Extracellular IL-37 Enhances Osteogenic and Odontogenic Differentiation of Human Dental Pulp Stem Cells via Autophagy Pathway

Na Li
Nanjing Medical University
Yan Chen
Nanjing University Medical School Affiliated Stomatological Hospital: Nanjing Stomatological Hospital
Ming Yan
Nanjing Medical University
Yanqiu Wang
Nanjing Medical University
Jintao Wu
Nanjing Medical University
Lin Fu
Nanjing Medical University
Jinhua Yu (yujinhua@njmu.edu.cn)
Nanjing Medical University https://orcid.org/0000-0003-4874-9910

Research

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Abstract

Background

The osteogenic and odontogenic differentiation of dental pulp stem cells (DPSCs) contributes to the restoration and regeneration of dental tissues. Previous study indicated that IL-37 has often been identified as an anti-inflammatory factor that affects other pro-inflammatory signals. It is known to be a factor capable of inducing *in vitro* osteogenic differentiation of bone marrow mesenchymal stem cells (BMMSCs). The aims of this study were to explore the effects of IL-37 on the differentiation of DPSCs.

Methods

DPSCs were cultured in growth medium with different concentration of IL-37, ALP activity was done to detect the optimal concentration for the following experiments. CCK-8 were conducted to assess the effect of IL-37 on proliferation of DPSCs. To assess differentiation, alkaline phosphatase activity, ALP staining, alizarin red S staining and real-time RT-PCR of DSPP, Runx2, ALP, and OSX were measured. Western blot was conducted to examine the levels of autophagy related markers (Beclin1, P62, LC3).

Results

Cells cultured with 1 ng/mL IL-37 owned the highest ALP activity. IL-37 enhanced the osteogenic and odontogenic differentiation of DPSCs following upregulated the expression of Beclin1, downregulated the expression of P62, and reduced the ratio of LC3II/I, whereas depletion of autophagy suppressed DPSCs osteogenic and odontogenic differentiation.

Conclusion

IL-37 increased osteogenic and odontogenic differentiation via autophagy.

1. Introduction

Dental pulp is the soft tissue inside tooth, which plays an indispensable role in the homeostasis of vital teeth [1]. The function of dental pulp is to support dentin formation and regeneration [2]. A range of injuries or diseases including caries, pulpitis, periapical periodontitis, tooth trauma, etc. can result in pulp necrosis, further teeth losing [3]. For therapeutic strategies for the kind of teeth above, vital pulp therapy (VPT) and regenerative endodontic treatment (RET) attracted recent attention to remain functional of teeth. The aims of VPT are to preserve and save dental pulp vitality, to induce dental pulp stem cells (DPSCs) to differentiate into osteoblasts and odontoblasts and ultimately form the hard tissue such as the tertiary dentin. RET known as revascularization aims to promote normal physiological development in teeth with necrotic pulp and act as a substitute for injured dental structures. DPSCs have been regarded as an important candidate for such treatment. Their capability of differentiation into odontogenic and osteogenic stem cells associated with biomaterials and growth factors is critical for dental pulp regeneration [4, 5]. Mesenchymal stem cells (MSCs) have a significant role in pulp regeneration therapy

for the reconstruction of tissues. Due to the easily available source of dental tissues, dental stem cells are considered good candidates for tissue engineering applications.

Human MSCs are a serviceable therapeutic tool for tissue engineering. DPSCs are identified as a type of MSCs that were originally isolated from human dental pulp tissue [6]. DPSCs have a lot of merits of easy access with the least invasive procedures and without any ethical controversy, and retain capacities of clonogenic formation, high proliferation, excellent regeneration, multilineage differentiation potential, and little inherent immunogenicity. As has been widely acknowledged, DPSCs can be induced to differentiate into a good many of directions, including osteogenic, dentinogenic, adipogenic, chondrogenic, myogenic, and neurogenic differentiation [7]. Compared with other MSCs from tissues as follows: bone marrow [8], peripheral blood [9], adipose tissue [10], and umbilical cord blood [11, 12], DPSCs demonstrate higher clonogenic and proliferative potential. Therefore, DPSCs become an engaging tool cell source for tissue engineering and regenerative medicine.

During the developmental process of MSCs, extracellular cues tend to exert its function in determining the fate of MSCs. Previous studies have revealed that in chronic inflammatory bone diseases, bone regeneration can be inhibited, and the osteogenic differentiation of DPSCs can be influenced by inflammatory microenvironments [13]. Interleukin 1 family member 7 (IL-1F7), a novel anti-inflammatory cytokine was recently proposed to be renamed Interleukin-37 (IL-37) [14]. IL-37 functions as a natural inhibitor of inflammatory and immune responses [15]. IL-37 is an anti-inflammatory factor that affects other pro-inflammatory signals, such as those mediated by tumor necrosis factor α (TNF-α), IL-1β, and IL-18 [16]. It was reported that IL-37 can promoted BMMSCs to differentiate into osteogenic lineage cells *in vitro* [17]. Recent studies have shown that abnormal expression of IL-37 in several autoimmune orthopedic diseases, such as ankylosing spondylitis and rheumatoid arthritis [18]. Most recently, studies demonstrated a connection between IL-37 and several bone metabolism-related inflammatory cytokines and reported that recombinant IL-37 (rhIL-37) inhibited the expression of pro-inflammatory cytokines, such as IL-6, TNF-α, IL-17, and IL-23 in patients with ankylosing spondylitis [19]. The new study has indicated that IL-37 suppresses osteoclast formation and bone resorption *in vivo* [20].

A better understanding of the molecular mechanisms that govern odontogenesis and osteogenesis might provide us with new perspectives on treatment of many oral diseases. Recent reports have provided evidence that macromolecular degradation in stem cells in a process of differentiation occurs through autophagy [21]. Autophagy or “self-eating”, is a conservative cellular degradation pathway that recycles cellular content [22]. Upon activation of autophagy, degradation of intracellular protein and organelles via a process that involves the delivery of cytoplasmic cargo to lysosomes and release of metabolites required for anabolic processes, such as cell growth, proliferation and differentiation [23]. When cells are subjected to external stress, such as nutrition deficiency, oxidative stress, hypoxia, tumor formation, aging or infection, autophagy plays an important role as a cell survival mechanism [24, 25]. Recent studies have indicated that autophagy is an essential part in the functioning and maintenance of stem cells, acting to maintain their stemness, regulate their self-renewal, and mediate their differentiation capacity [26, 27] [28]. In addition, accumulating evidence has demonstrated that autophagy is also involved in

osteogenesis and bone development [29, 30]. Autophagy is also an essential process that maintains mineralizing capacity, and balance the population of osteoblasts and osteoclasts [31]. The members in our team have demonstrated that autophagy is involved in the odontogenic and osteogenic differentiation in stem cells of apical papilla and rBMMSCs [32, 33].

We hypothesis that autophagy might be involved in IL-37-mediated DPSCs osteogenic and odontogenic differentiation. Therefore, the aim of the present study was to verify whether IL-37 is able to promote the osteogenic and odontogenic differentiation of DPSCs and whether autophagy was involved in the regulation of osteo/odontogenic differentiation of DPSCs *in vitro.*

2. Materials And Methods

2.1. Isolation and Cell culture

Fresh extracted third molars were collected from patients (age:17–23 years old) with the patient's consent. Tooth was cleaned with PBS (Gibco, USA). The pulp tissue was minced into smaller fragments and then immersed into a solution (3 mg/mL collagenase type I (Sigma, USA) and 4 mg/mL dispase (Gibco, USA)) for 1 h. Cell suspensions were seeded into culture dishes containing complete medium.

2.2. Cell phenotype analysis

2.2.1. Immunofluorescence staining

Single cell suspensions were seeded on top of coverslips. After removing the medium, cells were fixed and washed. Briefly, after permeabilization with Triton X-100 for 12 min, cells were blocked with BSA (Boster, China) overnight, and then incubated with primary antibodies (anti-STRO-1, 1:100) overnight at 4°C. Cells were incubated with Cy3-conjugated secondary antibodies (Abcolonal, China). Following counterstained with DAPI, cells were photographed using a fluorescence microscope (Leica, Germany).

2.2.2 Flow cytometric analysis

Cells were contained the following monoclonal antibodies for 1 h on ice: CD90/FITC, CD105/PerCP-Cy5.5, CD34/PE, CD45/PE, and CD73/PE (1:100, BD Biosciences, USA). Cell were washed and resuspended for analysis using a FACScan. The data were analyzed on the software (FlowJo, USA).

2.2.3. Colony-forming assay

After cultured for 10 days later, cells were stained with toluidine blue (Beyotime, China). Photographs were taken under the microscope (Leica, Germany).

2.2.4. Multiple lineage differentiation

Cells were cultured in osteoinductive medium (OM) of 50 mg/ml ascorbic acid, 10 mM sodium β-glycerophosphate, 10 nM dexamethasone (Sigma, USA). After 2 weeks, cells were stained with Alizarin Red S (Sigma, USA) to detect mineral deposits.

Cells were incubated in adipogenic medium (Cyagen Biosciences Inc, USA) for 4 weeks. Then, cells were stained with Oil Red O reagent of the kit to reveal lipid droplets.

Cell pellets were prepared for a three-dimensional culture system. Cells were cultured in chondrogenic differentiation medium (Cyagen Biosciences Inc, USA) for 28 days. Alcian blue staining was utilized to examine the cartilage nodules.

2.3 ALP activity assay

Cells were incubated with different concentrations of IL-37 (0, 0.1, 1, 10, and 100 ng/mL) for 5 days. ALP activity of DPSCs was detected according to manufacturer's instruction.

2.4 ALP staining

Cells were seeded on 12-well plates and incubated with different cultured medium in different groups for 5 days. ALP staining was performed using an ALP staining kit (Beyotime Biotechnology, China) according to the manufacturer's instruction.

2.5 Alizarin red staining

DPSCs were cultured in OM for 14 days in the presence or absence of IL-37. The formation of mineralized nodules of DPSCs was evaluated by alizarin red S staining. For quantitative analysis, 10% cetylpyridinium chloride (CPC) was added and the absorbance was measured at 562 nm.

2.6 CCK-8

To assess the effect of IL-37 on the proliferation of DPSCs, cells were incubated with different groups concentrations of IL-37 (0, 1 ng/mL) for 0, 1, 3, 5, or 7 days. Then, the cells were incubated in the mixture (CCK-8: α-MEM = 1:9) for 2 h.

2.7 EdU assay

Cell proliferation ratio was measured using EdU Cell Proliferation Assay Kit according to the protocol of manufacturer.

2.8 RNA extraction and PCR analysis

Total RNA was isolated and reversed to cDNA by the RT Reagent Kit (Vazyme, China). Real-time qPCR reactions were carried out SYBR Green Mix (Vazyme, China). The gene specific primers are listed in Table 1.

Table 1

Primer sequences for real-time quantitative PCR analysis of gene expression.

Target gene	Sequences (5' – 3')	Product size (bp)	GenBank accession number
RUNX2	Forward, TCTTAGAACAAATTCTGCCCTTT	136	NM_001024630.3
	Reverse, TGCTTTGGTCTTGAAATCACA		
OSX	Forward, CCTCCTCAGCTCACCTTCTC	148	NM_001173467.1
	Reverse, GTTGGGAGCCCAAATAGAAA		
ALP	Forward, GACCTCCTCGGAAGACACTC	137	NM_000478.4
	Reverse, TGAAGGGCTTCTTGTCTGTG		
DSPP	Forward, ATATTGAGGGCTGGAATGGGGA	136	NM_014208.3
	Reverse, TTTGTGGCTCCAGCATTGTCA		
GAPDH	Forward, GAAGGTGAAGGTCGGAGTC		
	Reverse, GAGATGGTGATGGGATTTC	225	NM_002046.3

2.9 Western Blot Analysis

Cells were lysed in lysis buffer radioimmunoprecipitation assay (RIPA: PMSF = 100:1). Western blot was conducted according to previous studies [33].

2.10 TEM

Cells were collected after treated with or without 1 ng/mL IL-37 for 12 h. After fixation, dehydration, embedding, sectioning and staining, samples were viewed with a Hitachi Model H-7500 TEM (Hitachi, Japan).

2.11 Immunofluorescence staining

After cell attachment, the culture solid was replaced by complete medium and IL-37 (1 ng/mL) for 12 h. Cells were stained with primary antibody including DSPP, RUNX2, ALP, LC3 (1:100) according to the steps above.

2.12 Statistical analysis

Data are shown as mean ± SEM and were tested for statistical significance using ANOVA.

3. Results

3.1 Characterization of SCAPs and screening for the optimal IL-37 concentration

DPSCs were isolated and displayed a typical cobblestone-like morphology. Primary DPSCs were observed as shown in Fig. 1A. PDSCs in passage 3 were typical fibroblast-like or spindle-like in Fig. 1B. Immunofluorescence staining indicated that isolated DPSCs were positive for the established MSCs-specific surface marker STRO-1 (Fig. 1C). Flow cytometry analysis showed a pronounced expression of mesenchymal stem cell markers including CD73, CD90, and CD105, while they also showed the negativity for hematopoietic cell marker CD34 and leucocyte maker CD45. Cell surface markers: CD34 (0.07%); CD45 (0.30%); CD73 (99.6%); CD90 (93.3%); CD105 (87.5%) (Fig. 1D). The result of colony-forming assay showed that DPSCs could form single cell colonies. A single colony stained with crystal violet was observed under the microscope (Fig. 1E). Furthermore, results of oil red O staining, alizarin red S (ARS) staining and Alcian blue staining showed that DPSCs could differentiate into adipocytes, osteoblasts, and chondrocyte (Fig. 1F, G, H).

3.2 Effect of IL-37 on the proliferation of DPSCs

At day 5, ALP activity assay showed that ALP activity was the highest in the 1 ng/mL IL-37 group (Fig. 2A; $P < 0.01$). As compared with other groups, the 1 ng/mL IL-37 group also presented the highest ALP protein and gene expression after being induced for 5 days (Fig. 2B, C, D; $P < 0.01$). Therefore, 1ng/mL IL-37 was selected to be the optimal concentration for our further study. To evaluate the impact of IL-37 on the proliferation of DPSCs, CCK-8 analysis and EdU assay were performed. As indicated by the results of EdU assay (Fig. 2E, F; $p > 0.05$), and CCK-8 (Fig. 2G; $p > 0.05$), 1ng/mL IL-37 exerted almost no significant effect on the proliferation of SCAPs as compared with the control group.

3.3 IL-37 induced the osteo/odontogenic differentiation of SCAPs

To analyze the influence of IL-37 on the osteo/odontogenic differentiation and mineralization of DPSCs, cells were treated with 1 ng/mL IL-37. The protein expression of osteogenic and odontogenic markers in hDPSCs were examined cultured with or without 1 ng/mL IL-37 for 0, 3 and 7 days. Cells cultured with 1 ng/mL IL-37 expressed markedly higher levels of DSPP, RUNX2, ALP and OSX than those control groups for 3, 7 days, while, there is no difference between control group and IL-37 group for 0 day (Fig. 3A and B). Consistently, qRT-PCR showed that the osteogenic and odontogenic markers *DSPP, RUNX2, ALP and OSX* were also increased in the IL-37 treatment groups for 3, 7 days (Fig. 3C; $p < 0.05$ *or* 0.01). To further confirm whether IL-37 mediate osteogenic and odontogenic differentiation of DPSCs, cells were then cultured in OM with and without IL-37. Compared with control group, ALP staining revealed that ALP activity was significantly higher with administration of 1 ng/mL IL-37 (Fig. 3D). ALP activity assay confirmed the results of staining (Fig. 3E, $p < 0.01$). A similar trend in Alizarin Red S staining was detected after induction for 14 days. DPSCs treated with 1 ng/mL IL-37 and IL-37 + MM presented more mineralized nodules than control group or MM group (Fig. 3F). CPC assay revealed that extracellular calcium deposition was significantly increased in IL-37 group at day 14 as compared with the control group (Fig. 3G; $p < 0.01$). In addition, immunofluorescence assay showed that the osteoblastic and

odontoblastic markers DSPP and RUNX2 were upregulated in 1 ng/mL IL-37-treated group (Fig. 2H, I). Above all, these results indicated that 1 ng/mL IL-37 enhanced the osteo/odontogenic differentiation of DPSCs.

3.4 1 ng/mL IL-37 triggered autophagy of DPSCs

To explored the mechanism of IL-37-enhanced odontoblastic and osteoblastic differentiation potential of DPSCs, western blot was conducted. DPSCs were treated with 1 ng/mL IL-37. Exposure of DPSCs to complete medium containing 1 ng/mL IL-37 resulted in elevated protein expression of autophagy-related protein Beclin1 and the expression ratio of LC3-II/I in a time-dependent manner while the expression of p62 was decreased (Fig. 4C, D). The images of immunostaining with LC3 showed that endogenous LC3 dot form in IL-37-treated DPSCs was significantly higher than control group (Fig. 4E). Meanwhile, to further confirm the induction of autophagy in IL-37-treated DPSCs, the formation of autophagosomes was observed by means of TEM. The photos of TEM showed more autophagosomes in 1 ng/mL IL-37-treated DPSCs group at 7 days than those in the control group (Figure F). These observations strongly suggested IL-37 activated autophagy in IL-37-treated DPSCs.

3.5 The autophagy inhibitor 3-MA suppresses IL-37-mediated enhancement osteoblastic and odontoblastic differentiation of DPSCs

To further validate our findings, autophagy was inhibited with specific inhibitor 3-MA, respectively. Western blot analysis indicated that the ratio of LC3-II to LC3-I, Beclin1 expression levels in IL-37 + 3-MA group were significantly lower than the IL-37 group, while 3-MA significantly up-regulated P62 protein levels, suggesting that autophagy was suppressed when autophagy inhibitor3-MA treatment was applied (Fig. 5A, B). The result indicated that 3-MA showed obviously autophagy inhibition. RT-PCR and western blot were conducted to investigate the effects of IL-37 on osteoblastic and odontoblastic differentiation of DPSCs following autophagy inhibitor treatment. Western blot analysis showed that the inhibitor 3-MA dramatically suppressed IL-37-mediated enhancement of DSPP, RUNX2, ALP and OSX expression in DPSCs at protein levels (Fig. 5C, D). Similarly, the inhibitor 3-MA suppressed IL-37-enhanced the expression of *DSPP*, *OCN*, *RUNX2* and *OSX* in DPSCs at gene level (Fig. 5E). To investigate the role of autophagy in IL-37-induced mineralization, ALP staining, ALP activity assay, ARS staining and CPC assay were performed. The results of ALP staining and ALP assay showed that ALP activity of IL-37-treated DPSCs was inhibited with the addition of autophagy inhibitor 3-MA on day 5 (Fig. 5F, G). After induction for 2 weeks, the amount of minerals was significantly decreased in the presence of autophagy inhibitor 3-MA compared with IL-37 treatment alone (Fig. 5H, I). As shown by immunostaining analysis, the expression of DSPP and RUNX2 increased remarkably in IL-37-treated DPSCs compared with IL-37 + 3-MA (Fig. 5J, K). These results indicated that IL-37-enhanced osteoblastic and odontoblastic differentiation of DPSCs were repressed by autophagy inhibition.

3.6. Upregulation of autophagy by rapamycin further promoted odontogenic and osteogenic differentiation of DPSCs

To further examine the effect of autophagy on osteogenic and odontogenic differentiation of DPSCs, cells were cultured in medium in the presence of rapamycin. The result also indicated that rapamycin treatment significantly up-regulated Beclin1 protein levels, increased the ratio of LC3-II to LC3-I and down-regulated P62 protein level compared with the IL-37 group (Fig. 5A, B). Besides, hDPSCs exposed to rapamycin significantly increased autophagy activity. Western blot analysis and qRT-PCR were conducted to detect the inducer of autophagy rapamycin on the osteogenic and odontogenic differentiation of DPSCs at the RNA and protein levels. The results of western blot showed that the expression of osteogenic and odontogenic markers DSPP, RUNX2, ALP and OSX were all up-regulated in IL-37 + rapamycin group comparing with IL-37 (Fig. 6C, D). PCR assay for mRNA expression of odontogenesis/osteogenesis-related molecules showed that autophagy activation by rapamycin enhanced IL-37-induces differentiation (Fig. 6E). ALP staining and ALP activity assay showed that rapamycin upregulated ALP activity compared with IL-37 groups (Fig. 6F, G). ARS staining indicated that the number of mineralized nodules was observed more in the rapamycin group (Fig. 6H). CPC assay showed that the group treated with IL-37 + rapamycin presented higher calcium contents as compared with IL-37 group (Fig. 6I). Besides, immunofluorescence assay showed that osteogenic and odontogenic markers DSPP, RUNX2 were upregulated in IL-37 + rapamycin-treated group (Fig. 6J, K). All these data indicated that autophagy plays a catalytic role in IL-37-induced osteogenic and odontogenetic differentiation of hDPSCs.

Discussion

Osteogenic differentiation and odontogenic differentiation are crucial characteristics of the oral stem cells pluripotency. They play key roles in the maintenance, tissue regeneration and engineering. In this study, we demonstrated that IL-37 and the related target autophagy pathway play an important role in the osteo/odontogenic differentiation of DPSCs. DPSCs consist of heterogeneous multipotent cell populations, which have the potential to differentiate into osteoblasts and odontoblasts. Osteogenic and odontogenic differentiation result in the expression of related genes and proteins such as DSPP, RUNX2, ALP, OSX. This study emphasized a novel and promising role of IL-37 in regulating the odontogenic and osteogenic differentiation of DPSCs.

IL-37 is recently identified as an anti-inflammatory cytokine [34]. A Chinese study reported that patients carrying a mutation in the coding region of IL-37 have lower disease activity scores and less pain, which indicated significant function for the anti-inflammatory effects of IL- 37[35]. Previous studies demonstrated that cytokines were associated with autophagy. For example, in addition to controlling the migration of macrophages into degenerative tissues, IL-33, one of cytokines, also regulated autophagy in these tissues [36]. The level of IL-37 is normally low, but this significantly increases under severe inflammatory conditions. It has been previously found that IL-37 plays an important regulatory role in the

development of several inflammatory and autoimmune diseases [37, 38]. Recent studies investigated that IL-37 mediates a variety of anti-cancer effects in multiple types of cancer [39]. Recently, it was reported that IL-37 promoted the osteogenic differentiation of BMSCs [17]. In this study, we found that extracellular IL-37 accelerated the osteogenic and odontogenic differentiation of DPSCs via autophagy when at 1 ng/mL, while there is no significant effect on the proliferation.

The autophagy pathway is normally inhibited by 3-MA and activated by rapamycin [40]. Autophagy is a major intracellular mechanism to protect cells from stress stimulation and maintain the properties of stem cells [30, 41]. Autophagy plays an important role in the osteogenic and odontogenic differentiation of hDPSCs under specific condition [1]. During cellular differentiation, autophagy occurs in order to meet metabolic needs associated with morphological and functional changes. Several proteins, including LC3, p62, and Beclin1 have been involved in autophagy and are used as markers for the activation of the process. LC3 is the most widely used autophagic marker. LC3 is usually produced by ubiquitin, the residues of which are exposed on the vesicular membrane surface after ATG4 homolog catalysis, and forms LC3-I in the cytoplasm [41]. LC3-I can specifically bind phosphatidylethanolamine on the vesicle membrane surface and eventually forms LC3-II, which is an integral membrane protein present in autophagosomes [42]. Accumulation of LC3 II is thus used as a marker for activation of autophagy, which can directly reveal autophagy. Beclin-1 is an autophagy-initiated protein. P62 possesses LC3-interacting region and serves as a signaling hub of autophagy. P62 is degraded through the process of autophagy, therefore the degradation of P62 expression level can serve as a marker of autophagic clearance [30]. The increases of Beclin1, an autophagy initiation protein, is also accompanied by activation of the process. In this study, IL-37 promoted LC3 I/II conversion, increased the protein levels of Beclin1 and decreased the protein expression of P62.

ALP has a vital function during mineralization of osteoblasts and odontoblast, and its activity is upregulated at an early stage of calcification [43]. In this study, 1 ng/ml was the optimal concentration of IL-37 to promote differentiation of DPSCs by detecting the ALP activity at day 5, as well as the mRNA level and protein level of ALP at day 5. Our results showed that the expression levels of osteogenic and odontogenic markers, including DSPP, RUNX2, ALP, and OSX were upregulated at 3, 7 days of differentiation, indicating that 1 ng/mL IL-37 can enhance the osteo/odontogenic differentiation of DPSCs. The autophagy pathway was shown to positively regulate differentiation of MSCs. We proceeded to investigate whether autophagy was activated by IL-37, the key proteins in autophagy signaling were evaluated. Consistent with pathway analysis, the protein expressions of Beclin 1, the ratio of LC3 II/I were significantly increased while the expression of P62 was reduced, when DPSCs were treated with IL-37 for 6, 12, 24 h. To further confirm whether autophagy signaling was implicated in the process of IL-37 enhanced osteogenic and odontogenic differentiation of DPSCs, we evaluated the expression of related markers above with or without autophagy inhibitor 3-MA. While 3-MA was used, we found that all the markers of osteogenesis and odontogenesis were dramatically reduced. These *in vitro* results demonstrated that autophagy is an important mechanism in IL-37 enhanced osteo/odontogenic differentiation of hDPSCs. Rapamycin, as autophagy activator was used to further detect the relationship

between autophagy and osteo/odontogenic differentiation of IL-37 treated DPSCs. Osteogenic and odontogenic differentiation of IL-37 enhanced DPSCs further increased with rapamycin treatment.

In summary, the present study revealed that IL-37 could induce osteogenic and odontogenic differentiation in DPSCs, which involved the upregulation of autophagy. The results expanded our knowledge on the role of IL-37 in osteogenesis and odontogenesis of DPSCs, and also suggested that IL-37 may be a potential drug for pulp treatment in the future and provided insight into the molecular mechanism of vital pulp therapy. Also, kinetic *in vivo* measurements should be carried out in future studies.

Conclusion

Overall, IL–37 enhances differentiation of DPSCs into odontoblasts and osteoblasts via autophagy signaling pathways.

Abbreviations

3-MA 3-methyladenine; ALP: alkaline phosphatase; ARS: alizarin red staining; α-MEM: alpha modified eagle's medium; BMMSCs: bone marrow mesenchymal stem cells; BSA: bovine serum albumin; CCK-8: cell counting kit -8 assays; DAPI 4-6-diamidino-2-phenylindole; DPSCs: dental pulp stem cells; DSPP dentin sialophosphoprotein; FBS fetal bovine serum; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; IL-37 Interleukin-37; LC3 microtubule-associated protein 1 light chain 3; MTOR mechanistic target of rapamycin; OSX: osterix; PBS: phosphate buffered saline; real time RT-PCR: real time reverse-transcription polymerase chain reaction; RIPA: radio immunoprecipitation assay; RUNX2: runt-related transcription 2.

Declarations

Acknowledgements

Not applicable.

Conflict of interest

The authors declare no conflicts of interest.

Authors Contributions

Na Li conceived and designed the study, collected and assembled data, and wrote the manuscript. Yan Chen and Ming Yan performed the data analysis and interpretation. Yanqiu Wang, Jintao Wu completed data analysis and interpretation. Lin Fu reviewed the data. Jinhua Yu conceived and designed the study, provided financial support and study material, performed the data analysis and interpretation, and approved the final version of the manuscript. All authors read and approved the manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

Studies were carried out in accordance with the Declaration of Helsinki and got the approval of the Ethical Committee of Nanjing Medical University.

Consent for publication

Not applicable.

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Figures

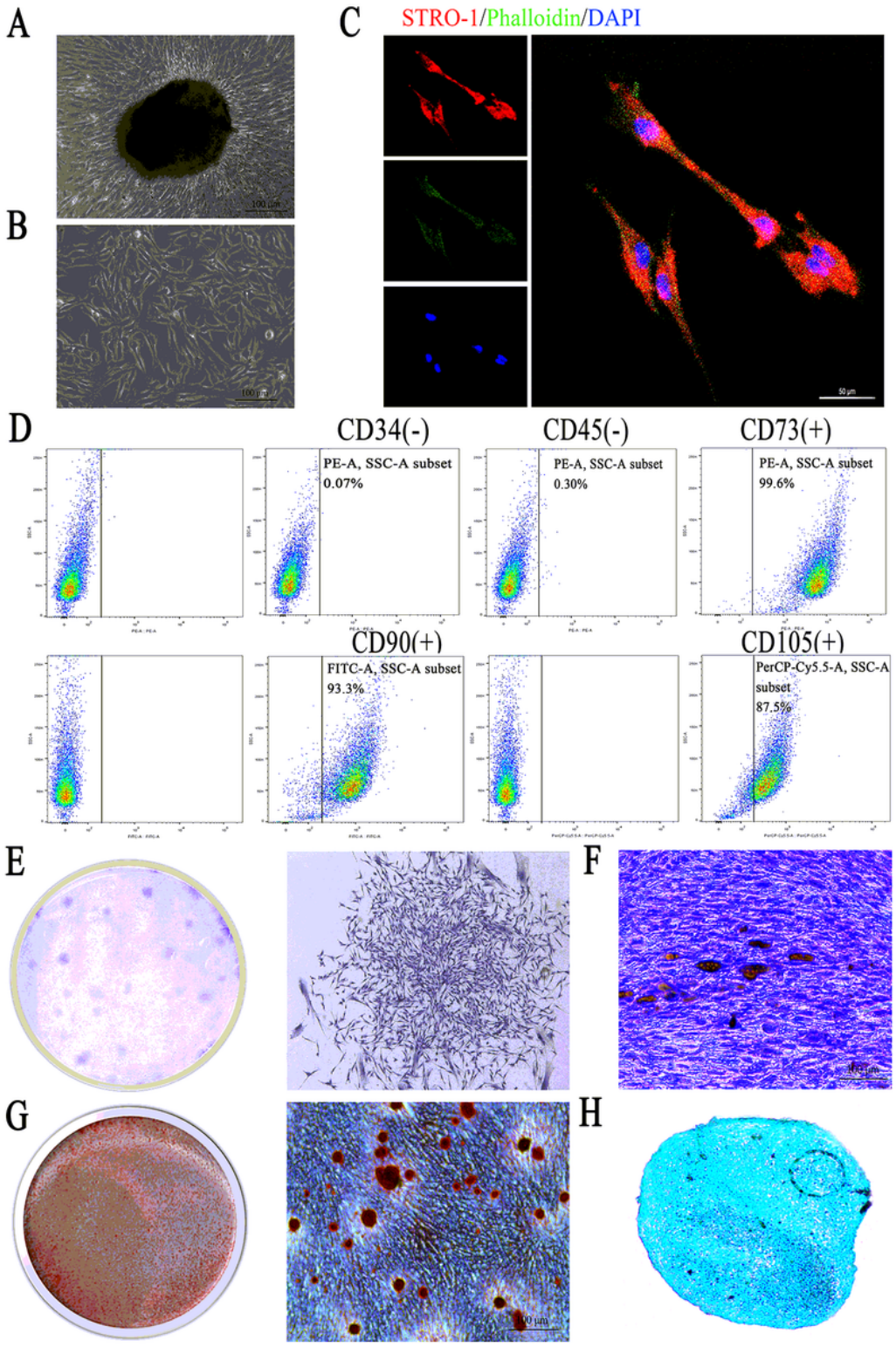

Figure 1

Identification of DPSCs (A) Morphology of primary DPSCs after 1 weeks of culture; (B) hDPSCs at passage 3 were grown in culture medium with a long spindle shape; (C) Immunofluorescence staining of STRO-1 of DPSCs; (D) Immunophenotype analysis of DPSCs was determined by flow cytometry assay; (E) Colonies derived from single cell were observed and photographed under the microscope; (F) Adipocyte staining by Oil Red O upon adipogenic induction for 4 weeks; (G) Mineral deposits stained by

Alizarin Red S in DPSCs following osteogenic differentiation for 2 weeks; (H) Chondrogenic staining by Alcian blue after cells were induced to differentiate into chondrogenic lineages for 28 days.

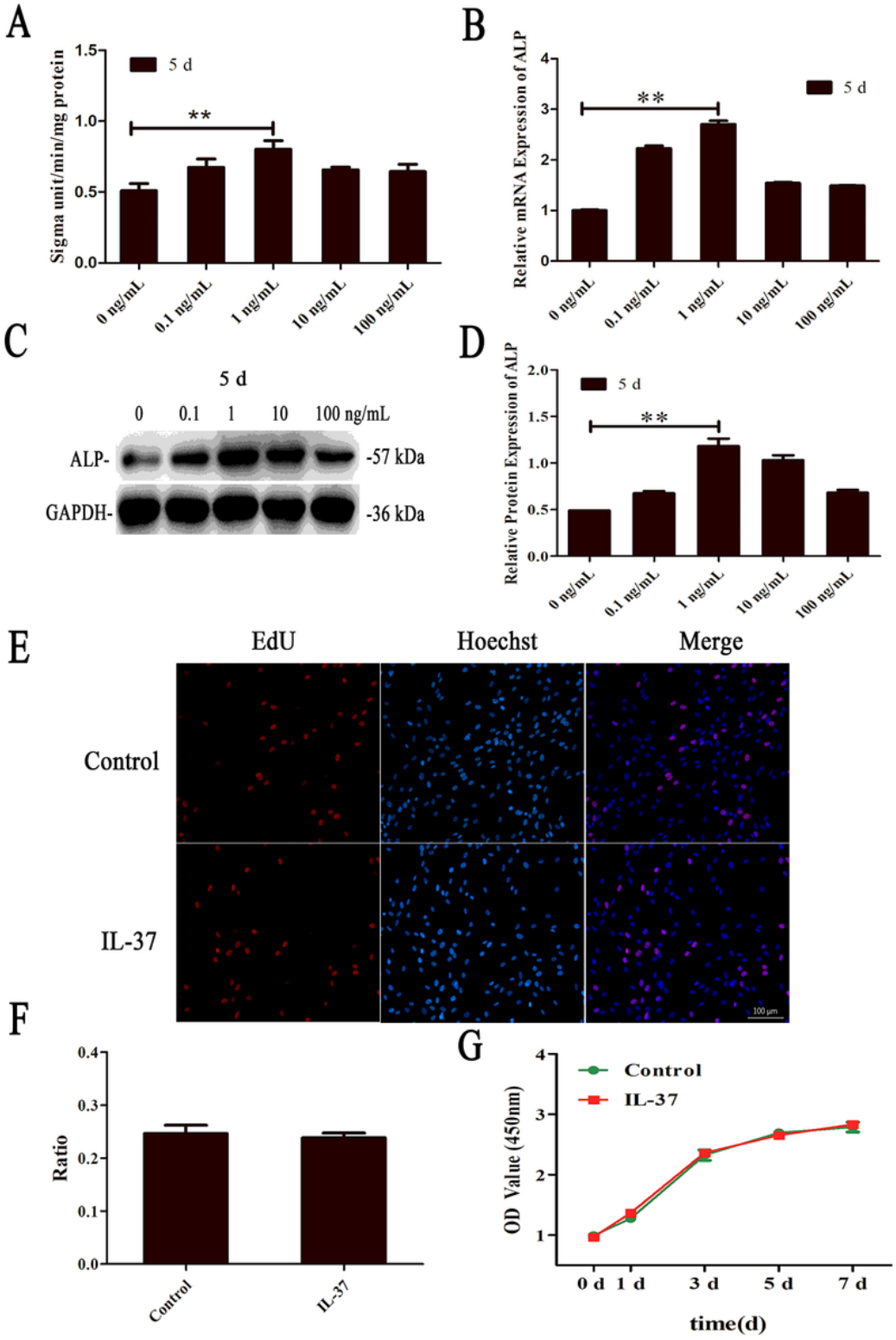

Figure 2

Screening for the optimal IL-37 concentration and effect of IL-37 on the proliferation of DPSCs (A) The effect of different concentrations of IL-37 on the ALP activity in DPSCs; (B)The effect of different concentrations of IL-37 on the mRNA expression level of ALP in DPSCs; (C) The effect of different

concentrations of IL-37 on the protein expression level of ALP in DPSCs; (D) Relative quantitative analysis of western blot analyses for ALP; (E) Cell viability was determined by EdU staining; (F) Relative quantitative analysis of EdU staining; (G) CCK-8 analysis showing that IL-37 has no significant effect on DPSC proliferation.

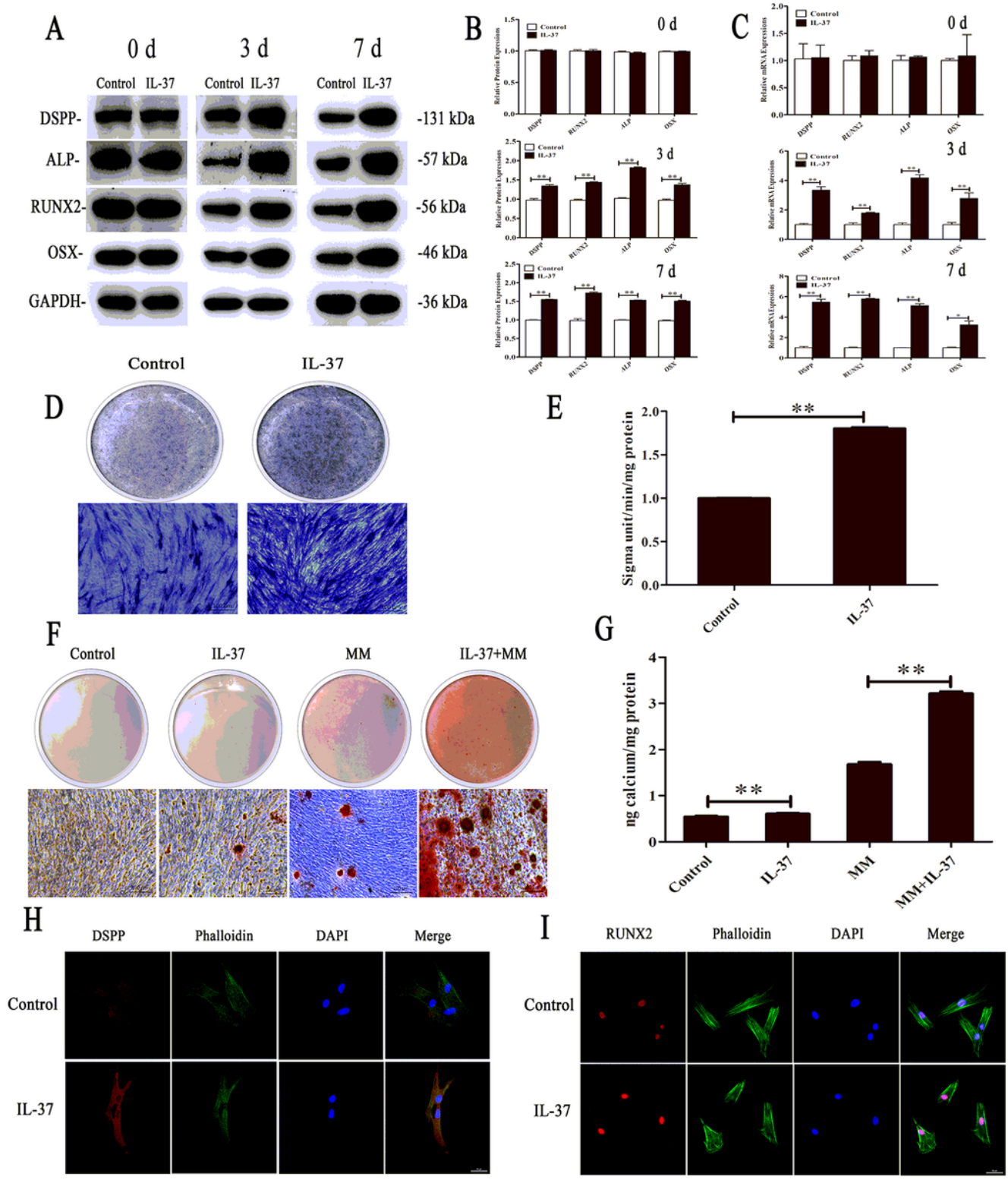

Figure 3

IL-37 enhances the osteo/odontogenic differentiation capacity of hDPSCs (A) Western blot analyses of osteo/odontogenic proteins including DSPP, RUNX2, ALP, and OSX; (B) Relative quantitative analysis of western blot (DSPP, RUNX2, ALP, and OSX); (C) Relative mRNA expression of osteo/odontogenic genes (DSPP, RUNX2, ALP, and OSX) on day 0, 3, and 7; (D) Results of ALP staining on day 5; (E) Relative ALP activity on day 5; (F) Results of ARS on day 14; (G) Relative quantitative analysis of the ARS; (H) Immunofluorescence detection indicated that IL-37 increased the protein expression of DSPP; (I) Immunofluorescence detection indicated that IL-37 increased the protein expression of RUNX2.

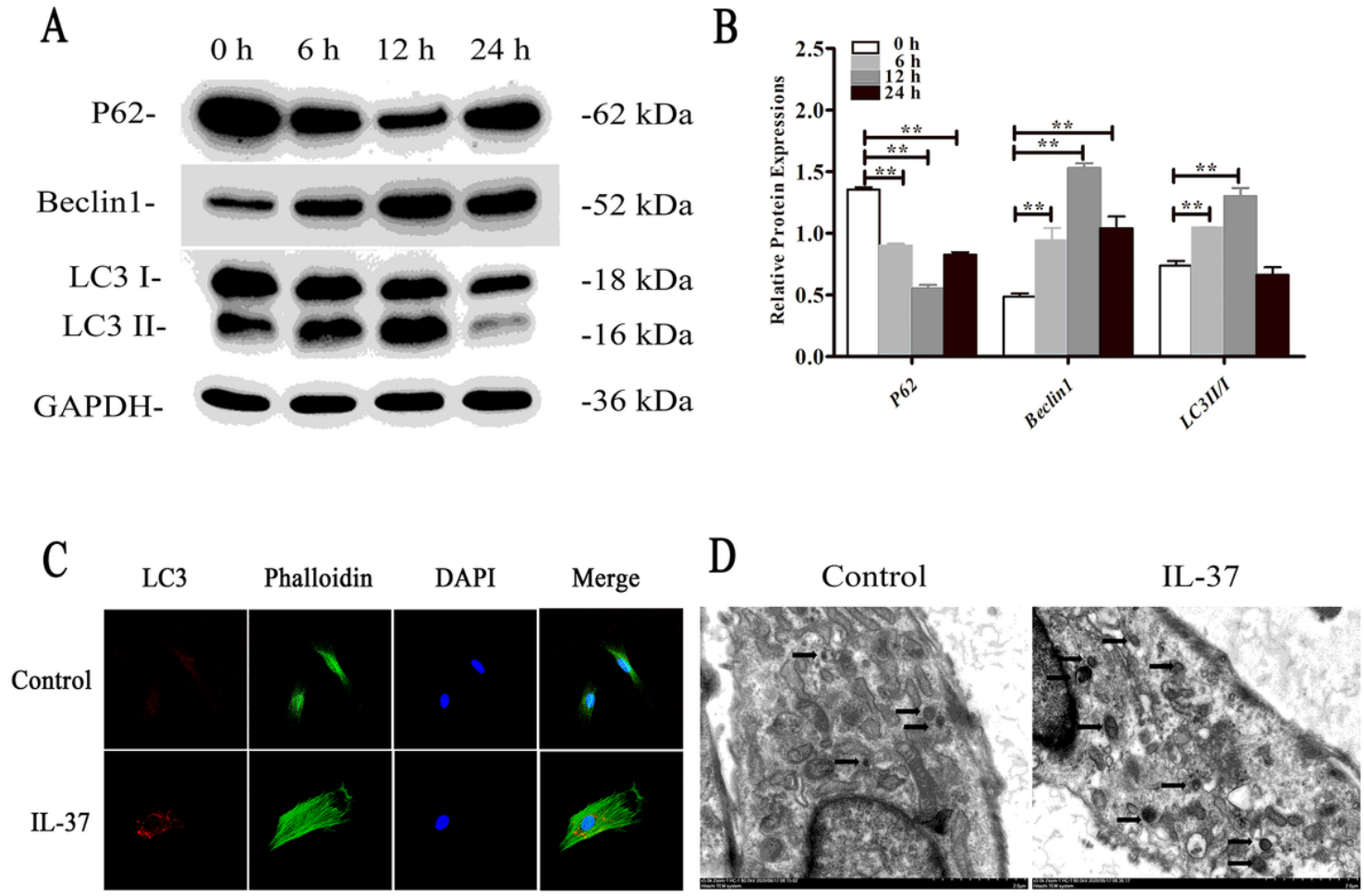

Figure 4

IL-37 activated autophagy of DPSCs (A) Western blot was conducted to detect the autophagy-related markers (LC3, Beclin1, and p62); (B) Quantification was done by ImageJ. $p<0.05$, $^{**}p < 0.001$; (C) The images of LC3 (red) and DAPI (blue) in DPSCs by immunofluorescence staining. Scale bar = 50 μm; (D) Transmission electron microscopy analysis of autophagosomes in IL-37 group and control group. Autophagosomes are indicated by black arrows.

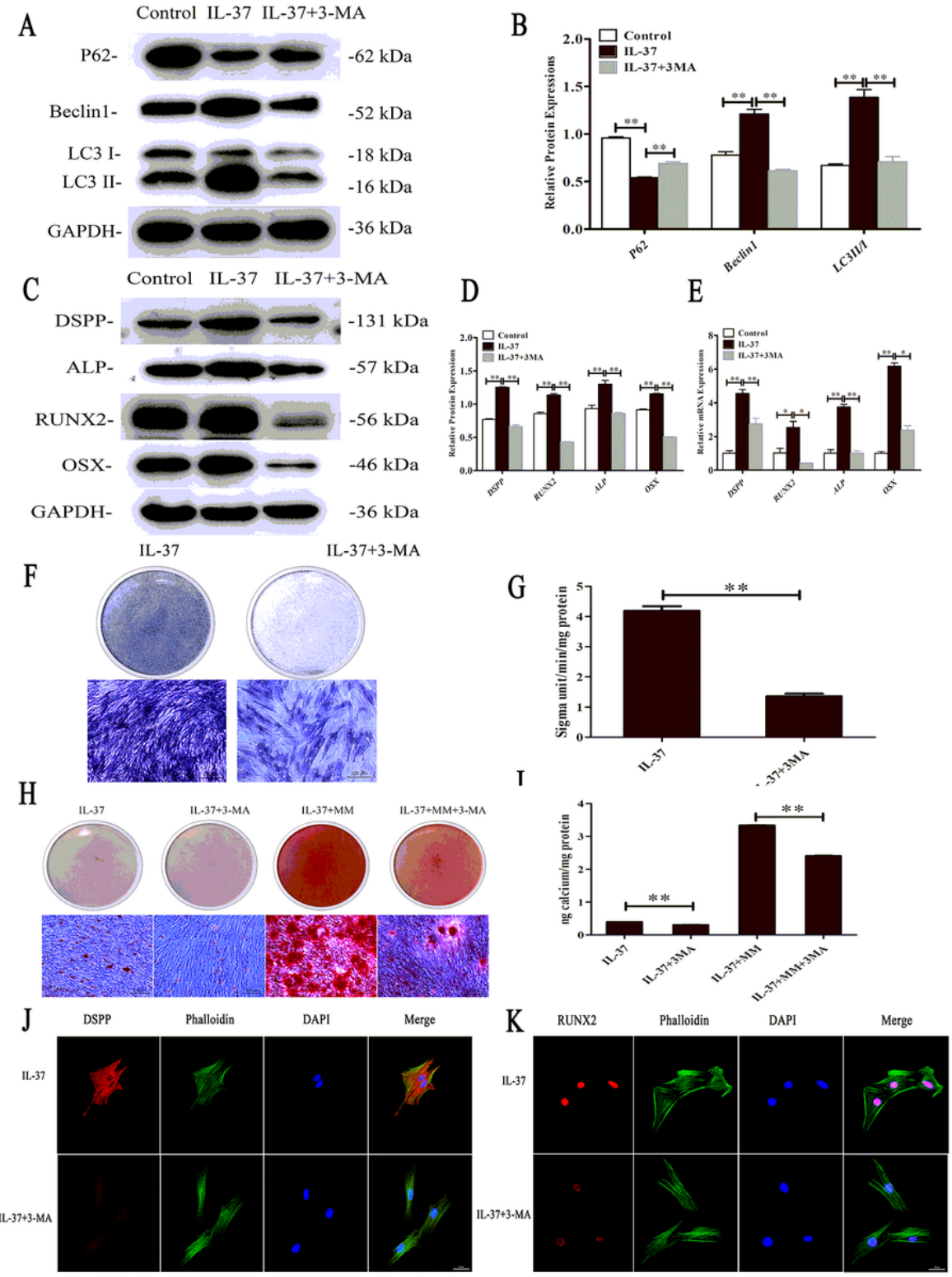

Figure 5

3-MA reverses IL-37-induced autophagy enhanced osteogenic and odontogenic effects in DPSCs (A) Western blot analysis of LC3, Beclin1 and p62 in DPSCs treated with or without 3-MA; (B) The ratio of LC3-II/LC3-I and quantification of P62, Beclin1 were done by Image J. * p< 0.05, **p< 0.01 C. (C) Western blot analysis of osteogenic and odontogenic markers in DPSCs; (D) Relative quantitative analysis of western blot analyses for DSPP, RUNX2, ALP, and OSX; (E) Relative mRNA expression of

osteo/odontogenic genes (DSPP, RUNX2, ALP, and OSX); (F) Results of ALP staining on day 5; (G) Relative ALP activity on day 5; (H) Results of ARS on day 14; (I) Relative quantitative analysis of the ARS; (J) Immunofluorescence detection indicated that 3-MA decreased the protein expression of DSPP; (K) Immunofluorescence detection indicated that 3-MA decreased the protein expression of RUNX2.

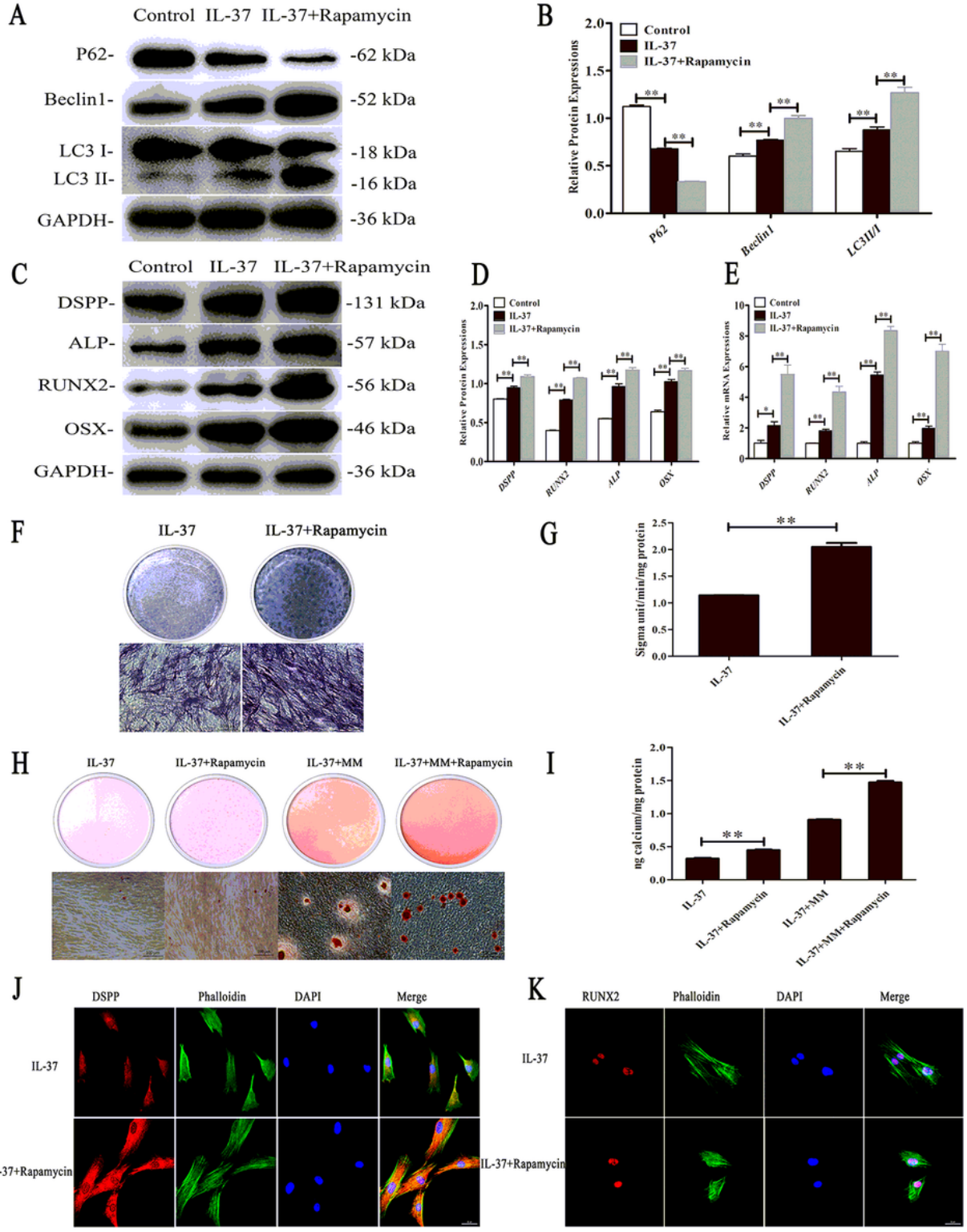

Figure 6

The effects of autophagy activator rapamycin on osteo/odontoblastic differentiation of IL-37-induced DPSCs (A) Western blot analysis of LC3, Beclin1 and p62 in DPSCs treated with or without rapamycin; (B) The ratio of LC3-II/LC3-I and quantification of P62, Beclin1 were analyzed by Image J. * $p < 0.05$, ** $p < 0.01$; (C) Western blot analysis of osteogenic and odontogenic markers in DPSCs; (D) Relative quantitative analysis of western blot analyses for DSPP, RUNX2, ALP, and OSX; (E) Relative mRNA expression of osteo/odontogenic genes (DSPP, RUNX2, ALP, and OSX); (F) Results of ALP staining on day 5; (G) Relative ALP activity on day 5; (H) Results of ARS on day 14; (I) The quantity result for ARS staining; (J) Immunofluorescence detection indicated that rapamycin increased the protein expression of DSPP; (K) Immunofluorescence detection indicated that rapamycin increased the protein expression of RUNX2.