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Original Article

Orthodontically induced changes to the genetic profile in periodontal ligament tissue and cytokine release in gingival crevicular fluid – A pilot investigation

Nutthakarn Ratanasereeprasert [a], Li-Fang Hsu [b], Shih-Kai Wang [c], Chung-Chen Jane Yao [a,d*]

[a] *Graduate Institute of Clinical Dentistry, School of Dentistry, National Taiwan University, Taipei, Taiwan*
[b] *Department of Dentistry, National Taiwan University Hospital, Hsin-Chu Branch, Hsin-Chu, Taiwan*
[c] *Department of Dentistry, School of Dentistry, National Taiwan University, Department of Pediatric Dentistry, National Taiwan University Children's Hospital, Taipei, Taiwan*
[d] *Division of Orthodontics and Dentofacial Orthopedics, Dental Department, National Taiwan University Hospital, Taipei, Taiwan*

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KEYWORDS
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Abstract *Background/purpose:* It has been known that genetic factors influence orthodontic tooth movement, however, scientific research on humans is lacking. Therefore, this study aimed to investigate dynamic changes to the genetic profile in human periodontal ligament (PDL) tissue and cytokine release in gingival crevicular fluid (GCF) during the first 28 days of orthodontic treatment.
Materials and methods: Fifteen teeth from three patients were recruited. Full-mouth fixed appliances with extraction of four premolars and one maxillary third molar was planned for orthodontic treatment. GCF collection and tooth extraction were performed following force application for 0, 1, 3, 7, and 28 days. GCF was analyzed using multiplex immunoassay for 27 cytokines. PDL tissue was collected after extraction and submitted for RNA exome-sequencing using Illumina sequencing platform. Further analysis of differentially expressed genes (DEGs), gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and heatmaps were conducted.
Results: GCF cytokine levels varied among three patients; some patients exhibited a peak cytokine level on Day 0 whereas others did so on Days 1–3. In RNA exome sequencing data, GO and KEGG analyses showed that genes associated with sensory receptors were upregulated

* Corresponding author. Graduate Institute of Clinical Dentistry, School of Dentistry, National Taiwan University, No. 1, Chang Te St., Zhongzheng Dist., Taipei 10048, Taiwan.
E-mail address: janeyao@ntu.edu.tw (C.-C. Jane Yao).

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on Day 1, genes involved in bone remodeling were upregulated on Days 3 and 28, and genes related to osteoclast differentiation were upregulated on Day 7.
Conclusion: RNA sequencing data demonstrate that the specific types of genes are expressed at different time points, whereas the data on cytokine changes show a large variation in concentration levels and dynamic change patterns among the patients.

Introduction

Orthodontic tooth movement (OTM) is the biomechanical process by which mechanical loading on the teeth causes local hypoxia and stimulates an aseptic inflammatory response in the periodontal tissue, leading to a coordinated process of bone remodeling.[1] It is a combination of cellular responses, including stress transfer from the periodontal ligament (PDL), bone deposition by osteoblasts, bone resorption by osteoclasts, and the inflammatory response of immune cells.[1] Although scientific evidence for the biologic reaction to OTM is extensive, individual variations play a critical role in the process, which affect orthodontic treatment times and outcomes for patients.[2] The era of precision medicine demands clarification of individual mechanisms including mRNA expression and cytokine release during treatment for OTM.

Gingival crevicular fluid (GCF) is an inflammatory exudate derived from blood vessels of the gingival plexus and surrounding periodontal tissues. It contains tissue breakdown products, cytokines, growth factors, and antibodies.[3] This can help determine the periodontitis biomarker profile,[4] predictors of periodontal disease progression,[5] and parameters for assessing periodontal therapy.[6] In orthodontics, GCF is considered a biological substance that can vary in amount and protein composition during OTM and is suitable for monitoring cytokine expression to observe the tissue response to OTM.[7] Proinflammatory cytokines have been reported as elevated during the early stage of OTM, indicating orthodontic force as the trigger for the signaling cascade to achieve tooth movement, whereas the level is stable in the linear stage because the system reaches new physiological homeostasis.[8]

RNA sequencing can thoroughly investigate the changes induced by OTM. Several studies have used genetic profiling to explore the putative genes participating in OTM in animals and humans.[9–11] However, no study has yet been conducted to investigate human mRNA expression during orthodontic treatment at set time points including the early (Days 1–7) and linear phases of OTM (Day 28).[8]

In our study, we focused on investigating the cytokine and genetic profile of human samples at different time points within a 28-day treatment duration because the initial phase and lag phase are highly dynamic as a result of acute inflammatory reactions. They involve typical innate immune responses from periodontal tissue-resident and extravasated immune cells and lead to high levels of various pro-inflammatory cytokines. Besides collecting GCF, we were able to extract premolars according to orthodontic treatment plan and explore individual differences using high-throughput screening. This study is the first to report changes at these time points in humans.

Materials and methods

Sample collection

This study was approved by the Institutional Review Board (IRB; 202011067RIND) of National Taiwan University Hospital, Taipei, Taiwan. The selection criteria were shown in Table 1. Patients were recruited from February 2019 to October 2021 in accordance with approved IRB protocols. Before patients could participate in the study, they underwent clinical examination for plaque accumulation, bleeding upon probing, and probing depth to confirm the absence of gingivitis and other periodontal diseases. Oral hygiene instruction was given before the study. Initially six patients were recruited for the study; however, ultimately, three patients (A, L, and X) with 14 teeth presenting good quality and quantity for RNA exome sequencing were analyzed.

Table 1 Inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
(1) Young female adults aged 19–24 years (2) Class I or mild Class II/III malocclusion (3) Treatment with fixed orthodontic appliances in the permanent dentition (4) Extraction of four premolars at Day 1, 3, 7, and 28 of force loading and one maxillary third molar as Day 0. (5) Good oral hygiene with healthy periodontal status (probing depth <4 mm, gingival index = 0, plaque index ≤1)	(1) Patients with poor oral hygiene or active periodontal disease (2) Severe skeletal discrepancy (3) Patients with systemic diseases affecting tooth movement, bone metabolism, or maxillofacial malformation (4) Use of any medication that could interfere with orthodontic tooth movement (e.g., antibiotics, antihistamines, cortisone, immunosuppressants, and hormones).

Biomechanics

Orthodontic force application was performed after full-mouth bonding with 0.018 slot MBT brackets (3M Unitek, Monrovia, CA, USA) by ligating a 0.014-inch nickel–titanium wire (PG supply Inc., Avon, CT, USA), which delivered light continuous force (70–100 g) with a deflection of 1–4 mm to the treatment group.[12] The force duration was set at 0 (control group), 1, 3, 7, and 28 days.

Gingival crevicular fluid collection for biomarker analysis

After the evaluation of periodontal health, the participants were scheduled for GCF collection and tooth extraction on Days 0, 1, 3, 7, and 28 after orthodontic force application. For GCF collection on Day 0, the procedure was performed on the maxillary first premolars, which were later used as Day 1 sample after force loading. GCF was collected using a filter paper strip (Periopaper, Oraflow, Plainview, NY, USA) inserted in the mesio-buccal gingival sulcus against the tooth and the amount was measured (Periotron 8000, Oraflow), placed in 100 μL of GCF buffer with Protease and Phosphatase Inhibitors (Medchemexpress, Monmouth Junction, NJ, USA), and stored at −80 °C.

Periodontal ligament tissue collection for RNA sequencing

After GCF was collected from the targeted tooth, the tooth was extracted as least traumatically as possible. For PDL collection on Day 0, tissue samples were taken from the maxillary third molars. The tooth was gently washed with cold normal saline and the PDL tissue at the middle third of the root was scraped with a sharp curette and immersed in RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) for RNA stabilization and stored at −80 °C.

Biomarker analysis

GCF samples were analyzed using a cytokine antibody assay (Bio-Plex Pro Human 27-plex Assays, Bio-Rad Laboratories, Inc., Hercules, CA, USA). The following cytokines/chemokines were measured: IL-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, Eotaxin, Basic FGF, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α, and VEGF. The graph of the standard curve of IL-1β between the controls and Sample A are shown in Supplementary Fig. 1.

RNA exome library preparation and sequencing

Purified RNA was used for the preparation of the sequencing library using the TruSeq RNA Exome Library Prep Kit (Illumina, San Diego, CA, USA). The quality of libraries was assessed using the Agilent Bioanalyzer 2100. The qualifying libraries were then sequenced on the Illumina NovaSeq 6000 with 150 bp paired-end reads generated by Genomics (BioSci & Tech Co., New Taipei City, Taiwan). However, periodontal tissue samples from one tooth (Day 7 from Sample X) were not able to undergo RNA sequencing analysis because the RNA was less than 0.2 μg. The remaining 14 samples consisted of three Day 0 samples (control; no orthodontic force) from maxillary third molars and the treatment groups were premolars that received orthodontic force for different times: three Day 1, three Day 3, two Day 7, and three Day 28 samples.

Heatmap

A heatmap was created from the top 100 most expressed genes with the highest transcripts per million (TPM) values to identify the highly expressed genes in human PDL tissue and their changes during OTM using the Next-Generation Clustered Heat Map tool.[13] To further investigate OTM-related genes within these 100 enriched genes, gene sets were categorized by gene ontology (GO) terms using the WEB-based Gene SeT AnaLysis Toolkit (WebGestalt).[14] OTM-related GO terms were then analyzed and data redundancy was reduced using the weight set cover method to identify top gene sets while maximizing gene coverage.[15] The processed data were used to create an OTM-related gene heatmap.

Bioinformatics

We investigated the differentially expressed genes (DEGs) among the control samples and four different treatment time points. To identify the most relevant DEGs among all three participants, a statistical significance indicated by a log2 fold change (± 1) and an adjusted *P-value* < 0.05 were chosen as the cut-off criteria. GO and KEGG pathway enrichment analysis was performed using WebGestalt and Panther v17.0 to demonstrate the most significant GO and KEGG pathways at each time point and also the trending changes of possible OTM-related GO and KEGG pathways.[14,16] The enrichment ratio in GO and KEGG pathways was calculated using the number of observed genes divided by the number of expected genes from each GO or KEGG category.

Results

Differences in cytokine profiles in the gingival crevicular fluid of orthodontically treated teeth at different time points

The cytokine profiles in GCF were divided into five types; pro-inflammatory cytokines: IL-1β and TNF-α (Fig. 1A), anti-inflammatory cytokines: IL-1Ra and IL-10 (Fig. 1B), growth factors: FGF and VEGF (Fig. 1C), adaptive immunity cytokines: IL-2 and IL-5 (Fig. 1D), and chemokines: IP-10 and MCP-1 (Fig. 1E). However, no common pattern indicates huge individual variations in GCF response up on orthodontic force loading.

The graph in Fig. 1 shows two representative cytokines of each type to increase readability. A complete graph of 27 cytokines is shown in Supplementary Fig. 2. Fig. 1 demonstrates that no distinct pattern was expressed at different treatment times. Large variations in cytokine expression

Figure 1 Dynamic changes of representative cytokine profiles in 28-day orthodontic treatment in all three samples. A) Pro-inflammatory cytokines, B) Anti-inflammatory cytokines, C) Growth factors, D) Adaptive immunity cytokines, E) Chemokines.

were found among the three patients. Most of the cytokine expression in Sample A (blue line) rose suddenly on Days 1–3, the level dropped on Day 7, and thereafter remained steady until Day 28. Most of the cytokine expression in Sample L (orange line) was the highest on Day 0 and decreased thereafter. Except for IL-5 and IP-10, the patterns showed increased levels on Days 1–3, with a decrease after Day 3. Most of the cytokine expression in Sample X (green line) was the highest on Day 0, dropped on Day 1, increased over Days 3–7, and gradually decreased on Day 28. Except for IL-2, the pattern showed no upregulation after the decrease on Day 1.

Heatmap of highly expressed genes during orthodontic tooth movement

Top 100 genes with the highest TPM score on Day 1 were chosen to investigate highly expressed genes in human PDL tissue (Fig. 2A) at early stage of OTM. Five top GO terms were obtained after data processing by using the weighted set cover method, including extracellular matrix (ECM) organization, ossification, protein localization to organelles, cellular response to endogenous stimuli, and protein-containing complex assembly (Fig. 2B). OTM-related gene heatmap (Fig. 2C) was created using 22 genes from ECM organization and ossification GO terms.

Differentially expressed genes of periodontal ligament tissue under orthodontic treatment at different time points

Statistical analysis revealed 320 genes differentially expressed in force-loaded PDL compared to control PDL (Day 0) in all three patients (197 upregulated genes and 123 downregulated genes). The numbers of genes that were significantly upregulated on Day 1, Day 3, Day 7, and Day 28 were 23, 40, 160, and 19 genes, whereas the numbers of downregulated genes were 6, 11, 103, and 12 genes, respectively (Supplementary Table 1). Raw data of DEGs were presented in Supplementary Table 2.

Gene ontology analysis for biological process during orthodontic tooth movement

The top GO biological processes during OTM are shown in Fig. 3. On Day 1, the majority of GO terms included photoreceptor cell maintenance and retina development (Fig. 3A). On Day 3, most GO terms were associated with skeletal system morphogenesis and bone mineralization (Fig. 3B). On Day 7, most were related to mitotic cell cycle (Fig. 3C). On Day 28, the top GO terms included cartilage development, skeletal system morphogenesis, organ development, and ion homeostasis (Fig. 3D).

Other possible OTM-related GO terms were analyzed and plotted into a graph to demonstrate changes within the 28-day treatment interval, including collagen fibril organization, bone growth, mechanoreceptor differentiation, ECM organization, blood vessel remodeling, and osteoclast differentiation (Fig. 3E). The graph in Fig. 3E demonstrates that most of the OTM-related GO terms were upregulated on Days 1–3, dropped on Day 7, and remained low until Day 28, except bone growth, blood vessel remodeling, and osteoclast differentiation. Bone growth and blood vessel remodeling were upregulated on Days 1–3, fell on Day 7, and increased again on Day 28. Osteoclast differentiation began to be upregulated on Day 3, peaked on Day 7, and dropped thereafter. The complete data of top GO biological process and possible OTM-related GO including the DEGs lists involved in each GO terms were shown in Supplementary Table 3.

Kyoto encyclopedia of genes and genomes pathways during orthodontic tooth movement

Top KEGG pathway enrichment at four different treatment times is shown in Fig. 4. On Day 1, the top KEGG pathway was maturity-onset diabetes of the young, prion disease, and ECM-receptor interaction (Fig. 4A). On Day 3, rheumatoid arthritis, IL-17 and the NF-kappa B signaling pathways, which are involved in inflammatory response, were significantly upregulated (Fig. 4B). On Day 7 it included glycosaminoglycan degradation, prostate cancer, rheumatoid arthritis, cancer, cell cycle, and osteoclast differentiation (Fig. 4C). On Day 28, they were similar to those on Day 3, which were associated with inflammatory response and parathyroid hormone synthesis (Fig. 4D).

Similar to GO enrichment, possible OTM-related KEGG pathways were analyzed and plotted on a graph to demonstrate the changes within the 28-day treatment interval, including ECM–receptor interaction, rheumatoid arthritis, the IL-17 signaling pathway, and the TNF signaling pathway (Fig. 4E). The graph in Fig. 4E demonstrates that ECM–receptor interaction and TNF signaling pathway had a similar dynamic change that they were highly upregulated at day 1, dropped at day 3–7, then slightly increased at day 28. Rheumatoid arthritis and the IL-17 signaling pathway shared a common pattern that they were upregulated at day 1–3, decreased at day 7, then rose up at day28. The complete data of top KEGG and possible OTM-related KEGG including the DEGs lists involved in each KEGG pathway were shown in Supplementary Table 4.

Discussion

In this study, we investigated cytokine profiles in GCF and mRNA expression upon orthodontic force application at five different time points (Day 0, Day 1, Day 3, Day 7, and Day 28) in the early and linear phases of tooth movement.[17] Multiplex bead immunoassays can measure multiple cytokines in a limited GCF volume, particularly in patients with healthy periodontal status.[18] However, because of the small sample size and large variation among patients, we could not identify a common and specific patterns at each time point. Previous studies have shown that inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) are significantly upregulated 1–3 days after orthodontic force administration, after which their levels fall rapidly.[7,8,19] Very few studies have investigated changes in other types of cytokines, chemokines, or growth factors after orthodontic treatment except EGF and IL-1Ra which have been shown to increase on Days 1–3 and decrease thereafter.[7,19] In our study, only Sample A exhibited a biomarker pattern that corresponded to those of previous studies, and Samples L

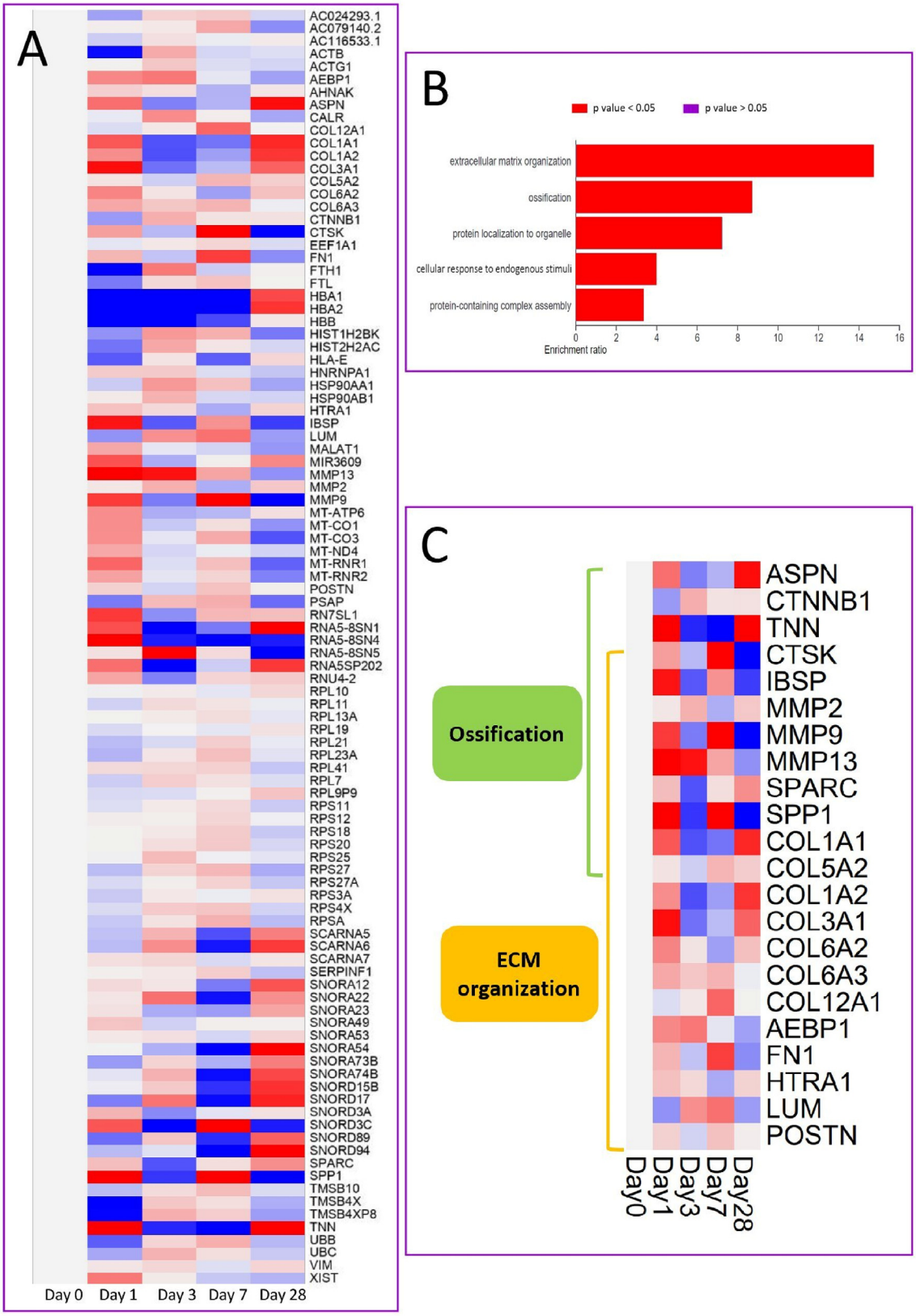

Figure 2 Heatmap representing differential expression of highly expressed genes in human PDL tissue underlying orthodontic treatment within a 28-day period. The fold change in the TPM values of genes are represented by different colors: fold change >2, red; fold change <0.5, blue. The TPM value indicates the relative expression of a transcript. A) Heatmap demonstrating the top 100 highly expressed genes in human PDL. B) Graph demonstrating the top GO terms from the top 100 highly expressed genes processed using the weighted set cover method. C) Heatmap representing differential expression of OTM-related genes from ECM organization and ossification GO terms. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and X exhibited the opposite pattern, with cytokine levels dropping on Days 1–3 after treatment. Therefore, research using GCF warrants detailed examination on individual responses but not means for the whole population.

New advancement in next generation sequencing can provide high through put information from minute PDL tissues under different loading periods of orthodontic force. A heatmap showed the top 100 highly expressed genes on Day1 in human PDL tissue, some of which were directly involved in OTM. After categorizing the enriched genes by their GO terms, 22 genes involved in the GO terms ECM organization and ossification were determined as OTM-related genes. Nine out of 12 genes involved in the ossification GO term overlapped with nine out of 19 genes involved in ECM organization, indicating that these two biological processes are highly correlated. Ossification is the process of bone development, which can be controlled by osteoblast-lineage cells, bone resorption cells, and the ECM, which acts as a bio-environment to regulate bone remodeling. The ECM structure and components induce vascular invasion, which recruits bone cells and growth factors to stimulate bone remodeling, whereas matrix metalloproteinases facilitate ECM degradation.[20] Therefore, ECM organization and remodeling are critical processes in ossification.

Mouse genetic profile of OTM from the top 50 DEGs revealed showed the ECM organization-related genes (*Col3a1*, *Col4a2*, *Col5a1*, *Col6a1*, *Eln*, *Loxl2*, and *Mmp14*) being downregulated by Day 3, but increased during Days 7–14.[21] Although human and mouse PDL tissues are both rich in genes involved in the ECM organization GO term, their changes after OTM differ. Human PDL has a faster response on Day 1, drops on Day 3, increases on Day 7, and falls on Day 28, whereas mouse PDL has a slower and more stable response that is only upregulated from Day 7 to Day 14.

In GO analysis, our study unexpectedly revealed the highest activation of genes involved in photoreceptor development and retina morphogenesis occurred in loaded PDL on Day 1. Nevertheless, a previous study showed that stem cells from human exfoliated deciduous teeth can be

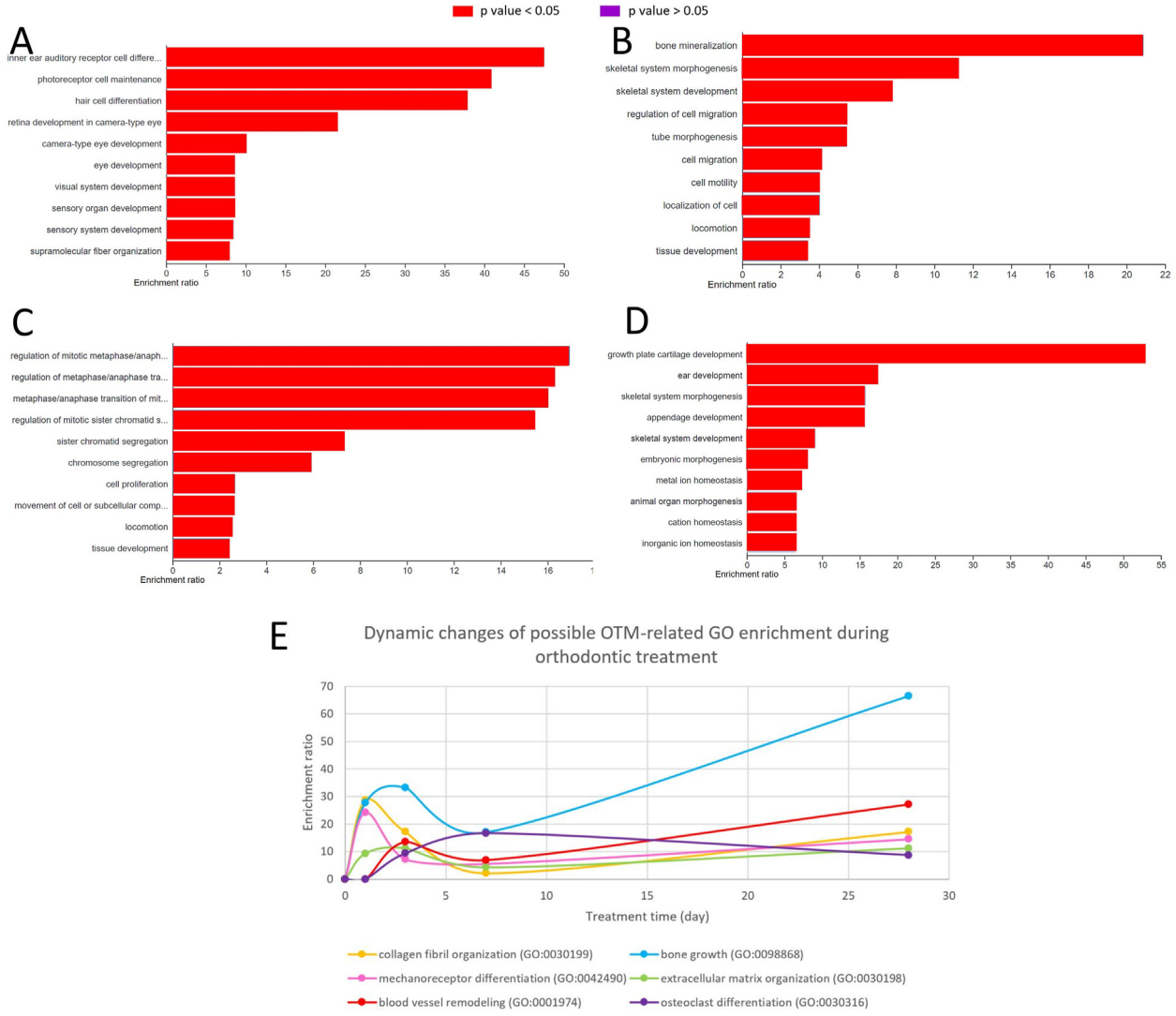

Figure 3 Top GO terms at different orthodontic time points and dynamic changes of possible OTM-related GO terms. A) Top GO terms on Day 1, B) Top GO terms on Day 3, C) Top GO terms on Day 7, D) Top GO terms on Day 28, E) Dynamic changes of possible OTM-related GO terms.

induced to transdifferentiate into retinal photoreceptor-like cells in vitro and maintain good viability in vivo after transplantation into mice.[22] PDL tissue, which is rich in stem cells, could exhibit photoreceptor gene expression. On Day 3 and Day 28, orthodontic force induced DEGs in similar GO terms such as skeletal system morphogenesis, which might indicate two waves of gene expression on bone development (an early phase on Day 3 and a late phase on Day 28) after orthodontic force administration.

On Day 7, the DEGs were related to mitotic cell cycle, which had some similarities to a previous study on transcriptional expression in human PDL tissue after 3-week administration of orthodontic force.[23] Although their DEGs had no commonality with those in our study, their gene set enrichment analysis revealed that their DEGs were related to cell cycle, cell mitosis, and DNA replication, which is similar to our results on Day 7 after force delivery. Combining our data with theirs could imply that 1—3 weeks after OTM, the mRNA expression indicated activation of cell cycle pathway.

In the possible OTM-related GO term graph pattern in Fig. 3E, there were two interesting findings:

1) The expression of mechanoreceptor differentiation genes rose rapidly on Day 1, subsequently dropped, and gradually increased once more on Day 28. This seems to represent the stimulation of specialized mechanoreceptor in PDL tissue 1—2 days after the start of force delivery (initial displacement phase). The expression of mechanoreceptor dropped as the tooth stopped moving during the lag phase, which lasted approximately 20 days, then slightly increased again on Day 28, which seems to be the post-lag phase in which the tooth begins moving after bone resorption in the lag phase.[24]
2) The top GO term associated with OTM on Day 7 was upregulated osteoclast differentiation. This delayed differentiation of osteoclasts possibly derived from adjacent bone marrow space may be responsible for the undermining resorption that removed the hyalinization

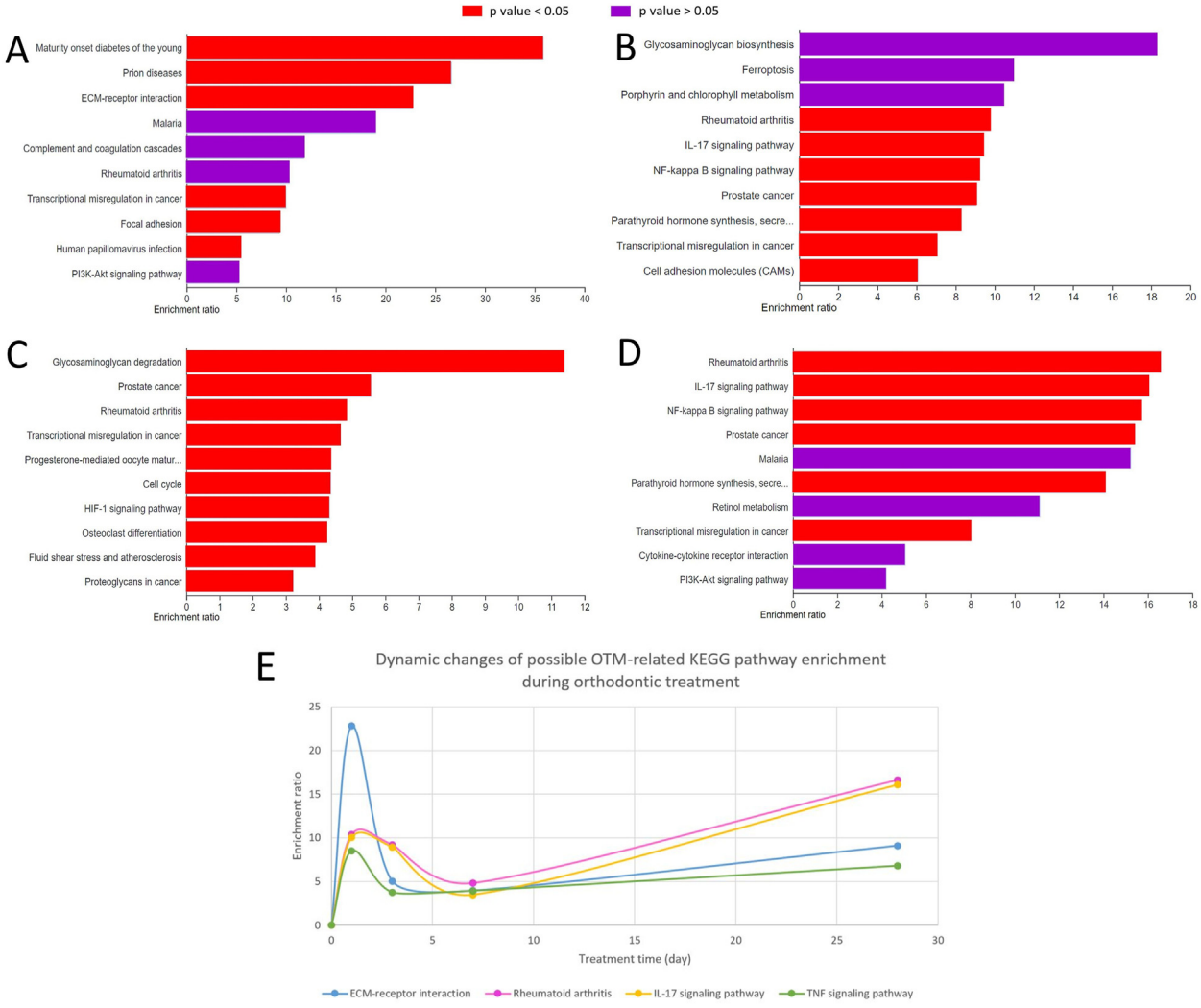

Figure 4 Top KEGG enrichment pathways at different orthodontic time points and dynamic changes of OTM-related KEGG pathways. A) Top KEGG terms on Day 1, B) Top KEGG terms on Day 3, C) Top KEGG terms on Day 7, D) Top KEGG terms on Day 28, E) Dynamic changes of possible OTM-related KEGG pathways.

of the compressed PDL, which indicates that heavy force was applied.[1] If light force was delivered, the blood flow would not be cut off, allowing faster osteoclast recruitment within 2 days to start frontal resorption. Nevertheless, avoiding PDL hyalinization in clinical situations is difficult; hence, OTM is likely mediated by both frontal resorption and undermining resorption.[25]

As for possible OTM-related KEGG pathways, ECM receptor interaction was upregulated on Day 1 and exhibited a dynamic pattern similar to the mechanoreceptor differentiation GO term in that its enrichment level peaked on Day 1, subsequently dropped, and slightly increased on Day 28. This correlation might be due to integrin, the ECM receptor in PDL tissue, acting as a mechanosensor, which can be stimulated by loading force to generate a signaling cascade altering gene expression, cytoskeletal organization, and ultimately tissue remodeling.[1,26]

Rheumatoid arthritis and the IL-17 signaling pathway were the top KEGG pathways on Day 3 and Day 28. Indeed, higher IL-17A levels were associated with a higher severity of arthritis; hence, it is considered a potential biomarker as well as a therapeutic target in rheumatoid arthritis.[27,28] Expression changes during the 28-day course were similar to the bone growth GO term because of their DEGs (*MMP3*, *MMP13*, and *TNFSF11*), which affected the bone remodeling process.[29,30] In a mouse study, KEGG pathways involved in rheumatoid arthritis and IL-17 signaling were also upregulated on Day 3 but decreased thereafter.[21]

One of the limitations of this study was that the control samples (Day 0) were maxillary third molars, whereas the experimental samples were premolars. These teeth have several differences, including dental anatomy, location, timing of eruption, and occlusal loading. Because our patients were all young adults, some of maxillary third molars were still erupting; therefore, the incomplete exposure to the oral cavity and the absence of occlusal loading might have caused differences in the transcriptome profiles between the third molars and premolars. Previously, mRNA expression of *Postn* and *Twist* in the PDL tissue of teeth without antagonists temporarily decreased 24 h after opposing tooth extraction, but recovered after 72 h, and the amount of expression was similar to the control teeth after 168 h.[31] Although the changes were short-term, the data suggested the importance of occlusal force to *Postn* and *Twist* gene expression, particularly in the case of *Postn*, because it was in top 100 highly expressed genes in our human PDL study; hence, the absence of occlusal function might influence our results.

With this novel but initial approach, we could identify some temporal patterns of differential regulation using small amounts of tissue obtained from orthodontic force loaded roots. However, limitations include the small sample size, human individual variations, unpredictable PDL tissue quantity, and difficult process of recruiting patients. Future studies should include more samples and investigate and correlate the OTM rates of each patient with their cytokines and genetic profiles.

Declaration of competing interests

The authors have no conflicts of interest relevant to this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jds.2023.07.038.

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