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ORIGINAL ARTICLE

TMT-labelled quantitative proteomic analysis to predict the target promoting human odontogenic inflammatory granulation tissue transform into reparative granulation tissue

Jianying Zhang[a]*, Dengke Li[a]*, Xueni Zheng[a], Wuyang Zhang[a], Rui Hou[a], Changkui Liu[b], Yu Zhang[a], Kaijin Hu[a], Hongzhi Zhou[a] and Yang Xue[a]

[a]Department of Oral and Maxillofacial Surgery, School of Stomatology, State Key Laboratory of Military Stomatology & National Clinical Research Center for Oral Diseases & Shaanxi Clinical Research Center for Oral Diseases, The Fourth Military Medical University, Xi'an, China; [b]Department of Oral and Maxillofacial Surgery, School of Stomatology, Xi'an Medical University, Xi'an, China

ABSTRACT

Objectives: Odontogenic inflammatory diseases are main causes for alveolar bone breakdown and teeth loss, leaving great difficulties in denture restoration. Local inflammatory granulation tissue (IGT) is considered as pathological tissue and required to be removed. However, there are many evidences supporting that under appropriate intervention, IGT in alveolar bone maybe transformed into reparative granulation tissue (RGT), followed by ossification. Therefore, this study aimed to discover a specific target to promote this transformation.

Materials and methods: After drawing out histological differences between IGT and RGT with haematoxylin and eosin (H&E) and immunohistochemical (IHC) assay staining, TMT-labelled quantitative proteomic analysis was applied to identify potential targets.

Results: The most striking histological property of RGT was found to be ECM deposition, which significantly decreased inflammatory cells, prominently increased fibroblasts as well as triggered changes of vascular types. Combined with histological findings and proteomic analysis, five KEGG pathways were associated with ECM, inflammation and angiogenesis and 49 pathways involved in differentially expressed proteins. COL1A1 was not only the most up-regulated protein, but also one of main hubs in protein–protein interaction regulatory network. Specific protease cathepsin K (CTSK) was identified. Level of CTSK in RGT was down-regulated to 69.10–76.97% ($p < .05$), with significantly up-regulated COL1A1, COL1A2, FN1 and TGFB1 included in focal adhesion, PI3K-Akt signalling pathways and angiogenesis. CTSK involved in transformation from IGT to RGT.

Conclusions: CTSK might be a target to regulate transformation from IGT to RGT in alveolar bone through ECM, stem cells and angiogenesis mechanisms. However, further research is also clearly required.

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KEYWORDS
Odontogenic inflammatory diseases; granulation tissue; proteomic; extracellular matrix; cathepsin K (CTSK)

Introduction

Odontogenic inflammatory diseases, such as periodontitis, periapical inflammation and pericoronitis of the third molar, are a series of severe chronic inflammatory diseases affecting majority of world's adult population [1,2]. These diseases are the main causes of alveolar bone breakdown and loss of teeth, leaving great difficulties in denture restoration, especially dental implant [1–5]. These diseases are commonly characterized by the eroded alveolar bone, which then replaced with the inflammatory granulation tissue (IGT). Therefore, the IGT is generally considered as the pathological tissue and required to be removed by operation, no matter the tooth should be preserved or not [1,6].

Since the concept of 'osteoimmune' was put forward in year of 2000, there have been more and more evidences supporting that odontogenic inflammatory diseases should be kinds of osteoimmune disorders [1,3–5]. Great efforts have been made to promote the recovery of inflammatory lesions by regulating local osteoimmune [2,5]. On the other hand, osteogenic cells as well as stem cells with osteogenic differentiation potential have been identified in both periodontal and periapical IGT [3,7–11]. In theory, under appropriate intervention, IGT in alveolar bone may be transformed into reparative granulation tissue (RGT), followed by ossification and repairing bone defect. Strong evidence could be found in clinical to support this assumption. For example, in many periapical inflammation patients, the destroyed bone would heal gradually after perfect root canal treatment. In a part of periodontitis patients, the damaged bone can regenerate after occlusal adjustment. In these patients, the IGT can transform to RGT and then be completely replaced by new

CONTACT Yang Xue xueyangfmmu@163.com; Kaijin Hu hukaijin2015@126.com; Hongzhi Zhou hongzhi.zhou@foxmail.com Department of Oral and Maxillofacial Surgery, School of Stomatology, State Key Laboratory of Military Stomatology & National Clinical Research Center for Oral Diseases & Shaanxi Clinical Research Center for Oral Diseases, The Fourth Military Medical University, 145 West Changle Road, Xi'an 710032, PR China
*These authors contributed equally to this work.

bone. However, in most of patients, the diseases are progressing rapidly and are difficult to achieve ideal results by dental treatment alone.

The oral tissues usually considered as the alternative and valid sources for the mesenchymal stem cells (MSCs) that play the similar biofunctions of MSCs derived from the bone marrow [12,13]. The orally derived MSCs also exhibit a series of characteristics or capabilities, including adherence to plastic substrate and fibroblast-like phenotype, ability to differentiate into adipogenic, osteogenic and chondrogenic lineages and capability to enhance bone formation [12,13]. Moreover, a few studies [12–15] have focussed on the stem cells derived from human oral cavity according to their easy access, high proliferative ability and multipotent differentiation potential. The regenerative endodontic procedures are commonly conducted to replace the IGT and damaged structures, such as root and dentin tissues or structures, and the pulp–dentin complex [16,17]. The oral tissue derived MSCs might act as the materials for the pulp–dentin regeneration. Therefore, the oral tissue derived stem cells might benefit to the transformation from the IGT to RGT. Presently, it is urgent to find a specific target which can stop inflammation, inhibit bone destruction, activate stem cells and finally promote transformation from IGT to RGT, resulting in bone regeneration and healing. In order to find this target, the differences between IGT and RGT should be detected first.

The present study applied TMT-labelled quantitative proteomic analysis to identify the differentially expressed proteins (DEPs) in RGT comparing with IGT to describe the involved biological processes (BPs) and molecular pathways. The results would contribute to find the potential targets that could stop and even reverse odontogenic inflammatory diseases, and provide a basis for understanding alveolar bone healing and regeneration in inflammatory microenvironments.

Materials and methods

Sample preparation

Human granulation tissues from alveolar bone were obtained from 10 patients (with age of 31.00 ± 9.59 years, six female and four males), who had undergone delayed autogenous tooth transplantation surgery in School of Stomatology, the Fourth Military Medical University. The IGT was collected during tooth extraction. The RGT which needed to be removed for treatment was collected during tooth transplantation 2 weeks later. Three pairs of samples for histological examination were fixed in 4% paraformaldehyde, while seven pairs of samples for analysis of DEPs were stored at −80 °C after collection. To reduce biological variation from patient to patient, three pooled samples both in the inflammatory group and the reparative group were used. Each pooled sample contained two granulation tissues, respectively.

This study has been authorized and approved by Ethic Committee of School of Stomatology, the Fourth Military Medical University, Xi'an China (approval no. IRB-REV-2019083). Informed consents for experiments were obtained from all the patients.

H&E and IHC staining

Samples were fixed in 4% paraformaldehyde overnight and embedded in paraffin. Paraffin sections were stained with haematoxylin and eosin (H&E) (Beyotime, Beijing, China) as instructed by the manufacturer. For immunohistochemical (IHC) analysis, the slides were pre-incubated with 3% H_2O_2 for 10 min to inhibit endogenous peroxidase. Goat serum was used to block the non-specific binding and then the sections were incubated with rabbit anti-cathepsin K (CTSK) antibody (1: 200, Cat. no. ab19027, Abcam, Cambridge, MA), rat anti-endomucin (EMCN) antibody (1:100, Cat. No. ab106100, Abcam, Cambridge, MA) and rabbit anti-CD31 antibody (1: 200, Cat. No. ab28364, Abcam, Cambridge, MA) at 4 °C overnight. After that, the sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 30 min at 37 °C. Colour for the staining was developed using a DAB substrate kit (Boster, Wuhan, China), and then counterstaining with haematoxylin. Then, we used the Image J to quantitative analysis the average optical density (AOD) of CTSK-staining.

TMT-labelled quantitative proteomic analysis

TMT-labelled quantitative proteomic analysis was implemented according to the literatures [18,19]. Briefly, proteins were extracted from both the IGT and RGT. Total protein concentrations were estimated with the BCA kit (Beyotime, Beijing, China) based on the protocols of the manufacturer. A quantity of 50 mg of protein from each sample was used following the manufacturer's protocol (Expedeon, San Diego, CA). Each pooled sample was digested with sequencing-grade trypsin (Promega, Fitchburg, WI) in 500 mM triethylammonium bicarbonate (TEAB) buffer overnight in an enzyme to substrate ratio of 1:100 (w/w) at room temperature with gentle shaking, followed by a second digestion for 4 h with the same amount of trypsin. Then, the digested peptides from different samples were labelled with tandem mass tags (TMTs) reagents (Thermo, Pierce Biotechnology, Waltham, MA). The labelled peptides were fractionated for MS analysis. For LC–MS/MS analysis, the MS/MS spectra from each LC–MS/MS run was searched against the selected database using an in-house Mascot or Proteome Discovery searching algorithm. The DEPs were selected with the criteria of fold change (RGT/IGT ratio)$\geq$1.2, $p\leq$.05.

Bioinformatics analysis and statistical analysis

Gene Ontology (GO) annotation including BP, cellular component (CC) and molecular function (MF) analysis was based on the Gene Ontology Database (http://www.geneontology.org/). The KEGG database (http://www.genome.jp/kegg/) was used to classify the DEPs, too. The two-tailed Fisher's exact test was employed to test the enrichment of the differentially abundant proteins versus all identified proteins, and a p value $<$.05 was considered significant. Protein–protein interactions (PPIs) and co-expressions were analysed on the Dr. Tom platform (http://biosys.bgi.com/#/report/login)

supplied by Beijing Genomics Institution (BGI, Shenzhen, China). PPI were selected with the criteria of score ≥500. Co-expressions were selected with the criteria of score ≥0.8.

Results

Histological examinations

According to the H&E staining results, the most striking property of RGT was extracellular matrix (ECM) deposition with significantly decreased cells. Another major difference between the two kinds of granulation tissues lied in cell types. There were so many eosinophils, lymphocytes and other inflammatory cells in IGT, while RGT was mainly composed of fibroblasts. Finally, there were a lot of capillaries in both IGT and RGT. But IHC staining showed that there were some differences in types of vessels in the two groups. Meanwhile, a lot of CD31 strong positive vessels could be seen in both groups (Figure 1). Moreover, there were a few EMCN positive expression cells in RGT group however, even no EMCN positive expression cells in the IGT group (Figure 1). In summary, compared with IGT, the RGT was characterized by ECM deposition, significantly decreased inflammatory cells, prominently increased fibroblasts as well as change of vascular types.

Protein identification and quantification based on TMT

A total of 4606 proteins were identified in the granulation samples, matched in database (uniprot_HomoSapiens). Among the 4606 identified proteins, 555 DEPs (259 up-regulated and 296 down-regulated) were identified in comparison between RGT and IGT (Figure 2(A)).

GO annotation of DEPs

GO annotation showed that DEPs belonged to 4927 GO terms, among which there were 460 and 59 over-represented GO terms with p value $<.05$ (Figure 2(B)) and $<.001$ (Figure 2(C)), respectively. According to the histological differences between IGT and RGT, further analysis was focussed on ECM, inflammation and angiogenesis. The results showed that 17 of 59 GO terms ($p < .001$) were related to the ECM, four were related to inflammation and only one was related to angiogenesis (Figure 2(D)).

KEGG pathway enrichment analysis of DEPs

KEGG pathway enrichment analysis showed that the DEPs belonged to 314 pathways including 35 over-represented and p value $<.05$ pathways. Further analysis was also focussed on ECM, inflammation and angiogenesis based on histological examination. As a result, there were three ECM, two inflammation and one angiogenesis related KEGG pathways (Figure 3(A)). Among ECM related pathways, focal adhesion was the most representative pathway, encompassing 25 DEPs, followed by the PI3K/Akt signalling and ECM-receptor interaction pathways, encompassing 21 and 16 DEPs, respectively (Figure 3(B)). FcγR-mediated phagocytosis pathway, including 13 DEPs, was related to inflammation (Figure 3(C)). Totally, 49 DEPs were involved in above five pathways.

Hierarchical clustering, co-expression and PPI analysis of interested DEPs

The 49 DEPs involved in above five pathways were identified as the interested DEPs for further analysis. The DEPs were

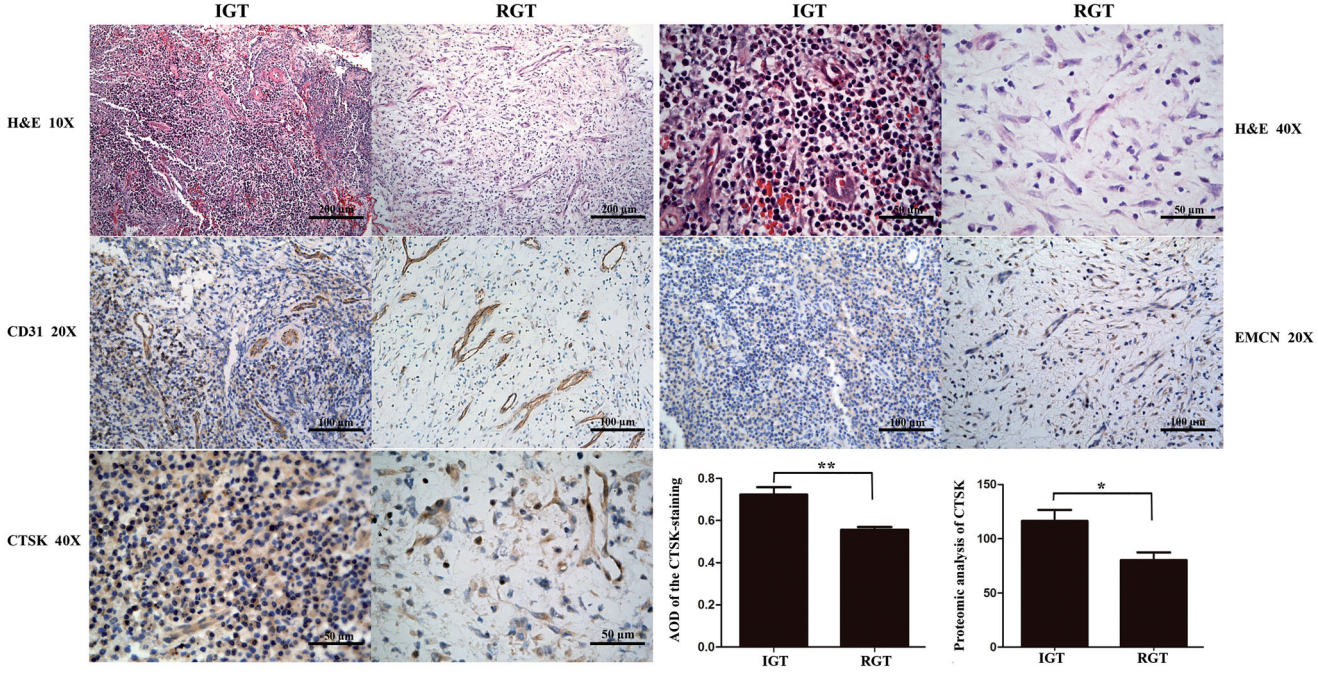

Figure 1. Histological analysis of IGT and RGT. H&E staining of IGT and RGT was shown in the first line. IHC staining against CD31 and EMCN was shown in the middle. IHC staining against CTSK, semi-quantitative analysis of the AOD of CTSK-staining and quantitative proteomic analysis of CTSK located at the bottom. The values represent mean ± SD. and obtained from three independent samples. $^{*}p < .05$; $^{**}p < .01$.

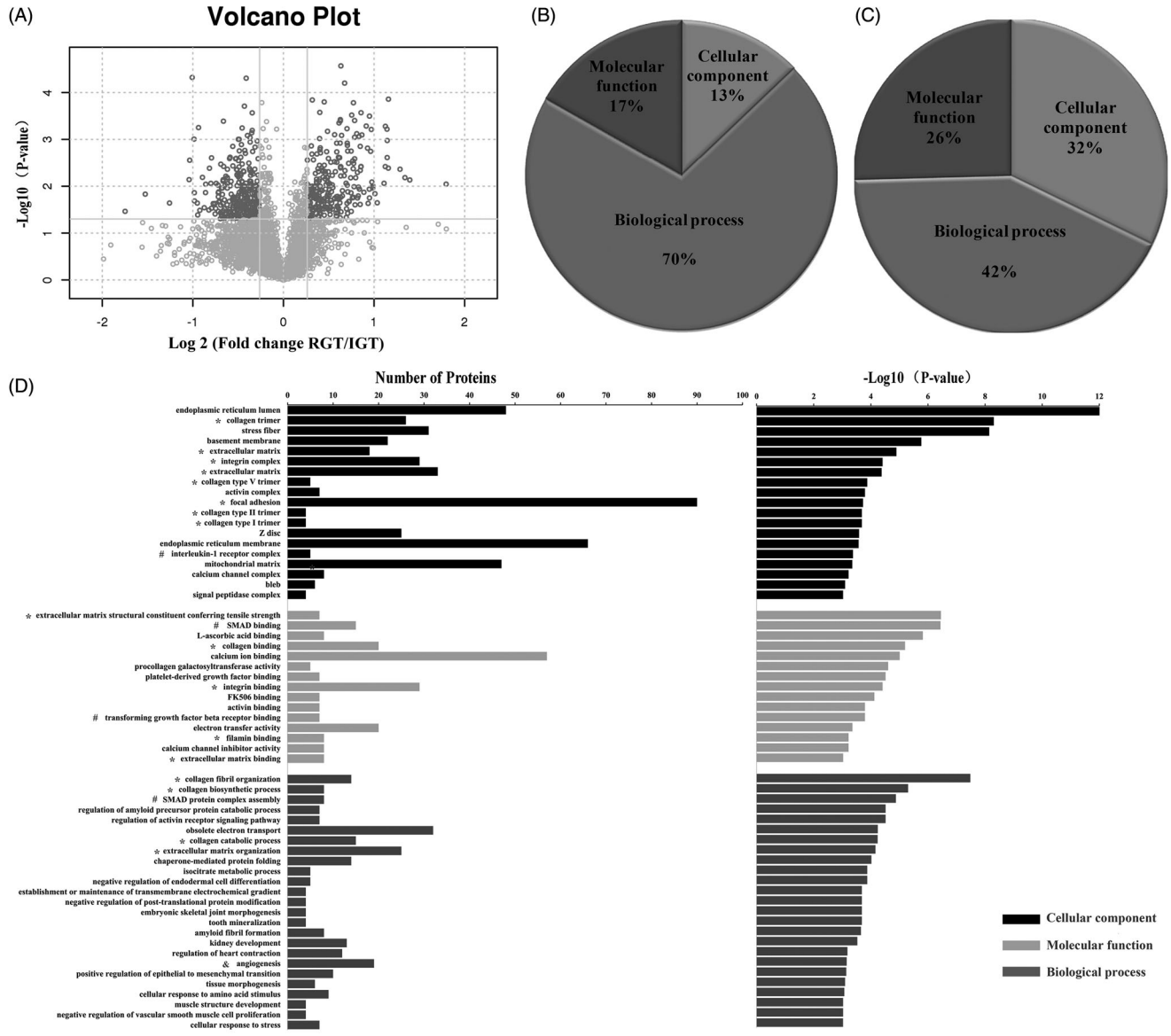

Figure 2. GO annotation of DEPs. (A) A volcano plot of the log2 fold-change (*x*-axis) versus –log10 *p* value (*y*-axis). The dark points in the upper right (ratio >1.2) and upper left (ratio < 0.833) sections with $p < .05$ represent proteins those were significantly dys-regulated in RGT. (B) The classification of 460 over-represented GO terms ($p < .05$). (C) The classification of 59 over-represented GO terms ($p < .001$). (D) Names (*y*-axis), number of involved proteins (*x*-axis) and –log10 *p* value (*x*-axis) of the 59 over-represented GO terms ($p < .001$). *: ECM related GO terms; #: inflammation related GO terms; &: angiogenesis related GO terms.

displayed in the form of a hierarchical clustering heat map (Figure 4(A)) and also used to analyse their co-expression (Figure 4(B)). The results indicated that COL1A1 was the most up-regulated protein. At the same time, COL1A1 and other three ECM proteins including COL1A2, FN1 and AKT1, were involved in 4/5 of the pathways.

To further clarify our understanding of the regulatory role of ECM in the transformation from IGT to RGT, all DEPs in three ECM related pathways were used to build a PPI regulatory network. The results (Figure 4(C)) demonstrated that these screened DEPs formed a complex regulatory network containing 28 nodes and 210 edges. FN1, COL1A1, COL1A2, ITGA6 and ITGA7 were found to be important hubs. On the other hand, DEPs in two inflammation related pathways were also used to build a regulatory network (Figure 4(D)). Though this network was less complex than the ECM related one, we were surprised to find that FN1, COL1A1, COL1A2 and three additional DEPs (LYN, PLCG1 and MMP2) were also the important hubs. Since the two groups of pathways share some of the same key molecules, all the 49 interested DEPs were used to build a regulatory network, too. As a result (Figure 4(E)), a complex regulatory network containing 42 nodes and 314 edges was found. FN1 was the most representative protein with 27 edges, followed by COL1A1 (16 edges), ITGA6 (16 edges), COL1A2 (14 edges) and MMP2 (13 edges). This result indicated that these proteins at least partially biologically connected as a group.

COL1A1 is not only one of the main hubs during the complex regulatory network, but also the most up-regulated protein. Its up-regulation may be caused by the increase of its synthesis and secretion and/or the decrease of its degradation. Therefore, CTSK, as the main protease to degrade COL1A1, was included in the PPI analysis, too. Furthermore, its expression in IGT and RGT was also detected with IHC staining.

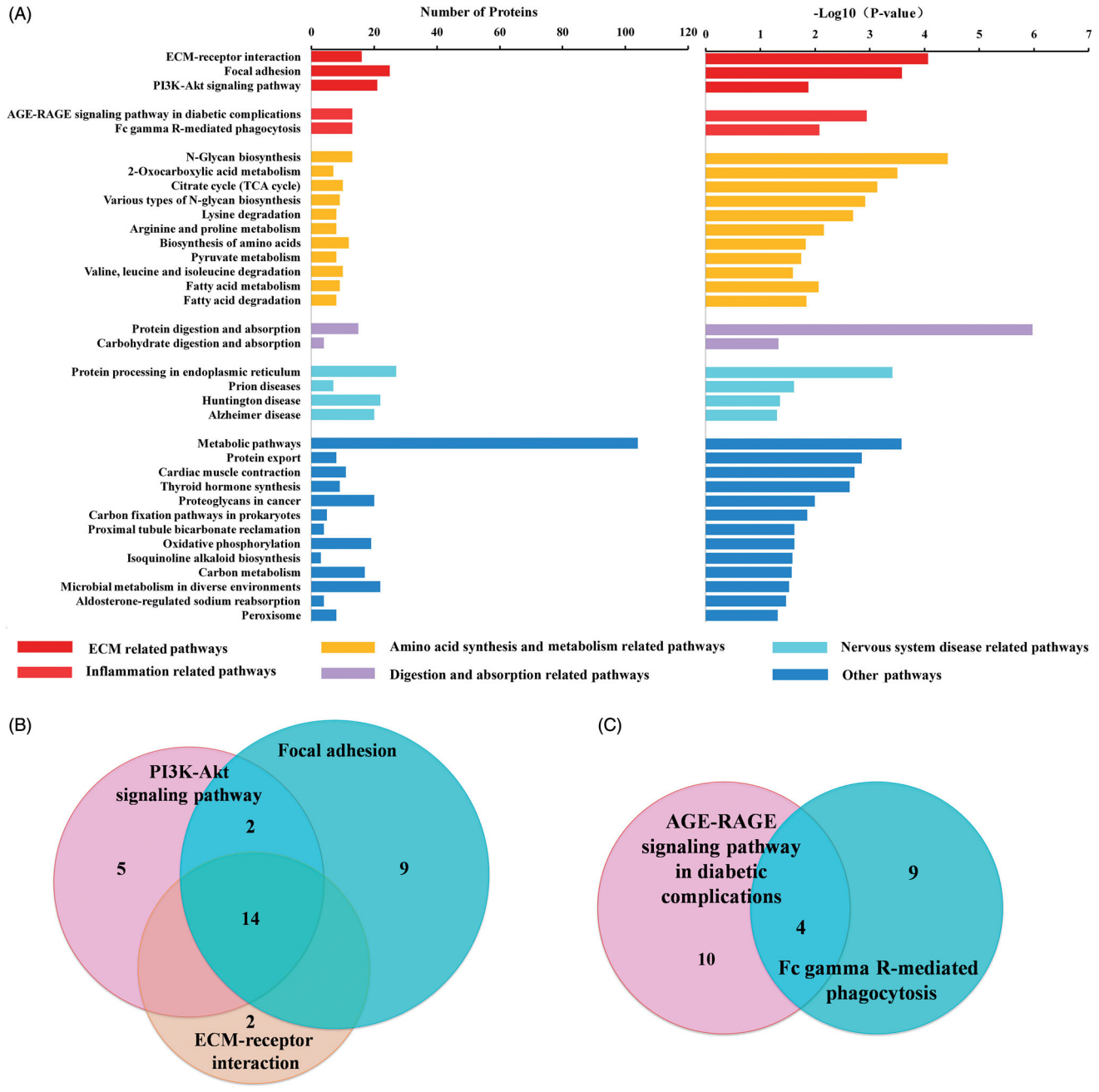

Figure 3. KEGG pathway enrichment analysis of DEPs. (A) Names (*y*-axis), number of involved proteins (*x*-axis) and –log10 *p* value (*x*-axis) of the 35 over-represented KEGG pathways ($p < .05$). (B) Number of involved proteins in the three ECM related pathways. (C) Number of involved proteins in the two inflammation related pathways.

Expression of CTSK

IHC staining result (Figure 1) showed that strong staining of CTSK was found in vascular endothelial cells in both IGT and RGT. At the same time, eosinophils in IGT as well as fibroblasts in RGT showed strong CTSK staining, too. Semi-quantitative analysis of the AOD of CTSK-staining showed that the level of CTSK in RGT was down-regulated to 76.97% ($p = .0039$) compared with that in IGT. This result was similar to the result of quantitative proteomic analysis, down-regulated to 69.10% ($p = .0462$). Further analysis found that CTSK was involved in five KEGG pathways, including osteoclast differentiation (map04380), lysosome (map04142), Toll-like receptor signalling (map04620), rheumatoid arthritis (map05323) and apoptosis pathways (map04210). These results suggested that CTSK might play critical roles in both regulating bone regeneration and immune response.

Involvement of CTSK in transformation from IGT to RGT

In this study, we also determined the expression of ALP, COL1A1 and RUNX2 in vascular endothelial cells undergoing treatment of CTSK inhibitor, odanacatib (ODN, 1 μM), using western blotting assay (Figure 5(A)). The expression of ALP (Figure 5(B), $p < .001$), RUNX2 (Figure 5(C), $p < .001$) and COL1A1 (Figure 5(D), $p < .01$) was significantly lower in IGT group compared to that in RGT group. However, when vascular endothelial cells in IGT group treating with ODN (IGT + ODN group), expression of ALP (Figure 5(B), $p < .001$), RUNX2 (Figure

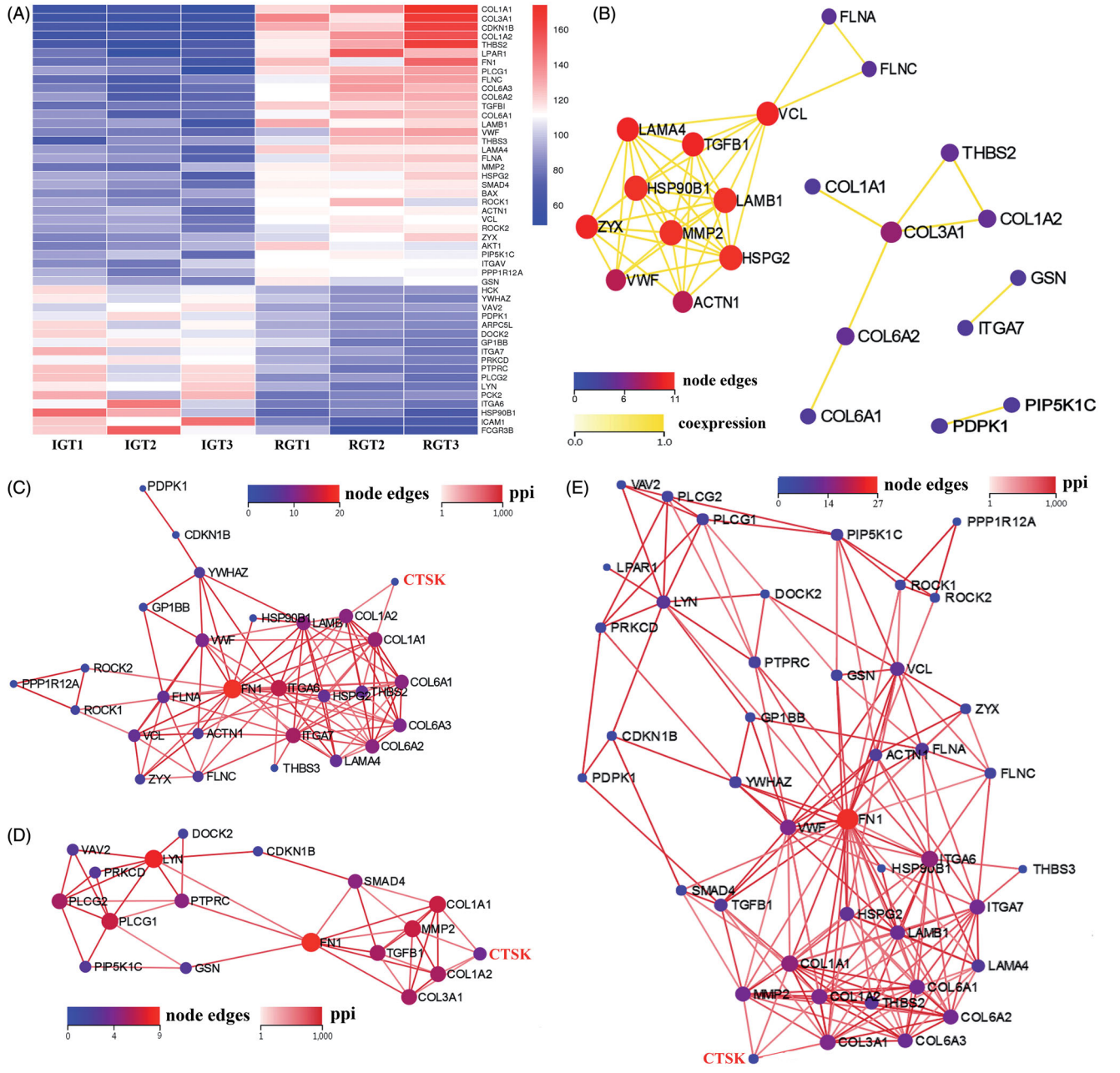

Figure 4. Hierarchical clustering, coexpression and PPI analysis of the interested DEPs. (A) Hierarchical clustering of the interested DEPs. (B) Coexpression of the interested DEPs. (C) PPI regulatory network of DEPs in ECM related pathways. (D) PPI regulatory network of DEPs in inflammation related pathways. (E) PPI regulatory network of all the interested DEPs.

5(C), $p < .001$) and COL1A1 (Figure 5(D), $p < .01$) was markedly increased compared to that in IGT group. Therefore, the CTSK was involved in the transformation from IGT to RGT.

Discussion

Odontogenic inflammatory diseases are a series of chronic inflammatory diseases mainly affecting periodontal tissues, leading to bone breakdown and loss of teeth. While great efforts have been made to understand their pathogenesis and treatment, there remains a pressing need for developing potent therapeutic strategies for targeting alveolar bone healing and regeneration [1,2]. For the first time, we suggest that promoting the transformation from IGT to RGT might be a new direction to breakthrough. Histological examination was the most accurate way to reveal the differences between the two kinds of granulations. A proteomics study is a more accurate way to close the feasibility of this transformation. Bioinformatics analysis of the proteomics data provides us with many directions for further studies and may help us to find the targets to promote the transformation of IGT to RGT. The EMCN has been proven to be a type-1 integral-membrane O-sialoglycoprotein expressing on surface of the endothelium in capillaries and venules [20]. The EMCN could prevent the leukocyte adhesion within the non-inflamed tissue, while downregulation for the EMCN during the inflammatory processes is critical for facilitating the migration for the inflammatory cells into the targeted tissues [20–22]. Therefore, we also identified the changes of EMCN expression in

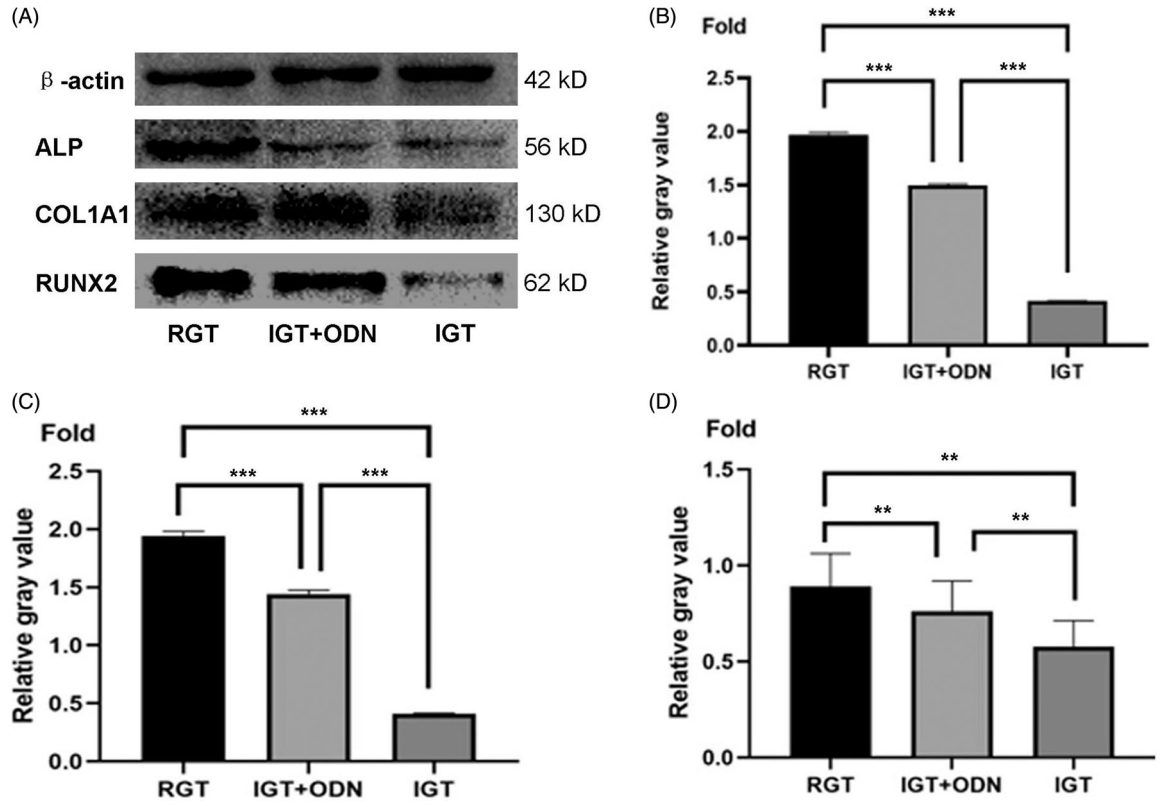

Figure 5. CTSK involved in the transformation from IGT to RGT. (A) Western blotting assay for determining expression of ALP, COL1A1 and RUNX2 in vascular endothelial cells treating with CTSK inhibitor, odanacatib (ODN) at final concentration of 1 μM. (B) ODN treatment reduced ALP expression in cells of IGT group. (C) ODN treatment reduced RUNX2 expression in cells of IGT group. (D) ODN treatment reduced COL1A1 expression in cells of IGT group. $^{*,**}p<.01$, $^{***}p<.001$.

both of IGT and RGT tissues. Our findings showed that there were a few EMCN positive expression cells in RGT group however, even no EMCN positive expression cells in IGT group, which suggest that EMCN involves in the inflammation of granulation tissues.

According to the histological examinations and proteomic analysis, RGT showed different histological appearance with IGT but shared similar molecular basis. Five hundred and fifty-five (12.05%) DEPs were found in 4606 identified proteins. This may be another evidence that IGT was able to transformed into RGT under suitable intervention.

Both GO annotation and KEGG pathway enrichment analysis showed that ECM, especially the COL1A1 was the most representative protein. Then, its specific protease CTSK draws our attention. CTSK is a member of the papain-like cysteine protease family. At the beginning, it was thought to be specifically expressed in osteoclasts and play a critical role in bone resorption [23–25]. In recent years, more and more studies have shown that CTSK is also abundantly expressed in MSCs, endothelial cells, fibroblasts, dendritic cells and $CD4^+$ T cells, and its role is not limited to osteoclast mediated bone resorption [26–30]. It has been reported that CTSK played important roles in dendritic cell-mediated immune response through TLR pathways [26,27]. Then, it demonstrated that silencing or inhibiting of CTSK could effectively prevent the periapical lesions, periodontitis and rheumatoid arthritis in mice by regulating osteoimmune [1,2,4,5]. However, it remains unknown whether CTSK could be a therapeutic target to treat above diseases. On the other hand, though it was proved that inhibiting CTSK can promote bone formation by stimulating the proliferation and migration of bone MSCs and the angiogenesis of type H ($CD31^{hi}EMCN^{hi}$) [25,31–33]. It is not clear whether this regulatory potential could be affected by the local inflammatory environment.

The present study confirmed that the level of CTSK in RGT was down-regulated to 69.10–76.97% ($p<.05$), with significantly up-regulated COL1A1, COL1A2, FN1 and other ECM proteins included in Focal adhesion and PI3K/Akt signalling pathways. Additionally, down-regulated CTSK was also accompanied with up-regulated TGFB1 (fold change = 1.55168, $p<.001$), a key molecule in angiogenesis (GO:0001525).

Moreover, the vascular endothelial cells treating with CTSK inhibitor ODN [4] (IGT + ODN group) demonstrated remarkably higher expression of ALP, COL1A1 and RUNX2 compared to that in IGT group. These results shed light on that CTSK might be a target to regulate the transformation of IGT to RGT (or involve in this transformation) in alveolar bone through ECM, stem cells and angiogenesis mechanisms.

Though our study discovered a few interesting results, there are also some limitations. First, this study only determined EMCN and CD31 molecule respectively, however without co-staining experiments for confirming the EMCN expression in vessels (CD31 molecule). Secondarily, the bioinformatics analyses in this study for proteomics data have provided many directions for the following investigations and might be beneficial to discover the targets for promoting transformation of IGT to RGT. However, we have not conducted sufficient evidences for further proving the CTSK protein involving in the processes of transformation.

Clinically, the local IGT is known as a pathological tissue for the oral diseases. While, the transformation from IGT to the RGT is possible when the patients undergoing appropriate intervention. Therefore, as our findings demonstrated, the TMT-labelled quantitative proteomic analysis discovered in this study might be benefit to the clinical intervention for transforming IGT tissues to RGT tissues in the early stage. Also, the CTSK as a potential target for regulating the transformation from IGT to RGT, might provide insights for the clinical therapy for the odontogenic inflammatory diseases.

In conclusion, the histological and bioinformatics' differences between IGT caused by odontogenic inflammatory diseases and RGT formed during tooth extraction socket healing process were identified for the first time. The transformation from IGT to RGT in alveolar bone may be promoted by inhibiting CTSK through ECM, stem cells and angiogenesis mechanisms, however, further research is clearly required.

Disclosure statement

The authors declare no conflict of interest.

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