ER) stress is induced by hypoxia, poor nutritional status, aging, and viral infection, and its response has been considered a stress defense system that eliminates abnormal proteins accumulated in the endoplasmic reticulum and protects against cell injury (1–4). The state of the ER lumen is monitored by a single transmembrane protein called the stress sensor, which resides on the ER membrane. Stress sensors include three ubiquitously expressed sensor proteins (inositol-requiring enzyme 1 (IRE1), PRK-like ER kinase (PERK), and activation transcription factor 6 (ATF6), called classical stress

sensors, and the old astrocyte specifically induced substance (OASIS) family of tissue-specific sensor proteins (2). In normal conditions, the classical stress sensor remains inactive by binding of the binding immunoglobulin protein (BiP)/78 kDa glucose-regulated protein (GRP78), an ER molecular chaperone, to the luminal domain of the ER. BiP/GRP78 has a greater binding affinity for unfolded proteins than for sensor proteins. Therefore, when defective proteins accumulate in the ER lumen due to ER stress, BiP/GRP78 dissociates from the sensor proteins, binds to the defective proteins, and mediates the refolding of the defective proteins. In contrast, the sensor protein from which BiP/GRP78 is dissociated is activated and sends a signal to trigger the unfolded protein response (UPR) (5, 6).

Recently, signals from ER stress sensors have been shown to play an important role not only in stress avoidance, but also in cell differentiation and maturation, cell proliferation, and promotion of bone formation (7–9). Previous studies have shown that sustained activation of ER stress sensors induces chronic inflammation and is involved in the development and progression of diabetes mellitus, neurodegenerative diseases, and cancer (10–12).

IRE1 has a kinase domain and an RNase domain in the cytoplasmic side region as functional domains. When BiP/GRP78 dissociates under ER stress, it dimerizes or oligomerizes and is activated by autophosphorylation. Activated IRE1 splices the mRNA of transcription factor XBP-1 with its RNase domain and excises 26 bp of RNA. The protein translated by the spliced X-box binding protein (XBP)-1 mRNA acts as the functional transcription factor XBP-1 and induces transcription of target molecules required for UPR (13–15). The kinase domain of activated IRE1 phosphorylates apoptosis signal-regulating kinase (ASK) 1 and IκB kinase (IKK), thereby regulating the expression of osteogenic genes, including Osterix, and inducing the transcription of molecules involved in inflammatory response (16). As described above, the IRE1-XBP-l pathway has been reportedly involved in various cellular functions, but the details are still unknown.

Pulpitis is an inflammation of pulp tissue caused by persistent biological (bacterial infection), physical (trauma, grinding, etc.), or chemical (dental materials, etc.) factors. In pulpitis, pulp cells and immunocompetent cells such as macrophages, neutrophils, and lymphocytes produce inflammatory and anti-inflammatory cytokines, and the degradation of extracellular matrix such as type I collagen is enhanced (17). One of the causes of pulpitis is dental caries. Dental pulp cells in dental caries-affected teeth are expected to be exposed to cellular stress, but the details of this exposure have not been clarified.

Cellular stresses include ER stress and oxidative stress (18), and it has been shown that oxidative stress reduces the calcification ability of pulp cells (19). In dental pulp tissue, the ER stress sensors ATF6 and PERK may participate in the differentiation of dental pulp cells into odontoblasts (20, 21). The relationship between pulpitis and ER stress remains unknown.

In this study, the hypothesis was that ER stress is involved in the onset of pulpitis, and the aim was to investigate the ER stress response in dental pulp cells to elucidate the pathogenesis of pulpitis.

## MATERIALS AND METHODS

### Reagents

Tunicamycin (TM), an inducer of ER stress, lipopolysaccharide (LPS) from Escherichia coli (#L2630), and 4μ8c, an inhibitor of IRE1, were purchased from FUJIFILM Wako Pure Chemicals Corporation (Osaka, Japan), SIGMA (St. Louis, MO, USA), and Selleck Biotech (Kanagawa, Japan), respectively. Human dental pulp cells (hDPCs) were obtained from Lonza (Basel, Switzerland).

### Human Teeth

Six human teeth (from 6 patients) extracted for orthodontic reasons were used in this study. Three of the extracted teeth had dental caries, whereas the other three did not. The teeth with dental caries did not show clinically irreversible acute pulpitis. The teeth were obtained with informed consent at Hiroshima University Hospital (Ethical approval number: E-2741).

### Tissue Preparation

The acquired teeth were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer at pH 7.2 and stored until the beginning of the experiment. Tissue preparation was performed according to the previous report (22). Briefly, after decalcification with EDT-X® (FALMA Co., Ltd., Tokyo, Japan) for 4 weeks, the tissues were embedded in paraffin. Serial sections were sliced throughout the mesial-distal plane of the teeth with a thickness of 5 μm. Sections for hematoxylin and eosin (HE) staining and immunochemical analysis were stored at room temperature and -20°C, respectively.

### Immunohistochemical Procedures

Immunohistochemical analysis was performed as described previously (22). Rabbit polyclonal anti-XBP1 (1:100; Nobus Biologicals, Centennial, CO, USA) and rabbit polyclonal anti-BiP/GRP78 (1:100; Proteintech, Rosemont, IL, USA) were used as primary antibodies. Goat anti-rabbit IgG-HRP (1:200; R&D systems, Minneapolis, MN, USA) was used as a secondary antibody. As controls, some sections were incubated without a primary antibody (data not shown).

### Cell Culture

hDPCs from passages 6 to 7 were grown in a growth medium (GM), which was Dulbecco's modified eagle medium (DMEM) supplemented with 10% heat-inactivated FBS (Fetal Bovine Serum; Thermo Fisher Scientific, Waltham, MA, USA), 100 IU/mL of penicillin, and 100 μg/mL of streptomycin. Penicillin and streptomycin were purchased from FUJIFILM Wako Pure Chemicals Corp. This growth medium is referred to as medium A. The cells were incubated in a humidified atmosphere of 5% $CO_2$ and 95% air at 37°C.

### WST Assay

Cell viability was evaluated using the WST assay with the Cell Counting Kit-8, following the manufacturer's instructions (CCK-8; Dojindo Laboratories, Kumamoto, Japan). To perform the test, the hDPCs were seeded in each well of a 96-well plate ($5.0\times10^3$ cells per well) and cultured in 0.1 mL of medium A. After 24 hours of incubation, the culture medium was replaced with serum-free medium A (medium B) containing TM at concentrations ranging from 0.005 to 5 μM (final concentration).

**TABLE 1.** Primers for real-time PCR

| Primer | Sequence (5′-3′) | Product length (bp) | Tm (°C) | GenBank code |
|---|---|---|---|---|
| GAPDH | | | | |
| Forward | AACGTGTCAGTGGTGGACCTG | 160 | 62 | NM_002046 |
| Reverse | AGTGGGTGTCGCTGTTGAAGT | | | |
| XBP1 | | | | |
| Forward | CTGGAACAGCAAGTGGTAGA | 395 | 62 | NM_005080 |
| Reverse | CTCCCTCCAGGCAGG | | | |
| Bip/GRP78 | | | | |
| Forward | CAACCCCGAGAACACGGTC | 70 | 58 | NM_005347 |
| Reverse | CTGCACAGACGGGTCATTC | | | |
| IL-6 | | | | |
| Forward | ATCTGGATTCAATGAGGAGACT | 108 | 58 | NM_000600 |
| Reverse | TGTTCCTCACTACTCTCAAATCTG | | | |
| IL-8 | | | | |
| Forward | GAATTCTCAGCCCGCGGCAAAAAC | 222 | 60 | NM_000584 |
| Reverse | GCCAAGGAGTGCTAAAGAACTTAG | | | |

PCR: Polymerase chain reaction, Tm: Tunicamycin, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, XBP1: X-box binding protein, GRP78: 78 kDa glucose-regulated protein

The cells were then incubated for another 24 hours. Next, the CCK-8 solution was added to each well, and the cells were incubated for an additional hour. Finally, the measurement of absorbance was carried out using a microplate reader (Multiskan™FC; Thermo Fisher Scientific).

### Isolation of Total RNA and Reverse Transcription

The hDPCs were seeded at a density of 1.0×10$^5$ cells per well in 6-well plates and cultured in medium A until subconfluent. The cells were treated with 0.1 µM TM or 1.0 µg/mL LPS for the indicated times before the end of incubation on day 7. An inhibition assay of IRE1 was performed by pretreating cells for 30 min with 1.0 µM 4µ8c (Selleck Biotech). RNA iso plus (Takara Bio Inc., Shiga, Japan) was used for total RNA extraction from each culture following the manufacturer's protocol. To synthesize cDNA, 500 ng of total RNA was taken and mixed with the Rever Tra Ace qPCR RT Master Mix with gDNA remover kit (Toyobo Co., Ltd, Osaka, Japan). The whole process was carried out in a thermal cycler (Veriti™ 96-Well Thermal Cycler; Life Technologies, Foster City, CA, USA) and the final volume achieved was 10 µL.

### Real-time PCR

THUNDERBIRD® Next SYBR® qPCR Mix (Toyobo Co., Ltd) was used for two-step qPCR on the StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific). The gene expression levels were normalized to the expression levels of GAPDH mRNA in the same samples. In the present experimental system, the gene expression of GAPDH showed generally constant Ct values in the presence or absence of LPS or other stimuli. This normalization process was carried out using the 2-ΔΔCt method. The relevant primers' sequences were listed in Table 1 (23–25).

### Immunoblotting

hDPCs were cultured in 6-well plates at a density of 1×10$^5$ cells/well in 2 mL of medium A. After 7 days of culture, the cells were treated with 1.0 mg/mL LPS for 24 hours with or without 1.0 mM 4µ8c, following the change of medium A to medium B. Immunoblotting was then performed as previously described (26). Primary antibodies were as follows: rabbit polyclonal anti-XBP1 (1.0 mg/mL; Nobus Biologicals, Centennial, CO, USA) or mouse anti-human β-actin antibody (1:1000; Cell Signaling Technology, Danvers, MA, USA. Secondary antibodies were as follows: anti-rabbit IgG horseradish peroxidase-conjugated antibody (1:2000; R&D Systems) or anti-rabbit IgG horseradish peroxidase-conjugated antibody (1:1000; Cell Signaling Technology).

### RNA-seq and Data Analysis

hDPCs were seeded into 6-well plates at a density of 1.0×10$^5$ cells/well and cultured in medium A until subconfluent. The cells were treated with 1.0 µg/mL LPS for 3 hours before the end of incubation on day 7. Total RNA from each culture was extracted using RNA iso plus (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. Construction of a cDNA library from the extracted total RNA were conducted using the TruSeq Stranded mRNA LT Sample Prep kit (Illumina, San Diego, CA, U.S.A.). Paired-end RNA sequencing and data analysis were performed at Macrogen Japan (Tokyo, Japan).

### Statistical Analysis

Each result is presented as the mean±standard error of the mean. Each experiment was conducted at least three times to ensure accuracy and reliability of the results. The results of the Shapiro-Wilk test showed that the data were consistent with a normal distribution. To compare two groups, we used an unpaired two-tailed Student's t-test. For comparisons of more than two groups, one-way ANOVA was followed by Dunnett's test (Fig. 1, Fig. 2a) or Tukey's test (Fig. 2b) for multiple comparisons. Significance was set at 0.05 ($p<0.05$).

## RESULTS

The photographs shown in Figure 3 and Figure 4 are representative of experiments performed on three extracted teeth with dental caries and three extracted teeth without dental caries, respectively. A radiograph showed dental caries on the proximal surface of a human mandibular left third molar (Fig. 3a). HE staining of pulp tissue adjacent to the caries showed mild inflammation, including pulpal hyperemia, but no significant inflammatory cell infiltration (Fig. 3b, c). Immunohistochemical analysis

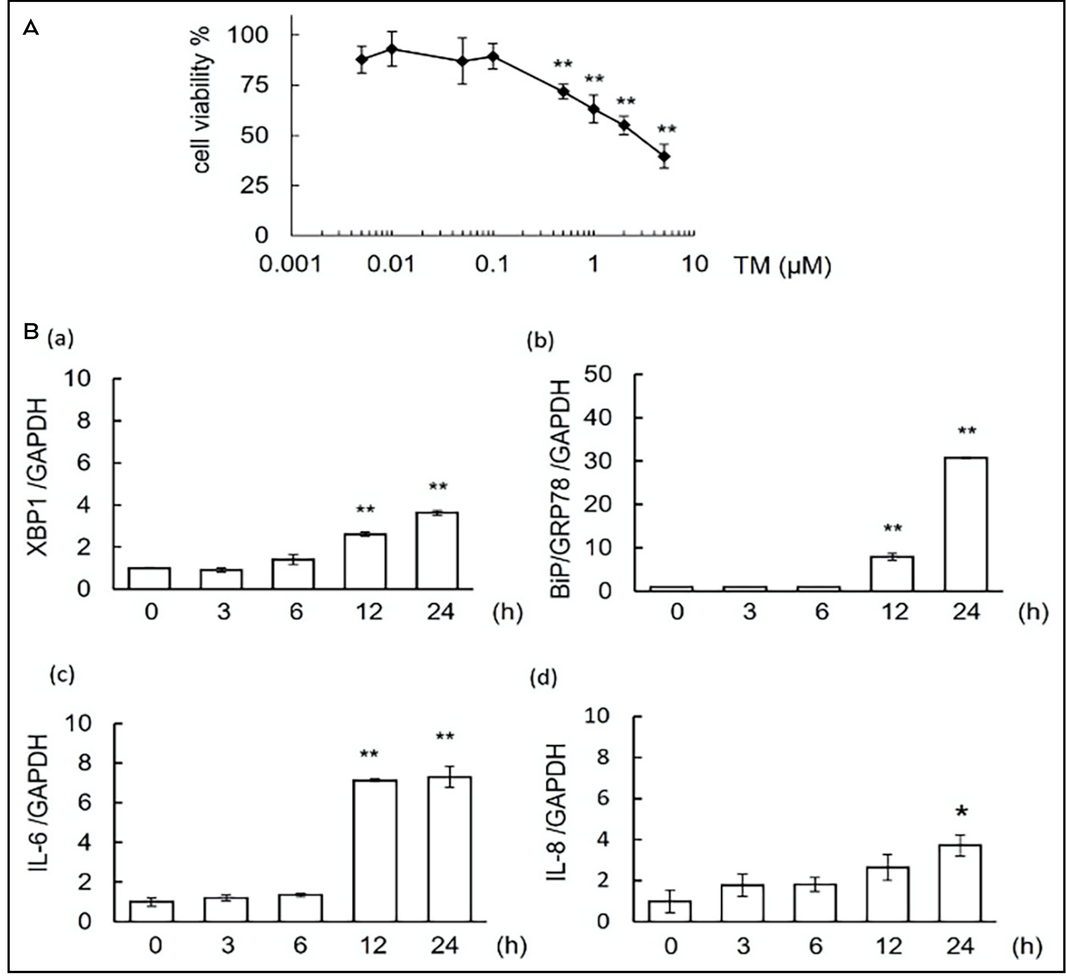


**Figure 1.** Effects of TM in hDPCs. (A) Effects of TM on cell viability of hDPCs. The hDPCs were treated with TM at concentrations of 0.005, 0.01, 0.05, 0.1, 0.5, 1, 2, and 5 μM. Cell viability is assessed by the WST assay using Cell Counting Kit-8. Data are presented as means±SD of three independent experiments. **p<0.01, one-way ANOVA with Tukey's test. (B) Time-course effects of TM on XBP1, BiP/GRP78, IL-6, and IL-8 mRNA expression levels. The hDPCs were exposed to TM (0.1 μM) for the indicated times before the end of incubation on day 7. The mRNA expression levels were determined by real-time PCR. Graphs show the ratio of XBP1(a), BiP/GRP78 (b), IL-6(c) and IL-8(d) mRNA to GAPDH mRNA.

Values represent means±SD of three cultures. *: p<0.05, **: p<0.01: Differs significantly from the control. TM: Tunicamycin, hDPCs: Human dental pulp cells, WST: Water soluble tetrazolium salts, SD: Standard deviation, ANOVA: Analysis of variance, XBP1: X-box binding protein, GRP78: 78 kDa glucose-regulated protein mRNA: Messenger ribonucleic acid

showed that many cells around the dentin surface and the blood vessels were positive for BiP/GRP78 (Fig. 3d, e) and XBP1 (Fig. 3f, g). In the caries-free human mandibular right third molar (Fig. 4a), no inflammatory findings were observed (Fig. 4b). Positive reactions to BiP/GRP78 (Fig. 4c) and XBP1 (Fig. 4d) were not observed around the dentin surface and the blood vessels.

TM at concentrations of 0.5, 1, 2, and 5 μM significantly decreased cell viability in hDPCs (p<0.01) (Fig. 1a). TM at concentrations of 0.005, 0.01, 0.05, and 0.1 μM did not decrease cell viability (Fig. 1a). TM at 0.1 μM had a higher ability to induce mRNA expression of ER stress markers than TM at 0.05 μM (data not shown). Therefore, in the present study, TM at 0.1 μM, which is thought to be enough to induce ER stress, was used.

TM at 0.1 μM increased ER stress marker mRNA levels of XBP1 and BiP/GRP78 and inflammatory cytokine mRNA levels of IL-6 and IL-8 in a time-dependent manner until 24 h in hDPCs (Fig. 1b). TM at 0.1 μM caused a 3.6-fold increase in XBP1 mRNA expression (p<0.01), a 30.7-fold increase in BiP/GRP78 mRNA expression (p<0.01), a 7.3-fold increase in IL-6 mRNA expression (p<0.01), and a 3.7-fold increase in IL-8 mRNA expression (p<0.01).

LPS at 1.0 μg/mL increased the level of XBP1 mRNA in hDPCs. The maximal effect was seen at 12 h (a 4.2-fold increase, p<0.01) (Fig. 2a(a)). However, LPS at 1.0 μg/mL did not affect BiP/GRP78 mRNA expression in hDPCs (Fig. 2a(b)). LPS at 1.0 μg/mL increased the levels of IL-6 and IL-8 mRNAs in hDPCs. The maximal effect was seen at 3 h (p<0.01) (Fig. 2a(c), (d)).

As expected from the above results, LPS at 1.0 μg/mL increased the production of XBP1 in hDPCs (Fig. 2b). The 4μ8c at 1.0 μM significantly abrogated the production of LPS-induced XBP1 (p<0.05) (Fig. 2b).

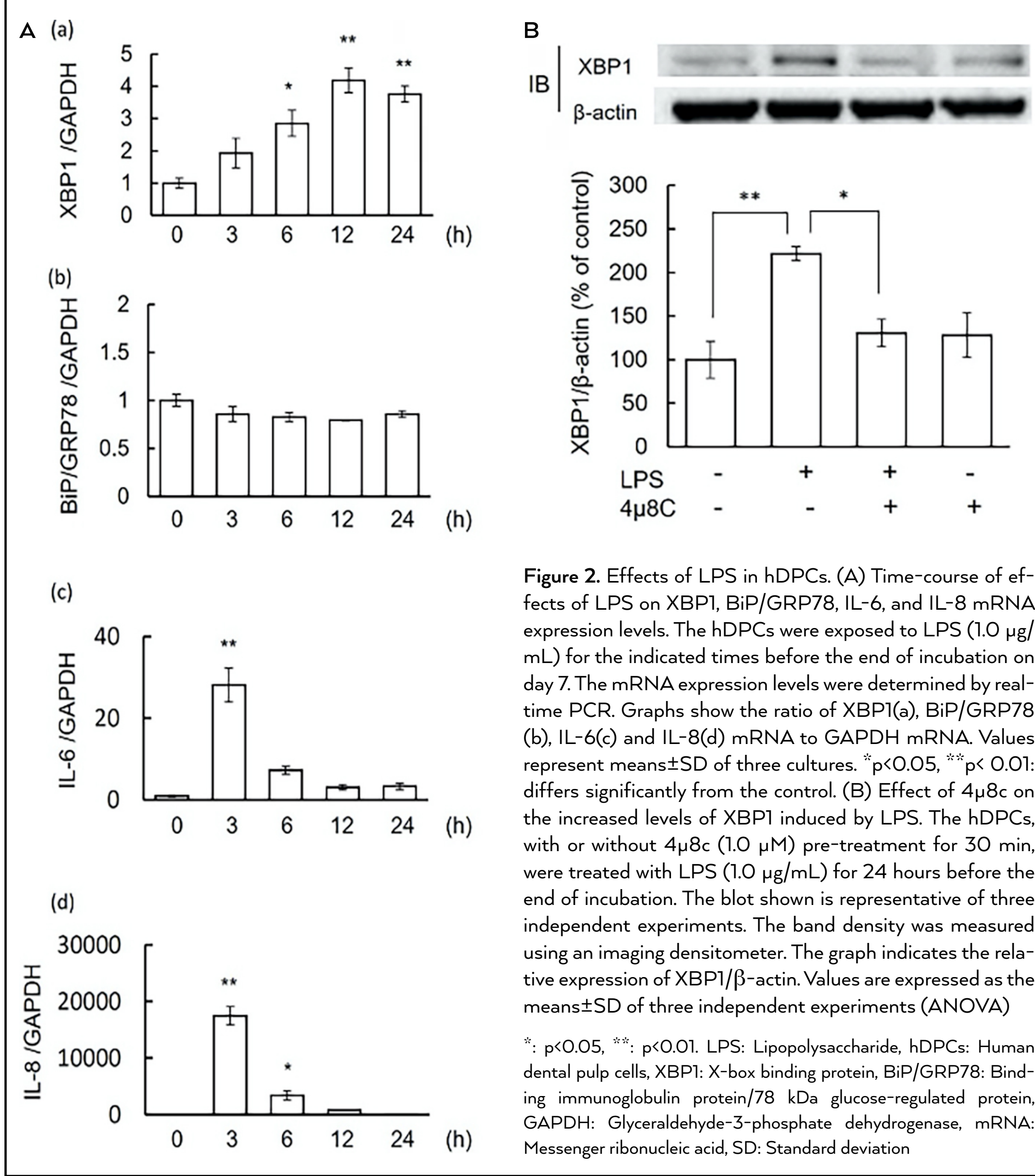


**Figure 2.** Effects of LPS in hDPCs. (A) Time-course of effects of LPS on XBP1, BiP/GRP78, IL-6, and IL-8 mRNA expression levels. The hDPCs were exposed to LPS (1.0 μg/mL) for the indicated times before the end of incubation on day 7. The mRNA expression levels were determined by real-time PCR. Graphs show the ratio of XBP1(a), BiP/GRP78 (b), IL-6(c) and IL-8(d) mRNA to GAPDH mRNA. Values represent means±SD of three cultures. *p<0.05, **p< 0.01: differs significantly from the control. (B) Effect of 4μ8c on the increased levels of XBP1 induced by LPS. The hDPCs, with or without 4μ8c (1.0 μM) pre-treatment for 30 min, were treated with LPS (1.0 μg/mL) for 24 hours before the end of incubation. The blot shown is representative of three independent experiments. The band density was measured using an imaging densitometer. The graph indicates the relative expression of XBP1/β-actin. Values are expressed as the means±SD of three independent experiments (ANOVA)

*: p<0.05, **: p<0.01. LPS: Lipopolysaccharide, hDPCs: Human dental pulp cells, XBP1: X-box binding protein, BiP/GRP78: Binding immunoglobulin protein/78 kDa glucose-regulated protein, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, mRNA: Messenger ribonucleic acid, SD: Standard deviation

In the present RNA-seq data analysis of hDPCs, based on fold change (|FC|>=2) and p-values (raw. p<0.05) of the comparison pair of 0 h vs 3 h, 71 genes were upregulated, and 35 genes were downregulated (Appendix 1a). Of the 71 upregulated genes, genes with a p-value smaller than 0.01 were selected for further investigation (Appendix 1b). Sec16B, which is required for organization of transitional ER sites and protein export, was included in the list (Appendix 1b). KEGG pathway functional analyses were performed to predict the role of DEGs. Of the top 10 pathways, the TNF signaling pathway and the NF-kappa B signaling pathway were enriched the most, as shown in Appendix 1c.

## DISCUSSION

This study using extracted human teeth is the first to demonstrate that ER stress is induced in pulp tissue prior to the onset of severe inflammatory cell infiltration caused by dental caries. The results indicate that regulating ER stress may lead to a reduction in the subsequent development of pulpitis. In recent years, there have been reports indicating that ER stress plays a role in various pathological processes, including diabetes, ischemic diseases, cancer, and viral infections. In the context of neurodegenerative diseases, ER stress has been found to be involved in the pathological processes of Alzheimer's disease and Parkinson's disease. Additionally, in psychiatric disorders, bipolar disorder is now being understood in terms of pathological mechanisms related to ER stress. With regard to neurodegenerative diseases, ER stress has been reported to be involved in the pathological processes of Alzheimer's disease and Parkinson's disease. Furthermore, in psychiatric disorders, bipolar disorder is now being explained in terms of pathological mechanisms related to ER stress (12, 27–29). This study suggests for the first time that pulpitis may be another disease in which ER stress is involved.

TM is the generic name for antibiotics produced by the Bacterium streptomyces, which inhibit glycosyltransfer reactions in the early stages of glycoprotein synthesis (30). In experimental biology, TM is used as a substance that causes ER stress. It has been reported that TM treatment of dental pulp cells increases the mRNA expression of ER stress markers (31). In the present study, TM also promoted mRNA ex-

 

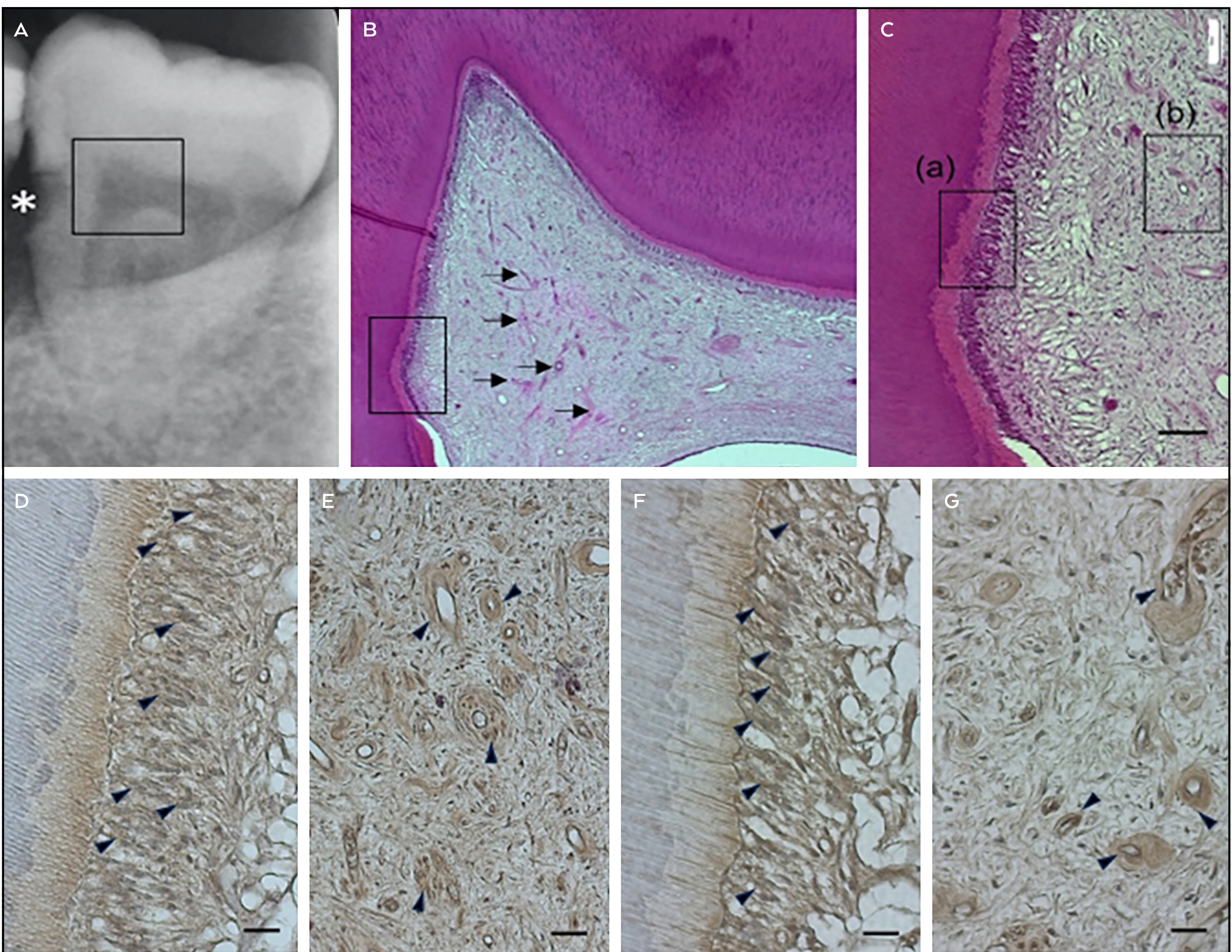


**Figure 3.** Expression of ER stress markers in a human tooth with dental caries. (A) Radiograph showing dental caries on the proximal surface of a mandibular left third molar. (B) HE staining of pulp tissue adjacent to dental caries, as shown in the rectangular area in A. Arrows: blood vessels. (C) Magnification of the rectangular area shown in B. HE staining. Bars: 50 μm. (D) Immunolocalization of BiP/GRP78 (arrowheads). Magnification of the rectangular area shown in C (a). Bars: 20 μm. (E) Immunolocalization of BiP/GRP78 (arrowheads). Magnification of the rectangular area shown in C (b). Bars: 20 μm. (F) Immunolocalization of XBP1 (arrowheads). Magnification of the rectangular area shown in C (a). Bars: 20 μm. (G) Immunolocalization of XBP1 (arrowheads). Magnification of the rectangular area shown in C (b). Bars: 20 μm

*: Dental caries. ER: Endoplasmic reticulum, HE: Hematoxylin eosin, BiP/GRP78: Binding immunoglobulin protein/78 kDa glucose-regulated protein, XBP1: X-box binding protein

pressions of ER stress markers in hDPCs. In addition, TM has been reported to increase the expression of inflammatory cytokines such as IL-6, IL-8, and TNF-α in bronchial epithelial cells via PERK-ATF4-C/EBP homologous protein (CHOP) signaling (32). The present study showed for the first time that TM upregulates mRNA expressions of inflammatory cytokines in dental pulp cells. This finding suggests that ER stress is involved in pulpal inflammation. Future studies will elucidate the mechanism by which TM promotes inflammatory cytokine expressions in dental pulp cells.

LPS is a component of the cell wall of Gram-negative bacteria and acts as an antigenic component of Toll-like receptor 4 (TLR4). LPS is commonly used to induce inflammation in various human tissues, including dental pulp (33). LPS has been shown to increase the expression of ER stress markers, BiP/GRP78 and CHOP, in periodontal ligament cells and institutional epithelial cells (34, 35). The present study is the first to examine this phenomenon in dental pulp cells. CHOP is a transcription factor belonging to the CCAAT/enhancer-binding protein (C/EBP) family, implicated in regulating processes related to cellular proliferation, differentiation, energy metabolism, and expression of cell type-specific genes (36). CHOP plays a central role in apoptosis in ER stress by promoting gene expression of apoptosis-inducing Bcl-2 antagonist/killer and Bcl-2-associated X protein and suppressing the production of apoptosis-suppressing Bcl-2 (37). In the present study, LPS increased the gene expression of XBP1, but did not affect the expression of BiP/GRP78. The gene expression of CHOP was slightly elevated at 24 hours (data not shown). Therefore, when the experimental design requires a longer exposure to LPS, apoptosis of the cells used needs to be considered. BiP/GRP78 expression was observed in pulp

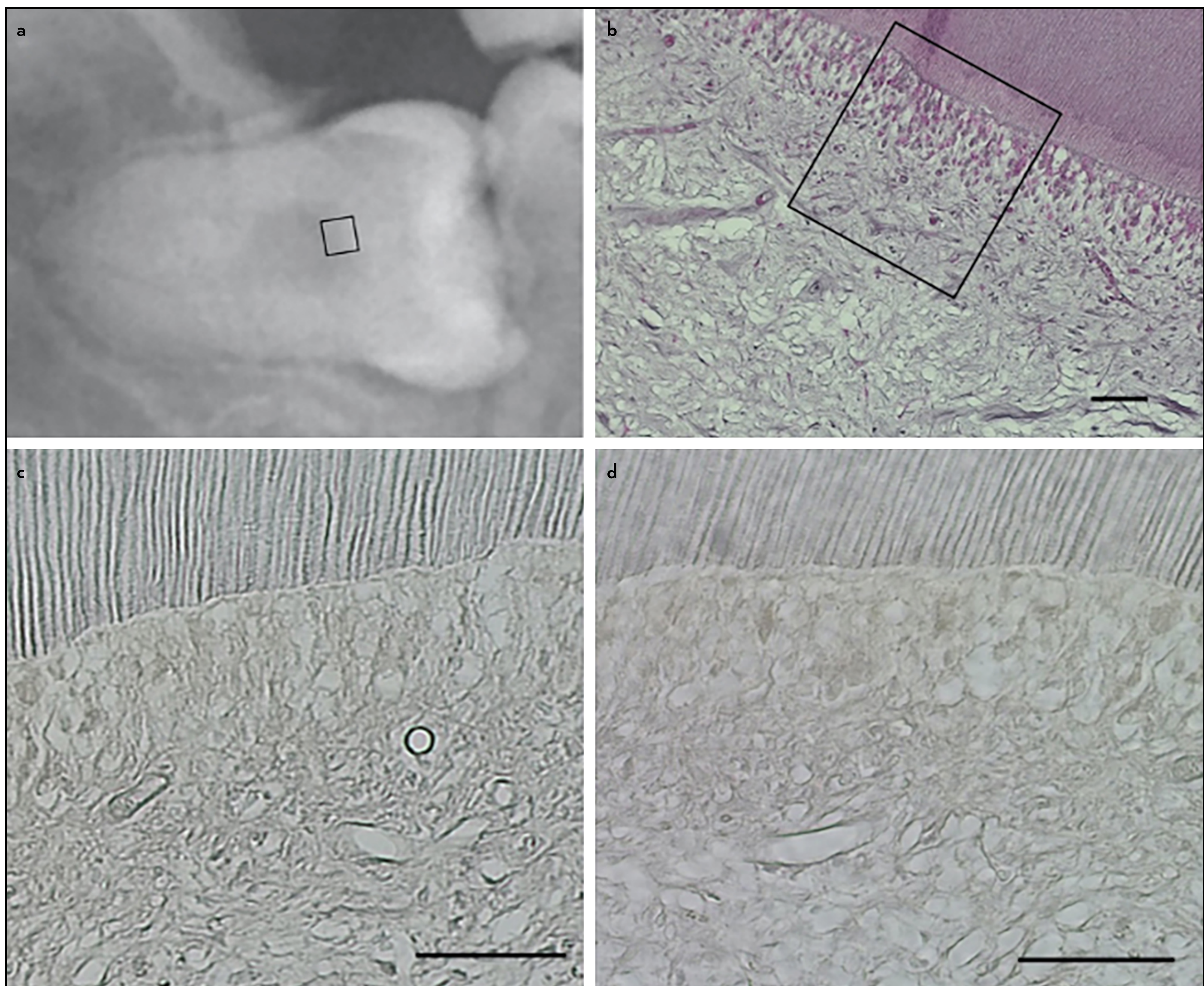


**Figure 4.** Expression of ER stress markers in a human impacted tooth without dental caries. (a) Radiographic view showing the proximal surface of a mandibular right third molar. (b) HE staining of pulp tissue of the rectangular area in a. Bars: 50 μm. (c) Immunolocalization of GRP78/BIP. Magnification of the rectangular area shown in b. Bars: 50 μm. (d) Immunolocalization of XBP1. Magnification of the rectangular area shown in b. Bars: 50 μm

ER: Endoplasmic reticulum, HE: Hematoxylin eosin GRP78 /BiP: 78 kDa glucose-regulated protein/Binding immunoglobulin protein, XBP1: X-box binding protein

tissues of dental caries-affected teeth by immunostaining, as shown in Figure 3d, e, but LPS did not promote BiP/GRP78 gene expression (Fig. 2b). This suggests that, in clinical practice, other pulpitis-initiating factors in addition to LPS may be involved in the induction of ER stress.

A previous study showed that IRE1 activation is indispensable for the sustained production of inflammatory mediators such as IL-6 in macrophages (38). IRE1 is a downstream target of TLRs, which induces XBP1 splicing and promotes generation of pro-inflammatory cytokines. This function of IRE1 helps maintain homeostasis and resolve acute infections (2, 39). In the present study, LPS promoted the production of XBP1 through IRE1. At present, the detailed mechanism, such as the involvement of TLR4 in this pathway, remains unknown. In addition, based on the results of the KEGG pathway functional analysis (Appendix 1c), NF-kB may be involved in the LPS-induced ER stress because it is a downstream target of XBP1. In addition, TNF signaling may also be involved.

Sec16 is a protein found on the transitional endoplasmic reticulum and ER exit sites (ERES). Its function is to aid in coat protein II (COP II) complex-mediated ER exit (40). Since Sec16 binds to a large number of COPII proteins at the ER exit site, it has been considered to function as a scaffold protein during vesicle formation. In addition, it is also thought that it may act as an organizer of the ER exit site (41). In the present RNA sequence data (Appendix 1b), Sec16 was included in the list of genes upregulated by LPS. LPS may affect the transport pathway that carries proteins out of the endoplasmic reticulum.

## CONCLUSION

This study demonstrated that ER stress may be involved in the onset of pulpitis. This leads to a more comprehensive understanding of the pathogenesis of pulpitis.

**Disclosures**

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**Ethics Committee Approval:** The study was approved by the Hiroshima University Hospital Ethics Committee (no: E-2741, date: 13//01/2022).

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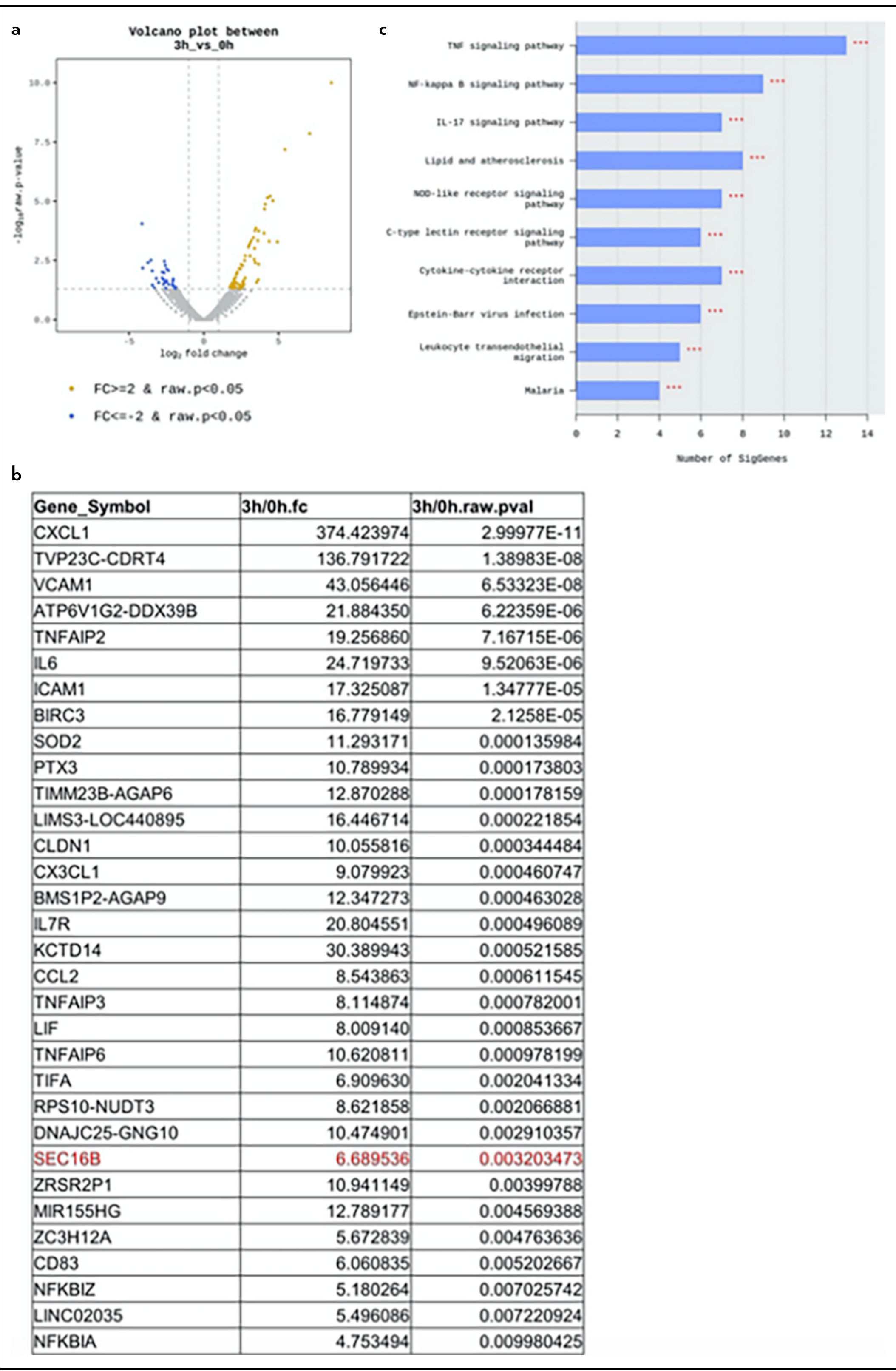


| Gene_Symbol | 3h/0h.fc | 3h/0h.raw.pval |
|---|---|---|
| CXCL1 | 374.423974 | 2.99977E-11 |
| TVP23C-CDRT4 | 136.791722 | 1.38983E-08 |
| VCAM1 | 43.056446 | 6.53323E-08 |
| ATP6V1G2-DDX39B | 21.884350 | 6.22359E-06 |
| TNFAIP2 | 19.256860 | 7.16715E-06 |
| IL6 | 24.719733 | 9.52063E-06 |
| ICAM1 | 17.325087 | 1.34777E-05 |
| BIRC3 | 16.779149 | 2.1258E-05 |
| SOD2 | 11.293171 | 0.000135984 |
| PTX3 | 10.789934 | 0.000173803 |
| TIMM23B-AGAP6 | 12.870288 | 0.000178159 |
| LIMS3-LOC440895 | 16.446714 | 0.000221854 |
| CLDN1 | 10.055816 | 0.000344484 |
| CX3CL1 | 9.079923 | 0.000460747 |
| BMS1P2-AGAP9 | 12.347273 | 0.000463028 |
| IL7R | 20.804551 | 0.000496089 |
| KCTD14 | 30.389943 | 0.000521585 |
| CCL2 | 8.543863 | 0.000611545 |
| TNFAIP3 | 8.114874 | 0.000782001 |
| LIF | 8.009140 | 0.000853667 |
| TNFAIP6 | 10.620811 | 0.000978199 |
| TIFA | 6.909630 | 0.002041334 |
| RPS10-NUDT3 | 8.621858 | 0.002066881 |
| DNAJC25-GNG10 | 10.474901 | 0.002910357 |
| SEC16B | 6.689536 | 0.003203473 |
| ZRSR2P1 | 10.941149 | 0.00399788 |
| MIR155HG | 12.789177 | 0.004569388 |
| ZC3H12A | 5.672839 | 0.004763636 |
| CD83 | 6.060835 | 0.005202667 |
| NFKBIZ | 5.180264 | 0.007025742 |
| LINC02035 | 5.496086 | 0.007220924 |
| NFKBIA | 4.753494 | 0.009980425 |

**Appendix 1.** The RNA-seq data indicating the differentially regulated transcripts between the control group and the LPS-treated group in hDPCs. (a) A volcano plot summarizing the RNA-seq data indicating the differentially regulated transcripts between the control group and the LPS-treated (3 hours) group. (b) A list of up-regulated genes selected based on fold change (|FC|>=2) and p-value (raw.p<0.01). (c) Top 10 terms of KEGG pathway functional analysis

***: p<0.001. RNA: Ribonucleic acid, LPS: Lipopolysaccharide, hDPCs: Human dental pulp cells, FC: Fold change, KEGG: Kyoto Encyclopedia of Genes and Genomes