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RAPID COMMUNICATION

PTGS2 identified as a biomarker of glucocorticoid-induced osteonecrosis of the femoral head and an enhancer of osteogenesis

Due to the use of more adjuvant therapies and the increasing prevalence of associated diseases, nontraumatic osteonecrosis of the femoral head (ONFH) has become a substantial worldwide health issue; while glucocorticoid administration was the highest-ranked risk factor, with a proportion ranging from 26.35 to 55.75%.[1] Albeit with similar radiological features, glucocorticoid-induced ONFH (GONFH) manifested a greater probability for advance-to-late-stage (post-collapse) lesions in comparison with its non-glucocorticoid-induced counterparts.[1] Therefore, an accurate and timely diagnosis of osteonecrosis following glucocorticoid therapy is of great significance to initiate nonoperative treatment regimens or joint-preserving procedures, slow disease progression, and defer or avert joint arthroplasty.

Glucocorticoids could affect circulating molecules at multiple levels in either excitatory or inhibitory methods, which reflects the systemic change during the development of GONFH. To identify potential circulating targets for the diagnosis and treatment of this disease, GEO (http://www.ncbi.nlm.nih.gov/geo/) database was systematically searched and eventually, the microarray data of GSE123568 were identified and downloaded, which enrolled a total of thirty patients with GONFH and ten participants without GONFH (following glucocorticoid administration) to develop the Affymetrix Human Gene Expression Array (a platform of GPL15207, Affymetrix, Inc., Santa Clara, CA, USA). Figure S1A demonstrated that after data normalization, the black lines were almost in the same position, indicating an excellent degree of standardization, ensuring the accuracy of subsequent data processing. Then, the limma eBayes method in Bioconductor was used to identify differentially expressed genes (DEGs) between GONFH and non-GONFH samples, following the criteria of the $|\log_2\text{fold change (FC)}| \geq 1.5$ with adjusted *P*-values (adj. *p*. val) < 0.05. A total of 18,835 genes were obtained following data preprocessing, among which, 118 (0.626%) were considered to be up-regulated, 59 (0.313%) were down-regulated and the expression of remaining transcripts were stable. In addition, a volcano plot and a heatmap of all DEGs were generated using the R ggplot2 package (Fig. 1A; Fig. S1B).

The up- and down-regulated DEGs were uploaded to an online biological information database, the Database for Annotation, Visualization, and Integrated Discovery (DAVID) version 6.8 Beta (https://david-d.ncifcrf.gov/), for further analysis. Gene ontology (GO) enrichment analysis of 177 DEGs between the GONFH group and the control group was performed to identify the most relevant biological processes (BPs), molecular functions (MFs), and cellular components (CCs). The top ten enriched terms in BPs, CCs, and MFs were presented in Figure 1B, S1C, and S1D as well as Table S1. In the present study, the inflammatory response was the top associated biological process term under GO analysis. Meanwhile, "positive regulation of NF-κB import into nucleus" and "immune response" were also enriched. Additionally, based on the KEGG pathway analysis, the DEGs were significantly enriched in 10 signaling pathways, such as cytokine–cytokine receptor interaction, rheumatoid arthritis, and phagosome (Fig. 1C). Multiple studies have proved that TLR4/NF-κB pathway plays a pivotal role in the pathogenesis of GONFH. NF-κB and its ligand (RANKL) are expressed on osteoblasts, and involve in the differentiation and proliferation of osteoclasts.

The interactions between the proteins expressed from DEGs, which consisted of 113 nodes and 289 edges (Fig. S2A), were constructed from STRING database (https://www.string-db.org/)[2] and visualized using Cytoscape 3.7.2 software. Three significant modules (Fig. 1D) were obtained by module analysis in the protein–protein interaction network using MCODE. In addition, 10 top

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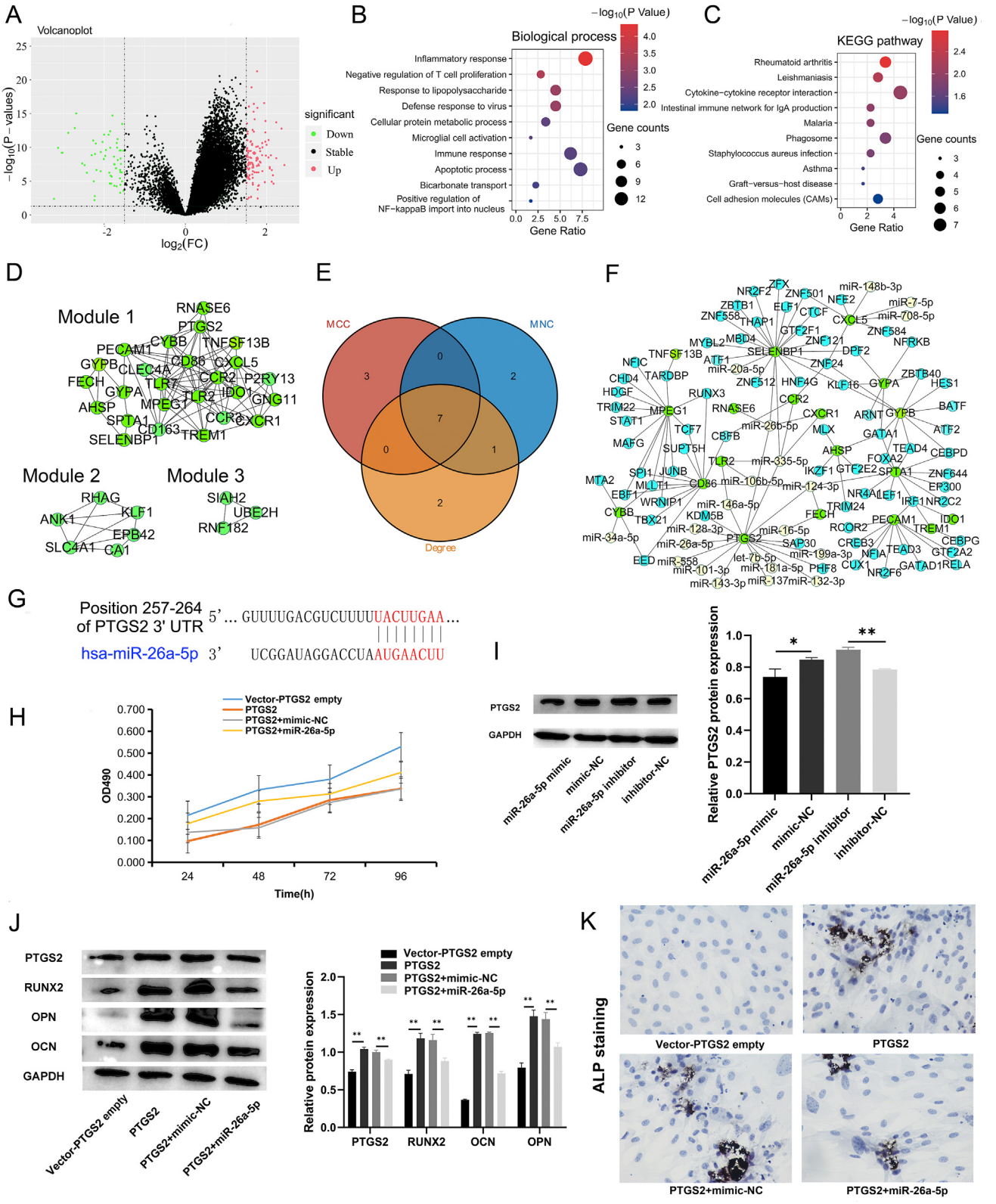

Figure 1 Transcriptomic analysis and experimental verification identified PTGS2 as a biomarker in the development of glucocorticoid-induced osteonecrosis of the femoral head (GONFH) and an enhancer of osteogenesis. **(A)** 177 DEGs are shown in the volcano plot, containing 118 upregulated genes in red and 59 downregulated genes in green, following the criteria of the $|\log_2 \text{fold change (FC)}| \geq 1.5$ with an adjusted *P*-values (adj. *p*. val) < 0.05. Black indicates no difference in expression. Gene ontology including biological process **(B)**, and Kyoto Encyclopedia of Genes and Genomes **(C)** enrichment analysis of these 117 DEGs between thirty patients with GONFH and ten participants without GONFH (following glucocorticoid administration). **(D)** Three significant

genes with relatively high connectivity degrees (≥12) were: TLR2 (degree = 30), CD86 (degree = 21), TLR7 (degree = 20), CCR2 (degree = 18), CYBB (degree = 17), SLC4A1 (degree = 17), EPB42 (degree = 16), AHSP (degree = 13), CD163 (degree = 13), and TREM1 (degree = 12). The top 10 hub genes, which were selected based on the three most commonly used classification methods (degree, MNC and MCC) in cytoHubba, were presented in Table S2. By overlapping the first 15 genes, 7 central genes (*TLR2*, *CCR2*, *CD86*, *TLR7*, *CYBB*, *SLC4A1* and *EPB42*) were consequently identified as presented in Figure 1E. We observed that the five hub genes (*TLR2*, *CCR2*, *CD86*, *TLR7* and *CYBB*) were up-regulated and gathered in module 1, while two hub genes (*SLC4A1* and *EPB42*) were down-regulated and gathered in module 2. TLR2 is a plasma membrane-bound pattern recognition receptor that signals as a heterodimer with either TLR1 or TLR6, which could induce proinflammatory cytokines, osteoclastogenesis, bone resorption and systemic bone loss.[3] The protein CD86 (Cluster of Differentiation 86) is a molecule expressed on antigen-presenting cells that provide costimulatory signals necessary for T cell activation and survival.[3] The percentage of $CD86^+$ B cells in patients with steroid-related, alcohol-related, or idiopathic ONFH were significantly higher than that in the healthy control, which may be associated with the development of ONFH.

Then these DEGs were uploaded to NetworkAnalyst 3.0 (https://www.networkanalyst.ca/faces/home.xhtml), a visual analytics platform for comprehensive gene expression profiling and meta-analysis, to obtain the transcription factors (TFs) and microRNAs (miRNAs) targeting the screened DEGs.[4] The miRNA—DEG pairs were identified through network analysis of DEGs using the TarBase and miRTarBase databases. Finally, a total of 34 associations between 22 miRNAs and only 12 DEGs were identified and visualized in Cytoscape (Fig. S2B). As is shown, PTGS2 regulated 15 interacting miRNAs (e.g., hsa-mir-558 and hsa-mir-26a-5p). According to TF binding site data and genetic coordinate position information provided on ENCODE, the potential regulatory network between DEGs and TFs was constructed to analyze the functional roles of the selected DEGs. A total of 104 associations between 75 TFs and 16 DEGs were predicted (Fig. S2C). The merged TF—gene—miRNA interacted network (Fig. 1F and Table S3) included 21 DEGs, 75 TFs, and 22 miRNAs, with 104 associations between the TFs and DEGs, and 34 associations between the miRNAs and DEGs. Then, we found that GATA1, ZNF24 and IRF1 regulated 4 interacting DEGs. Simultaneously, hsa-mir-335-5p regulated 5 interacted DEGs.

The diagnostic accuracy of the identified DEGs was analyzed using the R pROC package. The receiver operating characteristic curves (ROCs) were constructed to calculate the area under the curve (AUC) and the Youden index (sensitivity + specificity-1). Five hub genes (*TLR2*, *CCR2*, *CD86*, *TLR7*, and *CYBB*) and a selected upregulated gene (*PTGS2*) were analyzed and revealed high diagnostic values (AUC > 0.8). For *TLR2*, *CCR2*, *CD86*, *TLR7*, *CYBB* and *PTGS2*, the AUC values were 0.930, 0.897, 0.917, 0.900, 0.873 and 0.970, respectively, while the maximum Youden indexes were 0.767, 0.900, 0.833, 0.800, 0.733 and 0.833, respectively (Fig. S3A—F).

Although *PTGS2* was not identified as a hub gene in the PPI network, its effects on osteogenesis of BMSCs were assessed *in vitro* experiments for several reasons. First, PTGS2 is significantly upregulated ($\log_2FC = 2.05$) in the serum of patients with GONFH. Second, in the miRNA-DEG network analysis, PTGS2 regulated 15 interacting miRNAs, which provided multiple candidates for disease intervention. Third, PTGS2 is highly expressed in the bone marrow (Fig. S3G) and could bind with miR-26a-5p according to the Starbase database (Fig. 1G) and published literature.[5] Last but not least, the AUC of PTGS2 for diagnosing GONFH is 0.970 (Fig. S3F), which is higher than the five selected hub genes. In the first place, miR-26a-5p expression in human BMSCs after transfection with miRNA mimics and inhibitors was confirmed by using qRT-PCR (Fig. S3H). After transfection of PTGS2, the cell viability was decreased, which could be ameliorated by miR-26a-5p (Fig. 1H). In Western blot analysis, the expression level of PTGS2 was significantly lower in the miR-26a-5p mimic group than in the mimic NC group, while considerably higher in the miR-26a-5p inhibitor group than in the inhibitor NC group (Fig. 1I). Meanwhile, the expression level of PTGS2 and osteogenic markers of RUNX2, OCN and OPN, and the viability of ALP was significantly increased in the Vector-PTGS2 group than in the Vector-PTGS2 NC group (Fig. 1J, K). The osteogenic effect of PTGS2 could be revised by miR-26a-5p. PTGS2, also known as cyclooxygenase 2 (COX-2), is a critical enzyme in prostaglandin biosynthesis. This enzyme is expressed in inflammation and could augment osteoblast differentiation in mesenchymal stem cells.[1] Therefore, PTGS2 may serve as a promising target for normal tissue repair and the treatment of ONFH.

The identified DEGs, functional terms and pathways in the present study can help shed light on the molecular mechanism underlying the pathogenesis of GONFH and provide potential diagnostic markers for early detection

modules in the protein—protein interaction (PPI) network using the ENCODE database. **(E)** Hub genes were identified by the overlap of the three methods (degree, MCC, and MNC) in CytoHubba. **(F)** Integrative regulatory network of TF-DEG-miRNA. Green indicates gene, and yellow indicates miRNA, and blue indicates transcription factor (TF). **(G)** Binding sites between PTGS2 and miR-26a-5p. **(H)** The cell viability of BMSCs in the Vector group, PTGS2 group, PTGS2+mimic-NC group and PTGS2+miR-26a-5p group. **(I)** The expression level of PTGS2 in the miR-26a-5p mimic group, mimic-NC group, miR-26a-5p inhibitor group and inhibitor-NC group. $^*P < 0.05$, $^{**}P < 0.01$. **(J)** The expression level of PTGS2 and osteogenic markers of RUNX2, OCN and OPN in the Vector group, PTGS2 group, PTGS2+mimic-NC group and PTGS2+miR-26a-5p group. **(K)** The viability of alkaline phosphatase (ALP) in the Vector group, PTGS2 group, PTGS2+mimic-NC group and PTGS2+miR-26a-5p group. DEGs, differentially expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; TF, transcription factor; DEG, differentially expressed gene; miRNA, microRNA; MCC, maximal clique centrality; MNC, maximum neighborhood component.

and treatment of this devastating disease. Combined with various bioinformatic analyses, PTGS2 may serve as a therapeutic candidate for GONFH.

Conflict of interests

All the authors declare that they have no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.01.005.

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