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WNT3A promotes the cementogenic differentiation of dental pulp stem cells through the FOXO1 signaling pathway

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Abstract

Background Dental pulp stem cells (DPSCs) possess capability of multidirectional differentiation, and their cementogenic differentiation potential enables them to participate in cementum repair and regeneration. The molecular mechanisms underlying cementogenic differentiation of DPSCs remain unclear.

Methods DPSC data set GSE138179 was retrieved from gene expression omnibus (GEO) database. Weighted gene co-expression network analysis (WGCNA) was employed to identify significant modules. Pathway enrichment exploration was conducted utilizing gene ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Set Enrichment Analysis (GSEA), and Metascape tools. CIBERSORT was utilized to analyze immune cell infiltration analysis. The comparative toxicogenomics database (CTD) was utilized for the validation of core targets. Subsequently, cell experiments were conducted to validate the core targets. Changes in protein expression related to the FOXO1 signaling pathway, cell cycle, and apoptosis were evaluated using western blotting (WB).

Results Differentially expressed genes (DEGs) associated with DPSC cementogenic differentiation were predominantly enriched in crucial pathways such as the signaling pathway, cell apoptosis, and Wnt signaling pathway. Bioinformatics analysis confirmed WNT3A as a pivotal biomarker for DPSC cementogenic differentiation, and WNT3A was highly expressed in the cementogenic differentiation group. Western blotting results demonstrated that compared to the DPSC group, molecules such as Caspase-3, Caspase-9, FAS, P53, and BAX were downregulated in the CDDPSC group, suggesting reduced apoptosis. Furthermore, upregulation of WNT3A expression in CDDPSC–OE further suppressed the expression of these apoptotic molecules, suggesting a mitigated apoptotic response. Downregulation of WNT3A expression in CDDPSC–KO resulted in increased expression of apoptosis-related molecules, thereby enhancing apoptosis.

Conclusions WNT3A is highly expressed in the cementogenic differentiation of DPSC, and WNT3A mediates FOXO1 pathway to promote differentiation of dental pulp stem cells into cementogenic differentiation, thus realizing the formation and maintenance of cementum tissue.

Keywords WNT3A, FOXO1, Cementogenic differentiation, Dental pulp stem cells, Apoptosis

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Introduction

Dental pulp stem cells (DPSCs) are a type of mesenchymal stem cells derived from dental pulp tissue of deciduous or permanent teeth. They possess the potential for self-renewal, multi-lineage differentiation, and high proliferative capacity. Under specific conditions, DPSCs can be induced towards cementogenic differentiation, thereby providing strong support for bone tissue regeneration and dental pulp regeneration. This includes the regulation of signaling pathways [1], the modulation of cellular, growth, and transcription factors, as well as the role of extracellular matrix [2]. Inflammatory reactions occurring around dental pulp are typically triggered by caries or trauma, and interleukins represent a class of anti-inflammatory factors that modulate cementogenic differentiation of human DPSCs in various ways. IL-37 activation enhances cementogenic differentiation of DPSCs by inducing autophagy in DPSCs [3]. Biological factors such as mineral trioxide aggregate and calcium-enriched mixture stimulate different gene expressions and growth factor release, thereby inducing cementogenic differentiation of human DPSCs [4]. Calcium silicate hydrate based dental pulp adhesive can activate the signaling pathway of dental pulp stem cells PMID: 33573840. Despite research exploring the molecular mechanisms underlying the differentiation of DPSCs into cementoblasts, controversies still exist. Studies have shown that periodontal ligament stem cells have important characteristics of osteogenesis and differentiation and high tissue regeneration ability, and GuttaFlow Bioseal can promote the spontaneous differentiation of human periodontal stem cells into cementoblast-like cells PMID: 30466731.Therefore, this study aims to explore specific molecular mechanism of cementum differentiation formed by DPSCs, and promote dental health and expand the application field of dental clinical treatment.

Over the past few decades, bioinformatics technology has advanced significantly, emerging as an indispensable tool in life sciences research and medicine [5, 6]. The continuous evolution and widespread adoption of next-generation sequencing technologies, such as Illumina, Ion Torrent, and PacBio platforms, have dramatically accelerated data generation in genomics, transcriptomics, and proteomics. High-throughput sequencing enables the rapid acquisition of large-scale genomic data, helping reveal the panorama of genetic variations, gene expression, and the proteome [7]. The progress in bioinformatics technology offers robust tools for life sciences and medical research, propelling scientific frontiers and unleashing immense potential for future investigations and medical applications [8, 9].

WNT3A, a member of WNT protein family, holds a pivotal position in various biological processes critical for embryonic development, cell proliferation, differentiation, and the determination of stem cell fate [10]. Its encompasses a secretory signal peptide and a C-terminal ubiquitination segment composed of 22 amino acids. WNT3A orchestrates diverse biological phenomena, including embryonic development, organogenesis, tissue regeneration, and cellular destiny determination [11]. WNT3A transmits signals by activating the WNT signaling pathway. The WNT signaling pathway is mainly divided into two types: "canonical" and "non-canonical," involving various cell surface receptors and internal signaling molecules [12]. For example, FOXO [13], β-catenin [14] and other channels. Due to its significant role in development and diseases, WNT3A has become a focus of biomedical research [15].

This study endeavors to employ bioinformatics technology to elucidate core biomarkers of associated with cementogenic differentiation in dental pulp stem cells, conducting comprehensive enrichment and pathway analysis. The pivotal role of WNT3A in cementogenic differentiation process of dental pulp stem cells will be validated using publicly available data sets. Experimental validation will be performed to corroborate the findings.

Methods

Human dental pulp stem cell data set

For this study, the configuration file for the human dental pulp stem cell data set GSE138179 was obtained from Gene Expression Omnibus (GEO) database, derived from platforms GPL6102 and GPL21827. GSE138179 comprises 3 samples of human DPSCs and three samples of human DPSCs undergoing cementogenic differentiation. This data set was utilized to identify differentially expressed genes (DEGs) associated with cementogenic differentiation process of human DPSCs.

DEGs selection

Probe summary and background correction of gene expression matrices in GSE138179 were performed using the R package limma. Subsequently, Benjamini–Hochberg method was used to adjust the raw p values and calculate the fold change (FC) using the false discovery rate (FDR). The criteria for identifying DEGs were set at a significance level of $p < 0.05$, $FC > 1.5$. Volcano plots were generated to visualize DEGs.

Weighted gene co-expression network analysis (WGCNA)

Initially, the gene expression matrix of GSE138179 underwent preprocessing steps, wherein median

absolute deviation (MAD) was computed for each gene. The bottom 50% of genes with the smallest MAD were excluded. Using the "goodSamplesGenes" method in the R package WGCNA, eliminate to outlier genes and samples. Subsequently, WGCNA was used to construct a scale-free co-expression network. To cluster genes with similar expression profiles into gene modules, average linkage hierarchical clustering based on the TOM dissimilarity measure was used, with a minimum module size of 30 genes and a sensitivity threshold of 3 genes. Further analysis included calculating the differences in module Eigen genes, determining the cutoff point in the module dendrogram, and merging some of the modules. Modules with a distance of less than 0.25 were merged. It is worth noting that grey module is considered a group of genes that cannot be assigned to any module.

Functional enrichment analysis

DEGs screened by Venn diagram were entered into KEGG API. The latest KEGG pathway's gene annotations were acquired to map genes into background set for enrichment analysis using R package cluster profiler to obtain results of gene set enrichment. GO annotations of genes were obtained from the R package. Map genes into background set as background, minimum gene set was 5, maximum gene set was 5000, p value < 0.05, FDR < 0.25. Metascape database (http://metascape.org/gp/index.html) was also used as an auxiliary analysis.

Gene set enrichment analysis (GSEA)

Samples were categorized into human dental pulp stem cell samples and samples undergoing cementogenic differentiation, and assess relevant pathways and molecular mechanisms. GSEA was conducted based on gene expression profiles and phenotype grouping, with a minimum gene set of 5, a maximum gene set of 5000, one thousand permutations, p value of < 0.05 and FDR of < 0.25 considered statistically significant.

Immune infiltration analysis

CIBERSORT stands as a widely utilized method for quantifying immune cell infiltration. In this study, we adopted an integrated bioinformatics approach, leveraging CIBERSORT package to analyze the gene expression matrix derived from human dental pulp stem cell data set GSE138179. The linear support vector regression principle was employed to deconvolute expression matrix of immune cell subtypes, estimating the abundance of immune cells. A confidence level of $p < 0.05$ was used as the cutoff criterion to filter out samples with sufficient confidence.

Gene expression heatmap

R package heatmap was employed to construct a heatmap depicting expression levels of differentially expressed genes identified within PPI network of GSE138179. This visualization serves to illustrate expression disparities of these genes between samples of human DPSCs samples and those undergoing cementogenic differentiation.

PPI network construction and analysis

STRING database serves the purpose of gathering, evaluating, and integrating all publicly accessible PPI data sources, supplementing them with predicted interactions. The list of differentially expressed genes was input into STRING database to construct PPI network. PPI network generated by STRING database was visualized, core genes were predicted using Cytoscape. MCODE was utilized to identify most significant modules. In addition, four algorithms (MCC, MNC, EPC, and EcCentricity) were used to calculate the genes with the best correlations. The resulting list of core genes was visualized and exported.

CTD analysis

Comparative Toxicogenomics Database (CTD) offering valuable insights into potential mechanisms of disease-related environmental exposures and drugs. Core genes were entered into CTD to identify the most relevant diseases. Radar charts illustrating expression differences for each gene were created using Excel.

miRNA

TargetScan serves as an online database utilized for predicting and analyzing miRNA-target gene interactions. TargetScan was employed to systematically screen for miRNAs potentially regulating central DEGs. This approach facilitated identification of potential regulatory networks involving miRNAs and their target genes, thereby enhancing our understanding of the regulatory mechanisms underlying gene expression alterations observed in our study.

Cell culture

Mouse dental pulp stem cells were purchased from Shanghai Biotech Biochemical Technology Co., Ltd. These cells were seeded into culture dishes using DMEM (high glucose medium) supplemented with an appropriate concentration of fetal bovine serum or synthetic additives. The cells were cultured at 37 °C with 5% CO_2.

Cell grouping

Cultured cells were divided into four groups:

Control Group (Dental pulp stem cell group, abbreviated as DPSC group).

Cementogenic differentiation Group of Dental Pulp Stem Cells (abbreviated as CDDPSC), with cementogenic induction added to dental pulp stem cells.

Cementogenic differentiation overexpression group (abbreviated as CDDPSC–OE), with cementogenic induction added to dental pulp stem cells and transfection with WNT3A overexpression plasmid.

Cementogenic differentiation knockout group (abbreviated as CDDPSC–KO), with cementogenic induction added to dental pulp stem cells and transfection with WNT3A knockout plasmid.

Construction of WNT3A overexpression plasmid

Acquire WNT3A cDNA: purchase the cDNA corresponding to WNT3A. Opt for a vector such as pCDNA3.1 (+) equipped with the necessary promoter and selection marker. Prepare the plasmid vector: cut WNT3A cDNA using DNA-cutting enzymes. Fuse WNT3A cDNA with vector: employ T4 DNA ligase to join the WNT3A cDNA with the linearized vector. Transform Escherichia coli cells and screen for plasmids containing correct insert. Confirm via sequencing: sequence resulting WNT3A overexpression plasmid to verify the correct insertion of the WNT3A cDNA sequence. Amplify the plasmid on a large scale: amplify confirmed plasmid to an adequate quantity suitable. Cell transfection: introduce the constructed WNT3A overexpression plasmid into the target host cells. Selection and isolation: add the appropriate selection marker to screen for successfully transfected cells. Perform single-cell cloning to ensure stable and consistent expression of the chosen cell line.

Construction of WNT3A knockout siRNA

Select a specific siRNA sequence targeting WNT3A, ensuring effective degradation or silencing of target gene's mRNA. Opt for a suitable plasmid vector. Linearize plasmid vector using appropriate restriction enzymes. Utilize DNA ligase to incorporate synthesized siRNA sequence into linearized plasmid vector. Screen for plasmids with correct insert. Amplify obtained plasmid to a sufficient quantity for subsequent experiments. Sequence amplified plasmid to ensure the correct siRNA sequence is inserted. The constructed siRNA plasmid was transfected into the target host cells.

Transfection conditions and reagent concentrations

Transfection conditions: prior to electrotransfection, the mixture of cells and siRNA was precooled on ice for 10 min with an electric field strength of 400 V/cm and pulse width of 510 μs. Reagent concentration: cell concentration was controlled at 80,000 cells/ml for inoculation.

Before transfection, ensure the cell density is approximately 50–70% confluence to maintain viability. Use 1 μg of plasmid DNA per well (6-well plate) as a starting point. The amount of transfection reagent should generally be 2–3 times the weight of the plasmid DNA. Mix the transfection reagent and DNA in serum-free medium and incubate for 15–30 min to form complexes. Gently add the mixture to the cell culture medium, ensuring even distribution. After 4–6 h of transfection, replace the medium with serum-containing medium to reduce the toxicity of the transfection reagent. Incubate the cells at 37 °C with 5% CO_2 for 24–48 h.

Western blot (WB) detection of FOXO1 signaling pathway, cell cycle-related proteins, and apoptosis-related proteins

Centrifuge cells at 2000 rpm, 4 °C for 5 min to pellet the cells. Collect the cell pellets and add approximately 250 μL RIPA lysis buffer per 10^6 cells ensuring the buffer contains various protease inhibitors. Vortex and oscillate the mixture thoroughly to ensure proper lysis of the cells.

Mix the protein solution with 5×reducing protein loading buffer at a ratio of 4:1. Subsequently, denature the mixture in a boiling water bath for 15 min. Once denatured, store the solution at − 20 °C until further use.

Prepare separation gels with varying concentrations tailored to the specific experimental requirements. Following the addition of TEMED, immediately mix the solution and pour it into the gel mold to the appropriate height. Before pouring the gel, ensure the comb is properly positioned, with the comb teeth approximately 5–8 mm from the liquid surface. Slowly and uniformly add distilled water to the top of the separation gel until full. After about 30 min, pour off the water on the top of the separation gel and absorb the remaining water with absorbent paper.

Begin by preparing six sheets of filter paper measuring 7×9 cm and a PVDF membrane of suitable size (0.45 um). Activate the PVDF membrane with by soaking it in methanol for 2 min prior to use. Assemble the transfer basin with clips, two sponges, a glass rod, filter paper, and the activated PVDF membrane. Open the clips, positioning the white one on the left and the black one on the right. Place a sponge on each side of the basin, followed by three layers of filter paper. Carefully remove the separation gel and position it on the filter paper. Attach the PVDF membrane to the gel, ensuring no bubbles between the membrane and the gel. Cover the membrane with three sheets of filter paper and

remove bubbles. For the transmembrane process, apply a constant current of 300 mA for 30 min. During this process, maintain the transmembrane equipment in an ice bath to ensure optimal conditions for the transfer.

After transferring membrane, place it in a container filled with Tris-buffered saline with Tween-20 (TBST), rinse it quickly, and then add a solution of 5% milk. Allow the membrane to block on a shaker at room temperature for 30 min.

Following the manufacturer's instructions for the primary antibody, dilute it accordingly. Once the blocking solution has been discarded from the container, add the diluted primary antibody to the membrane. Incubate the membrane on a shaker at 4 °C overnight with gentle agitation.

Collect the primary antibody, rinse the membrane three times with TBST, then add TBST, place it on a shaker for quick washing, 5 min each time, and wash three times.

Dilute secondary antibody with TBST at a ratio of 1:5000. Add diluted secondary antibody to the container and place it on a shaker for gentle shaking at room temperature for 30 min.

After incubation, rinse membrane three times with TBST. Then, add TBST to the container and place it on a shaker for quick washing, with each wash lasting for 5 min. Repeat this washing process three times.

Mix ECL A and B liquids at a ratio of 1:1. Take out the PVDF membrane after washing, add mixed ECL luminescent liquid, completely submerge membrane in the liquid, wait for 1 min, use absorbent paper to remove excess liquid, place it in chemiluminescence instrument. After exposure, save original image. Use AIWBwell TM analysis software to analyze the saved tiff-format original images.

Results

DEGs analysis

Employing predefined cutoff values, we identified DEGs within gene expression matrix of human DPSCs data set GSE138179. 434 DEGs were identified (Fig. 1).

Functional enrichment analysis

DEGs

According to GO analysis, in Biological Process (BP), they were mainly enriched in processes such as regulation of gene expression, apoptosis, and stromal cell proliferation (Fig. 2A). In Cellular Component (CC) analysis, they were predominantly enriched in cytoplasm and cytoplasmic ribosomes (Fig. 2B). In Molecular Function (MF) analysis, they were exhibited enrichment in G protein-coupled receptor kinase activity, transcription factor activity, and growth factor binding (Fig. 2C). In KEGG analysis, they were primarily enriched in pathways

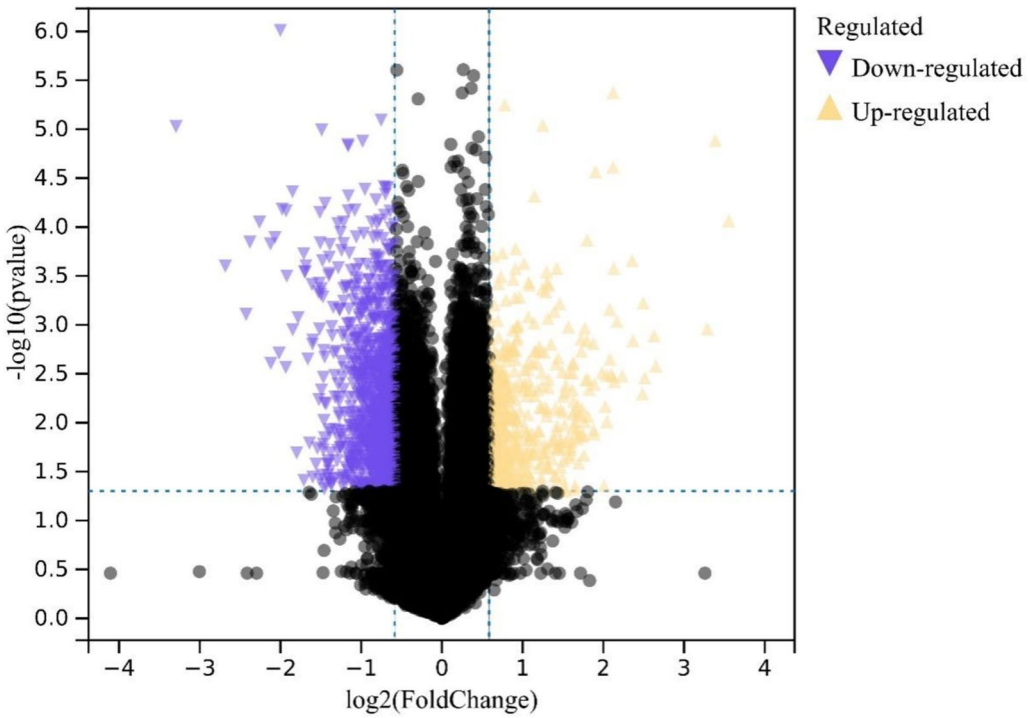

Fig. 1 Differential gene analysis. A total of 434 DEGs

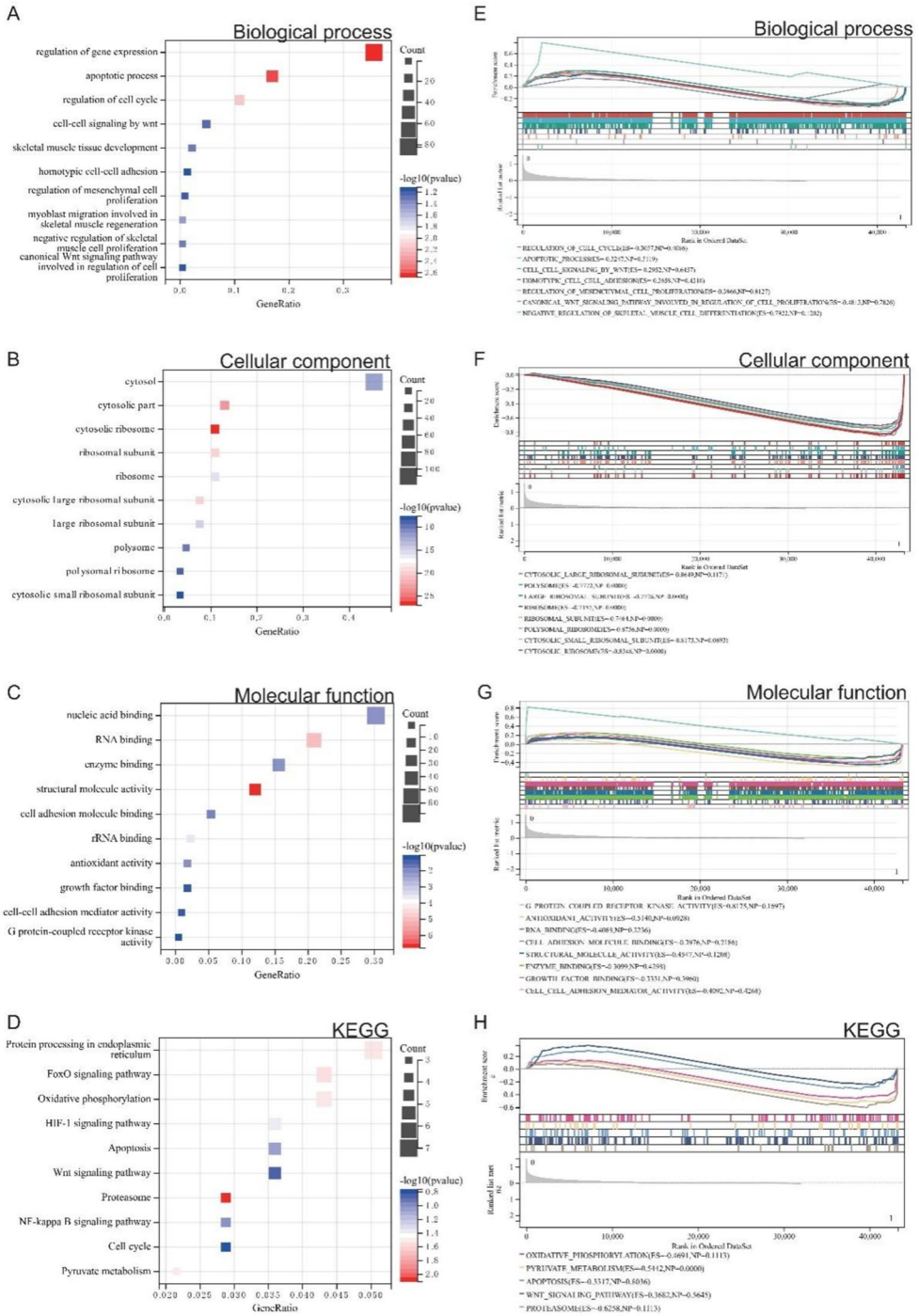

Fig. 2 **A**–**D** GOKEGG enrichment analysis of DEGs. **A** Biological process analysis. **B** Cellular component analysis. **C** Molecular function analysis. **D** KEGG enrichment analysis. **E**–**H** GSEA of DEGs. **E** Biological process analysis. **F** Cellular component analysis. **G** Molecular function analysis. **H** KEGG enrichment analysis

including FOXO signaling pathway, oxidative phosphorylation, HIF-1 signaling pathway, cell apoptosis, and Wnt signaling pathway (Fig. 2D).

GSEA

The intersection of enrichment terms with DEGs in GO and KEGG is depicted in the figure. The enrichment results of gene expression matrix from the human

dental pulp stem cells data set GSE138179 revealed that differentially expressed genes are primarily enriched in regulation of cell cycle, regulation of stromal cell proliferation, cytoplasmic ribosomes, growth factor binding, cell apoptosis, and Wnt signaling pathway (Fig. 2E, F, G, H).

Metascape enrichment analysis

GO enrichment items encompass processes such as regulation of hematopoiesis, maintenance of protein localization, and positive regulation of protein processing (Fig. 3A). Furthermore, we generated enrichment networks colored by enrichment terms and *p* values (Fig. 3B, C, D), offering a visual representation of the associations and confidence levels associated.

Immune infiltration analysis

The CIBERSORT software package was used to analyze the gene expression matrix of human dental pulp stem cells data set GSE138179. The proportion of immune cells in entire gene expression matrix was obtained. The results revealed a notably elevated proportion of Plasma cells in samples (Fig. 4A). Subsequently, a correlation analysis of infiltrating immune cells was performed to generate a co-expression pattern diagram. The findings indicated a strong positive correlation between activated Mast cells activated and T follicular helper cells when the expression of activated Mast cells was high, potentially influencing the cementogenic differentiation process of human DPSCs (Fig. 4B).

Differential gene expression heatmap

The expression levels of DEGs in gene expression matrix of human DPSCs data set GSE138179 and generated a heatmap (Fig. 5). Significant differences in gene expression were observed between human dental pulp stem cell samples and samples undergoing cementogenic differentiation. We speculated that DEGs may contribute to promoting the cementogenic differentiation process of dental pulp stem cells.

WGCNA

Through network topology analysis, we determined optimal soft threshold power, which was set to 14 for this analysis (Fig. 6A). Subsequently, we constructed a hierarchical clustering tree for all genes analyzed interactions between important modules (Fig. 6B). 25 modules were identified (Fig. 6C), along with a module–phenotype correlation heatmap (Fig. 6D), a scatter plot of GS versus MM for relevant hub genes (Fig. 6E, F, G). By applying a cutoff criterion ($|MM| > 0.8$), we identified three highly connected genes within clinically significant modules as hub genes. Venn diagram was generated a by intersecting WGCNA results with DEGs (Fig. 6H).

Protein–protein interaction (PPI) network construction and analysis

PPI network of DEGs was constructed using STRING and analyzed using Cytoscape (Fig. 7A). Subsequently, 4 algorithms were employed, a Venn diagram was created to obtain the intersection as core genes (Fig. 7B). The results of MCC, MNC, EPC, and EcCentricity algorithms are presented in Fig. 7C, D, E, F. Ultimately, three core genes (UBC, SKP1, and WNT3A) were identified.

CTD analysis

Exploring diseases associated with core genes using the list of core genes on the CTD website has enhanced the understanding of gene–disease associations. UBC, SKP1, WNT3A were associated with periodontal diseases, osteoporosis, developmental skeletal disorders, and necrosis (Fig. 8).

Prediction and functional annotation of miRNAs associated with hub genes

We employed TargetScan to identify relevant miRNAs by inputting hub gene list (Table 1). Our findings revealed that for UBC gene, the related miRNAs included hsa-miR-130a-3p, hsa-miR-301a-3p, hsa-miR-4295; for the SKP1 gene, they were hsa-miR-101-3p.2 and hsa-miR-101-3p.2; and for the WNT3A gene, they were hsa-miR-216a-3p, hsa-miR-128-3p, and hsa-miR-3681-3p.

WNT3A activates the FOXO1 signaling pathway

The Western blot (WB) analysis revealed notable differences in WNT3A expression among the experimental groups. Specifically, WNT3A exhibited significantly higher expression levels in CDDPSC group compared to DPSC group. Upon introducing the WNT3A overexpression plasmid in CDDPSC group, the expression of WNT3A significantly increased. Conversely, when WNT3A siRNA was introduced in the CDDPSC group, the expression of WNT3A significantly decreased. Moreover, key node proteins within the signaling pathway, including FOXO1, CyclinB, p15, p130, PLK1, GADD45, FasL, demonstrated upregulated in the CDDPSC relative to the DPSC group. In addition, with the upregulation of WNT3A expression in CDDPSC–OE, the expression of key node proteins in the pathway also increased. Conversely, with the downregulation of WNT3A expression in CDDPSC–KO, the expression of key node proteins in the pathway decreased. This result indicates a positive correlation between WNT3A and the FOXO1 signaling pathway, with WNT3A activating the FOXO1 signaling pathway (Fig. 9).

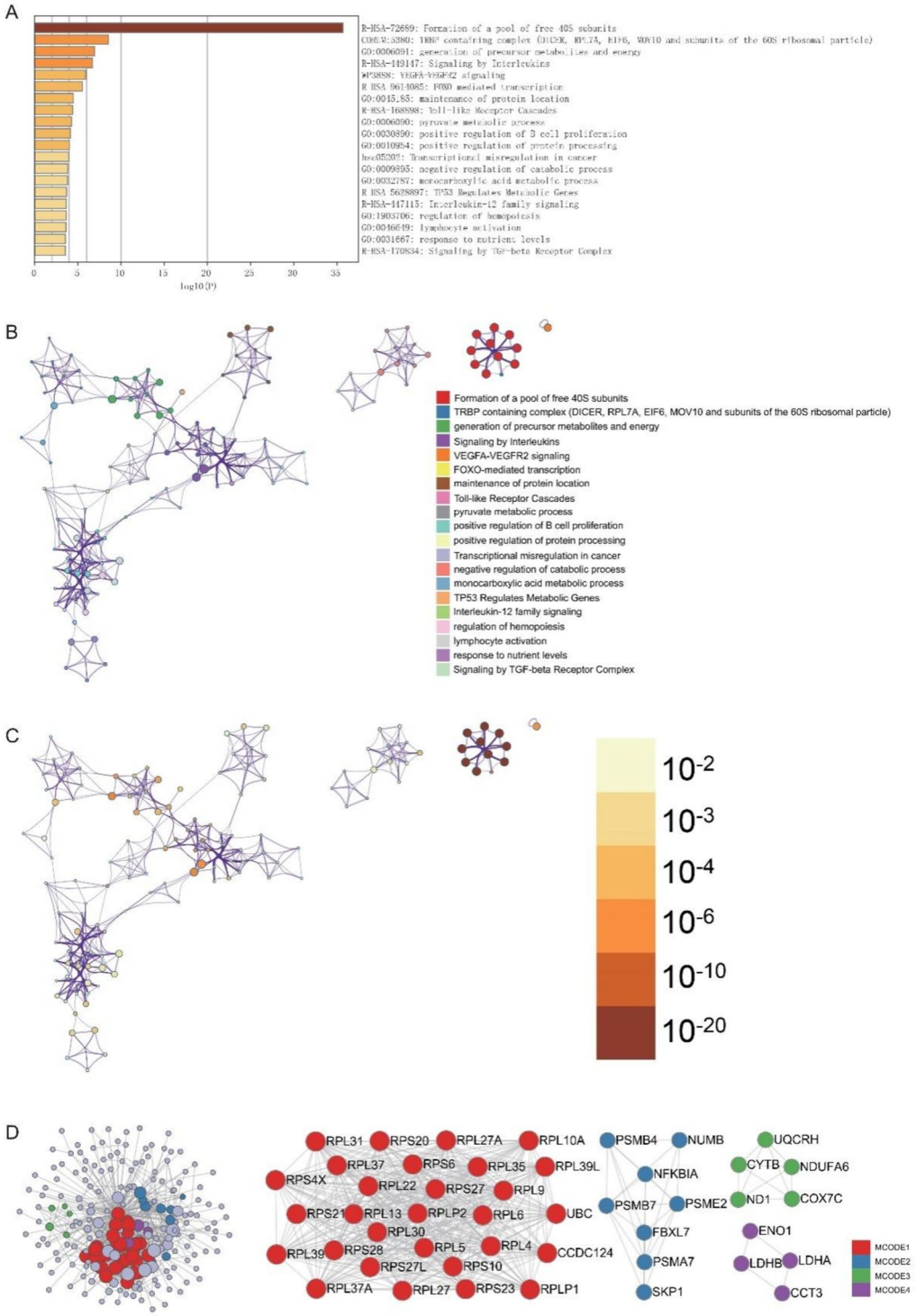

Fig. 3 Metascape enrichment analysis. **A** Bar graph of enriched terms across input gene lists, colored by *p* values. **B** Network of enriched terms: colored by cluster ID, where nodes that share the same cluster ID are typically close to each other. **C** Colored by *p* value, where terms containing more genes tend to have a more significant *p* value. D Protein–protein interaction network. MCODE components identified in the gene lists

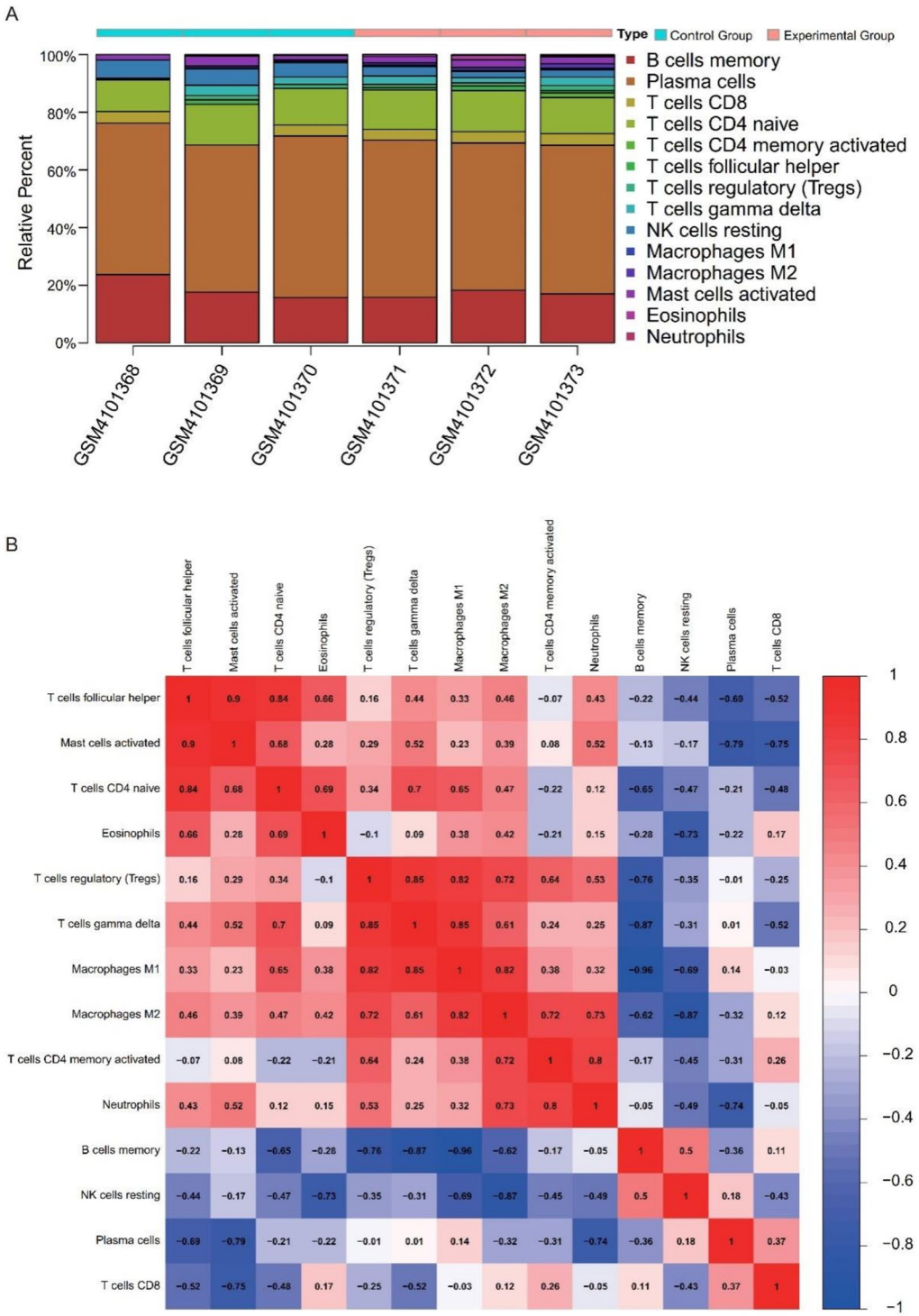

Fig. 4 Immunoinfiltration analysis. **A** Whole gene expression matrix results in proportion of immune cells. **B** Map of co-expression patterns between immune cell components

WNT3A promotes the expression of cell cycle proteins

The WB analysis revealed elevated expression levels of Cyclin-D1 and c-Myc molecules in CDDPSC group compared to DPSC group. Furthermore, as WNT3A expression increased in CDDPSC–OE, there was a corresponding augmentation in the expression levels

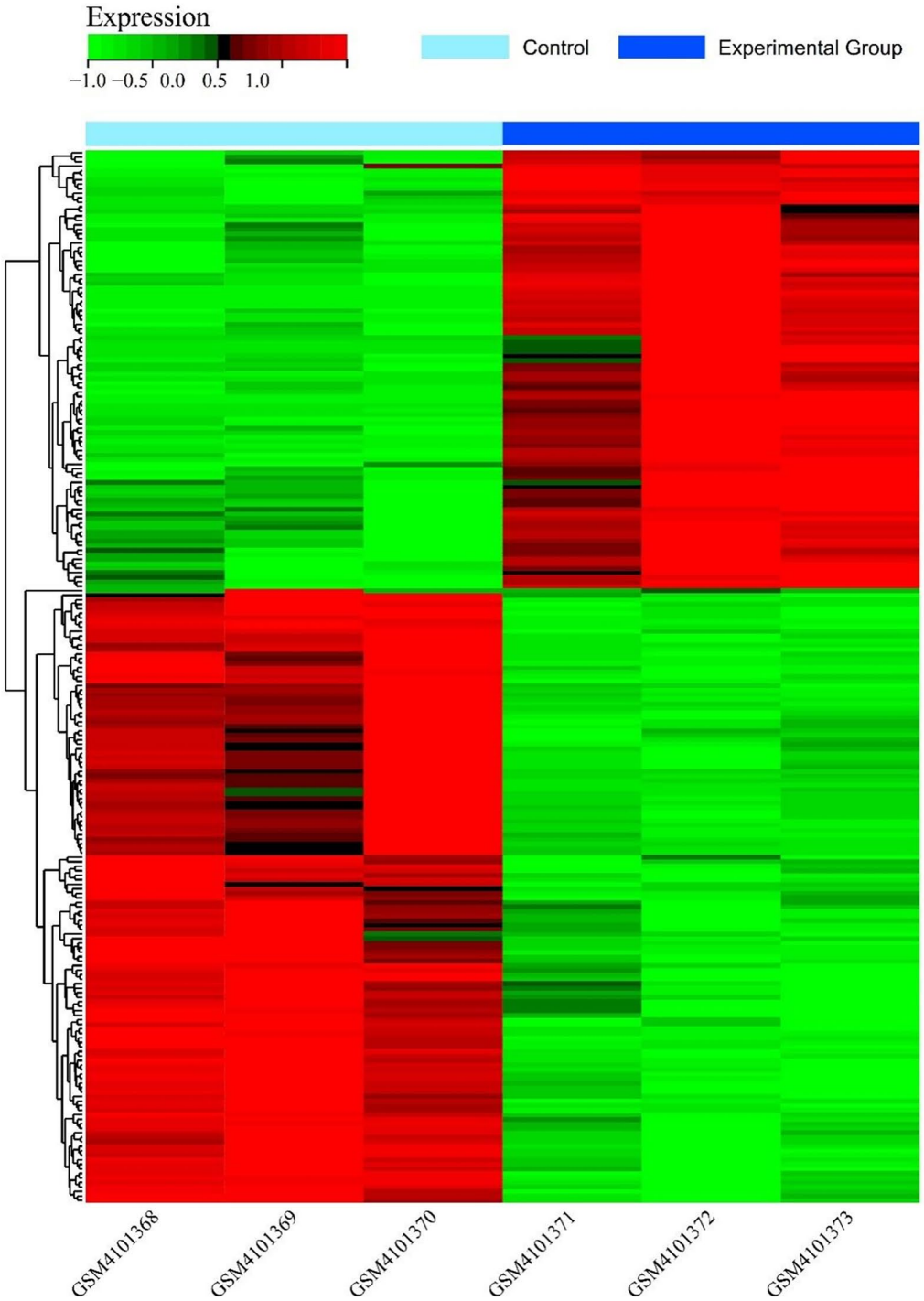

Fig. 5 Heat map of the differential gene in the gene expression matrix of the data sets GSE138179

of Cyclin-D1 and c-Myc, thereby enhancing the cell cycle progression. Conversely, upon downregulation of WNT3A expression in CDDPSC–KO, Cyclin-D1 and c-Myc expression levels decreased, consequently weakening the cell cycle. WNT3A plays a pivotal role in promoting cell cycle progression, as evidenced by its positive correlation with the expression levels of Cyclin-D1 and c-Myc (Fig. 10).

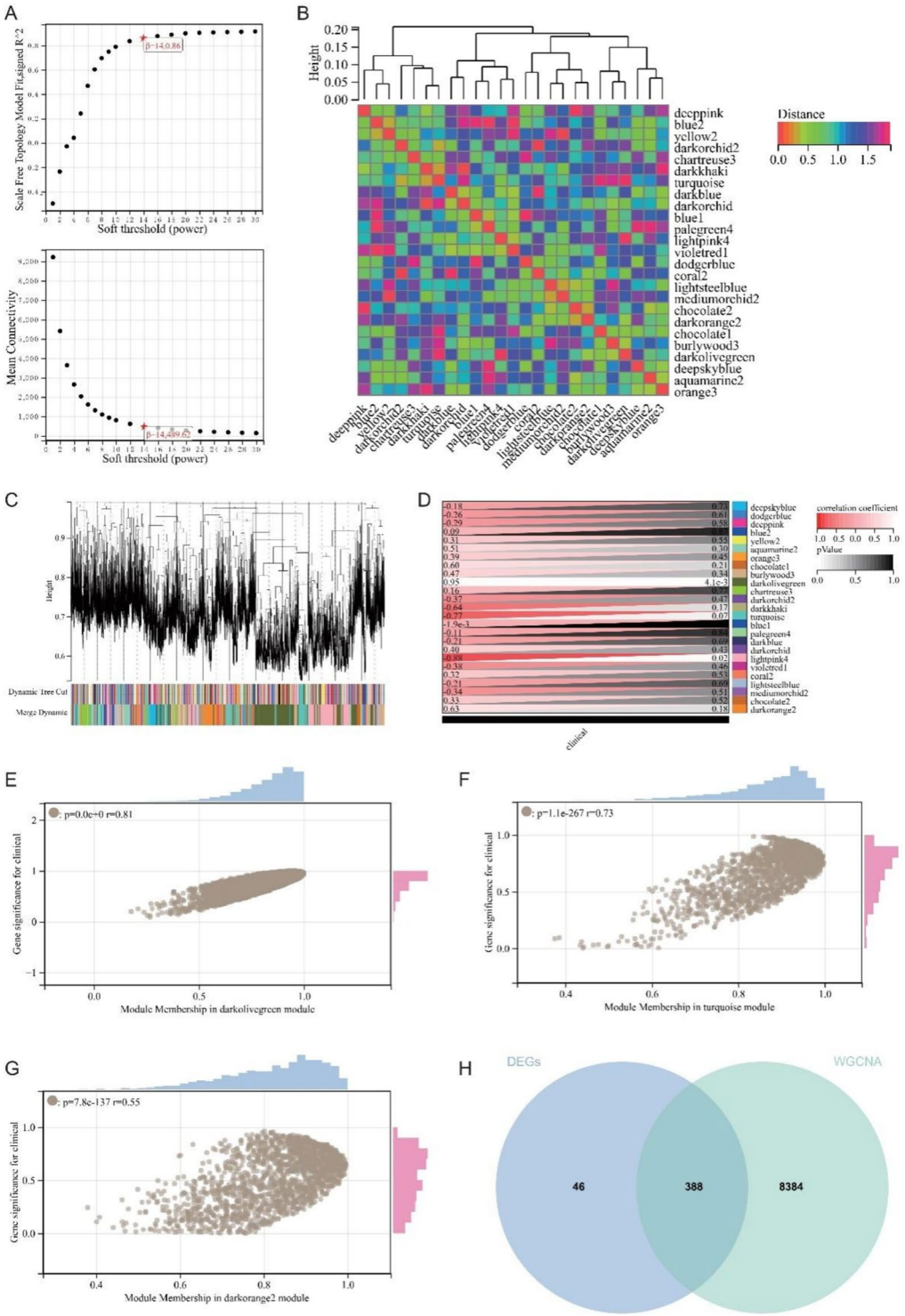

Fig. 6 WGCNA. **A** β = 14,0.86. β = 14,489.62. **B**, **C** Hierarchical clustering tree of all genes was constructed, and 25 important modules were generated. **D** Heat map of correlation between modules and phenotypes. **E**–**G** Scatter map of correlation between GS and MM of related hub genes. **H** DEGs screened by WGCNA and DEGs was used to obtain Venn map. 388 intersection genes were obtained

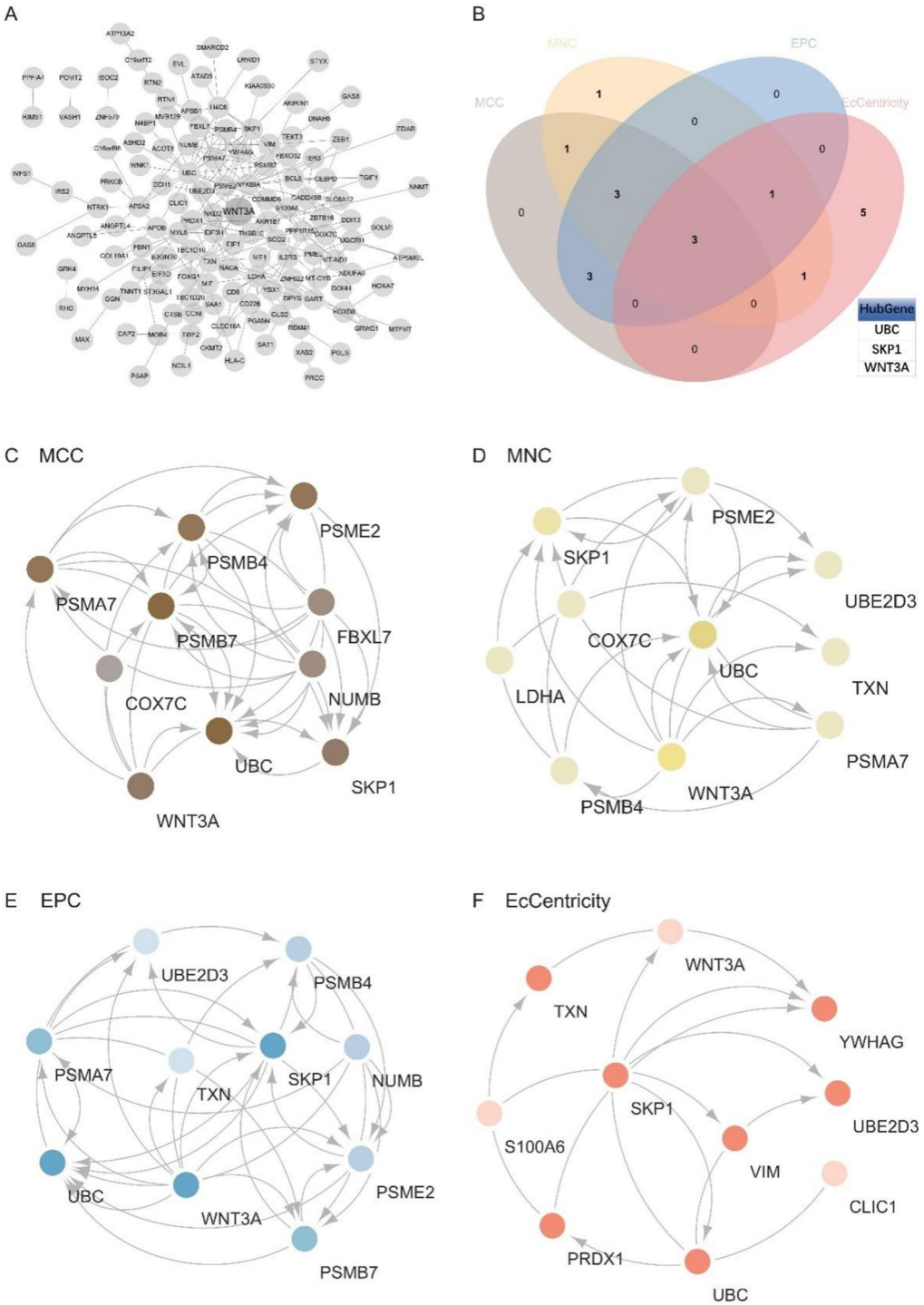

Fig. 7 Construction and analysis of protein–protein interaction (PPI) networks. **A** Construct the PPI network of DEGs using STRING online database and utilize Cytoscape software for analysis. **B** Core genes (UBC, SKP1, and WNT3A) were obtained by merging using Venn diagrams. **C** MCC was used to identify the central gene. **D** MNC was used to identify the central gene. **E** EPC was used to identify the central gene. **F** EcCentricity was used to identify the central gene

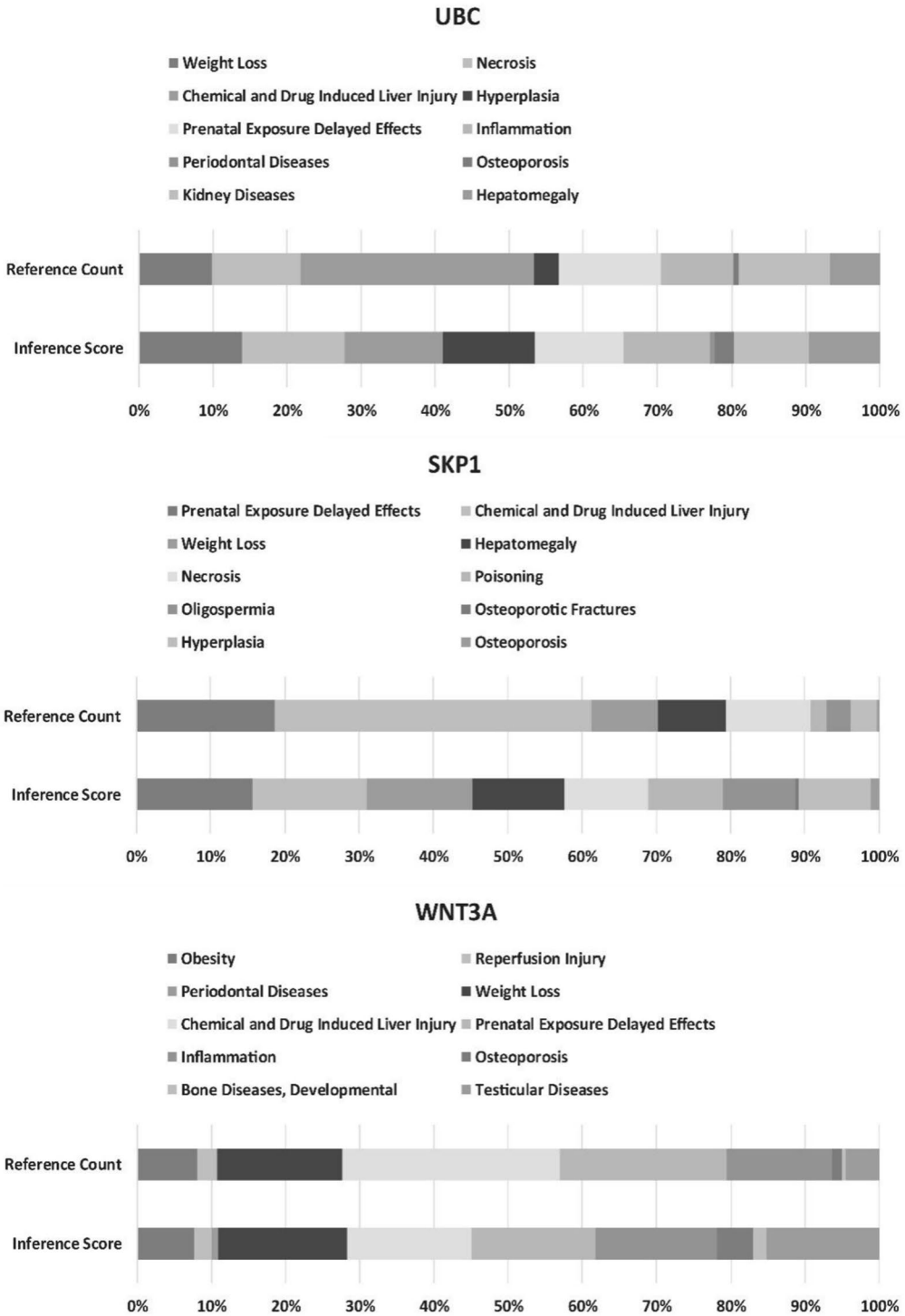

Fig. 8 CTD analysis. Three core genes (UBC, SKP1, and WNT3A) are associated with periodontal disease, osteoporosis, developmental bone disease, and necrosis

WNT3A reduces the expression of apoptosis-related proteins

The WB analysis demonstrated that in comparison to the DPSC group, in comparison to the Caspase-3, Caspase-9, FAS, P53, and BAX molecules were downregulated in the CDDPSC group. Moreover, as WNT3A expression

Table 1 A summary of miRNAs that regulate hub genes

	Gene	miRNA		
1	**UBC**	hsa-miR-130a-3p	hsa-miR-301a-3p	hsa-miR-4295
2	**SKP1**	hsa-miR-101-3p.2	hsa-miR-101-3p.2	
3	**WNT3A**	hsa-miR-216a-3p	hsa-miR-128-3p	hsa-miR-3681-3p

increased in CDDPSC–OE, the expression of Caspase-3, Caspase-9, FAS, P53, and BAX also decreased, leading to a reduction in apoptosis. Conversely, with the down-regulation of WNT3A expression in CDDPSC–KO, the expression of Caspase-3, Caspase-9, FAS, P53, and BAX increased, enhancing apoptosis. This result indicates that WNT3A reduces apoptosis (Fig. 11).

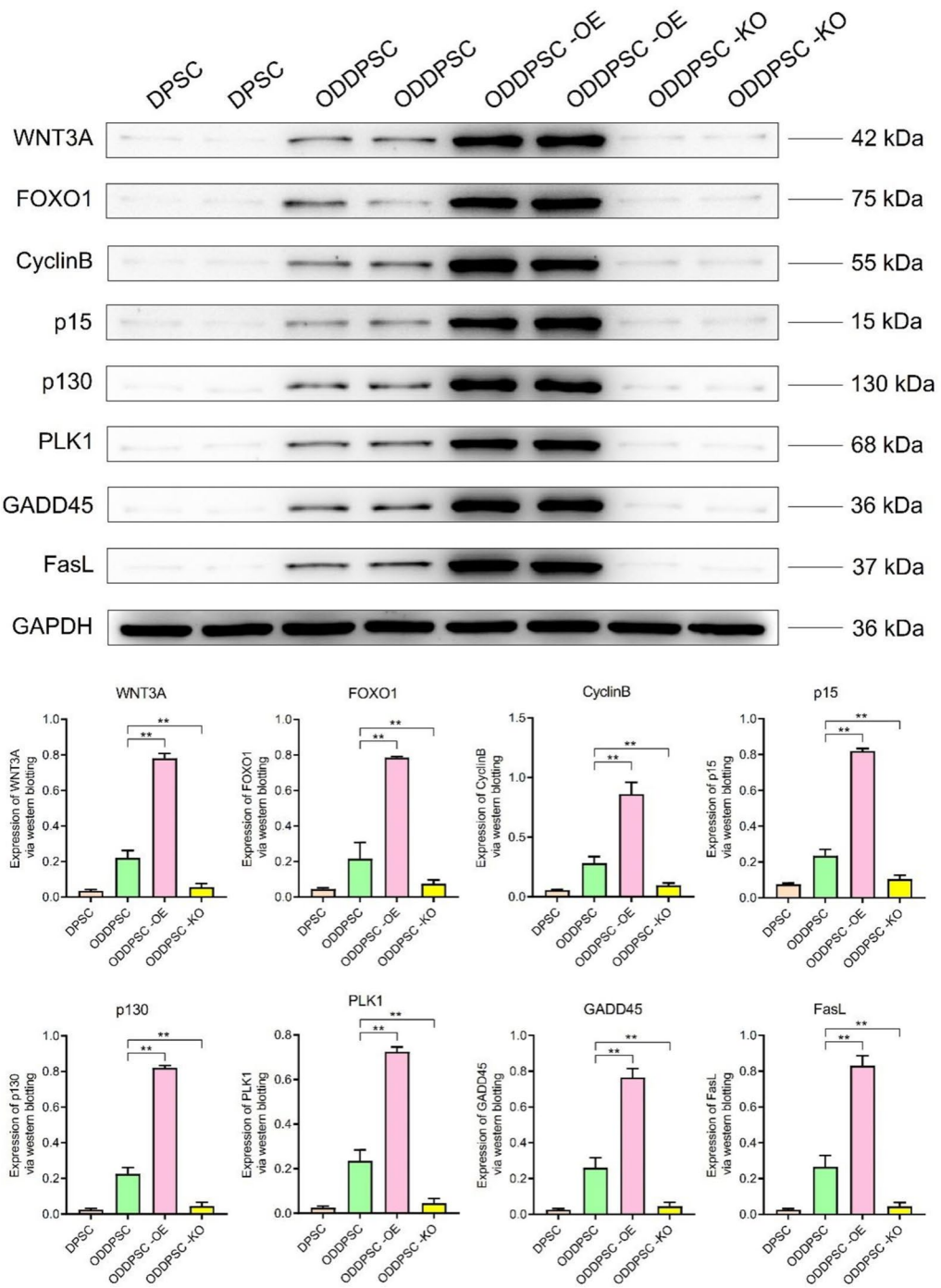

Fig. 9 WNT3A activates the FOXO1 signaling pathway

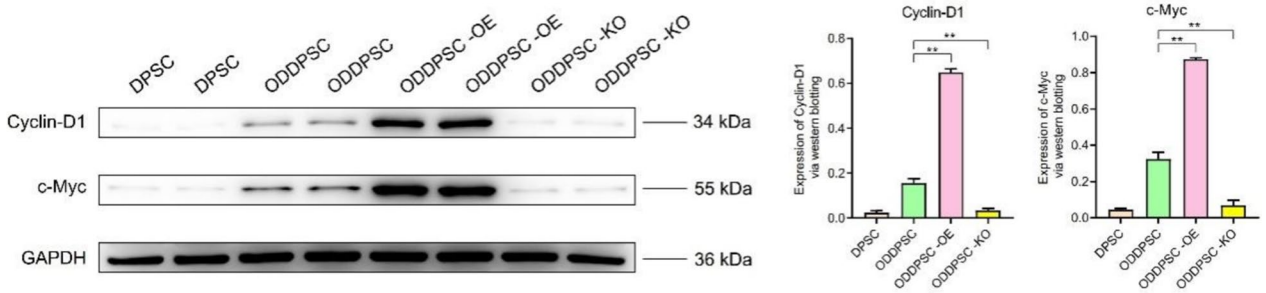

Fig. 10 WNT3A promotes the expression of cyclin

Discussion

The dental pulp is the soft tissue located at center of tooth, containing nerves, blood vessels, and mesenchymal cells [16]. Under normal conditions, dental pulp stem cells play crucial roles in maintaining the homeostasis of dental pulp tissue, repairing damaged tissue, and participating in tooth regeneration [17, 18]. Cementum is an essential component of tooth structure, playing a critical role in stability and function of teeth [19]. By inducing differentiation of dental pulp stem cells through various methods, they can develop into multiple cell types including cartilage, fat, vascular endothelial cells, and odontoblasts [20, 21]. This includes processes such as phenotypic transformation and matrix deposition, as well as the role of cementogenic functions [22–24]. Previous studies have found that WNT signaling has multiple inducing effects on DPSCs [25]. It can also be loaded into composite materials to promote DPSC oxidative stress resistance, enhance migration, and induce cementogenic differentiation. The potential mechanism is closely related to various biological processes and signaling pathways involved in dental pulp/dentin regeneration [26].

The results of this study demonstrate high expression of WNT3A during the cementogenic differentiation process of DPSCs. Increased expression of WNT3A facilitates the promotion of cementogenic differentiation of DPSCs, thereby playing a role in treatment of cementum diseases such as periodontitis and cementum resorption. WNT3A is a crucial member of the WNT signaling pathway, extensively involved in key biological processes such as cell differentiation, proliferation, tissue formation [25]. Its Gene Ontology (GO) annotations encompass critical roles in signal receptor binding and protein domain-specific interactions. As an integral part of the WNT protein family, WNT3A orchestrates cell fate determination and developmental processes by orchestrating both "canonical" and "non-canonical" WNT signaling cascades [27]. WNT3A plays a critical role in embryonic development, and its expression and function in adult tissues are crucial for maintaining tissue structure, regeneration, and determining stem cell fate [28]. Disruptions in WNT3A signaling have been implicated in a spectrum of diseases. The signaling mechanism of WNT3A involves binding to WNT receptors on the cell membrane, leading to the activation of downstream signaling molecules. This process regulates gene expression, cell proliferation, and cell fate determination, crucial for maintaining tissue homeostasis and adapting to environmental changes [29].

In addition, this study revealed that the upregulation of WNT3A also activates the Forkhead box O1 (FOXO1) signaling pathway. FOXO1 is a transcription factor that may regulate expression of cell cycle-related proteins in dental pulp stem cells, inhibiting cell proliferation and promoting differentiation towards odontoblastic lineage. Research has indicated that the FOXO1 pathway can be downregulated by ROR2, thereby promoting aging in dental pulp stem cells [30]. The study investigated role and mechanism of the FOXO1 pathway in cementogenic differentiation. Western blot experiments showed that key protein FOXO1 was upregulated in CDDPSCs compared to DPSCs, and conversely downregulated in CDDPSC–KO, indicating a positive correlation between WNT3A and FOXO1 signaling pathway. FOXO1 is known to regulate various diseases, depending on modulation of downstream targets [31]. Ren et al. [32] demonstrated that curcumin enhances Akt phosphorylation and inhibits Foxo1 acetylation, leading to increased apoptosis in diabetic rat cardiac cells. These findings suggest that the FOXO1 signaling pathway plays role in apoptosis, and other processes, consistent with the results of this study.

The results of this study indicate that increased expression of WNT3A in CDDPSC–OE leads to upregulation of Cyclin-D1 and c-Myc, enhancing the cell cycle. Cheng et al. [32] demonstrated that under low-nutrient conditions, FOXO1 induces Cyclin D1 to promote β-cell proliferation and triggers antioxidant mechanisms to prevent β-cell failure during oxidative

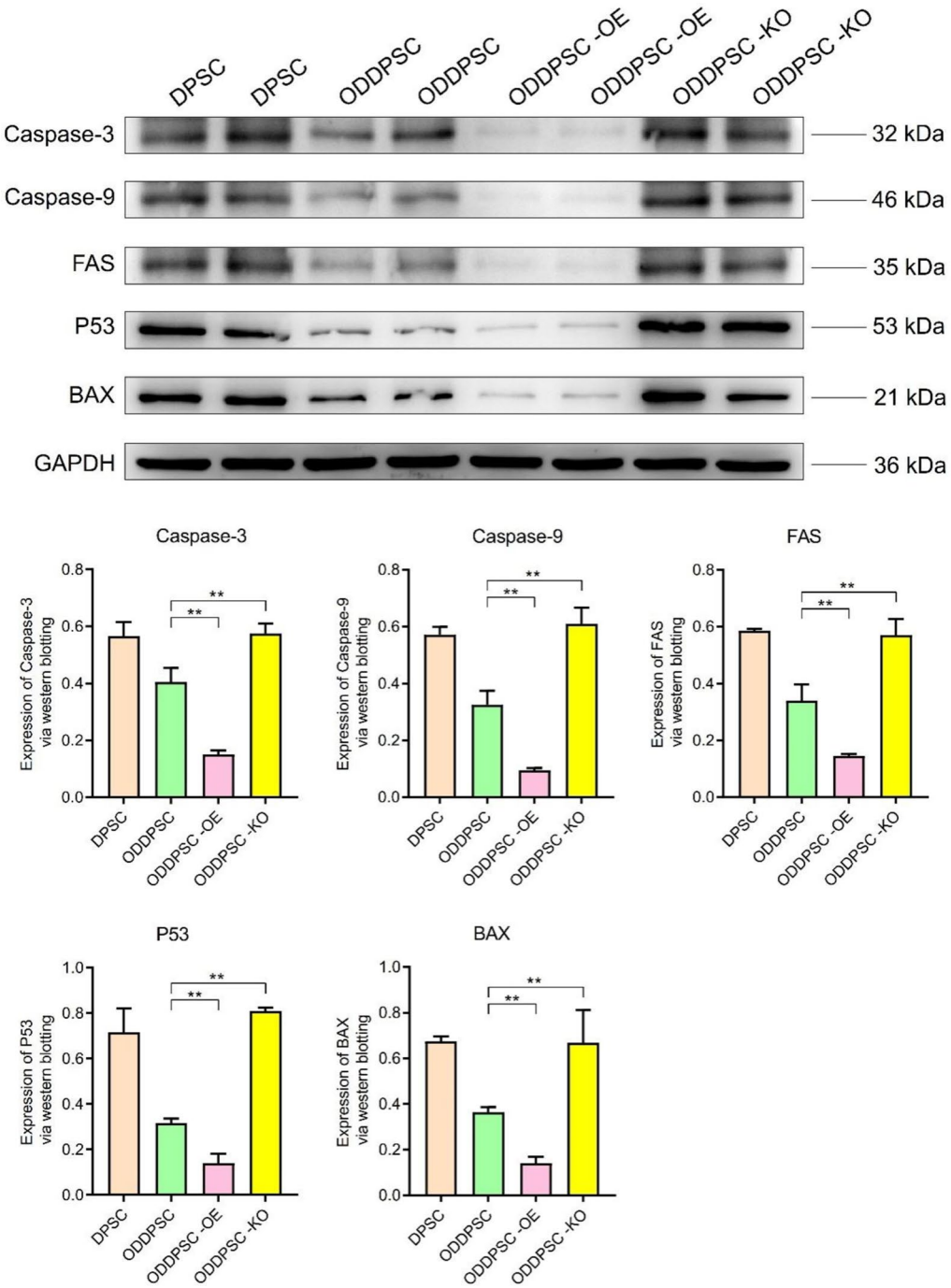

Fig. 11 WNT3A decreased the expression of apoptosis protein

stress. Peng's study [33] suggested that FOXO1 inhibits osteoclast formation mediated by MYC. In summary, high expression of WNT3A activates FOXO1, which regulates the expression of cell cycle-related proteins.

In CDDPSC–OE, increased expression of WNT3A leads to a decrease in expression of FAS, P53, and BAX, which are crucial factors in regulating the process of cell apoptosis. The reduced expression of these factors may inhibit cell apoptosis, resulting in a decrease in apoptosis. Studies have indicated that ERK1/2 and caspase signaling pathways trigger apoptosis in odontoblasts upon stimulation by exogenous ciliary neurotrophic factor

[34]. FOXO1 participates in regulating cell apoptosis by promoting the expression of apoptosis-related genes, thereby facilitating cell apoptosis and maintaining tissue homeostasis and health.

In dental pulp stem cells, activated FOXO1 regulates the expression of cell cycle-related genes, inhibiting cell proliferation, and promoting cementogenic differentiation. Similarly, FOXO1 may promote apoptosis of improperly differentiated cells by regulating expression of apoptosis-related genes, ensuring normal progression of differentiation. In addition, oxidative stress, DNA repair, and other mechanisms are also crucial in WNT3A activating the FOXO1 signaling pathway to influence the differentiation process of dental pulp stem cells. FOXO1 regulates cellular metabolic pathways, affecting cellular energy metabolism and nutrient signaling, thereby influencing the differentiation direction and functional expression of dental pulp stem cells. For instance, in cardiomyocytes, FOXO1 can activate increased expression of KLF5 and NOX4, as well as accumulation of ceramides, leading to diabetic cardiomyopathy [35].

The activation of FOXO1 signaling pathway by WNT3A influences differentiation process of various cells, including dental pulp stem cells. Research has demonstrated that the activation of FOXO signaling by WNT3A induces a transition of fiber types from fast-twitch to fatigue-resistant fibers in C2C12 cells. WNT signaling may thus represent a novel therapeutic target for preventing chronic heart failure-related skeletal muscle diseases [36]. Similarly, WNT signaling also serves as a promoting factor for differentiation into odontoblasts [37].

Dental pulp stem cells (DPSCs), with their easer of acquisition, higher proliferation and differentiation potential, and specificity for cementogenesis than other stem cells, such as PDSCS.DPSCs provide a solid foundation for studying the WNT3A–FOXO1 signaling pathway. Their unique advantages in regenerative medicine make them a core cell source for research in oral tissue engineering and periodontal disease treatment. In addition, dental pulp stem cell injection has been successfully developed by our research group and applied in clinical treatment research in the early stage. Dental pulp stem cells are used in cementum regeneration research, which is simpler, easier to obtain, and more convenient for clinical transformation of scientific research results in the later stage. Based on this, we believe that DPSCs may be more ideal seed cells for cementum regeneration. In this study, WNT3A promotes cementogenesis of DPSCs via the FOXO1 signaling pathway, enriching our understanding of cementogenesis mechanisms and offering new perspectives for the potential treatment of cementum-related diseases. WNT3A activation of the FOXO1 signaling pathway enhances the cementogenesis capability of DPSCs, suggesting that external regulation of WNT3A expression or FOXO1 activity may promote the regeneration of damaged cementum. FOXO1, as a key stress-response factor, plays a critical role in regulating cellular responses to oxidative stress and inflammation. Enhancing the WNT3A or FOXO1 pathway may provide novel therapeutic strategies for cementum repair in chronic inflammatory conditions. Furthermore, the WNT3A signaling pathway can further enhance regenerative effects when combined with biomaterials. Incorporating WNT3A-carrying molecules or FOXO1 activators into scaffold materials can significantly promote the targeted differentiation of DPSCs at damaged sites. This combination of materials and cell therapy offers a highly controllable approach to repairing cementum defects. Regulating the WNT3A–FOXO1 pathway to guide normal cementum formation also holds promise for promoting tooth and root development.

In summary, WNT3A regulates fate, and differentiation of dental pulp stem cells, thereby influencing their potential for odontogenesis and contributing to the maintenance of dental bone tissue. Moreover, WNT3A interacts with FOXO1 and other signaling pathways and molecular factors, forming a complex regulatory network that further modulates the cementogenic differentiation of DPSCs. This discovery provides a new perspective for understanding formation and maintenance of cementum, helping to elucidate the mechanisms underlying related dental bone diseases and offering new targets and strategies for their treatment.

Although this study has yielded valuable findings, uncovering the mechanism by which WNT3A promotes cementogenesis of DPSCs through the FOXO1 signaling pathway and opening new avenues for cementum regeneration therapy, its limitations must be acknowledged. Further research is required to validate its mechanisms within complex tissue microenvironments, explore precise regulation of this signaling pathway, and evaluate its safety and efficacy in animal models and clinical trials. In the future, by integrating gene editing technologies, biomaterials, and efficient delivery systems, these research outcomes hold promise for clinical translation, offering innovative solutions for the treatment of cementum-related diseases.

In conclusion, during the cementogenic differentiation of DPSCs process, WNT3A is highly expressed and interacts with signaling pathways and molecular factors such as FOXO1. This interaction guides the cementogenic differentiation of DPSCs.

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Author contributions
Author Contributions Dongmei Cheng: Conceptualization; data curation; methodology; project administration; formal analysis; writing—original draft; Yang Bao: writing—original draft; writing—review & editing; visualization;Xue Wang,Tianyuan Guo,Haidong Xiang,Yong Du,Zhiyong Zhang: data curation; formal analysis; visualization; Chunmei Zhang: methodology; investigation; formal analysis; Han Guo: Conceptualization; methodology; formal analysis; supervision; funding acquisition; validation; resources; writing—review & editing. All authors gave final approval and agreed to be accountable for all aspects of the work.

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Availability of data and materials
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Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

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