Role of YAP1 gene in proliferation and osteogenic differentiation of human periodontal ligament stem cells induced by TNF-α

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Abstract

Background: Periodontitis is a chronic inflammatory disease that occurs in periodontal tissues and can cause tooth loosening and loss in severe cases. As the main effector of downstream of Hippo signaling pathway, Yes-related protein 1 (YAP1) plays an important role in cell proliferation and differentiation. However, the role of YAP1 in periodontitis has not been reported. **Methods:** Cell activity was detected by CCk-8. YAP1 was overexpressed by cell transfection, and then RT-qPCR and western blot were used to detect the expression of YAP1. The cell proliferation was determined by clone formation assay, and the expression of proliferation-related proteins was determined by western blot. The cell differentiation was detected by Elisa kit of ALP and alizarin red staining. Finally, western blot was used to detect the expression of differentiation-related protein and Hippo signaling pathway-related proteins. **Results:** With the increase of concentration induced by TNF-α, the cell survival rate of human periodontal ligament stem cells (HPDLSC) decreased significantly. After the overexpression of YAP1, cell proliferation and proliferation-related protein expression increased. Overexpression of YAP1 can

improve the differentiation and the formation of osteoblasts of HPDLSCs induced by TNF-α. The expression of Hippo signaling pathway-related proteins transcriptional coactivators with PDZ binding domains (TAZ), TEA domain family member (TRED) increased and proliferation-related protein P27 decreased. **Conclusion**: TNF-α can inhibit proliferation and osteogenic differentiation of HPDLSCs, which can be ameliorated by the YAP1 gene through the Hippo signaling pathway. Our paper suggested that YAP1 may be a potential therapeutic target for periodontitis.

Introduction

Periodontal disease refers to the disease occurring in the gingival tissue and deep periodontal tissue (such as periodontal membrane, alveolar bone, cementum), which is divided into two categories: gingival disease and periodontitis(1). Periodontitis is the most common periodontal disease, mainly manifested by gingival redness and bleeding, periodontal pocket formation and pyorrhea, tooth loosening, periodontal abscess and other symptoms, which can eventually lead to tooth loss(2). It is a chronic infectious disease caused by bacteria attached to the periodontium to form plaque biofilm. At the same time, due to the long-standing chronic inflammation, periodontal pathogens and their metabolites are more likely to enter the bloodstream and spread to other organs, causing systemic diseases such as diabetes, glomerulonephritis and atherosclerosis(3). Periodontitis has been acknowledged as the systemic infection risk factors, and treatment of periodontitis disease is imminent.

Yes-related protein 1 (YAP1) is an intracellular connexin and transcriptional coactivator discovered in 1994(4). There are multiple domains and specific amino acid sequences in YAP1, including TEAD (TEA domain family member) domain, tryptophan-tryptophan (WW) domain and transcriptional activation domain(5). Through these domains, YAP1 can interact with a variety of proteins and participate in multiple intracellular signal transduction pathways. As a major downstream effector of Hippo signaling pathway, YAP1 also plays an important role in regulating organ size, promoting tissue regeneration and maintaining self-renewal of stem cells. YAP1 regulates cell proliferation and apoptosis-related factors to ensure normal organ

size(6). Under normal circumstances, YAP1 is activated in fetal cardiomyocytes, which proliferate obviously. However, after blocking the binding of YAP1 to TEAD transcription factor in cardiomyocytes, the activity of proliferation of YAP1 was inhibited, indicating that YAP1 is an important regulator of cardiomyocyte proliferation, myocardial morphogenesis and trabecular formation (7). Moreover, YAP1 activated the downstream target genes through TEAD binding domain, thus maintaining the pluripotency of stem cells (8). In addition, it has been reported that YAP1 is actively involved in embryonic development, which means that YAP1 regulates the differentiation of skeletal muscle, osteoblasts and neural crest cells (9-11). Therefore, YAP1 plays an important role in the maintenance of normal physiological activities. Once abnormal expression occurs, it will directly lead to the occurrence of a variety of human diseases. YAP1 activates the expression of Slug by transcriptional binding to TEAD and promotes the invasion and metastasis of lung cancer cells (12). Overactivation of endogenous YAP1/TAZ can inhibit the formation of SOX9 mediated by TEAD transcription factors, thereby damaging the proliferation and differentiation of chondrocytes, resulting in chondrodysplasia (17). In the final analysis, the abnormal expression of YAP1 promotes the proliferation or differentiation of relevant cells in the disease, leading to the occurrence and development of the disease. However, the study of YAP1 in periodontitis has not been reported so far.

Hippo signaling pathway is highly conservative, which consists of a series of kinase cascade. Hippo signaling pathway is involved in regulating a variety of cell biological processes, including cell polarity, cell differentiation and death, tumor development, etc. As one of the key cytokines in Hippo signaling pathway, YAP1 plays an important role in promoting tissue regeneration and maintaining self-renewal of stem cells. Studies have reported that the Hippo pathway transcriptional co-activator, YAP, confers resistance to cisplatin in human oral squamous cell carcinoma(13). In addition, YAP1 in Hippo signaling pathway drives proliferation, survival, and migration of oral squamous cell cancer cells in vitro(14). Thus, we speculate whether

YAP1 is involved in the proliferation and differentiation of periodontal stem cells through Hippo signaling pathway.

In this paper, we firstly used TNF-α to induce cells to form a periodontitis cell model at the cellular level, and then studied the specific effect of YAP1 on TNF-α-induced Human periodontal ligament stem cells (HPDLSCs) and its mechanism, which can provide a new therapeutic strategy for the treatment of periodontitis.

Materials and methods

Cell culture

HPDLSCs were purchased from Shanghai Cell Collection (Shanghai, China), and the cells were maintained at 37 °C and 5% carbon dioxide in DMEM (Gibco; Thermo Fisher Scientific) medium added with 10% FBS (Gibco; Thermo Fisher Scientific) and antibiotics in a humidified incubator. The medium was changed every 2–3 days.

Reagents

TNF-α (cat.no.1F3F3D4, Invitrogen; ; Thermo Fisher Scientific, Inc.) powder was dissolved in DMSO and the final concentration of cells was 0μg/ml, 5μg/ml, 10μg/ml and 40μg/ml. YAP1(cat.no.AF2371) was purchased from Beyotime. **Cell Counting Kit-8 (CCK-8)**

Cell proliferation was assessed using CCK-8 (Dojindo Molecular Technologies, Inc.) according to the instructions of the manufacturer. We seeded HPDLSCS at a density of 8,000 cells/well in a plate with 96 wells in complete medium overnight. Different concentrations of TNF-α were added to the cells in 5% CO_2 at 37°C for 72 h. 10 μl CCK-8 solution was added to each well and the absorbance at 450 nm in 4 h was measured using a microplate reader.

Cell transfection.

Cells ($1x10^5$ cells/well) were seeded into 6-well plates one day prior to transfection and cultured in an atmosphere containing 5% CO_2 at 37˚C. Subsequently, the cells

were transfected with YAP1 or negative control (NC) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), following the manufacturer's protocol. Cells from the blank control group (Control) did not receive any treatment. Following incubation for 48 h, cells were used for the following experiments.

RNA Isolation and Quantitative Real-Time Reverse Transcriptase PCR

Total RNA was isolated from HPDLSCS using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was synthesized using TaqMan™ Gene Expression Cells-to-CT™ Kit (Takara Biotechnology Co., Ltd., Dalian, China). Then cDNA was analyzed using a TaqMan® Universal PCR Master Mix kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. Amplification conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Primer sequences were obtained from GenScript (Piscataway, NJ, USA). The primer sequences are as follows: YAP1 forward: 5'- AGGACAGCCAGGACTACACAG□3' and YAP1reverse: 5'□ CACCAGCCTTTAAATTGAGAAC□3'; GAPDH forward :5'-GGTCGGAGTCAACGGATTTG-3' and GAPDH reverse: 5'-GGAAGATGGTGATGGGATTTC□3'. The relative gene expression level quantification was analyzed using the $2^{-\Delta\Delta Cq}$ method, and normalized to GAPDH expression (15).

Western blot

Cells were seeded at $1x10^6$ cells/well in six-well plates. After various treatments, proteins were extracted from cells using a protein lysis buffer (RIPA; Cell Signaling Technology) on ice for 15 min followed by centrifugation at 14,000 rpm for 15 min at 4 °C. The concentration of protein was detected through a bicinchoninic acid assay protein assay kit (Beyotime Institute of Biotechnology). Proteins (25 μg/lane) were resolved using 10% SDS-PAGE and transferred to PVDF membranes (EMD Millipore). These membranes were then incubated with primary antibodies and goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:5000; cat. no. ab181658; Abcam) at room temperature for 1 h. Proteins were visualized using Image

Quant™ LAS 4000 (GE Healthcare Life Sciences) and quantified using Image J (Version146; National Institutes of Health). Anti-YAP1 (1:1000, cat. no. ab52771, Abcam), anti-CyclinE (1:1000; cat. no. ab5980), anti-cyclinD1 (1:1000; cat. no. ab16663), anti- CTGF (1:1000; cat. no. ab6992), anti- Oct4 (1:1000; cat. no. ab19857), anti- Sox2 (1:1000; cat. no. ab93689) and anti-Runx2 (1:1000; cat. no. ab192256) were purchased from Abcam. and anti-GAPDH (1:1000; cat. no. 5174S) antibodies were obtained from Cell Signaling Technology, Inc.

Clone formation assay

Plate cloning formation experiment

Cells were seeded at 500 cells/well into six-well plates and cell culture was performed for 2 weeks and was terminated when macroscopic apophyses were found in culture dishes. Phosphate-buffered saline (PBS) were used to wash the cells and fixation with 20% methanol for 15 min was performed. Then, crystal violet was added and staining was performed for 40 min. After washing and air drying at room temperature, clones were counted using a cloning counter.

Alkaline Phosphatase Activity

Supernatant of cells that have been treated before were collected by centrifugation at 400 g for 5 minutes. ALP activity was calculated for each group by using an ELISA kit (Sigma, Shanghai, China). Finally, the amount of ALP activity was reported as IU/L.

Alizarin Red staining

Cells were seeded at $1*10^4$ cells/ml into 24-well plates. Mineralized medium (A-MEM medium containing 8% FBS, 10-7 mol/L dexamethasone, 50 ug/mL ascorbic acid, 10mmol/L deoxyribonucleic acid) was used to culture when cells were cultured to over 60% fusion. When cell stratification and black opaque nodules were observed outside the cells, cell culture continued and alizarin red staining was performed at 18 days. Discard the supernatant, rinse cells twice with PBS, dry it

slightly in the ultra-clean table, fix cell with filtered 4% paraformaldehyde for 10min, remove paraformaldehyde, rinse it twice with PBS, air dry it, dye it with alizarin red, incubate it in CO2 incubator for 20min, discard alizarin red, rinse it twice with PBS, and at last take photos under phase contrast microscope.

Statistical analysis

Data were presented as means ± standard deviation (SD). SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. The comparison in two groups was analyzed using student's t-test or one-way analysis of variance followed by Tukey's test. $P<0.05$ was considered to indicate a statistically significant difference. All experiments were repeated at least three times.

Results

TNF-α inhibited the activity of the HPDLSCs

0 ug/ml, 5 ug/ml, 10 ug/ml, 40 ug/ml of TNF-**α** was used to induce HPDLSCs for 72 h, and CCK-8 technology was used to detect cell activity. With the increase of TNF-**α** concentrations, cell activity decreased significantly and dropped to 60% at 10ug/ml, so we chose TNF-**α** with treatment condition of 10ug/ml and treatment time of 72h for the following experimental study (Fig1)**.**

Overexpression of YAP1 improved TNF-α-induced proliferation of the HPDLSCs

YAP1 was overexpressed by cell transfection and transferred to HPDLSCs. The expression level of YAP1 was detected by RT-qPCR and western blot. As shown in Figure 2 A and B, cell transfection was successful. Subsequently, the cells were divided into four groups: control, TNF-**α**, TNF-**α** +NC, and TNF-**α** + YAP1. The proliferation ability of cells was detected through clone formation assay. We found that the proliferation of TNF-**α** group decreased significantly compared with the control group. Compared with the TNF-**α** +NC group, the proliferation capacity of TNF-**α**+YAP1 group was obviously increased (Fig2C). We also detected the expression of proliferation-related proteins cyclinE, cyclinD1 and CTGF, and found that the trend was consistent with Fig2C (Fig2D). The results showed that

overexpression of YAP1 improved TNF-a-induced proliferation of HPDLSCs.

Overexpression of YAP1 improved TNF-α induced differentiation of the HPDLSCs

After overexpression of YAP1, cells were divided into four groups: control, TNF-a, TNF-α +NC, and TNF-α+YAP1. We detected the activity of ALP, and found that compared with the control group, the ALP activity of TNF-α group significantly decreased. Compared with TNF-α + NC group, ALP activity of TNF-α+YAP1 group increased. Then alizarin red staining was used to observe the bone mineralization ability of HPDLSCs. As shown in Fig3B, compared with the control group, osteogenesis ability of the rest of the three groups of cells decreased, but the osteogenic capacity and the ability of the formation of mineralized nodules of TNF-α+YAP1 group were higher than that of TNF-α and TNF-α+NC group. Moreover, there was no difference between TNF-α group and TNF-α +NC group. We also examined the expression of differentiated proteins Oct4, Sox2 and Runx2. The results showed that compared with the control group, the protein levels of Oct4, Sox2 and Runx2 in the other three groups decreased, but that in the TNF-α + YAP1 group were higher than that in TNF-α and TNF-α +NC groups, and there was no difference between the TNF-α and TNF-α +NC groups. These results indicated that overexpression of YAP1 can improve TNF-α-induced differentiation of HPDLSCs.

YAP1 participates in the proliferation and differentiation of HPDLSCs through the Hippo signaling pathway

In order to explore the mechanism of proliferation and differentiation of HPDLSCs which YAP1 was involved in, we tested the Hippo signaling pathway-related proteins TAZ, TRED and P27. Our experimental results showed that compared with the control group, the protein of TAZ and TRED in the TNF-α group decreased and the expression of proliferation-related protein P27 increased. Compared with the TNF-α +NC group, the protein expression of TAZ and TRED in the TNF-α + YAP1 group increased and the expression of P27 decreased. There was no difference between the TNF-α and TNF-α +NC groups (Fig4). This suggests that YAP1 may participate in the proliferation and differentiation of HPDLSCs through Hippo

signaling pathway.

Discussion

Human periodontal ligament stem cells (HPDLSCs), derived from adult periodontal membrane tissues, have a high proliferation capacity to form cell clones, and have the functions of self-renewal and multi-differentiation to form gingiva, periodontal membrane, cementum and alveolar bone(16). Under normal circumstances, HPDLSCs are in a resting state to maintain the stable state of cell replacement and function in the periodontal membrane. When the cementoblasts are damaged due to various reasons, the cells can differentiate into osteoblasts and participate in the repair and reconstruction of periodontal tissues(17, 18). When periodontitis occurs, HPDLSCs are exposed to an inflammatory environment dominated by TNF-α, which contains a variety of inflammatory cytokines(19, 20). These inflammatory factors stimulate HPDLSCs, reduce their ability of differentiation and osteogenesis, and affect bone tissue regeneration(21). Studies have reported that high concentration of TNF-α can inhibit osteogenic differentiation of HPDLSCs and form an in vitro pathological model of chronic periodontitis(22). In this study, we induced the HPDLSCs with TNF-α to form the model of periodontitis, and then screened optimal treatment conditions of TNF-α for the following experimental study.

Hippo signaling pathway is highly conservative, which consists of a series of kinase cascade. Hippo signaling pathway regulates cell proliferation, apoptosis, and differentiation mainly through the downstream effector molecules yes-associated proteins(YAP1) and transcriptional coactivators with PDZ binding domains(TAZ), so as to control the size of organ and tissue development steady-state(23). Moreover, Hippo signaling pathway is involved in regulating a variety of cell biological processes, including cell polarity, cell differentiation and death, tumor development, etc. As one of the key cytokines in Hippo signaling pathway, YAP1 plays an important role in promoting tissue regeneration and maintaining self-renewal of stem cells. It was reported that mice with YAP1 gene mutation died about 10 days of embryonic stage(10). Existing studies have shown that YAP1 is widely involved in the

development of various organs or tissues, including skin(24), kidney(25), lung(26), skeletal muscle(27), etc.. However, the role of YAP1 in periodontitis has not been reported. In this paper, the over-expression of YAP1 in periodontitis model cells showed that the enzyme activity of ALP in periodontitis cells increased significantly and promoted the formation of mineralized nodules in the cells. The enzyme activity of ALP was an obvious feature of osteoblast differentiation, and the activation of ALP enzyme promotes osteoblast differentiation(28). Our results showed that YAP1 could promote the proliferation and differentiation of HPDLSCs, thus inhibiting or slowing down periodontitis. At the same time, we also detected the expression of related proteins TAZ,TRED and P27 in the Hippo signaling pathway. We found that overexpression of YAP1 could promote the expression of TAZ and TRED proteins and inhibit the expression of P27 proteins, indicating that the Hippo signaling pathway was activated in this process. Therefore, we preliminarily concluded that YAP1 participated in the proliferation and differentiation of HPDLSCs through hippo signaling pathway.

In conclusion, TNF-α can induce proliferation and osteogenic differentiation of HPDLSCs, which can be ameliorated by the YAP1 gene through the Hippo signaling pathway. Our paper suggests that YAP1 may be a potential therapeutic target for periodontitis**.**

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Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Patients consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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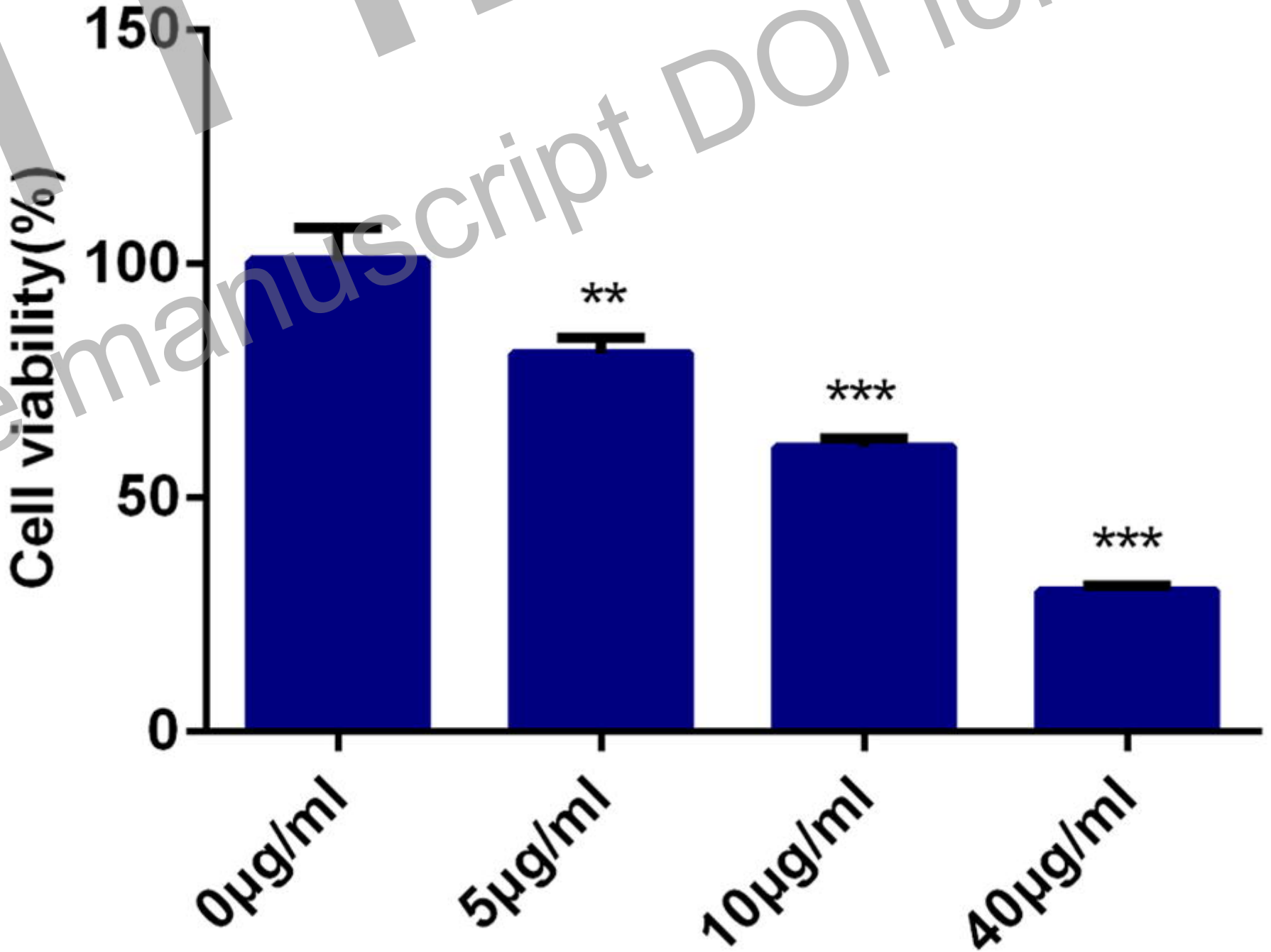
Figure legends

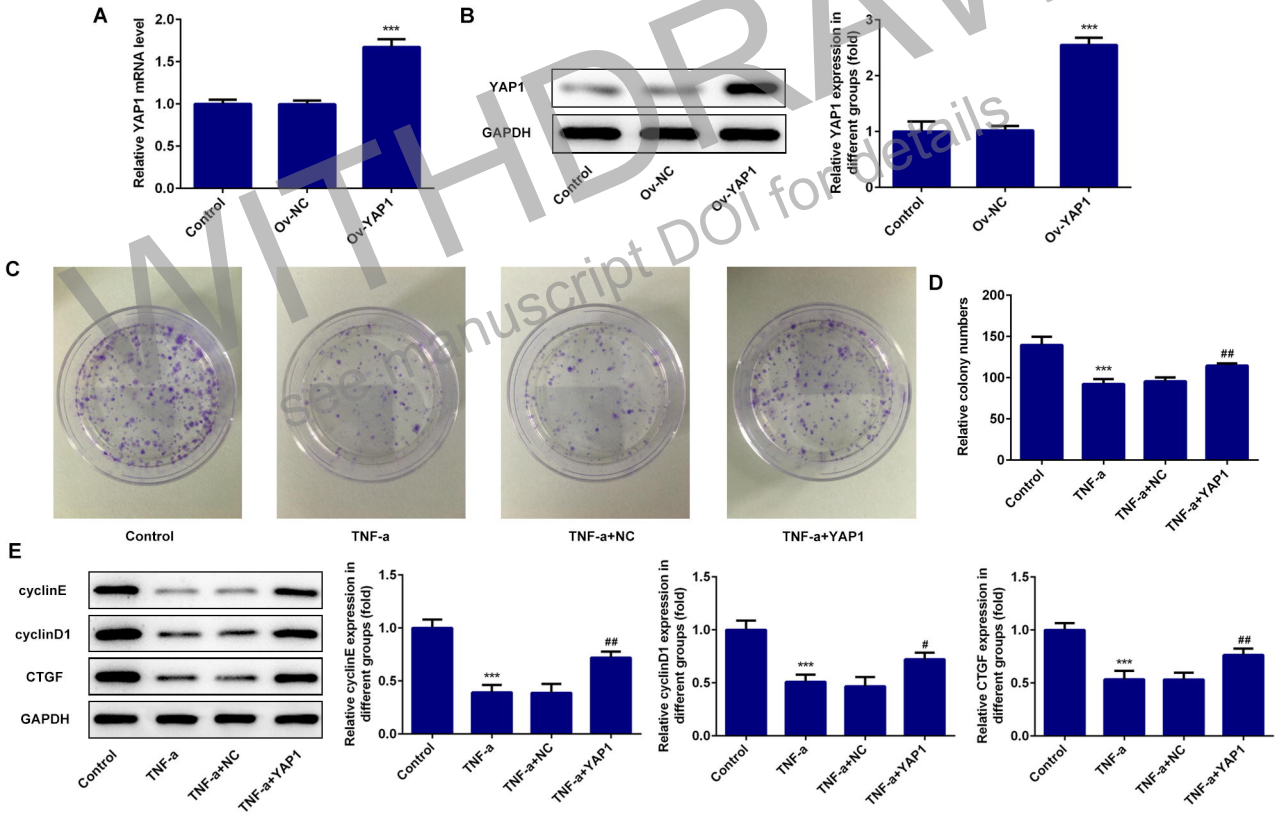
Fig1: TNF-α inhibited the activity of the HPDLSCs. The cell survival rate was measured by CCK-8 assay. **p<0.01,***p<0.001 vs 0μg/ml.

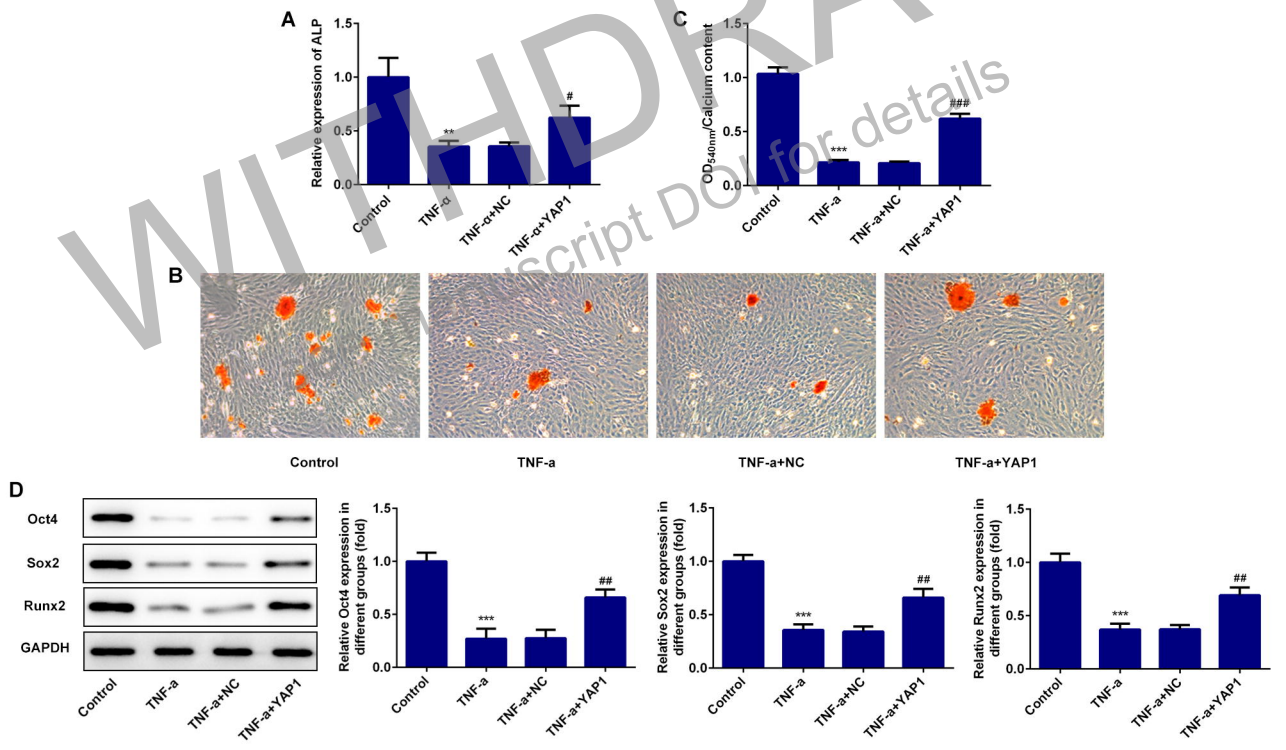
Fig2: Overexpression of YAP1 improved TNF-α induced proliferation of the HPDLSCs. (A) the expression of YAP1 was detected by RT-qPCR. (B) the expression of YAP1 was detected by western blot. (C) clone formation assay was used to detect cell proliferation. (D) expression of cyclinE, cyclinD1 and CTGF was detected by western blot. ***p<0.001 vs Control; #p<0.05, ##p<0.01 vs TNF-α+NC.

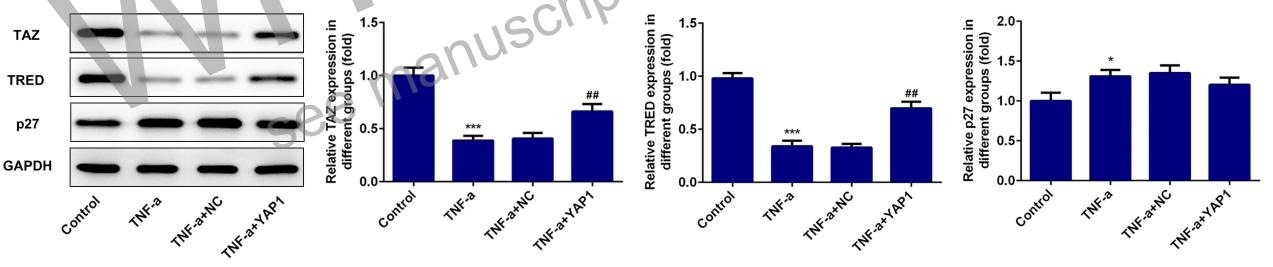
Fig3: Overexpression of YAP1 improved TNF-α induced differentiation of the HPDLSCs. (A) The expression of ALP activity was detected by ELISA. (B) Alizarin red staining was used to detect the osteogenic capacity of cells. (C) western blot was used to detect the expression of Oct4, Sox2 and Runx2. **p<0.01,***p<0.001 vs Control ; #p<0.05, ##p<0.01 vs TNF-α+NC.

Fig4:YAP1 participates in the proliferation and differentiation of HPDLSCs through the Hippo signaling pathway. Western blot was used to detect the expression of Oct4, Sox2 and Runx2. *p<0.05,***p<0.001 vs Control ; ##p<0.01 vs TNF-α+NC.








TAZ
TRED
p27
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Control
TNF-a
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Relative TAZ expression in different groups (fold)
1.5
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##
Control
TNF-a
TNF-a+NC
TNF-a+YAP1
Relative TRED expression in different groups (fold)
1.5
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##
Control
TNF-a
TNF-a+NC
TNF-a+YAP1
Relative p27 expression in different groups (fold)
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Control
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