Progress in Orthodontics

RESEARCH Open Access

Influence of sex and age on the gene expression of periodontal and pulp tissues during orthodontic tooth movement

Zuodong Zhao[1*], Steffen Fieuws[2], Catia Attanasio[3], Mariano Simón Pedano[4] and María Cadenas de Llano-Pérula[1]

Abstract

Background Orthodontic tooth movement (OTM) is a complex biological process triggered by orthodontic forces (OF). This study aims to study the influence of sex and age on the gene expression of the dental pulp (DP) and periodontal ligament (PDL) of human premolars subjected to 7 and 28 days of OF in vivo.

Methodology Linear mixed and negative-binomial models were used on previously published RNA sequencing (RNA-seq) datasets of DP and PDL tissue subjected to OF for 7 days and 28 days to verify if the effect of OF depends on sex and age. Differentially expressed genes (DEGs) were identified using false discovery rate and functional analysis was performed.

Results The datasets consisted of 69 DP and 63 PDL samples from 46 and 41 patients respectively, with similar sex and age distribution. RNA-seq showed that sex did not influence the DP's gene expression profile, since only one DEG related to immune response was detected after 28-days of OF. In contrast, sex significantly affected PDL: 505 DEGs were found after 7 days of OF, related to bone homeostasis, osteoclastic activity and immune response. Age impacted both tissues; in DP, 18 DEGs related to Ca^{2+} regulation and DNA damage repair were found at 7 days, and 10 DEGs associated with repair and adaptive capacities emerged at 28 days. In PDL, 181 genes related to bone regeneration were identified at 28 days, with no DEGs noted at 7 days.

Conclusion Our study demonstrates that under OF, DP's reaction is not sex-based, whereas PDL's is, particularly in the early phase of OTM, with women showing a more pronounced osteoclastic response. Age-related effects in DP tissue primarily influence Ca^{2+} homeostasis and DNA damage repair in early phases, and tissue repair and adaptive responses later. In contrast, age impacts PDL tissue mainly in the later stages of OTM, affecting its regenerative capacity.

Keywords Age, Dental pulp, Orthodontic tooth movement, Periodontal ligament, RNA-seq, Sex

*Correspondence:
Zuodong Zhao
zuodong.zhao@kuleuven.be
[1]Department of Oral Health Sciences-Orthodontics, KU Leuven, Leuven, Belgium
[2]Interuniversity Institute for Biostatistics and Statistical Bioinformatics, KU Leuven, Leuven, Belgium
[3]Laboratory of Gene Regulation and Disease, Department of Human Genetics, KU Leuven, Leuven, Belgium
[4]Department of Oral Health Sciences-Endodontics and BIOMAT–Biomaterials Research group, KU Leuven, Leuven, Belgium

Introduction

Orthodontic tooth movement (OTM) is a complex biological process triggered by orthodontic forces (OF). These forces provoke substantial biomechanical and biological responses within the dentoalveolar complex, impacting the alveolar bone, the periodontal ligament (PDL) and the dental pulp (DP) [1]. The PDL is an essential link between the teeth and the alveolar bone, orchestrating OTM by altering its shape, gene expression and protein secretion in response to mechanical load [2, 3]. Although to a lesser degree, the DP is also impacted by OF due to a reduction in pulp blood flow [4, 5]. However, these reactions are not universal, since research has shown that male and female patients respond differently to orthodontic treatment. Female patients tend to exhibit faster responses to OF, potentially due to hormonal influences on bone remodeling and their generally lower alveolar bone density compared to males [6, 7]. Sex hormones such as estrogen, progesterone, and androgen play significant roles in bone metabolism, thereby affecting OTM [8]. Additionally, variations in these hormones can influence the pulp's blood flow and inflammatory responses [9]. Estrogen, in particular, is known to regulate the activity of DP cells and the production of inflammatory mediators [10], which could lead to different DP tissue responses to OF in women.

Sex is not the only factor influencing OTM. As individuals age, the periodontium undergoes structural and functional changes, [11, 12] such as a decrease in Sharpey's fibers. These fibers present more irregular insertion into bone [13] and thicker principal fibers in older patients [14], which lead to slower bone metabolism, reduced osteoclast activity, and decreased proliferation of PDL cells [15–17], ultimately contributing to slower OTM [18]. Additionally, the DP exhibits age-related changes, such as reduced blood flow, increased fibrosis, and calcification [19].

Understanding how the dental tissues of men and women with different ages respond to OF could improve the selection of force systems and the definition of personalized treatment protocols, ultimately enhancing treatment efficacy and safety. More than 70% of the patients seeking orthodontic treatment are female [20]. Many of these patients may present unique hormonal conditions, such as pregnancy, oral contraceptive use, lactation or menopause [21], which makes the understanding of the sex-dependent responses of dental tissues to OF absolutely crucial. Also, according to the American Association of Orthodontists [22], the age range of the orthodontic population has been increasing steadily over the past decades. This is possibly due to the longer life expectancy, the gaining importance of oral health-related quality of life, the fact that older patients often need prerestorative orthodontics to harmonize their occlusion before comprehensive dental treatment and replacement of missing teeth, the simplification of orthodontic techniques or the introduction of clear aligners [23]. This underscores the necessity for efficient treatment strategies that preserve tissue health. However, research on the age- and sex-related dental tissues responses to OF has primarily been conducted in vitro or in animal models. Clinical studies tend to focus on histological changes and target genes, but to the best of our knowledge, exploration of transcriptomic alterations in the human dental tissue under OF in men and women with different ages has not been performed yet [24].

The present study explores the influence of sex and age on the transcriptomic profiles of PDL and DP tissues subjected to different lengths of OF application in vivo.

Material and methods

Study approval

This study obtained approval from the Commission of Medical Ethics prior to the start and has been conducted in strict adherence to the principles of Helsinki Declaration and the ethical guidelines for Medical and Health Research Involving Human Subjects ('World Medical Association declaration of Helsinki: Ethical principles for medical research involving human subjects', 2013). Informed consent was obtained from all participants before inclusion. In case they were minors, consent was also obtained from their parents or legal guardians.

Data collection and processing

RNA-seq datasets of DP and PDL tissue subjected to OF in vivo were produced by the authors in previous works [3, 5] and are publicly available at the Sequence Read Archive (SRA). The experimental settings of these datasets were as follows: healthy patients seeking orthodontic treatment who needed premolar extractions due to orthodontic reasons were recruited. The transcriptomic profiles of the pulp and periodontal tissues from the premolars extracted before orthodontic treatment (controls) were compared those extracted after 7- and 28-days of orthodontic force application respectively. Details regarding study design, patient recruitment, OTM model, and sequencing workflow are available in the Supplementary Methods. For the present study, the complete transcriptomic datasets of PDL and DP tissue (PRJNA1137899, PRJNA1136245) were downloaded from SRA with raw sequence files (.fastq files) and checked for quality by using FastQC v0.11.7 [25]. Adapter sequences were filtered out with ea-utils fastq-mcf v1.05 [26]. For accurate mapping, splice-aware alignment was performed with HISAT2 [27] against the human hg38 genome using default parameters. Reads that mapped to multiple loci in the reference genome were discarded. The resultant BAM alignment files were processed using

Samtools v1.5. Quantification of reads per gene was carried out with HT-seq Count v0.10.0, Python v2.7.14 [28].

Differential analysis of gene expression

Differential analysis of gene expression was performed with SAS software version 9.4 of the SAS System for Windows, as well as R-Software (R Core Team 2024). R: A language and environment for statistical computing. (R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/). For each gene, linear mixed models (LIN) with a random effect for subject were used to compare the log-transformed normalized count between groups. Analyses were performed separately for PDL and DP tissue and for the comparisons of 7 days versus control and 28 days versus control, respectively. Only genes with at least three subjects having at least a count equal to 10 were included in the analysis. Each model contained main effects of batch (reflecting that samples were sequenced across different experimental runs), sex, age and experimental group. Two extensions of the model were considered to evaluate the research questions. The first extension additionally modelled the interaction between sex and experimental group, which allows to verify if the effect of OF on the tissues differed between males and females, while the second extension modelled the interaction between age and experimental group. For the interaction with age, two versions were considered: (1) assuming linearity for the effect of age, (2) allowing nonlinearity using restricted cubic splines. If evidence for nonlinearity was present, conclusions were based on the model using splines for the effect of age. P-values were adjusted with the Benjamini–Hochberg procedure to control the false discovery rate (FDR). FDR < 0.05 was used to identify differentially expressed genes (DEGs). As a sensitivity analysis, results were also verified using the negative binomial model as implemented in the DESeq2 package. Note however that this approach did not allow to add a random effect of subject to handle the potential correlation between both jaws.

Bioinformatics

Principal component analysis (PCA) was applied to all normalized counts to visually assess transcriptome variation between samples. To identify key cellular and biological processes associated with DEGs unique to our comparative datasets, we applied Gene Ontology (GO) enrichment analysis. Signaling pathways associated with these DEGs were further examined using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. GO and KEGG enrichment analyses were performed using the Database for Annotation Visualization and Integrated Discovery (DAVID) [29].

Results

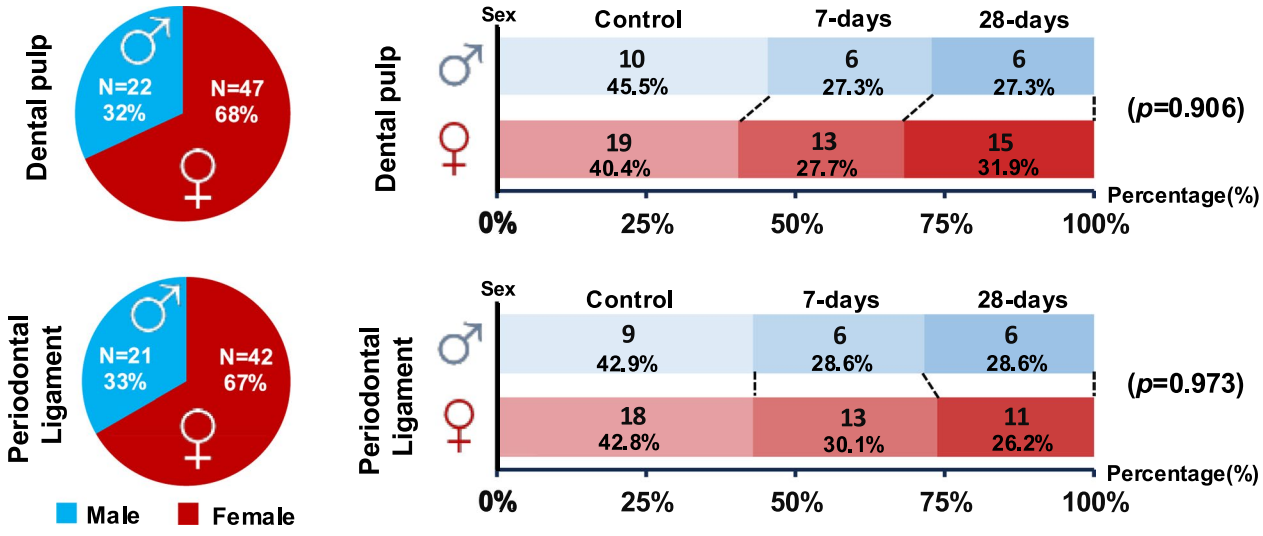
The previously published DP and PDL RNA-seq datasets were downloaded [3, 5]. The sex and age distribution per group (control without OF, 7 and 28 days of OF) and per tissue type (dental pulp and periodontal ligament) are presented in Fig. 1. In total, 69 DP (47 females/22 males, 16.81 ± 6.66 years old) and 63 PDL (42 females/21 males, 16.51 ± 6.67 years old) samples were included in the datasets. Sex distribution across experimental groups was compared using the Chi-square test of independence. No significant differences were found in sex distribution for either dental pulp ($p = 0.906$) or periodontal ligament samples ($p = 0.973$). To assess age distribution across experimental groups, we performed one-way ANOVA. No significant differences were found between the control, 7-day, and 28-day groups for either DP or PDL tissues (DP: $p = 0.517$; PDL: $p = 0.562$), indicating comparable age distributions. The number of sex- and age-related DEGs in both tissues after 7 and 28 days of OF application compared to the control group, are presented in Table 1.

Influence of sex on the DP transcriptomic patterns during OTM

To investigate the potential sex-dependent differences in the transcriptomic responses of DP during OTM, we analyzed PCA results of male and female DP tissues subjected to 7 days and 28 days of OF, as well as control groups without OF application (CG). The PCA results revealed minimal differences in the transcriptomic profiles of male and female pulp tissue subjected to 7 days of OF, 28 days of OF and CG (Fig. 2A). When comparing the 17,338 genes found between CG vs. 7-days with the 16,966 genes found between CG vs. 28-days, only one gene (ZNF711) was found to exhibit differential expression between males and females, 28 days after the application of OF (Table 2). This gene was downregulated in females (log2FC: – 0.26) and upregulated in males (log2FC: 0.51) with an FDR less than 0.05 (Fig. 2B).

Influence of sex on the PDL transcriptomic patterns during OTM

To study the sex-related differences in transcriptomic profiles of PDL tissue during OTM, we also conducted PCA. (Fig. 2A) The two principal components of the PCA, which account for 32.4% of the total data variance, show that the female and male samples are clearly separated after 7 days of OTM. However, this was not the case in the CG and after 28 days of OTM. Within the 15,807 genes compared between the CG vs. 7 days, 505 genes were expressed significantly different between females and males based on the FDR < 0.05. From these, a total of 444 genes (87.92%) exhibited opposing expression trends in men and women: 239 DEGs were upregulated



Tissue (N=Sample size)	Variable		Control Group	7-days of Orthodontic Force	28-days of Orthodontic Force
Dental Pulp (N = 69)	Batch	1	20/29 (68.97%)	0/19 (0.00%)	14/21 (66.67%)
		2	9/29 (31.03%)	19/19 (100.00%)	7/21 (33.33%)
	Sex	Female	19/29 (65.52%)	13/19 (68.42%)	15/21 (71.43%)
		Male	10/29 (34.48%)	6/19 (31.58%)	6/21 (28.57%)
	Age	Mean ± SD	17.55 ± 8.40	15.32 ± 3.71	17.14 ± 6.24
Periodontal Ligament (N = 63)	Batch	3	22/27 (81.48%)	0/19 (0.00%)	17/17 (100.00%)
		4	5/27 (18.52%)	19/19 (100.00%)	0/17 (0.00%)
	Sex	Female	18/27 (66.67%)	13/19 (68.42%)	11/17 (64.71%)
		Male	9/27 (33.33%)	6/19 (31.58%)	6/17 (35.29%)
	Age	Mean ± SD	17.48 ± 8.71	15.32 ± 3.71	16.29 ± 5.74

Fig. 1 Sex and age distribution of the patients included per group (control, 7- and 28-days of Orthodontic Force) and per dental tissue (dental pulp and periodontal ligament). Values of $p < 0.05$ were considered statistically significant different. *SD* standard deviation

in females and downregulated in males and vice-versa in 205 DEGs. From the 61 remaining genes, 29 DEGs were upregulated in both females and males and 32 were downregulated in both sexes. (Fig. 2B) GO analysis reveals significant sex differences in biological processes such as upregulation of angiogenesis in females, including "positive regulation of angiogenesis" and "regulation of angiogenesis." Additionally, other cell activities, such as "mRNA splicing" "chromatin remodeling" and "negative regulation of autophagy" were upregulated in males, further highlighting variation between sexes. KEGG analysis shows that the signal pathways "endocytosis", "focal adhesion" and "ECM-receptor interaction" are different between sexes. Within the 15,414 genes compared between the CG vs. 28 days, only 4 genes (MNS1, PIH1D1, NMNAT1, RPS19P7) were found to be differentially expressed. (Table 2, Fig. 3A).

Influence of age on the DP transcriptomic patterns during OTM

To explore the age-related DEGs in transcriptomic profiles of DP tissue during OTM, we also applied LIN model to assess the effect of age. For some genes, restricted cubic splines model was used where evidence for nonlinearity was present. As shown in Table 3, 18 DEGs (STIMATE, STXBP6, KCTD9P1, NACA3P, FBXL9P, RASSF8-AS1, PITPNM2, GABRG1, SPIRE2, HMGB1P21, SYT2, PSMD1, VPS26A, DUSP5, SYTL3, TLL1, ASB7, LAMTOR4) were found to be significantly associated with age 7 days after the application of OF. At 28 days, only 10 DEGs (ARHGEF34P, SNRPA1P1, SIN3A,

Table 1 Number of sex- and age-related differentially expressed genes (DEGs) found in dental pulp and periodontal ligament after 7 and 28 days of orthodontic force application compared to the control group without orthodontic force

			With FDR correction		Number of genes	Percentage
			$p < 0.05$ (NB)	$p < 0.05$ (LIN)		
Number of sex—related differentially expressed genes	Dental pulp	7-days vs. Control	No	No	17,325	99.92%
			Yes	No	13	0.07%
			No	Yes	0	0.00%
			Yes	Yes	0	0.00%
		28-days vs. Control	No	No	16,963	99.99%
			Yes	No	2	0.01%
			No	Yes	0	0.00%
			Yes	Yes	1	0.01%
	Periodontal ligament	7-days vs. Control	No	No	15,014	94.98%
			Yes	No	288	1.82%
			No	Yes	53	0.34%
			Yes	Yes	452	2.86%
		28-days vs. Control	No	No	15,409	99.97%
			Yes	No	1	0.01%
			No	Yes	2	0.01%
			Yes	Yes	2	0.01%

Table 1 (continued)

			p < 0.05 (NB)	p < 0.05 (LIN)	Evidence of nonlinearity	Number of genes	Percentage
Number of age—related differentially expressed genes	Dental pulp	7-days vs. Control	No	No	No	15,667	90.37%
			No	No	Yes	1653	9.54%
			Yes	No	No	2	0.01%
			Yes	No	Yes	0	0.00%
			No	Yes	No	0	0.00%
			No	Yes	Yes	15	0.09%
			Yes	Yes	No	1	0.01%
			Yes	Yes	Yes	0	0.00%
		28-days vs. Control	No	No	No	13,700	80.75%
			No	No	Yes	3256	19.19%
			Yes	No	No	4	0.02%
			Yes	No	Yes	0	0.00%
			No	Yes	No	0	0.00%
			No	Yes	Yes	6	0.03%
			Yes	Yes	No	0	0.00%
			Yes	Yes	Yes	0	0.00%
	Periodontal ligament	7-days vs. Control	No	No	No	15,319	96.91%
			No	No	Yes	488	3.09%
			Yes	No	No	0	0.00%
			Yes	No	Yes	0	0.00%
			No	Yes	No	0	0.00%
			No	Yes	Yes	0	0.00%
			Yes	Yes	No	0	0.00%
			Yes	Yes	Yes	0	0.00%
		28-days vs. Control	No	No	No	14,541	94.34%
			No	No	Yes	687	4.45%
			Yes	No	No	177	1.15%
			Yes	No	Yes	5	0.03%
			No	Yes	No	0	0.00%
			No	Yes	Yes	2	0.01%
			Yes	Yes	No	1	0.01%
			Yes	Yes	Yes	1	0.01%

Note that age has been considered as a continuous variable. Significant p-values refer to significant interactions with sex and age, respectively. *NB* negative binomial model, *LIN* linear mixed model

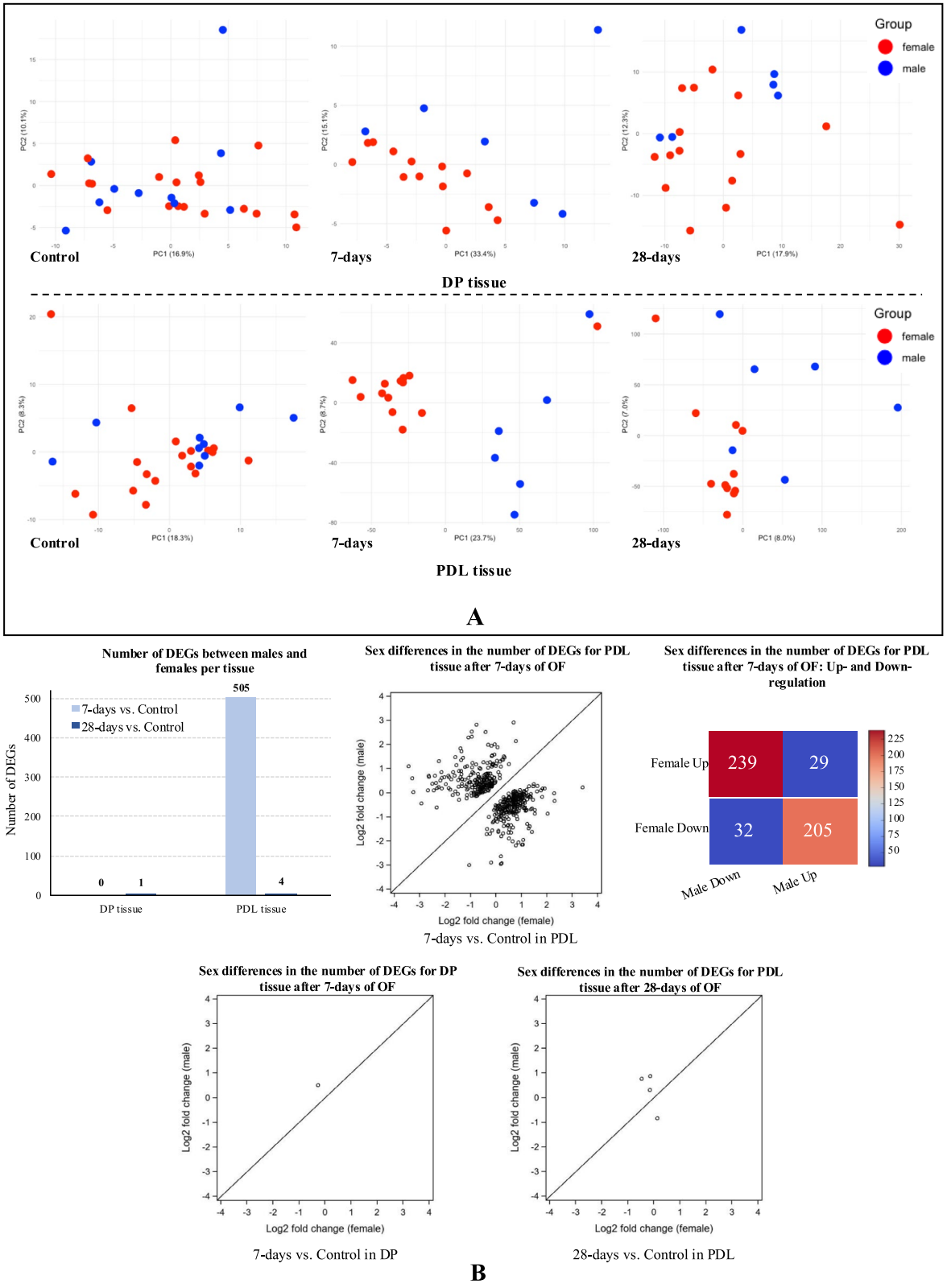


Fig. 2 **A** Principal component analysis (PCA) of the comparisons between females and males in the three different experimental groups. **B** Number of differentially expressed genes (DEGs) between the females and males in the two different tissues, volcano plot of 7-days vs. Control and 28-days vs. Control in PDL tissue and 7-days vs. Control in DP tissue

Table 2 Top 25 differentially expressed genes (DEGs) between female and male patients in dental pulp and periodontal ligament tissues after 7 and 28 days of orthodontic force application compared to the control group without orthodontic force

Tissue	Group	Gene	Female			Male			P-value interaction (FDR)
			Log2fold	Ratio	P-value (FDR)	Log2fold	Ratio	P-value (FDR)	
Dental pulp	7-days vs. Control	/	/	/	/	/	/	/	/
	28-days vs. Control	ZNF711	− 0.26	0.83	0.8258	0.51	1.42	0.1879	0.0089
Periodontal ligament	7-days vs. Control	C11orf58	− 1.02	0.49	< .0001	0.43	1.35	0.3971	< .0001
		ISG20L2	− 0.66	0.63	0.0080	0.65	1.57	0.1183	< .0001
		GIT1	0.82	1.76	0.0178	− 0.97	0.51	0.0816	< .0001
		REEP4	1.15	2.22	0.0003	− 0.41	0.75	0.5377	< .0001
		CFDP1	− 0.34	0.79	0.1258	0.72	1.64	0.0355	0.0001
		YJU2B	0.23	1.17	0.3451	− 0.87	0.55	0.0092	0.0001
		FASTK	0.72	1.64	0.0177	− 0.73	0.60	0.1570	0.0001
		WDR82	− 0.49	0.71	0.0213	0.54	1.46	0.1301	0.0002
		LTB4R	1.22	2.33	0.0216	− 1.29	0.41	0.1566	0.0002
		TNS2	0.65	1.57	0.0383	− 0.82	0.56	0.1089	0.0002
		DCAF15	0.65	1.57	0.2877	− 2.02	0.25	0.0224	0.0003
		RPL17P26	− 0.41	0.75	0.5559	2.52	5.72	0.0068	0.0003
		LZIC	− 0.66	0.63	0.0084	0.51	1.42	0.2773	0.0003
		PRPF4	− 0.70	0.62	0.0189	0.66	1.59	0.2151	0.0003
		GLYR1	− 0.71	0.61	0.0005	0.22	1.17	0.6182	0.0003
		FLNA	0.44	1.36	0.0110	− 0.33	0.79	0.3046	0.0005
		SLC27A3	0.20	1.15	0.4996	− 0.98	0.51	0.0131	0.0005
		SCYL1	1.17	2.25	0.0061	− 0.71	0.61	0.3937	0.0006
		LTBP4	0.19	1.14	0.5629	− 1.12	0.46	0.0123	0.0006
		R3HDM4	1.03	2.04	0.0004	− 0.25	0.84	0.7106	0.0006
		MTND6P4	− 1.87	0.27	0.0014	0.70	1.63	0.5756	0.0006
		FUZ	0.59	1.51	0.2756	− 1.65	0.32	0.0407	0.0007
		VPS28	0.75	1.68	0.0009	− 0.23	0.85	0.6565	0.0008
		RPL26P36	− 0.42	0.75	0.1362	0.76	1.70	0.0907	0.0008
		TVP23C	− 2.26	0.21	< .0001	0.23	1.17	0.8867	0.0008
	28-days vs. Control	MNS1	0.14	1.10	0.9836	− 0.83	0.56	0.0006	0.0072
		PIH1D1	− 0.14	0.91	0.9836	0.86	1.82	0.0027	0.0251
		NMNAT1	− 0.47	0.72	0.3490	0.76	1.70	0.0862	0.0345
		EMP2	− 0.15	0.90	0.5182	0.31	1.24	0.0675	0.0400

PCOLCE2, MARK1, CYP1B1-AS1, MROCKI, PARP1P1, MRPS35-DT, TXNL4B) were significantly associated with age. (Table 3, Fig. 3B) Plots with the predicted relation for both models (assuming linearity or not) are given for the top genes in the Fig. 4A and B, depicting the trend in up- or down-regulation of the genes affected by ages.

Influence of age on the PDL transcriptomic patterns during OTM

To investigate the age-related DEGs in transcriptomic profiles of PDL tissue during OTM, we applied the same models as DP tissue. No genes were found to be significantly associated with age after 7 days of OF on PDL tissue. However, after 28 days of OF, 181 genes were found significantly associated with age. Plots showing the predicted relationship for both models (assuming linearity or allowing non-linearity) are provided for the top genes in Table 4 and Fig. 4C. GO analysis indicates that biological processes such as "chromatin organization", "angiogenesis", "ribosomal small subunit biogenesis" and "negative regulation of the Notch signaling pathway" are influenced by age. Similarly, KEGG analysis highlights age-related associations in signaling pathways including "Ribosome", "Ribosome biogenesis in eukaryotes", and "Cytoskeleton in muscle cells" (Fig. 3C).

A summary of the findings of this study can be found in Supplementary Tables S1 and S2.

Discussion

This study investigates the influence of sex and age on the transcriptomic profiles of DP and PDL tissue subjected to different lengths of OF. Most of the previous

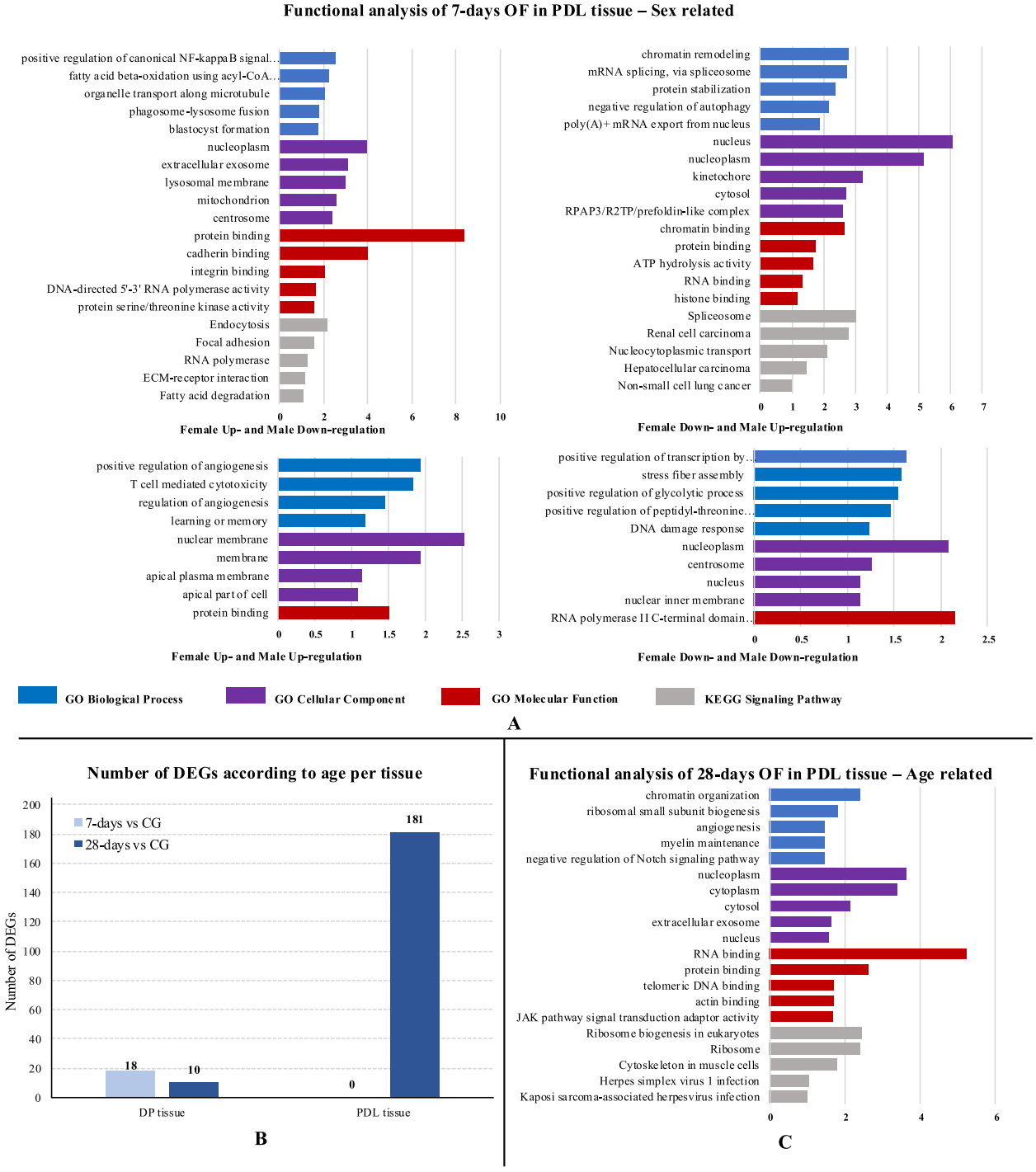


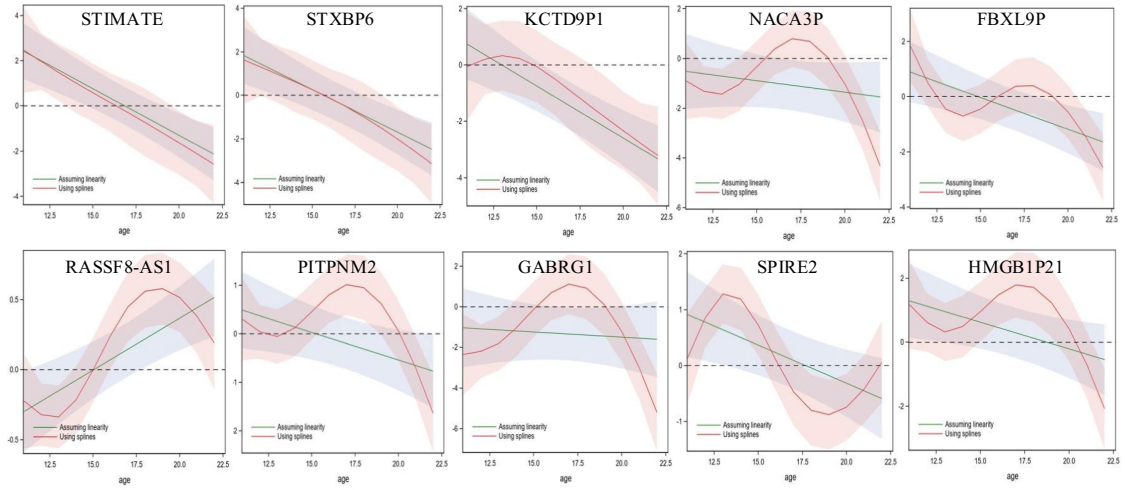
Fig. 3 **A** Top 5 biological processes (BP), cellular components (CC), molecular functions (MF) and signal pathways (KEGG) associated with sex-related genes after 7-days OTM in PDL tissue. **B** Number of differentially expressed genes (DEGs) between the different age in two different tissues. **C** Top 5 BP, CC, MF and KEGG associated with age-related genes after 28-days OTM in DP tissue

transcriptomic studies investigating sex differences in medicine typically perform DESeq2 package analyses for each sex group independently, identifying DEGs based on adjusted *P*-values and log2FC thresholds, followed by a comparison of DEGs between sexes. However, since the underlying model of DESeq2 is a negative binomial model (NB), the downside of this approach is that the potential correlation between data from the same subject cannot be taken into account. This was specifically important in our datasets since they contain upper and lower, first and second premolars, which could introduce bias. Therefore, ignoring the correlation might yield

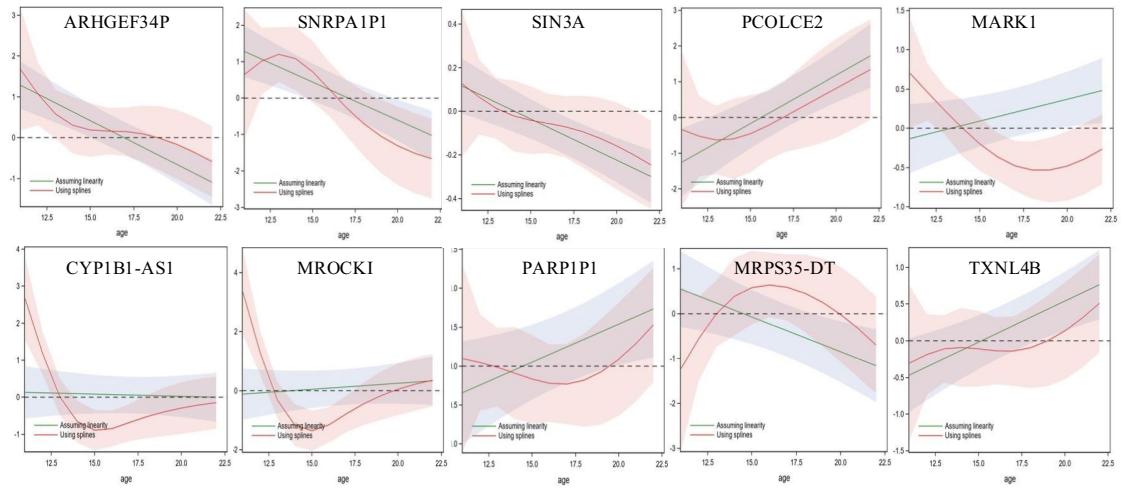
Table 3 Top 25 age-related differentially expressed genes (DEGs) in dental pulp tissue after 7 and 28 days of orthodontic force application compared to the control group without orthodontic force

Group	Gene	Log2 fold changes assuming linearity							Log2 fold changes using splines							*P splines*
		At 12yrs	P	At 17yrs	P	At 21yrs	P	P int	At 12yrs	P	At 17yrs	P	At 21yrs	P	P int	
7-days vs. Control	STIMATE	2.00	0.0005	− 0.06	0.8832	− 1.71	0.0016	0.0049	1.96	0.0020	− 0.28	0.6974	− 2.10	0.0042	0.0295	0.8887
	STXBP6	1.42	0.0185	− 0.53	0.2408	− 2.08	0.0003	0.0328	1.28	0.0543	− 0.54	0.4770	− 2.56	0.0008	0.0912	0.8576
	KCTD9P1	0.36	0.5339	− 1.49	0.0006	− 2.96	< 0.0001	0.0328	0.21	0.7443	− 0.95	0.1838	− 2.78	0.0001	0.3561	0.5209
	NACA3P	− 0.62	0.3761	− 1.08	0.0360	− 1.45	0.0288	0.9996	− 1.32	0.0100	0.79	0.1713	− 2.51	0.0000	0.0006	0.0000
	FBXL9P	0.65	0.1979	− 0.49	0.1897	− 1.41	0.0036	0.4967	0.48	0.2739	0.37	0.4512	− 1.45	0.0041	0.0014	0.0011
	RASSF8-AS1	− 0.22	0.0927	0.15	0.1416	0.44	0.0006	0.3274	− 0.32	0.0047	0.45	0.0004	0.38	0.0044	0.0014	0.0015
	PITPNM2	0.37	0.3098	− 0.20	0.4609	− 0.66	0.0607	0.8724	0.04	0.8852	1.01	0.0015	− 0.71	0.0326	0.0021	0.0000
	GABRG1	− 1.09	0.2235	− 1.35	0.0424	− 1.55	0.0718	0.9996	− 2.20	0.0011	1.12	0.1369	− 3.02	0.0001	0.0032	0.0000
	SPIRE2	0.77	0.0276	0.09	0.7236	− 0.45	0.1762	0.6593	0.86	0.0015	− 0.47	0.1226	− 0.42	0.1775	0.0059	0.0000
	HMGB1P21	1.13	0.0365	0.29	0.4645	− 0.38	0.4626	0.8724	0.61	0.1846	1.79	0.0006	− 0.71	0.1835	0.0068	0.0003
	SYT2	− 0.45	0.3202	− 0.47	0.1667	− 0.48	0.2710	0.9996	− 0.47	0.1528	0.76	0.0426	0.53	0.1647	0.0148	0.0000
	PSMD1	0.15	0.1553	0.25	0.0015	0.33	0.0012	0.9996	0.06	0.4675	0.53	0.0000	0.22	0.0166	0.0161	0.0000
	VPS26A	− 0.17	0.1346	− 0.15	0.0820	− 0.13	0.2395	0.9996	− 0.11	0.2028	− 0.56	0.0000	− 0.26	0.0123	0.0161	0.0000
	DUSP5	0.18	0.5959	− 0.05	0.8554	− 0.22	0.4861	0.9996	0.49	0.0483	− 0.71	0.0113	0.27	0.3409	0.0171	0.0000
	SYTL3	0.57	0.3527	− 0.26	0.5669	− 0.92	0.1142	0.9587	0.05	0.9179	1.00	0.0851	− 1.69	0.0044	0.0171	0.0011
	TLL1	− 0.21	0.5653	0.12	0.6657	0.38	0.2787	0.9996	0.06	0.8492	− 0.96	0.0036	0.32	0.3477	0.0378	0.0001
	ASB7	0.13	0.4487	0.00	0.9963	− 0.10	0.5301	0.9996	0.18	0.1903	− 0.45	0.0038	− 0.18	0.2397	0.0378	0.0001
	LAMTOR4	0.05	0.7530	0.13	0.2436	0.19	0.1726	0.9996	− 0.10	0.4281	0.15	0.2812	− 0.15	0.2878	0.0472	0.0001
28-days vs. Control	ARHGEF34P	1.06	< 0.0001	− 0.01	0.9425	− 0.88	0.0008	0.0004	1.06	0.0063	0.15	0.6192	− 0.36	0.4127	0.0567	0.4239
	SNRPA1P1	1.06	0.0011	0.02	0.9367	− 0.82	0.0095	0.0221	1.01	0.0294	− 0.27	0.4828	− 1.51	0.0070	0.1651	0.4939
	SIN3A	0.08	0.1848	− 0.11	0.0120	− 0.26	< 0.0001	0.0221	0.06	0.4619	− 0.07	0.3227	− 0.20	0.0541	0.2233	0.9402
	PCOLCE2	− 0.98	0.0269	0.37	0.2709	1.46	0.0005	0.0221	− 0.52	0.3596	0.03	0.9560	1.08	0.1332	0.4482	0.1585
	MARK1	− 0.08	0.7138	0.20	0.1969	0.43	0.0278	0.5510	0.46	0.0128	− 0.48	0.0031	− 0.39	0.0882	0.0346	0.0000
	CYP1B1-AS1	0.12	0.7169	0.06	0.8142	0.01	0.9715	0.9569	1.19	0.0001	− 0.68	0.0073	− 0.21	0.5626	0.0346	0.0000
	MROCKI	− 0.08	0.8495	0.13	0.6783	0.29	0.4460	0.8576	1.29	0.0007	− 0.79	0.0127	0.22	0.6288	0.0346	0.0000
	PARP1P1	− 0.24	0.4304	0.25	0.2937	0.64	0.0288	0.4956	0.05	0.8836	− 0.23	0.3803	0.29	0.4458	0.0346	0.0000
	MRPS35-DT	0.39	0.3166	− 0.38	0.1941	− 1.00	0.0079	0.3929	− 0.56	0.2428	0.59	0.1192	− 0.34	0.5442	0.0346	0.0030
	TXNL4B	− 0.35	0.1287	0.20	0.2493	0.65	0.0033	0.1895	− 0.19	0.4994	− 0.14	0.5603	0.31	0.3717	0.0346	0.0113

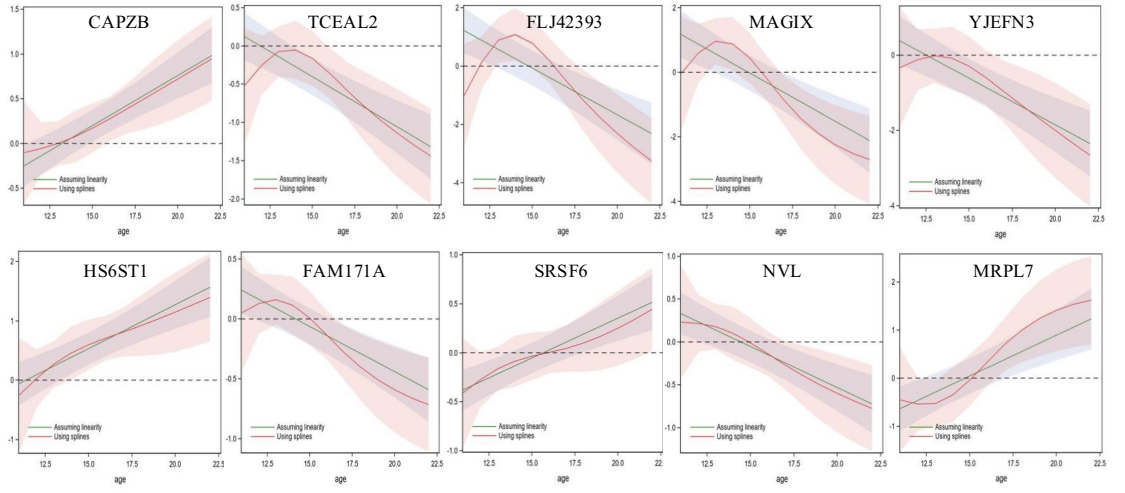
Estimates are given at arbitrarely chosen age levels (12, 17 and 21 years). P int: P-value for the interaction, indicating if the log2 fold change depends on age. P splines: P-value for the evidence of nonlinearity, i.e. comparing the model assuming linearity with the model using splines for the effect of age



A. DP Tissue 7-days vs. Control



B. DP Tissue 28-days vs. Control



C. PDL Tissue 28-days vs. Control

Fig. 4 Plots with the predicted relation of top 10 age-related differential expressed genes obtained from the model assuming linearity and from the model allowing nonlinearity (using splines). **A** Dental pulp (DP) tissue 7-days vs. Control, **B** DP tissue 28-days vs. Control, **C** Periodontal ligament (PDL) tissue 28-days vs. Control. Shaded area refers to the pointwise 95% confidence interval. Note that there are no DEGs in PDL tissue 7-days vs. control

Table 4 Top 25 age-related differentially expressed genes (DEGs) in periodontal ligament tissue after 7 and 28 days of orthodontic force application compared to the control group without orthodontic force

Group	Gene	Log2 fold changes assuming linearity							Log2 fold changes using splines							*P splines*
		At 12yrs	P	At 17yrs	P	At 21yrs	P	P int	At 12yrs	P	At 17yrs	P	At 21yrs	P	P int	
7-days vs. Control	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/
28-days vs. Control	CAPZB	− 0.14	0.2002	0.42	< 0.0001	0.87	< 0.0001	< 0.0001	− 0.06	0.7087	0.38	0.0062	0.83	0.0003	0.0561	0.5571
	TCEAL2	− 0.01	0.9499	− 0.66	< 0.0001	− 1.19	< 0.0001	0.0016	− 0.25	0.2010	− 0.57	0.0022	− 1.29	< 0.0001	0.1049	0.3436
	FLJ42393	0.89	0.0126	− 0.71	0.0323	− 1.99	< 0.0001	0.0018	0.11	0.8173	− 0.51	0.2410	− 2.80	0.0001	0.0023	0.0914
	MAGIX	0.87	0.0093	− 0.62	0.0401	− 1.82	< 0.0001	0.0043	0.60	0.1937	− 0.87	0.0258	− 2.51	0.0002	0.1423	0.1144
	YJEFN3	0.13	0.6649	− 1.11	< 0.0001	− 2.10	< 0.0001	0.0043	− 0.12	0.7684	− 0.96	0.0209	− 2.33	0.0006	0.0758	0.6944
	HS6ST1	0.09	0.5838	0.83	< 0.0001	1.42	< .0001	0.0043	0.03	0.9099	0.81	0.0001	1.27	0.0004	0.3156	0.8889
	FAM171A1	0.16	0.0693	− 0.21	0.0102	− 0.51	< 0.0001	0.0075	0.13	0.3115	− 0.28	0.0158	− 0.66	0.0006	0.2631	0.5431
	SRSF6	− 0.30	0.0020	0.11	0.2047	0.43	0.0010	0.0075	− 0.28	0.0539	0.04	0.7099	0.34	0.0984	0.1720	0.9083
	NVL	0.23	0.0382	− 0.24	0.0167	− 0.63	< 0.0001	0.0075	0.22	0.2026	− 0.27	0.0610	− 0.69	0.0050	0.3685	0.9616
	MRPL17	− 0.46	0.0324	0.38	0.0582	1.06	0.0003	0.0075	− 0.54	0.0537	0.70	0.0132	1.54	0.0008	0.1720	0.2227
	TMED3	− 0.31	0.0015	0.10	0.2587	0.42	0.0015	0.0078	− 0.33	0.0267	0.11	0.3821	0.43	0.0472	0.3685	0.9781
	LRRK2	0.34	0.0340	− 0.31	0.0336	− 0.84	0.0002	0.0096	0.30	0.1649	− 0.40	0.0403	− 1.03	0.0018	0.3685	0.1040
	ISCA1	− 0.47	0.0034	0.19	0.1940	0.72	0.0012	0.0096	− 0.59	0.0099	0.16	0.4075	0.60	0.0686	0.1505	0.2666
	PRDX6	− 0.28	0.0147	0.19	0.0669	0.57	0.0003	0.0096	− 0.35	0.0387	0.23	0.1023	0.58	0.0176	0.3265	0.7749
	SPART	0.07	0.2709	− 0.17	0.0021	− 0.36	< 0.0001	0.0096	− 0.02	0.8545	− 0.15	0.0598	− 0.43	0.0012	0.3322	0.4113
	RCN1	0.01	0.9209	0.46	< 0.0001	0.81	< 0.0001	0.0096	0.11	0.5025	0.39	0.0221	0.80	0.0035	0.5190	0.7690
	TK2	0.12	0.3399	− 0.40	0.0006	− 0.81	< 0.0001	0.0109	0.05	0.7819	− 0.42	0.0098	− 0.91	0.0010	0.4528	0.8817
	VEGFB	− 0.27	0.1178	0.41	0.0076	0.96	< 0.0001	0.0113	− 0.22	0.3806	0.38	0.0795	1.04	0.0045	0.1806	0.7554
	MALAT1	0.39	0.0123	− 0.19	0.1916	− 0.65	0.0020	0.0113	0.31	0.1538	− 0.25	0.2439	− 0.94	0.0078	0.3325	0.7175
	UBE2Q2P2	0.49	0.0343	− 0.37	0.0825	− 1.06	0.0007	0.0117	0.52	0.0955	− 0.39	0.2115	− 1.04	0.0395	0.5184	0.4433
	PPP1R12B	0.10	0.4123	− 0.38	0.0008	− 0.78	< 0.0001	0.0125	0.01	0.9696	− 0.30	0.0674	− 0.66	0.0160	0.6492	0.5418
	YTHDC1	0.18	0.2286	− 0.37	0.0096	− 0.82	< 0.0001	0.0125	0.08	0.6895	− 0.39	0.0712	− 0.95	0.0064	0.5625	0.6002
	MRC2	− 0.19	0.0223	0.13	0.0926	0.38	0.0008	0.0160	− 0.30	0.0135	0.19	0.0818	0.38	0.0335	0.1806	0.5571
	DMTF1	0.34	0.0614	− 0.31	0.0793	− 0.83	0.0009	0.0162	0.31	0.1978	− 0.37	0.1405	− 1.01	0.0127	0.5272	0.3616
	WDR19	0.05	0.7182	− 0.48	0.0001	− 0.91	0.0000	0.0162	− 0.13	0.5109	− 0.42	0.0127	− 0.92	0.0014	0.5520	0.4901

Estimates are given at arbitrarely chosen age levels (12, 17 and 21 years). P int: P-value for the interaction, indicating if the log2 fold change depends on age. P splines: P-value for the evidence of nonlinearity, i.e. comparing the model assuming linearity with the model using splines for the effect of age

too liberal p-values and thus inflate the type-1 error (for the evaluation of OF effect). As an alternative approach, a linear mixed model with a random effect for subject was used in our study, with log-transformed normalized count as outcome [30]. Regarding age differences, previous research primarily groups patients into categories based on chronological age, overlooking the physiological age variations possibly caused by individual differences. Treating age as a categorical parameter also fails to capture the gradual changes that age can present in clinical settings. Instead, our study treated age as a continuous variable, exploring whether the effect of OF depended on age in a linear way. Additionally, allowing the effect of age to be nonlinear (using splines), a distinction was made between settings where linearity was plausible and where splines were required to model the effect of age and its interaction.

Influence of sex on the transcriptomic pattern of the DP during OTM

Previous studies have shown that the DP exhibits a marked response during the early phase of OTM, involving immune activation, hypoxia, and DNA damage. In contrast, later phases are characterized by cell adhesion, migration, tissue organization, repair, and dentin formation [5]. In our study, ZNF711 was the only gene found to be differentially expressed between males and females after 28 days of OTM. ZNF711 (Zinc Finger Protein 711) is a transcriptional activator located on the X chromosome, which can bind to the promoters of target genes and recruit PHF8 (PHD Finger Protein 8) histone demethylase, thereby activating the expression of genes involved in neuronal development, such as KDM5C. KDM5C also plays a role in immune- and inflammatory related pathways [31]. Our results showed downregulation of ZNF711 in females and upregulation in males, suggesting enhanced pulp tissue repair and inflammatory regulation in men following OF application, possibly resulting in a better recovery process. Previous studies suggest that varying levels of estrogen can lead to differences in dental pulp blood flow and affect the odonto/osteogenic differentiation of dental pulp stem cells [9, 10]. However, the present study did not find a significant influence of sex hormones on the gene expression of DP tissue. Additionally, while our comparison of sex differences in DP tissue under OF did not yield many DEGs meeting the threshold of statistical significance, they hint at the underlying biological changes occurring within the DP tissue. Among the top 100 DEGs, identified biological processes related to inflammation, including "DNA repair", "positive regulation of T cell proliferation", "positive regulation of leukocyte migration" and "positive regulation of interleukin-4 production" can be identified. These findings underscore the role of inflammatory regulation in the differential response of DP to OF in males and females, suggesting a direction for further research (Figs. 5 and 6).

Influence of sex on the transcriptomic pattern of the PDL during OTM

In contrast to DP tissue, PDL tissues exhibited significant sex-specific expression after 7 days of OTM (505 genes).

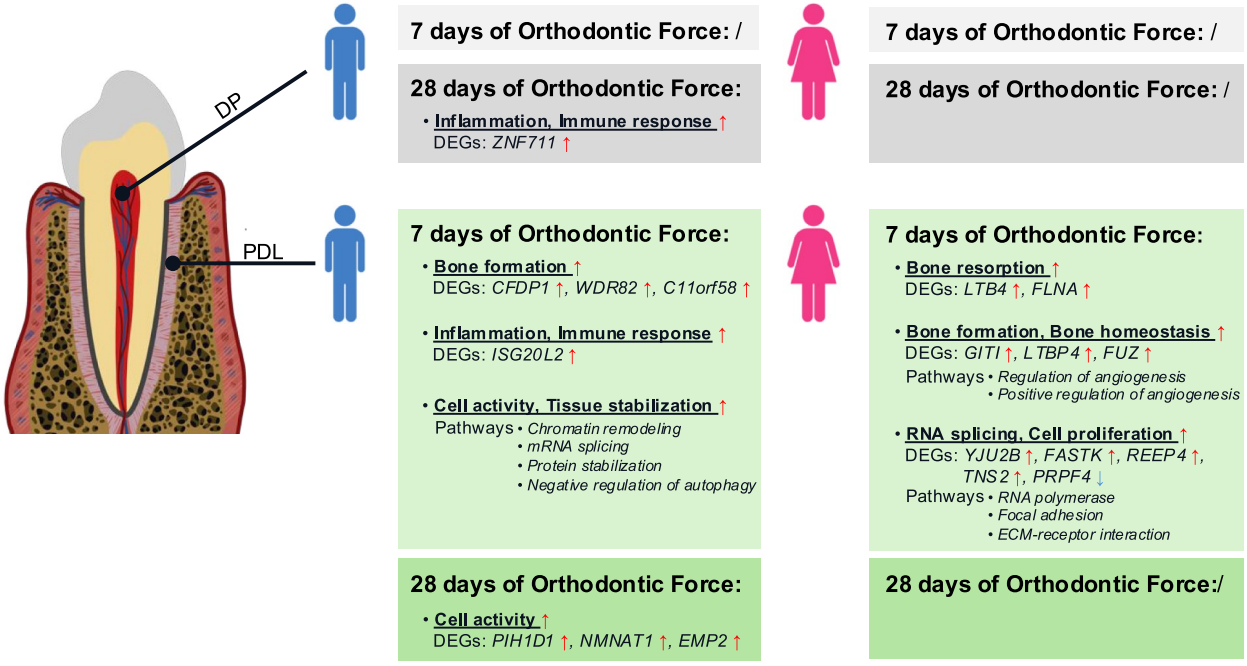


Fig. 5 Sex-related differences in dental pulp and periodontal ligament responses to orthodontic force

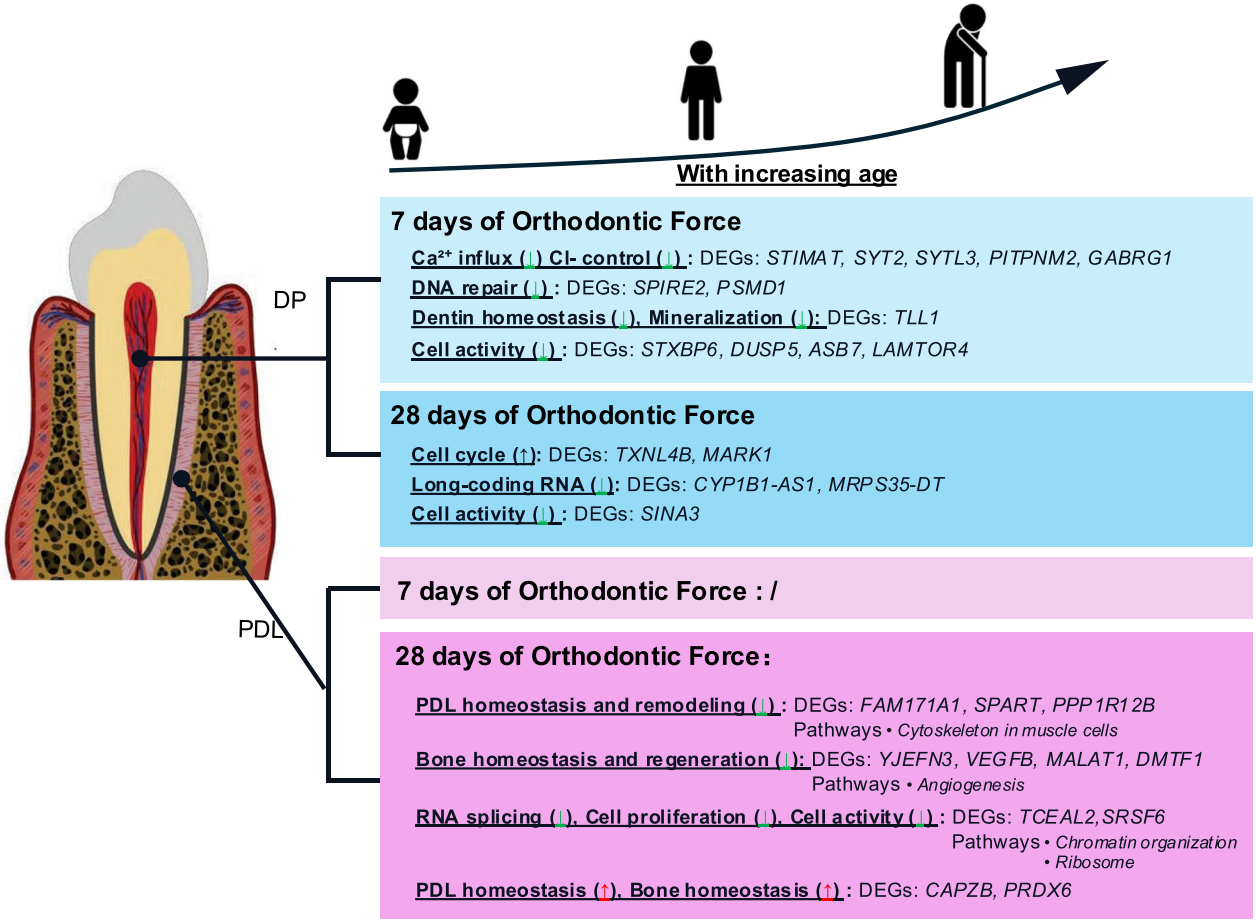


Fig. 6 Age-related differences in dental pulp and periodontal ligament responses to orthodontic force

Among the top genes, CFDP1 (Craniofacial Development Protein 1) and WDR82 (WD Repeat Domain 82) are linked to craniofacial development, osteogenesis and osteoblast differentiation [32–34]. Both genes exhibit decreased expression in females. In contrast, genes associated with bone resorption, such as LTB4 (Leukotriene B4 Receptor), which promotes osteoclast differentiation [35], and FLNA (Filamin A), linked to osteoclastogenesis [36], show upregulation in females, suggesting higher osteoclastic activity in early OTM stages. Interestingly, some osteogenic genes, including GIT1 (GIT ArfGAP 1) and LTBP4 (Latent Transforming Growth Factor Beta Binding Protein 4), are also upregulated in females. GIT1 plays a key role in bone regeneration through RAC1 signaling and the ERK1/2/NRF2 pathway [37], while LTBP4 is essential for TGF-β1-mediated osteogenesis and alveolar bone remodeling [38], supporting bone homeostasis during OTM. Previous research indicates that estrogen can influence bone remodeling through several mechanisms, including reducing oxidative stress and enhancing the survival and activity of bone-forming cells via the ERK1/2/NRF2 pathway [39]. Additionally, estrogen affects bone remodeling by interacting with TGF-β [40]. Thus, the findings of this experiment regarding the altered expression of GIT1 and LTBP4 provide theoretical support for prior studies, demonstrating that hormone levels indeed impact the metabolism and overall bone remodeling dynamics, thereby affecting OTM rate.

Additionally, C11orf58 (Chromosome 11 Open Reading Frame 58) is a bone mineral density (BMD)-associated gene [41, 42]. Although its role in bone metabolism is not yet clear, the upregulation of C11orf58 in males and downregulation in females suggest its potential involvement in bone metabolic processes, making it a novel candidate gene for OTM research. The data suggest that in the early stages of OTM, under conditions of stable bone homeostasis, both females and males exhibit increased bone formation, but females exhibit a more pronounced osteoclastic trend. Interestingly, while bone formation increased in both sexes, the genes involved are completely different, suggesting potentially different mechanisms of bone formation in men and women. In addition, we observed sex-specific differences in the expression of genes related to immune response and inflammation, such as ISG20L2 [43] and VPS28 [44] which indicate an enhanced immune response in men. Furthermore, genes

such as YJU2B, FASTK and PRPF4 also exhibited differential expression, with women showing increased cell proliferation. Interestingly, although significant differences in bone resorption were found in the current study, according to literature the incidence of orthodontically induced root resorption is not significantly different in men and women [45, 46], which suggests the involvement of different pathways. However, after 28 days of OF only 4 DEGs were different between males and females, suggesting that sex differences are more evident in the early stages of OTM.

In addition to the well-established influence of hormonal levels on metabolism and overall bone remodeling dynamics, the observed sex-specific gene expression differences during OTM could also reflect the effects of X-chromosome dosage and/or escape from X-inactivation (XCI), which may impact the rate and biological response to OTM. In female mammals, one of the two X chromosomes is randomly inactivated during early embryonic development (a process known as X-chromosome inactivation or Lyonization) to balance gene dosage between sexes. However, approximately 15–25% of X-linked genes are known to escape XCI and remain transcriptionally active on the otherwise inactive X chromosome. This can lead to increased expression of these genes in females compared to males, resulting in potential dosage effects and biological sex differences in immune response, metabolism, and tissue remodeling [47].

In our study, ZNF711, which was differentially expressed in the DP after 28 days of OTM, is located at Xq21.1 and encodes an X-linked transcription factor. It has been reported to partially escape XCI and influence the expression of genes involved in immune and neurodevelopmental pathways. On the other hand, FLNA, which showed differential expression in the PDL after 7 days of OTM, is located at Xq28 and encodes Filamin A, a cytoskeletal protein involved in cell migration and osteoclastogenesis. FLNA has been reported to subject to XCI, which indicated that its observed sex-biased expression in our study is more likely due to hormonal or regulatory mechanisms [48]. Therefore, the sex-related transcriptional divergence observed in this study likely results from the combined effects of X-chromosome dosage, escape of XCI, and systemic hormonal regulation. Future research should further investigate the contribution of these mechanisms to sex-specific transcriptional dynamics during orthodontic tooth movement.

Influence of age on the transcriptomic pattern of the DP during OTM

After applying OF for 7 days, we identified 18 genes with age-related expression patterns. Among these, STIMAT (STIM Activating Enhancer) acts as a positive regulator of Ca^{2+} influx, enabling the movement of calcium ions from the extracellular matrix into the cell [49]. Additionally, SYT2 (Synaptotagmin 2), together with SYT3, supports interactions between STIM2 and Orai1 at endoplasmic reticulum–plasma membrane contacts sites, which modulate Ca^{2+} influx under resting conditions [50]. Furthermore, PITPNM2 (Phosphatidylinositol Transfer Protein Membrane Associated 2), a protein tyrosine kinase, is activated in response to various extracellular stimuli that similarly elevate intracellular Ca^{2+} concentrations [51, 52]. Maintaining Ca^{2+} homeostasis is essential for the health of DP tissue. Increased intracellular Ca^{2+} concentration, via calcium channel influx, initiates a cascade of Ca^{2+} signaling, which upregulates DSPP expression in dental pulp stem cells (DPSCs) and promotes odontoblast differentiation [53, 54]. This represents the fact that pulp tissue reacts with reparative dentin formation to the stimulus which is an initial reparatory reaction to a certain stimulus. Our findings reveal that, under OF, STIMAT expression declines with age. Also, SYT2, SYT3 and PITPNM2 show a progressive decline with aging, after an initial increase between ages 12 and 17. This trend suggests that the mechanisms for maintaining Ca^{2+} stability in DP tissue evolve over time, potentially reducing the tissue's repair capacity and increasing susceptibility to pulp inflammation in older individuals. Additionally, previous research has shown that OTM induces DNA damage repair. In this study, we identified several genes involved in this process. For instance, SPIRE2 (Spire Type Actin Nucleation Factor 2), along with SPIRE1, promotes the assembly of nuclear actin filaments in response to DNA damage, aiding the mobilization of chromatin and repair factors to damaged sites [55]. PSMD1 (Proteasome 26S Subunit, Non-ATPase 1), a component of the 26S proteasome, is involved in essential cellular processes, including cell cycle regulation, apoptosis, and DNA damage repair [56]. Both SPIRE2 and PSMD1 exhibit a decline in expression with age, suggesting a reduced adaptive capacity of DP tissue to OF as individuals age. We also observed that STXBP6 (Syntaxin Binding Protein 6), which is involved in cellular exocytosis and endocytosis processes, and TLL1 (Tolloid-Like Protein 1), which plays a role in regulating dentin extracellular matrix (ECM) homeostasis and mineralization, display age-dependent expression patterns. These findings underline the age-related changes in Ca^{2+} regulation and decrease in the responsiveness to orthodontic-induced DNA damage repair, potentially affecting tissue resilience during orthodontic interventions.

Among the age-dependent expression patterns observed after 28 days of OF, several genes show upregulation with age: PCOLCE2, MARK1, MROCKI, and TXNL4B. Notably, TXNL4B (Thioredoxin Like 4B) plays a crucial role in pre-mRNA splicing, necessary for cell

cycle progression, while MARK1 (Microtubule Affinity Regulating Kinase 1) is involved in regulating cell polarity and microtubule dynamics [57]. Conversely, several genes exhibit a down- regulation, including SINA3, CYP1B1-AS1, and MRPS35-DT. While CYP1B1-AS1 and MRPS35-DT are long non-coding RNAs (lncRNAs) with currently undefined functions, SINA3 (SIN3 Transcription Regulator Family Member A) acts as a transcriptional repressor in cellular activities [58]. These suggest that DP tissue exhibits a different response with age during the later stages of OTM, potentially reflecting altered repair and adaptive capacities.

Influence of age on the transcriptomic pattern of the PDL during OTM

In contrast with the DP findings, no statistically significant age-related gene expression patterns were observed in PDL tissue after 7-days OF application, at least in the age range included in this study (16.51 ± 6.67 years old). However, by day 28, we identified 181 DEGs, indicating that age-related transcriptional changes become more pronounced as orthodontic treatment progresses. Among the top 25 genes identified, CAPZB, FAM171A1, SPART and PPP1R12B are notably involved in cellular realignment and microfilament rearrangement within the PDL, which is essential for the PDL tissue remodeling during OTM. CAPZB (Capping Actin Protein of Muscle Z-Line Subunit Beta) plays a key role in regulating cell morphology and organizing the cytoskeleton [59]. FAM171A1 (Family with Sequence Similarity 171 Member A1) contributes to cytoskeletal dynamics and is essential for actin stress fiber formation [60], while SPART may influence microtubule dynamics. Besides, PPP1R12B (Protein Phosphatase 1 Regulatory Subunit 12B) encodes a regulatory subunit of protein phosphatase-1, which appears to be upregulated as part of a self-protective mechanism. This response aids stretched cells in disassembling incompatible cytoskeletal structures to prevent excessive deformation and injury during the initial stages of stretch loading. Our results show that, aside from CAPZB, the expression of the other genes declines with age. This pattern underscores age-related changes in adaptive cytoskeletal modifications within PDL cells, suggesting a potential decline in PDL homeostasis and remodeling capacity in reaction to OF with age.

Additionally, genes related to bone homeostasis and regeneration exhibit distinct age-dependent expression patterns. YJEFN3 (YjeF N-Terminal Domain Containing 3), also known as apoA-I binding protein 2 (Aibp2), regulates angiogenesis [61]. PRDX6 (Peroxiredoxin 6) is essential for guiding human DPSCs toward osteoblast differentiation and has been closely associated with osteogenic differentiation, bone regeneration, and development [62]. VEGFB (Vascular Endothelial Growth Factor B) is a growth factor involved in vasculogenesis under hypoxic conditions, while MALAT1 (Metastasis Associated Lung Adenocarcinoma Transcript 1) has been shown to regulate the osteogenic differentiation of periodontal ligament stem cells (PDLSCs) [63]. DMTF1 (Cyclin D Binding Myb Like Transcription Factor 1) functions as an early osteocyte-selective marker, playing a role in matrix mineralization, hydroxyapatite formation, and phosphate homeostasis [64]. Our results reveal that, with the exception of PRDX6, which increases in expression with age, other genes display a declining trend. This suggests that periodontal tissue's bone regeneration capacity in response to OF may diminish with age, potentially impacting the PDL's regenerative response.

Furthermore, functional analysis shows that GO categories related to biological processes and pathways highlight aspects of gene expression homeostasis, including terms like "ribosomal small subunit biogenesis," "ribosome biogenesis in eukaryotes," and "ribosome". These processes, alongside pathways associated with "angiogenesis" and "cytoskeletal in muscle cells" relate to tissue regeneration. Thus, functional analysis indicates that PDL homeostasis and regenerative capacity during OTM are likely reduced with age.

Final considerations and clinical remarks

One limitation of this study is the use of bulk RNA-seq, which does not provide detailed insights into the specific cellular functions of individual cell types embedded in DP and PDL tissues. Future research utilizing single-cell RNA-seq or spatial transcriptomics could address this limitation by providing cell-type-specific resolution. Proteomics could provide more definitive evidence of the functional biological processes involved, as it is downstream of transcriptomics in the biological cascade. Additionally, the current study is constrained by a predominance of female patients and a small number of participants over 30 years old. Although this is a good reflection of the orthodontic population, it limits our ability to fully investigate the effects of aging on tissue response to orthodontic force. Furthermore, when dividing the available samples into experimental groups and further by sex, the subgroup sizes become relatively small. This reduced sample size limits the statistical power to detect moderate differences in gene expression with high confidence, particularly in sex-specific comparisons at individual timepoints. As such, the absence of significant findings in some subgroups may reflect limited power rather than true biological similarity. Future prospective studies with larger, better-balanced cohorts are needed to validate and expand upon these observations. Moreover, due to technical constraints during data acquisition, the 7-day and 28-day samples were sequenced in different experimental batches. As

a result, we did not perform a direct differential expression analysis between these two timepoints, since any differences observed could be confounded by batch effects rather than reflecting true temporal biological responses. If batch effects were not a concern, a direct comparison between the 28-day and 7-day samples could indeed provide valuable insights into the direction and dynamics of gene expression changes over time. We consider this a meaningful approach for future studies with improved batch balance and longitudinal design.

Despite these limitations, the genes identified in this study provide a wider insight into the biological background of OTM. To the best of our knowledge, this is the first study to investigate the transcriptomic profiles of both DP and PDL tissues subjected to OF, comparing them between men and women as well as among different ages, examining temporal changes across early and later phases of OTM. Results could offer a theoretical foundation for more precise orthodontic treatment strategies, carrying direct clinical implications for personalized patient care. For instance, tailored, sex- and age-based force application protocols could be developed. Based on our results, the first orthodontic force application should be lighter in range and applied for a longer time in women, due to their more active, longer bone resorption processes and their less efficient pulp recovery. Additionally, older patients may benefit from longer force application of the first archwire, to allow more time for bone regeneration, supporting healthier tooth movement while addressing the reduced pulp repair and adaptive capacities associated with aging. Recognizing both sex- and age-related differences in dental tissue responses to OF could play a critical role in preserving tissue health during orthodontic treatment. Personalized approaches could minimize potential orthodontic side effects such as pain, exaggerated alveolar bone resorption, tooth mobility, pulp inflammation, pulp necrosis or root resorption, which are key clinical concerns for orthodontists, endodontists, periodontists and more importantly, patients.

Conclusion

The gene expression of DP tissue subjected to OF shows no significant sex differences, while PDL tissue does, mostly in the early phase of OTM where women display a stronger osteoclastic response. Lower cell activity and proliferation is seen in both tissues with age. Age impacts DP tissue primarily by affecting calcium ion homeostasis and DNA damage repair in early phases of OTM, and tissue repair and adaptive capacities in later phases. Finally, age-related effects on PDL tissue emerge mainly in later phases of OTM, influencing its regenerative response by a lower bone and PDL homeostasis.

Abbreviations

CG	Control groups
DAVID	Database for Annotation Visualization and Integrated Discovery
DEGs	Differentially expressed genes
DP	Dental pulp
FDR	False discovery rate
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
LIN	Linear mixed models
OF	Orthodontic force
OTM	Orthodontic tooth movement
PCA	Principal component analysis
PDL	Periodontal ligament
RNA-seq	RNA sequencing
SRA	Sequence Read Archive
XCI	X chromosome inactivation

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40510-025-00596-w.

Supplementary material 1.

Supplementary material 2.

Supplementary material 3.

Acknowledgements

Not applicable.

Author contributions

ZZ: contributed to conception and design, data acquisition, analysis, and interpretation and drafted and critically revised the manuscript; SF: Data acquisition, analysis, and interpretation and critically revised manuscript; CA: Design, interpretation and analysis, and critically revised manuscript; MSP: Data acquisition, Conception, data interpretation, and critically revised the manuscript; MCLP: Conception and design, data interpretation, critically revised the manuscript. All authors gave final approval and agreed to be accountable for all aspects of the work.

Funding

This research was supported by the International Orthodontic Foundation (IOF) Young Research Grants Award of International Orthodontics Foundation (2022) and Start-up Grant for Young Professors of KU Leuven University (2017).

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

This project was approved by the Commission for Medical Ethics of KU Leuven University and University Hospitals (UZ) Leuven (file number S-60530). The work was carried out in accordance with the Helsinki Declaration. Prior to participation, all participants and their parents/legal representatives provided signed informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 11 August 2025 / Accepted: 10 November 2025
Published online: 24 November 2025

References

1. Krishnan V, Davidovitch Z. Cellular, molecular, and tissue-level reactions to orthodontic force. Am J Orthod Dentofacial Orthop. 2006. https://doi.org/10.1016/j.ajodo.2005.10.007.
2. Li Y, Zhan Q, Bao M, Yi J, Li Y. Biomechanical and biological responses of periodontium in orthodontic tooth movement: up-date in a new decade. Int J Oral Sci. 2021;13(1):20.
3. Zhao Z, Tarce M, Georgopoulou M, Zong C, Van Holm W, Attanasio C, et al. Periodontal ligament reactions to orthodontic force: a transcriptomic study on maxillary and mandibular human premolars. J Periodontal Res. 2024. https://doi.org/10.1111/jre.13352.
4. Vitali FC, Cardoso IV, Mello FW, Flores-Mir C, Andrada AC, Dutra-Horstmann KL, et al. Association between orthodontic force and dental pulp changes: a systematic review of clinical and radiographic outcomes. J Endod. 2022;48:298–311.
5. Zhao Z, Attanasio C, Zong C, Pedano MS, de Canas Llano-Pérula M. How does orthodontic tooth movement influence the dental pulp? RNA-sequencing on human premolars. Int Endod J. 2024;57:1783–801.
6. Haruyama N, Igarashi K, Saeki S, Otsuka-Isoya M, Shinoda H, Mitani H. Estrous-cycle-dependent variation in orthodontic tooth movement. J Dent Res. 2002;81:406–10.
7. Ono R, Katsumata A, Fujikawa Y, Takahira E, Yamamoto T, Kanamura N. Sex differences and age-related changes in the mandibular alveolar bone mineral density using a computer-aided measurement system for intraoral radiography. Sci Rep. 2024. https://doi.org/10.1038/s41598-024-57805-5.
8. Shiau HJ, Aichelmann-Reidy ME, Reynolds MA. Influence of sex steroids on inflammation and bone metabolism. Periodontol 2000. 2014;64:81–94.
9. Dzeletovic B, Grga D, Krsljak E, Stratimirovic D, Brkovic B, Stojic D. Dental pulp blood flow and its oscillations in women with different estrogen status. J Endod. 2012;38:1187–91.
10. Wang Y, Yan M, Yu Y, Wu J, Yu J, Fan Z. Estrogen deficiency inhibits the odonto/osteogenic differentiation of dental pulp stem cells via activation of the NF-κB pathway. Cell Tissue Res. 2013;352:551–9.
11. Krieger E, Hornikel S, Wehrbein H. Age-related changes of fibroblast density in the human periodontal ligament. Head Face Med. 2013;9:22.
12. Wu RX, Bi CS, Yu Y, Zhang LL, Chen FM. Age-related decline in the matrix contents and functional properties of human periodontal ligament stem cell sheets. Acta Biomater. 2015;22:70–82.
13. Severson JA, Moffett BC, Kokich V, Selipsky H. A histologic study of age changes in the adult human periodontal joint (Ligament). J Periodontol. 1978;49:189–200.
14. Reitan K. Some factors determining the evaluation of forces in orthodontics. Am J Orthod. 1957;43:32–45.
15. Kyomen S, Tanne K. Influences of aging changes in proliferative rate of PDL cells during experimental tooth movement in rats. Angle Orthod. 1997;67:67–72.
16. Misawa-Kageyama Y, Kageyama T, Moriyama K, Kurihara S, Yagasaki H, Deguchi T, et al. Histomorphometric study on the effects of age on orthodontic tooth movement and alveolar bone turnover in rats. Eur J Oral Sci. 2007;115:124–30.
17. Ren Y, Maltha JC, Van't Hof MA, Von Den Hoff JW, Kuijpers-Jagtman AM, Zhang D. Cytokine levels in crevicular fluid are less responsive to orthodontic force in adults than in juveniles. J Clin Periodontol. 2002;29:757–62.
18. Zheng Y, Zhu C, Zhu M, Lei L. Difference in the alveolar bone remodeling between the adolescents and adults during upper incisor retraction: a retrospective study. Sci Rep. 2022;12:9161.
19. Bernick S, Nedelman C. Effect of aging on the human pulp. J Endod. 1975;1:88–94.
20. Saccomanno S, Saran S, Laganà D, Mastrapasqua RF, Grippaudo C. Motivation, perception, and behavior of the adult orthodontic patient: a survey analysis. Biomed Res Int. 2022. https://doi.org/10.1155/2022/2754051.
21. Zhao Y, Qian S, Zheng Z, Peng J, Liu J, Guan X, et al. Consideration of hormonal changes for orthodontic treatment during pregnancy and lactation - a review. Reprod Biol Endocrinol. 2024;22(1):106.
22. American Association of Orthodontists. The Economics of Orthodontics. 2013. https://www.aaoinfo.org/news/2015/12/economics-orthodontics-survey-indicates-practice-management-data-mostly-stable-growth.
23. Torsello F, D'Amico G, Staderini E, Marigo L, Cordaro M, Castagnola R. Factors influencing appliance wearing time during orthodontic treatments: a literature review. Appl Sci. 2022;12(15):7807. https://doi.org/10.3390/app12157807.
24. Wang J, Huang Y, Chen F, Li W. The age-related effects on orthodontic tooth movement and the surrounding periodontal environment. Front Physiol. 2024;15:1460168.
25. Andrews S. FastQC: a quality control tool for high throughput sequence data. 2010. http://www.bioinformatics.babraham.ac.uk/projects/fastqc
26. Erik Aronesty. ea-utils: Command-line tools for processing biological sequencing data. 2011. https://github.com/ExpressionAnalysis/ea-utils.
27. Kim D, Paggi JM, Park C, Bennett C, Salzberg SL. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. Nat Biotechnol. 2019;37:907–15.
28. Anders S, Pyl PT, Huber W. HTSeq-a Python framework to work with high-throughput sequencing data. Bioinformatics. 2015;31:166–9.
29. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009;4:44–57.
30. Zhang Z, Ersoz E, Lai C-Q, Todhunter RJ, Tiwari HK, Gore MA, et al. Mixed linear model approach adapted for genome-wide association studies. Nat Genet. 2010;42:355–60.
31. Kleine-Kohlbrecher D, Christensen J, Vandamme J, Abarrategui I, Bak M, Tommerup N, et al. A functional link between the histone demethylase PHF8 and the transcription factor ZNF711 in X-linked mental retardation. Mol Cell. 2010;38(2):165–78. https://doi.org/10.1016/j.molcel.2010.03.002.
32. Tuo Y, Hu L, Gu W, Yuan X, Wu J, Ma D, et al. Identification of bone morphometric protein-related hub genes and construction of a transcriptional regulatory network in patients with ossification of the ligamentum flavum. Spine (Phila Pa 1976). 2023. https://doi.org/10.1097/BRS.0000000000004763.
33. Celauro E, Carra S, Rodriguez A, Cotelli F, Dimitri P. Exp Cell Res. 2017;361:236–45.
34. Bustos-Valenzuela JC, Fujita A, Halcsik E, Granjeiro JM, Sogayar MC. Unveiling novel genes upregulated by both rhBMP2 and rhBMP7 during early osteoblastic transdifferentiation of C2C12 cells. BMC Res Notes. 2011;4:370.
35. Moura AP, Taddei SRA, Queiroz-Junior CM, Madeira MFM, Rodrigues LFD, Garlet GP, et al. The relevance of leukotrienes for bone resorption induced by mechanical loading. Bone. 2014;69:133–8.
36. Wang YM, Ruan WY, Chi DD, Duan XH. Clinical and genetic analysis of multiple idiopathic cervical root resorption. Chin J Dent Res. 2024;27(1):89–99.
37. Li L, Tang P, Zhou Z, Wang Q, Xu T, Zhao S, et al. GIT1 regulates angiogenic factor secretion in bone marrow mesenchymal stem cells via NF-κB/Notch signalling to promote angiogenesis. Cell Prolif. 2019;52(6):e12689. https://doi.org/10.1111/cpr.12689.
38. Ayilavarapu S, Doctor A, Lee C-T, Tribble GD, Chiu Y, Weltman RL, et al. Altered human alveolar bone gene expression in type 2 diabetes—a cross-sectional study. J Periodontal Res. 2022;57(1):142–51. https://doi.org/10.1111/jre.12947.
39. Jin C, Yu X, Yang J, Lin Z, Ma R, Lin B, et al. Corynoline suppresses osteoclastogenesis and attenuates ROS activities by regulating NF-κB/MAPKs and Nrf2 signaling pathways. J Agric Food Chem. 2024;72:8149–66.
40. Hughes DE, Dai A, Tiffee JC, Li HH, Mundy GR, Boyce BF. Estrogen promotes apoptosis of murine osteoclasts mediated by TGF–β. Nat Med. 1996;2:1132–6.
41. Zhu W, Xu C, Zhang J-G, He H, Wu K-H, Zhang L, et al. Gene-based GWAS analysis for consecutive studies of GEFOS. Osteoporos Int. 2018;29(12):2645–58.
42. Tan L, Liu R, Lei S, Pan R, Yang T, Yan H, et al. A genome-wide association analysis implicates SOX6 as a candidate gene for wrist bone mass. Sci China Life Sci. 2010;53(9):1065–72.
43. Rodríguez-Galán A, Dosil SG, Hrčková A, Fernández-Messina L, Feketová Z, Pokorná J, et al. ISG20L2: an RNA nuclease regulating T cell activation. Cell Mol Life Sci. 2023;80(9):273.
44. Stefani F, Zhang L, Taylor S, Donovan J, Rollinson S, Doyotte A, et al. UBAP1 is a component of an endosome-specific ESCRT-I complex that is essential for MVB sorting. Curr Biol. 2011;21(14):1245–50. https://doi.org/10.1016/j.cub.2011.06.028.
45. Jiang R, McDonald JP, Fu M. Root resorption before and after orthodontic treatment: a clinical study of contributory factors. Eur J Orthod. 2010;32(6):693–7. https://doi.org/10.1093/ejo/cjp165.
46. Sameshima GT, Sinclair PM. Predicting and preventing root resorption: Part I. Diagnostic factors. Am J Orthod Dentofacial Orthop. 2001;119:505–10.
47. Tukiainen T, Villani AC, Yen A, Rivas MA, Marshall JL, Satija R, et al. Landscape of X chromosome inactivation across human tissues. Nature. 2017;550:244–8.
48. Balaton BP, Cotton AM, Brown CJ. Derivation of consensus inactivation status for X-linked genes from genome-wide studies. Biol Sex Differ. 2015;6:35.

49. Jing J, He L, Sun A, Quintana A, Ding Y, Ma G, et al. Proteomic mapping of ER–PM junctions identifies STIMATE as a regulator of Ca2+ influx. Nat Cell Biol. 2015;17:1339–47.
50. Redondo PC, Lopez JJ, Alvarado S, Jardin I, Nieto-Felipe J, Macias-Diaz A, et al. Extended synaptotagmins 1 and 2 are required for store-operated calcium entry, cell migration and viability in breast cancer cells. Cancers (Basel). 2024. https://doi.org/10.3390/cancers16142518.
51. Lev S, Moreno H, Martinez R, Canoll P, Peles E, Musacchio JM, et al. Protein tyrosine kinase PYK2 involved in Ca2+-induced regulation of ion channel and MAP kinase functions. Nature. 1995;376(6543):737–45. https://doi.org/10.1038/376737a0.
52. Lev S, Hernandez J, Martinez R, Chen A, Plowman G, Schlessinger J. Identification of a novel family of targets of PYK2 related to *Drosophila* retinal degeneration B (rdgB) protein. Mol Cell Biol. 1999;19:2278–88. https://doi.org/10.1128/MCB.19.3.2278.
53. Li S, He H, Zhang G, Wang F, Zhang P, Tan Y. Connexin43-containing gap junctions potentiate extracellular Ca2+-induced odontoblastic differentiation of human dental pulp stem cells via Erk1/2. Exp Cell Res. 2015;338(1):1–9. https://doi.org/10.1016/j.yexcr.2015.09.008.
54. An S. The emerging role of extracellular Ca2+ in osteo/odontogenic differentiation and the involvement of intracellular Ca 2+ signaling: from osteoblastic cells to dental pulp cells and odontoblasts. J Cell Physiol. 2019;234(3):2169–93. https://doi.org/10.1002/jcp.27068.
55. Belin BJ, Lee T, Mullins RD. DNA damage induces nuclear actin filament assembly by Formin-2 and Spire-1/2 that promotes efficient DNA repair. Elife. 2015;4:e07735. https://doi.org/10.7554/eLife.07735.
56. Kanayama H, Tamura T, Ugai S, Kagawa S, Tanahashi N, Yoshimura T, et al. Demonstration that a human 26S proteolytic complex consists of a proteasome and multiple associated protein components and hydrolyzes ATP and ubiquitin-ligated proteins by closely linked mechanisms. Eur J Biochem. 1992;206(2):567–78. https://doi.org/10.1111/j.1432-1033.1992.tb16961.x.
57. Gu GJ, Lund H, Wu D, Blokzijl A, Classon C, von Euler G, et al. Role of individual MARK isoforms in phosphorylation of Tau at Ser262 in Alzheimer's disease. Neuromolecular Med. 2013;15(3):458–69. https://doi.org/10.1007/s12017-013-8232-3.
58. Icardi L, Mori R, Gesellchen V, Eyckerman S, De Cauwer L, Verhelst J, et al. The Sin3a repressor complex is a master regulator of STAT transcriptional activity. Proc Natl Acad Sci U S A. 2012;109(30):12058–63.
59. Mukherjee K, Ishii K, Pillalamarri V, Kammin T, Atkin JF, Hickey SE, et al. Actin capping protein CAPZB regulates cell morphology, differentiation, and neural crest migration in craniofacial morphogenesis†. Hum Mol Genet. 2016;25:1255–70.
60. Rasila T, Saavalainen O, Attalla H, Lankila P, Haglund C, Hölttä E, et al. Astroprincin (FAM171A1, C10orf38): a regulator of human cell shape and invasive growth. Am J Pathol. 2019;189(1):177–89. https://doi.org/10.1016/j.ajpath.2018.09.006.
61. Gu Q, Yang X, Lv J, Zhang J, Xia B, Kim J, et al. AIBP-mediated cholesterol efflux instructs hematopoietic stem and progenitor cell fate. Science (1979). 2019;6431:1085–8.
62. Park K-R, Yun H-M, Yeo IJ, Cho S, Hong JT, Jeong YS. Peroxiredoxin 6 inhibits osteogenic differentiation and bone formation through human dental pulp stem cells and induces delayed bone development. Antioxid Redox Signal. 2018;30:1969–82.
63. Gu N, Wang Y, Li L, Sui X, Liu Z. The mechanism of lncRNA MALAT1 targeting the miR-124-3p/IGF2BP1 axis to regulate osteogenic differentiation of periodontal ligament stem cells. Clin Oral Investig. 2024;28:219.
64. Woo SM, Rosser J, Dusevich V, Kalajzic I, Bonewald LF. Cell line IDG-SW3 replicates osteoblast-to-late-osteocyte differentiation in vitro and accelerates bone formation in vivo. J Bone Miner Res. 2011;26(11):2634–46. https://doi.org/10.1002/jbmr.465.

Publisher's Note