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ORIGINAL ARTICLE

Basic Study

Yes-associated protein-mediated melatonin regulates the function of periodontal ligament stem cells under oxidative stress conditions

Ke Gu, Xiao-Mei Feng, Shao-Qing Sun, Xing-Yao Hao, Yong Wen

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Abstract

BACKGROUND

Human periodontal ligament stem cells (PDLSCs) regenerate oral tissue. *In vitro* expansion causes replicative senescence in stem cells. This causes intracellular reactive oxygen species (ROS) accumulation, which can impair stem cell function. Tissue engineering efficiency is reduced by exogenous ROS stimulation, which causes premature senescence under oxidative stress. Melatonin (MT), a powerful free radical scavenger, can delay PDLSCs senescence but may not maintain stemness under oxidative stress. This experiment examined the effects of hydrogen peroxide-induced oxidative stress on PDLSCs' apoptosis, senescence, and stemness.

AIM

To determine if MT can reverse the above effects along with the underlying molecular mechanisms involved.

METHODS

PDLSCs were isolated from human premolars and cultured in different conditions. Flow cytometry was used to characterize the cell surface markers of

PDLSCs. Hydrogen peroxide was used to induce oxidative stress in PDLSCs. Cell cycle, proliferation, apoptosis, differentiation, ROS, and senescence-associated β-galactosidase activity were assessed by various assays. Reverse transcription-polymerase chain reaction and western blot were used to measure the expression of genes and proteins related to stemness and senescence.

RESULTS

MT increases Yes-associated protein expression and maintains cell stemness in an induced inflammatory microenvironment, which may explain its therapeutic effects. We examined how MT affects PDLSCs aging and stemness and its biological mechanisms.

CONCLUSION

Our study reveals MT's role in regulating oxidative stress in PDLSCs and Yes-associated protein-mediated activity, providing insights into cellular functions and new therapeutic targets for tissue regeneration.

Key Words: Human periodontal ligament stem cells; Melatonin; Reactive oxygen species; Senescence; Yes-associated protein

Core Tip: While the use of mesenchymal stem cells in preclinical and clinical studies for the treatment of various diseases has provided promising results, the microenvironment of oxidative stress inhibits the efficacy of mesenchymal stem cells-mediated tissue regeneration, which remains a major challenge limiting clinical applications. Our findings provide new insights into melatonin (MT) regulation in the biological functions of oxidative stress-induced periodontal ligament stem cells and elucidate the potential mechanism of Yes-associated protein-mediated MT action. These findings strengthen our comprehension of the role of MT during cellular oxidative stress and provide potential targets for the development of new therapeutic strategies to promote tissue regeneration.

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INTRODUCTION

Human periodontal ligament stem cells (PDLSCs) are a source of oral tissue and the dominant seed cells in the oral tissue regeneration field[1]. With the extension of the human lifespan and the rise of an aging society, the aging of the body has become a major clinical factor that needs to be addressed through tissue regeneration. Aging microenvironments inhibit tissue regeneration mediated by mesenchymal stem cells (MSCs). Additionally, after multiple passages, the cells undergo mitotic arrest, replicative senescence, and stemness changes[2]. The changes over time include abnormal stem cell morphology, limited self-renewal ability, decreased differentiation potential, and a shortened lifespan.

Due to its simple operation and low cost, small- molecule drug therapy has become a recent research and development hotspot. Melatonin (MT), a pineal gland-secreted neurohormone peptide, is a powerful free radical scavenger and small-molecule drug detected at multiple sites in the body[3]. Prior studies have shown that MT regulates MSC proliferation, apoptosis, and stemness. Furthermore, MT effectively reverses the premature aging of bone marrow-MSCs caused by hydrogen peroxide (H_2O_2), in a dose-dependent manner[4] and can also restore the damaged osteogenic differentiation potential of senescent cells[4,5]. Under oxidative stress conditions, the self-renewal ability of MT-treated MSCs is enhanced, and apoptosis is reduced[6].

Yes-associated protein (YAP) is a downstream effector molecule of the Hippo signaling pathway[7]. As an evolutionarily conserved protein kinase signaling cascade, the Hippo pathway is closely associated with organ formation and tumorigenesis during growth and development. Many prior studies have confirmed that YAP plays a key role in the regulation of self-renewal and stemness in various cells[8]. YAP is highly expressed in embryonic stem cells, and during differentiation, YAP is degraded and inactivated in the cytoplasm, thus suppressing many major stem cell-related genes. In contrast, when YAP is overexpressed, embryonic stem cell differentiation is inhibited, and stem cell properties are maintained, even under culture conditions that induce differentiation[9,10]. The molecular mechanism by which MT protects stem cells from oxidative stress is unknown. While exploring mechanisms underlying the therapeutic effects of MT, we found that MT increases YAP expression and maintains cell stemness in an induced inflammatory microenvironment[11]. Here, we investigated the effect of MT on PDLSCs aging and stemness and explored the underlying biological mechanisms.

MATERIALS AND METHODS

PDLC isolation and culture

The dental samples evaluated here consisted of complete premolars taken from 20 systemically healthy donors who required extraction for orthodontic treatment. The individuals were between 12 and 14 years of age and provided informed consent. All procedures were approved by the Research Ethics Committee of Shandong University (No. GR201902). From the middle third of the root surface, periodontal ligament tissue was scraped and inoculated into flasks and maintained in a complete medium, composed of α-MEM (HyClone) and 10% fetal bovine serum (Gibco), at 37 °C in 5% CO_2. The culture medium was replaced every 2-3 days, and the PDLSCs were utilized between passages 3 and 4 (P3-4) to avoid passage-dependent changes in cell behavior.

Flow cytometry analysis

Flow cytometry (BD Biosciences) was used to analyze PDLSCs cell surface markers according to the manufacturer's instructions. Briefly, after cleaning with phosphate buffered saline (PBS) containing 3% fetal bovine serum, cells were incubated with monoclonal antibodies against human CD90, CD105, CD29, CD44, CD73, CD31, CD34, and CD45 for 1 hours at 4 °C in the dark. Cells that were not antibody-pretreated served as blank controls. Subsequently, the cells were washed with PBS and analyzed by flow cytometry.

H_2O_2-treated PDLCs

Human PDLCs at a density of 2 × 10^5 cells/well were inoculated in 6-well plates and incubated at 37 °C in 5% CO_2 for 24 hours. When the cell confluence reached 60%, 50 μM, 100 μM, 200 μM, 300 μM, or 400 μM H_2O_2 (a gradient dilution in complete medium) was added, and the samples were allowed to incubate for 2 hours. Next, the cells were washed in PBS to remove residual H_2O_2 and then incubated in a fresh complete medium, which was replaced every 2-3 days. These samples were used for subsequent experiments. Different concentrations of MT (1, 10, or 100 μM) dissolved in anhydrous ethanol were added to the medium, along with different concentrations of verteporfin (25 or 200 nM) dissolved in dimethyl sulfoxide.

Cell cycle distribution assay

Cell cycle distribution was quantified using a DNA quantitative analysis kit (Solarbio), according to the manufacturer's instructions. Cells at a density of 2 × 10^5 cells/well were seeded in 6-well plates, and 500 μL of pre-cooled 70% ethanol was added to allow the cells to fix overnight at 4 °C. Then, 100 μL of RNase A solution was added, and the samples were incubated in a water bath at 37 °C for 30 minutes. Subsequently, 400 μL of propidium iodide (PI) staining solution was added in a dark room, and the samples were incubated for 30 minutes at 4 °C, protected from light. The resulting fluorescence intensity was measured by flow cytometry using a PerCP laser.

Cell proliferation assay

A Cell Count Kit 8 (CCK-8) (Biosharp) assay was used to quantify cell proliferation according to the manufacturer's instructions. Briefly, in the CCK-8 assay, PDLSCs were seeded in 96-well culture plates (2 × 10^3 cells/well) and cultured for 1, 2, or 3 days. At each specified time point, the medium was replaced with fresh MEM supplemented with 10% CCK-8, and the samples were incubated at 37 °C for 2 hours in the dark. The resulting absorbance was measured at 450 nm using a microplate reader (SPECTROstar Nano, BMG Labtech).

Cell apoptosis assay

The percentage of apoptotic cells was determined using an annexin V-FITC/PI staining kit (Multisciences, Hangzhou, China). PDLSCs were seeded into six-well culture plates (1 × 10^5 cells/well), and after reaching 80%-90% confluence, the cells were washed with cold PBS and treated with binding buffer. Lastly, the cells were harvested and resuspended in 100 μL of binding buffer containing 5 μL annexin V-FITC and 10 μL PI for 15 minutes in the dark at room temperature. The percentage of apoptotic cells was determined using flow cytometry.

Osteogenic differentiation potential assay

To assess osteogenic differentiation potential, 1 × 10^5 PDLSCs were inoculated into 6-well plates and cultured in the osteogenic induction medium (complete medium supplemented with 50 mg/L vitamin C, 10 mmol/L sodium-glycerophosphate, and 10 mmol/L dexamethasone). After 7 days, PDLSCs were stained with alkaline phosphatase (ALP), and according to the manufacturer's instructions, an ALP activity assay kit (Nanjing Jiancheng Institute of Biological Engineering) was used to measure the ALP activity level, and a microplate reader was used to measure the absorbance at 520 nm. After 21 days, the mineralized nodules were stained with 2% alizarin red and measured at 560 nm using a microplate reader after solubilization in 10% acetyl pyridinium chloride (CPC, Sigma-Aldrich) for 30 minutes at room temperature.

Adipogenic differentiation potential assay

To assess the adipogenic differentiation potential, 1 × 10^5 PDLSCs were seeded in 6-well plates and cultured in the adipogenic induction medium (complete medium supplemented with 1 mol/L dexamethasone, 200 mol/L indomethacin, 10 mg/L insulin, and 500 mol/L IBMX). After 14 days, the lipid droplets were stained with oil-red O for evaluation.

Table 1 Gene primers

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
C-MYC	TTCGGGTAGTGGAAAACT	AGCAGCTCGAATTTCTTCC
OCT-4	CCCCTGGTGCCGTGAAGG	GCAAATTGCTCGAGTTCT
NANOG	AAAGAATCTTCACCTATG	GAAGGAAGAGGAGAGAC
P21	CGATGGAACTTCGACTTTGTCA	GCACAAGGGTACAAGACAGTG
GAPDH	AGAAGGCTGGGGCTGAGA	AGGGGCCATCCACAGTCT
RB	CTTCCTCATGCTGTTCAGGAG	TGCATGAAGACCGAGTTATAGAAT
ALP	TCCATCTGTAAAGGGCGG	AATACCAGCTACGCTGCA
COL1	GCTGATGATGCCAATGTA	CCAGTCAGAGTGGCACAT
YAP	GCTACAGTGTCCCTCGAACC	CCGGTGCATGTGTCTCCTTA

ALP: Alkaline phosphatase; YAP: Yes-associated protein.

Cellular reactive oxygen species assay

We analyzed cellular reactive oxygen species (ROS) levels in PDLSCs using a fluorescent intracellular ROS kit (Beyotime), according to the manufacturer's instructions. Briefly, the cells were incubated with the ROS assay reagent stock solution for 30 minutes at 37 °C in 5% CO_2 under light-protected conditions. After utilizing the FITC channel to excite green fluorescence, all subgroups were photographed in different fields of view under the same exposure conditions, and the single-channel fluorescence intensity was measured using ImageJ software. In addition, fluorescence intensity was measured using a flow cytometer with a FITC laser.

Senescence-associated β-galactosidase staining and quantitative analysis

PDLCs (1×10^5 cells) were inoculated on 24-well plates at 37 °C in 5% CO_2 overnight. In accordance with the manufacturer's protocol, we processed the cells using the cell senescence-specific β-galactosidase assay kit (Beyotime), with incubation at 37 °C for 12 hours. Cell observation and counting under a microscope (Olympus) allowed us to identify cells expressing senescence-associated-galactosidase (SA-gal) by their blue color.

Reverse transcription-polymerase chain reaction analysis

Total RNA was isolated from PDLSCs using the RNAios Plus reagent (Takara) and reverse-transcribed to cDNA using the PrimeScript TM RT kit with gDNA Eraser (Takara). cDNA was amplified by polymerase chain reaction under the following thermocycler conditions: 95 °C for 2 minutes, followed by 95 °C for 15 seconds, and 60 °C for 1 minutes, for 40 cycles. The relative expression levels of the target gene were normalized to GAPDH levels and determined by the $2^{-\Delta\Delta Ct}$ method. The primer sequences are shown in Table 1.

Western blot analysis

After washing PDLSCs three times with ice-cold PBS, the cells were lysed with RIPA reagent containing 1% PMSF and 1% phosphatase inhibitor cocktail. After centrifugation at 14000 × *g* for 15 minutes, the total protein concentration was measured using a BCA protein assay kit (Solarbio). Proteins were separated based on molecular weight using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 5% milk for 1 hour and then incubated with primary antibodies overnight at 4 °C. The membranes were then washed three times with tris-buffered saline and tween-20 and then incubated with secondary antibodies for 1 hour at room temperature. Protein strips were chromogenized using Pierce ECL Western Blotting substrate (Thermo Fisher Scientific), and densitometry was performed on each strip using ImageJ software (National Institutes of Health). The following primary antibodies were used: P21 (#2947, CST), phospho-Rb (Ser807/811) (#8516, CST), NANOG (#4903, CST), OCT-4A (#2840, CST), C-MYC (#5605, CST), and GAPDH (#8884, CST).

Statistical analysis

In all of the experiments, which were performed at least three times independently, the data were expressed as mean ± SD. A one-way analysis of variance was performed to compare results between three or more groups, and a student's *t*-test was used to compare results between two groups. Statistical analysis was performed using Prism (GraphPad Prism v7.02), and statistical significance was set at a threshold of $P < 0.05$.

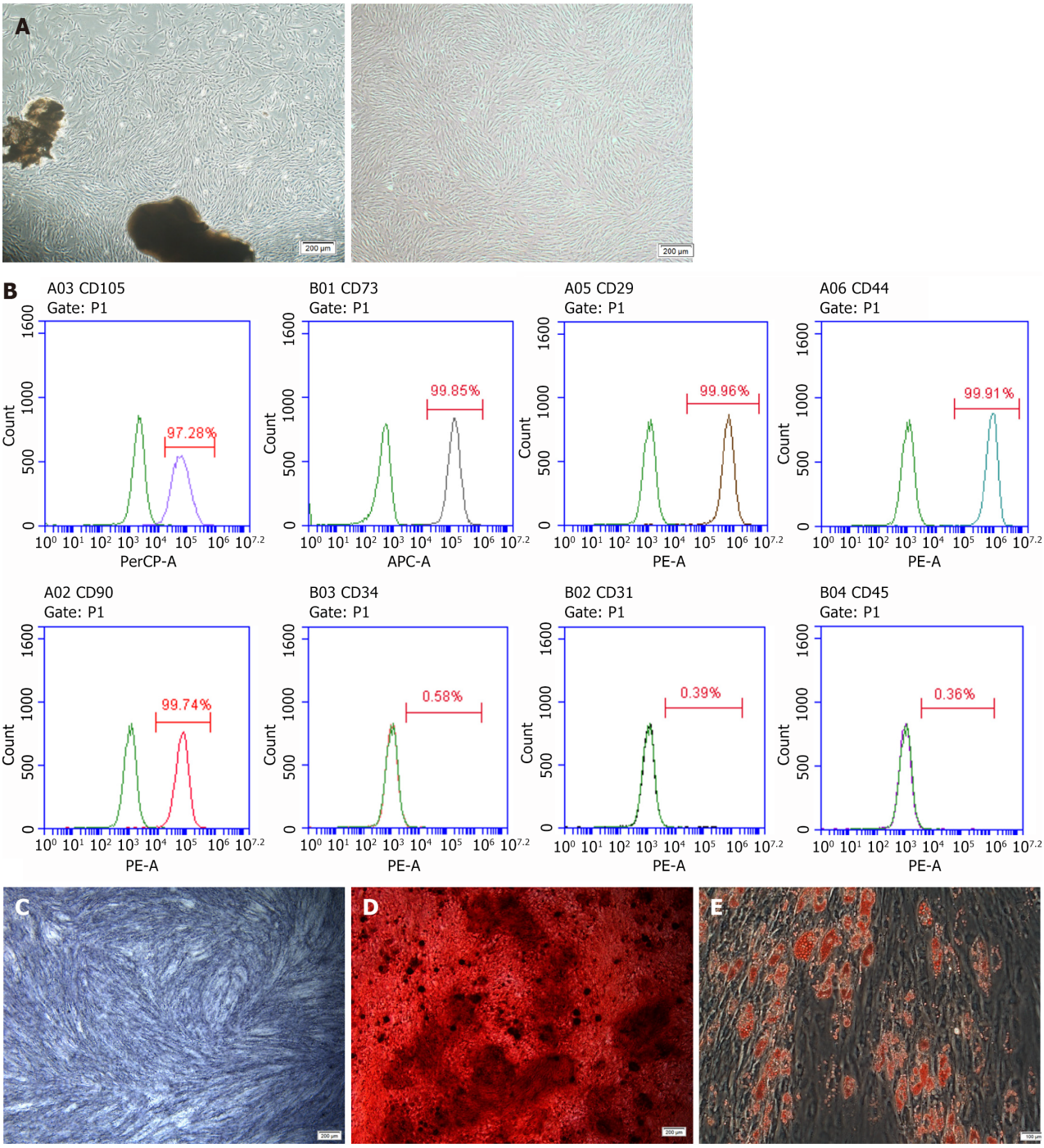


Figure 1 Characterization of periodontal ligament stem cells. A: Periodontal ligament stem cells (PDLSCs) were cultured (scale bar = 200 μm); B: PDLSCs expressed CD105, CD73, CD29, CD44 and CD90 positively and expressed CD34, CD31 and CD45 negatively; C: Alkaline phosphatase staining of PDLSCs; D: Alizarin Red staining of PDLSCs; E: Oil Red O staining of PDLSCs. Data are presented as mean ± SD, *n* = 3.

RESULTS

Characterization of PDLCs

PDLC evaluation using an inverted microscope revealed that the cells had long spindle shapes with abundant cytoplasm and well-defined cell margins (Figure 1A). Positive expression of the MSC-specific surface markers CD105, CD73, CD29, CD44, and CD90 was observed. Meanwhile, expression of the hematopoietic stem cell-specific molecule CD34, the platelet endothelial cell-specific molecule CD31, and the leukocyte-specific molecule CD45 was negative (Figure 1B). After 7 days of osteogenesis induction, the PDLSCs were uniformly and consistently stained dark blue upon ALP staining analysis (Figure 1C). After 21 days of osteogenic induction, PDLSCs were visible as a large number of reddish-brown mineralized nodules following alizarin red staining (Figure 1D). After 14 days of adipogenic induction, a large number of grape-vesicle-like lipid droplets were visible in the PDLSCs following oil-red staining (Figure 1E). Taken together, these results reveal that the cells used in this study possess multi-directional differentiation potential.

H_2O_2-induced oxidative stress in PDLCs

Microscopy observations revealed that the higher the concentration of H_2O_2, the lower the number of PDLSCs that adhered to the wall. Furthermore, the cell volume increased, and the morphology changed from the original short spindle shape to an elongated fibroblast-like appearance (Figure 2A). Notably, the apoptosis rate was proportional to the H_2O_2 concentration (Figure 2B). Furthermore, the ROS assay results showed that the number of ROS-positive PDLSCs increased with increasing H_2O_2 concentrations in a concentration-dependent manner (Figure 2C and D). In summary, we successfully established a model of H_2O_2-induced oxidative stress in PDLSCs.

H_2O_2 inhibits stemness and promotes senescence in PDLCs

Compared with the control group, the protein expression levels of the stemness-related proteins C-MYC, OCT-4, and NANOG were significantly decreased in the H_2O_2 group in a concentration-dependent manner (Figure 3A). Furthermore, the mRNA expression levels of these genes were consistent with the protein expression results (Figure 3B). This indicates that H_2O_2 inhibits the ability of PDLSCs to maintain their stemness.

To investigate the effect of H_2O_2 on the osteogenic differentiation potential of PDLSCs, we examined the ALP activity and mineralized nodules. Compared to the control group, the ALP staining intensity in PDLSCs decreased with increasing H_2O_2 concentration, and ALP activity followed a similar trend (Figure 3C). After 21 days of osteogenic induction, alizarin red staining and quantitative analysis showed that the PDLSCs accumulated fewer mineralized nodules with increasing H_2O_2 concentrations than the control group (Figure 3D). These results suggest that H_2O_2 has an inhibitory effect on PDLSCs osteogenic differentiation. Senescence-associated β-galactosidase (SA β-gal) staining showed that the number of SA β-gal-positive cells was significantly higher in the 200 μM H_2O_2-treated PDLSCs group than in the control group (Figure 3E). Consistent with this phenotype, p-RB protein expression decreased and that of p21, a molecular marker of cellular senescence, increased significantly in PDLSCs with increasing H_2O_2 concentrations (Figure 3F). These results suggest that H_2O_2 accelerates senescence in PDLSCs. Based on the above results, we selected 200 μM as the most suitable H_2O_2 concentration for subsequent experiments.

MT improves proliferation and reduces oxidative stress in H_2O_2-induced PDLCs

Microscopically, PDLSCs in the MT-treated group were in a good growth state, with a higher percentage of spindle-shaped cells compared to the H_2O_2 group (Figure 4A). The 3-day growth curves revealed that MT significantly enhanced the proliferation of H_2O_2-induced PDLSCs in a concentration-dependent manner (Figure 4A). Additionally, MT significantly reduced the proportion of H_2O_2-induced PDLSCs in the G0/G1 phase and increased the proportion of cells in the G2/M phase (Figure 4B). The ROS assay results showed that the number of H_2O_2-induced ROS-positive PDLSCs decreased significantly with increasing MT concentrations (Figure 4C and D). These findings suggest that MT effectively attenuates H_2O_2-induced oxidative stress in PDLSCs.

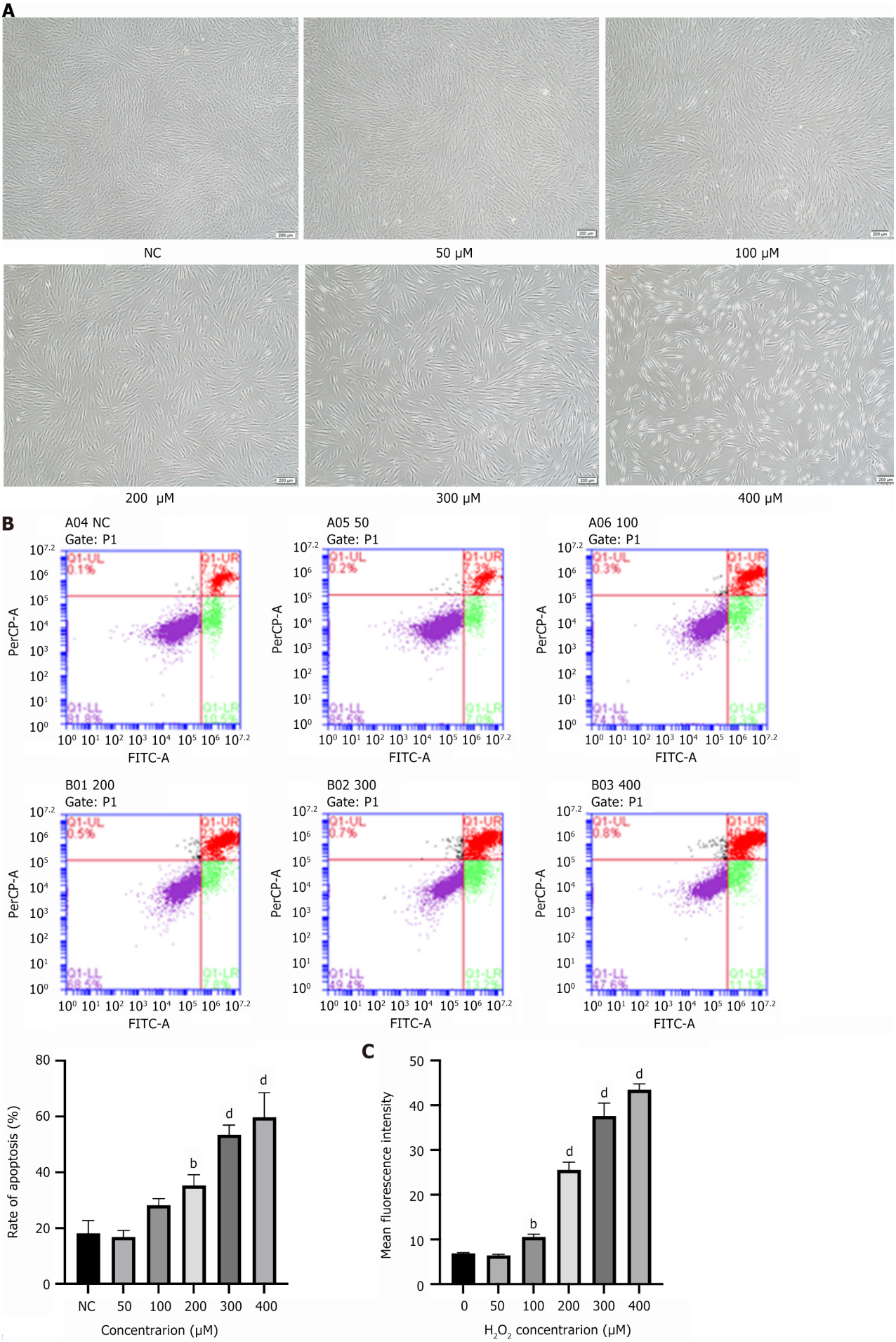
MT increases stemness and delays senescence in H_2O_2-induced PDLCs

MT significantly increased the protein expression levels of the stemness-related proteins C-MYC, OCT-4, and NANOG in H_2O_2-induced PDLSCs in a concentration-dependent manner, compared to the corresponding levels in the H_2O_2 group (Figure 5A). This observation suggests that MT enhances the ability of PDLSCs to maintain their stemness. To investigate the effect of MT on the osteogenic differentiation potential of H_2O_2-induced PDLSCs, we measured ALP activity in mineralized nodules. Compared to the H_2O_2 group, the intensity of ALP staining in PDLSCs became darker with increasing MT concentrations, and ALP activity followed a similar trend (Figure 5B). After 21 days of osteogenic induction, alizarin red staining and quantitative analysis showed that the PDLSCs accumulated more mineralized nodules with increasing MT concentrations (Figure 5C). These results suggest that MT enhances the osteogenic differentiation potential of H_2O_2-induced PDLSCs. Furthermore, SA β-gal staining of H_2O_2-induced PDLSCs showed that MT significantly decreased the number of SA β-gal-positive cells (Figure 5D). Consistent with this phenotype, the protein expression level of p-RB increased and that of p21 decreased significantly with increasing MT concentrations (Figure 5E). These results suggest that MT delays the senescence of PDLSCs.

Through a YAP-mediated pathway, MT enhances stemness and reduces senescence in H_2O_2-induced PDLCs

Compared with the control group, YAP mRNA expression and protein levels were decreased in the PDLSCs of the H_2O_2 group (Figure 6A and B), which indicates that oxidative stress inhibits YAP expression in PDLSCs. Furthermore, MT significantly elevated YAP protein levels in H_2O_2-induced PDLSCs in a concentration-dependent manner (Figure 6C). This finding suggests that YAP mediates the role of MT in enhancing stemness and reducing senescence in H_2O_2-induced PDLSCs. To further elucidate the underlying mechanism, verteporfin was used to antagonize YAP expression in MT-upregulated PDLSCs (Figure 6D). We also found that verteporfin significantly reduced the protein expression levels of C-MYC, OCT-4, and NANOG in MT-upregulated PDLSCs (Figure 6E). This observation suggests that YAP-mediated MT enhances the ability of H_2O_2-induced PDLSCs to maintain stemness.

ALP activity and mineralized nodules were examined to determine if YAP mediates MT's osteogenic differentiation potential of H_2O_2-induced PDLSCs. The results showed that verteporfin decreased the intensity of ALP staining in MT-treated PDLSCs, with ALP activity following a similar trend (Figure 6F). After 21 days of osteogenic induction, alizarin red staining showed that verteporfin decreased the MT-induced accumulation of mineralized nodules in PDLSCs (Figure 6G). These results suggest that through a YAP-mediated pathway, MT enhances the osteogenic differentiation potential of H_2O_2-induced PDLSCs. Furthermore, SA-gal staining revealed that verteporfin significantly increased the number of SA β-gal-positive, MT-downregulated PDLSCs (Figure 6H). Consistent with this phenotype, verteporfin decreased the protein expression level of p-RB in MT-upregulated PDLSCs and elevated the expression level of p21 in


A
NC
50 μM
100 μM
200 μM
300 μM
400 μM
B
A04 NC
Gate: P1
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Gate: P1
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Mean fluorescence intensity
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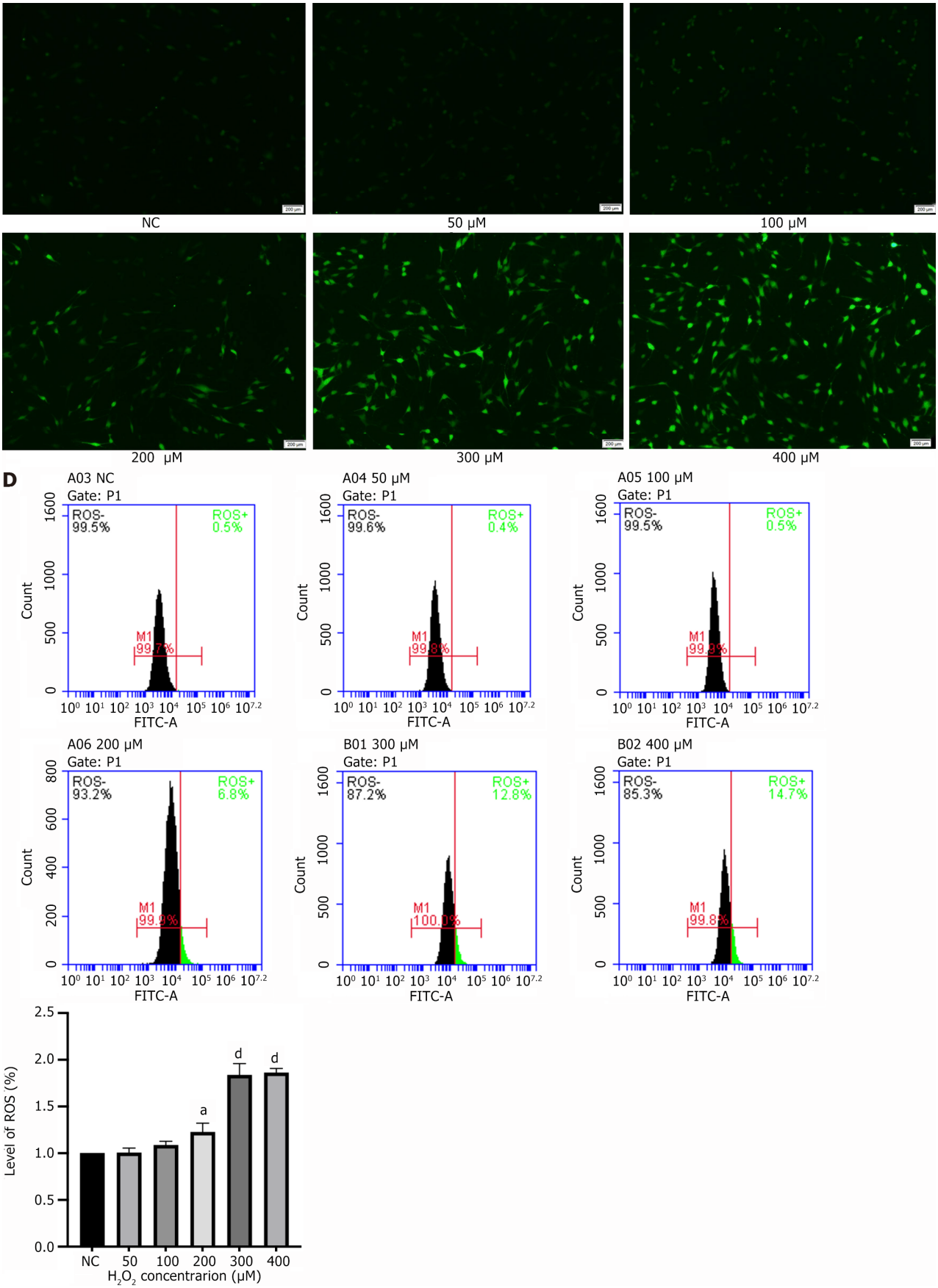
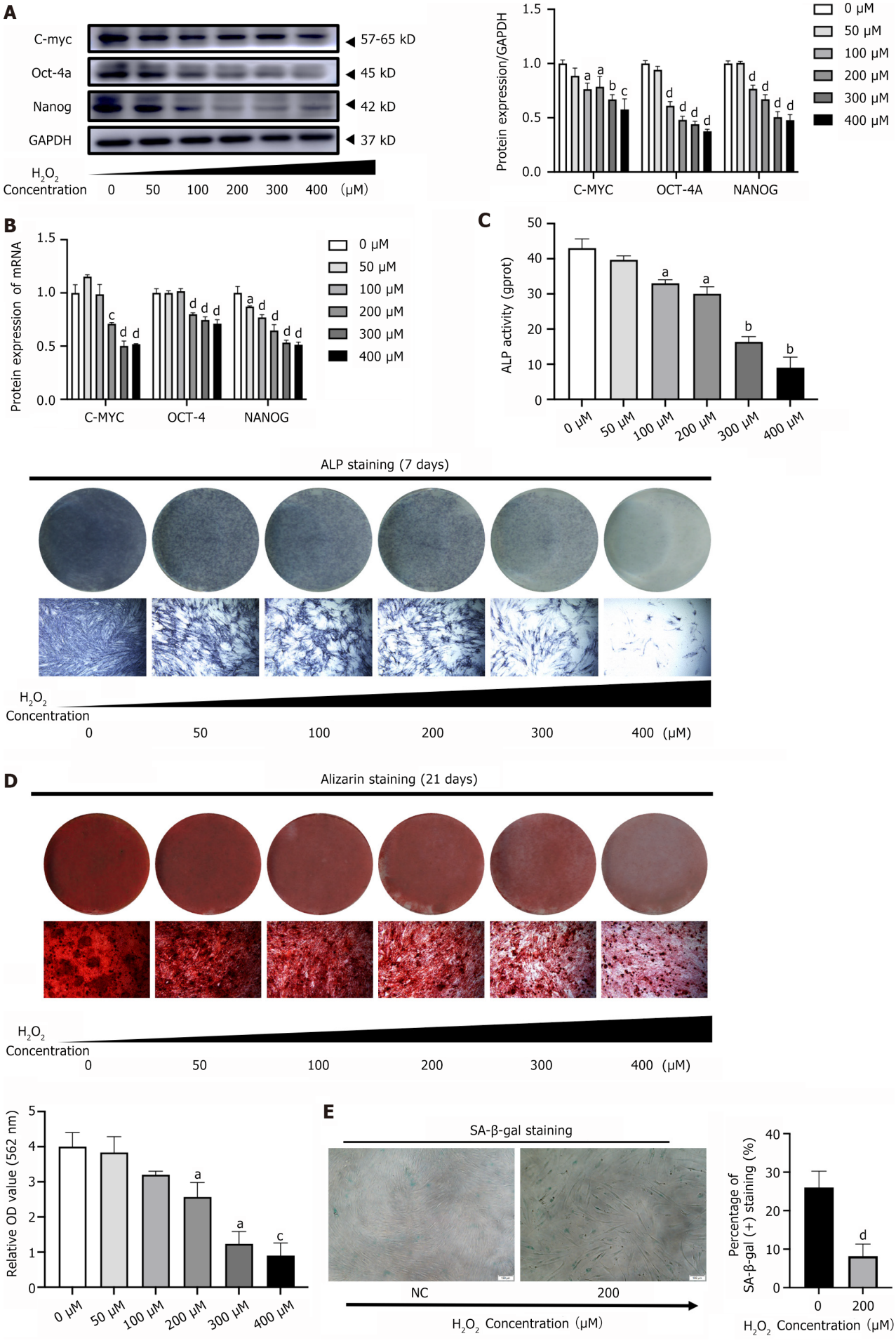


Figure 2 Hydrogen peroxide-induced oxidative stress in periodontal ligament stem cells. A: Hydrogen peroxide (H_2O_2) affected the growth state and cell morphology of periodontal ligament stem cells (PDLSCs); B: H_2O_2 promoted apoptosis of PDLSCs; C: H_2O_2 increased the reactive oxygen species fluorescence intensity of PDLSCs; D: H_2O_2 increased the proportion of reactive oxygen species-positive cells in PDLSCs. Data are presented as mean ± SD, $n = 3$. [a] $P < 0.05$; [b]$P < 0.01$; [d]$P < 0.0001$. NC: Negative control; ROS: Reactive oxygen species; H_2O_2: Hydrogen peroxide.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C-myc
Oct-4a
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GAPDH
57-65 kD
45 kD
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H2O2 Concentration 0 50 100 200 300 400 (μM)
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C-MYC
OCT-4A
NANOG
0 μM
50 μM
100 μM
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300 μM
400 μM
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C-MYC
OCT-4
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ALP activity (gprot)
ALP staining (7 days)
H2O2 Concentration 0 50 100 200 300 400 (μM)
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H2O2 Concentration (μM)

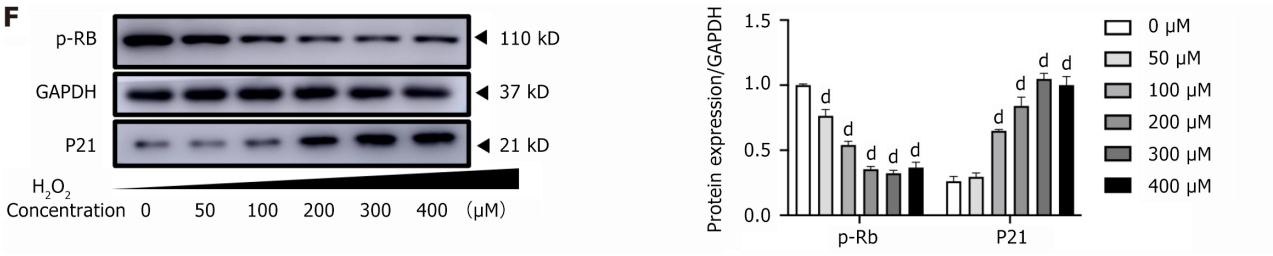


Figure 3 Hydrogen peroxide inhibited stemness and promoted senescence in periodontal ligament stem cells. A: Hydrogen peroxide (H_2O_2) inhibited the expression level of stemness-related proteins C-MYC, OCT-4, and NANOG in periodontal ligament stem cells (PDLSCs); B: H_2O_2 suppressed the expression levels of stemness-related genes C-MYC, OCT-4, and NANOG in PDLSCs; C: Alkaline phosphatase staining and alkaline phosphatase activity showed that the osteogenic differentiation ability of the H_2O_2-induced PDLSCs was weaker than that of the control group; D: Alizarin Red staining and quantitative analysis showed that the osteogenic differentiation ability of the H_2O_2-induced PDLSCs was weaker than that of the control group; E: Senescence-associated β-galactosidase staining indicated significantly more senescence-associated β-galactosidase-positive cells in H_2O_2-induced PDLSCs compared with the control group; F: Western blot results showed that H_2O_2 inhibited the expression of p-RB but increased the expression of p21 in PDLSCs. Data are presented as mean ± SD, $n = 3$. $^aP < 0.05$; $^bP < 0.01$; $^cP < 0.001$; $^dP < 0.0001$. NC: Negative control; H_2O_2: Hydrogen peroxide; ALP: Alkaline phosphatase; SA β-gal: Senescence-associated β-galactosidase.

MT-downregulated PDLSCs (Figure 6I). These results also suggest that MT delays the senescence of H_2O_2-induced PDLSCs through a YAP-mediated mechanism.

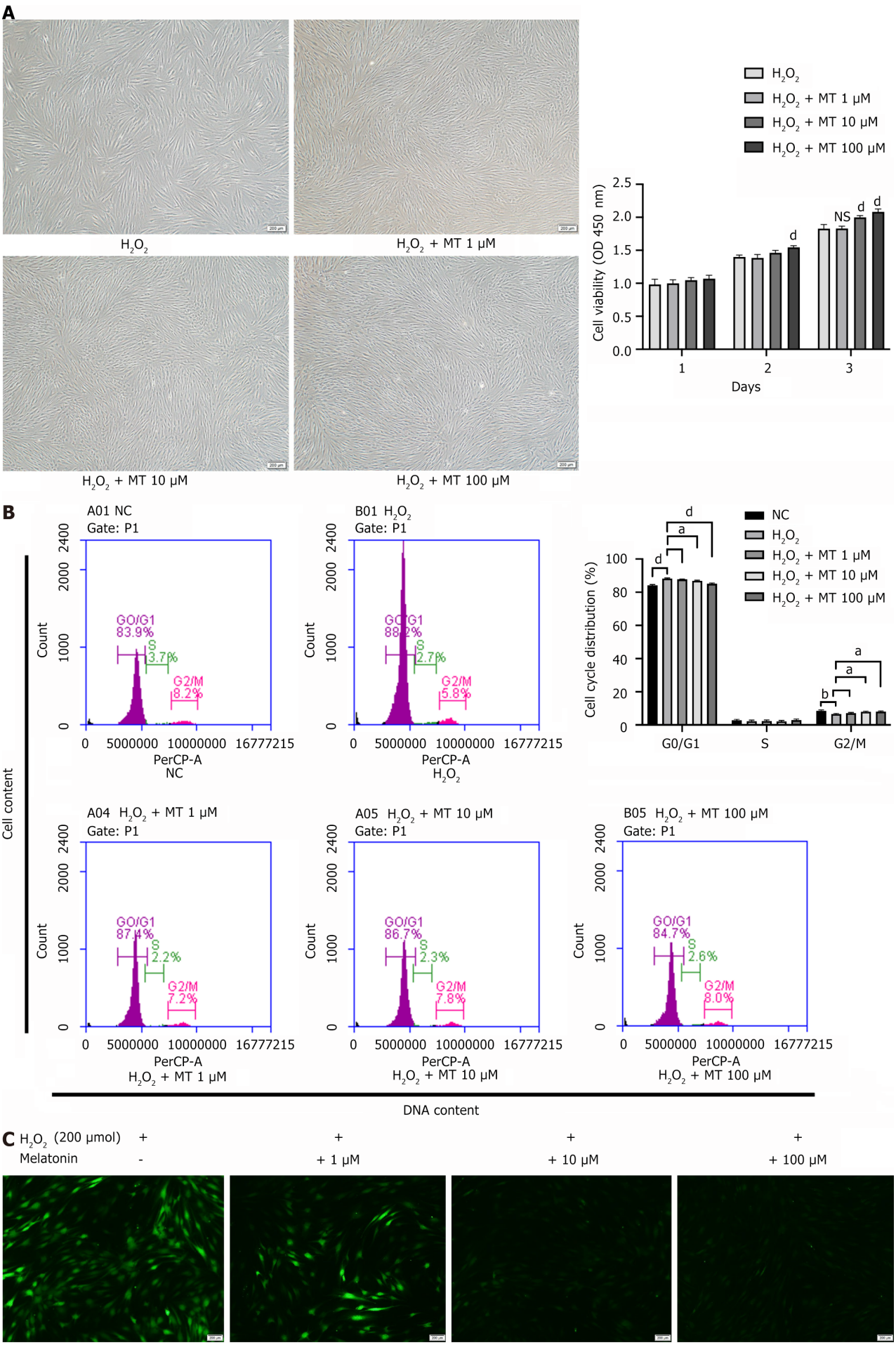
DISCUSSION

Previous studies have shown that PDLSCs have self-renewal, multi-directional differentiation, and immune regulation abilities, while oxidative stress-induced impaired regenerative capacity can lead to tissue dysfunction and organismal aging[1-3]. Therefore, it is necessary to identify ways to modulate PDLSC dysfunction and promote tissue regeneration. Small-molecule drugs have gradually become a research hotspot for delaying cellular senescence because of their simple production, low cost, and relatively mature understanding[4]. MT, a small-molecule drug, is a powerful free radical scavenger that regulates MSC proliferation and apoptosis[5]. However, its effects and mechanisms on PDLSCs remain unclear. Therefore, the present study investigated the role and mechanisms of MT in regulating the biological characteristics of PDLSCs under oxidative stress.

The incomplete intracellular oxidation of MSCs during *in vitro* expansion generates high levels of ROS, including H_2O_2 and hydroxyl radicals. Although it has been shown that H_2O_2 is a component of cellular signaling pathways and is necessary for the growth, development, and adaptation of the organism[6], long-term exogenous exposure to H_2O_2 induces cellular oxidative stress damage and impairs biological functions, thus leading to senescence[7-9]. In this study, we established an oxidative stress model by observing a gradual increase in PDLSCs-generated ROS with increasing H_2O_2 concentration. Our results showed that under oxidative stress conditions, PDLSCs' morphology gradually changed from a short spindle-like appearance to a slender fibroblast-like appearance. This may be due to the downregulated expression of dynamic actin cytoskeleton gene clusters, which would slow actin renewal[10], along with changes in the number and size of nuclei and organelles, including the mitochondria, endoplasmic reticulum, and lysosomes under oxidative stress conditions[11], resulting in morphological changes in PDLSCs.

Furthermore, the proliferative capacity of PDLSCs was reduced under oxidative stress, whereas apoptosis significantly increased, in agreement with previous findings[8]. Notably, it has been shown that MSCs are highly resistant to injury-induced apoptosis and preferentially activate premature cellular senescence in response to injury[12]. Cellular senescence is followed by increased lysosomal content and enhanced activity of SA β-gal, which is the current gold standard for cellular senescence assays. Notably, our results show a significant increase in the number of SA β-gal-positive PDLSCs under oxidative stress conditions. Additionally, the RB protein is a tumor suppressor protein that regulates cell proliferation, apoptosis, and differentiation and modulates cell biological functions[13]. Cell cycle-dependent protein kinases can phosphorylate RB[14], thereby advancing cell cycle progression[15]. P21 is a negative regulatory protein of the cell cycle that is closely associated with cellular senescence and is involved in regulating the G1/S phase transition[16]. Our results showed that p-RB expression was significantly downregulated and p21 expression was significantly upregulated in PDLSCs under oxidative stress, suggesting that oxidative stress induces senescence. Our results also showed that the stemness and osteogenic differentiation potential of PDLSCs were reduced under oxidative stress, consistent with the results of previous studies[17]. In the future, it will be crucial to identify small-molecule drugs that can counteract the effects of oxidative stress to delay cellular senescence and enhance cell stemness.

There are various types of antioxidants, including glutathione, MT, metformin, and resveratrol[3,8,18,19]. Among these, MT, a powerful endogenous free radical scavenger, has promising anti-aging applications. Our results showed that MT reduced oxidative stress-induced ROS in PDLSCs, indicating that it can scavenge free radicals and reduce intracellular oxidative stress. Moreover, MT restored the normal morphology of PDLSCs, enhanced their proliferation, and relieved the negative cell cycle regulatory effects of oxidative stress. Notably, previous studies have shown that MT enhances the proliferation and migration capacity of adipose-derived MSCs that have been passaged for a long time, thus delaying cellular senescence and maintaining cell stemness[18]. MT has also been shown to delay the senescence of MSCs


A
H2O2
H2O2 + MT 1 μM
H2O2 + MT 10 μM
H2O2 + MT 100 μM
Cell viability (OD 450 nm)
Days
NS
B
A01 NC
B01 H2O2
A04 H2O2 + MT 1 μM
A05 H2O2 + MT 10 μM
B05 H2O2 + MT 100 μM
Gate: P1
GO/G1
S
G2/M
Count
PerCP-A
Cell content
DNA content
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G0/G1
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G2/M
C
H2O2 (200 μmol)
Melatonin
+ 1 μM
+ 10 μM
+ 100 μM

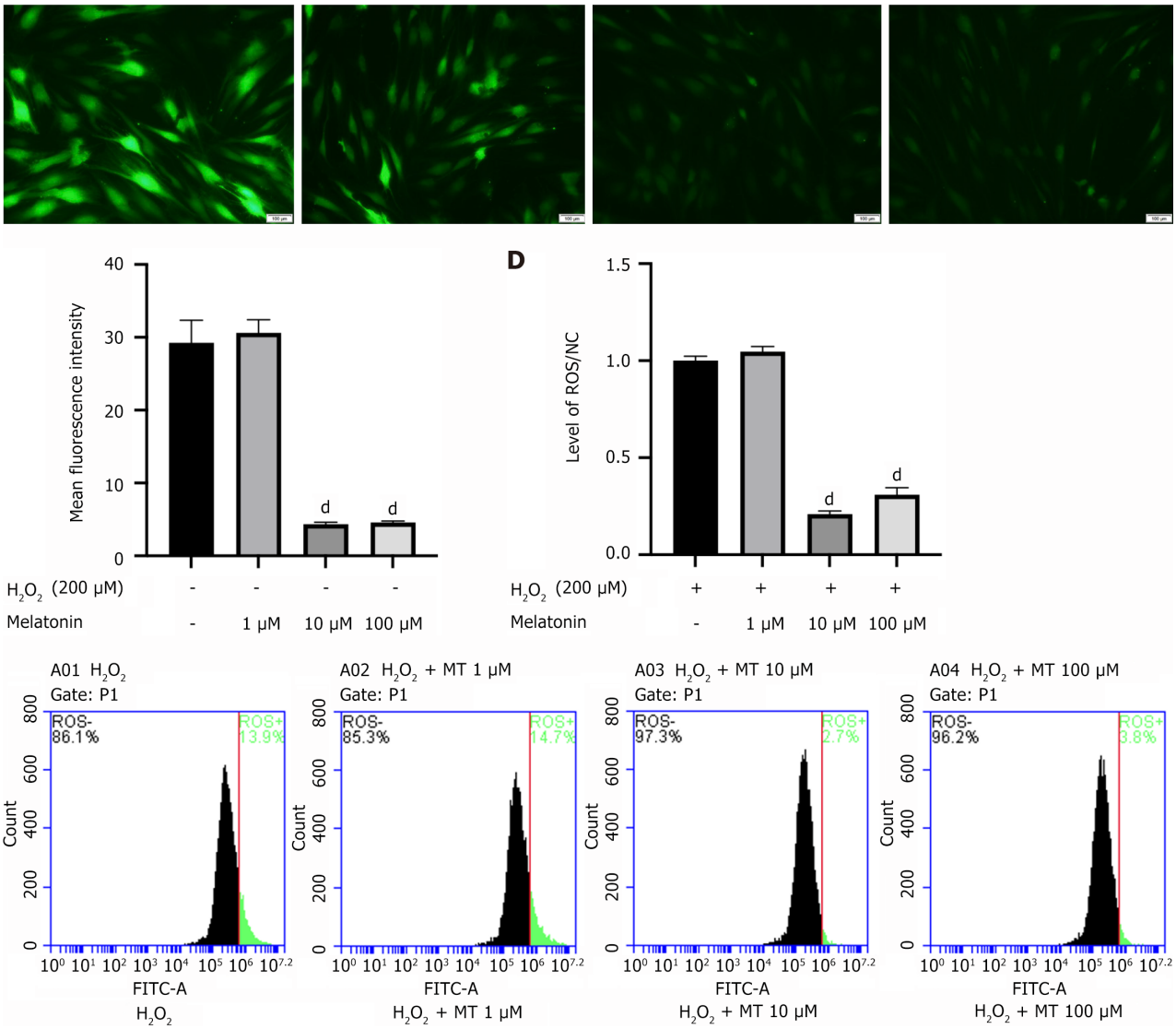
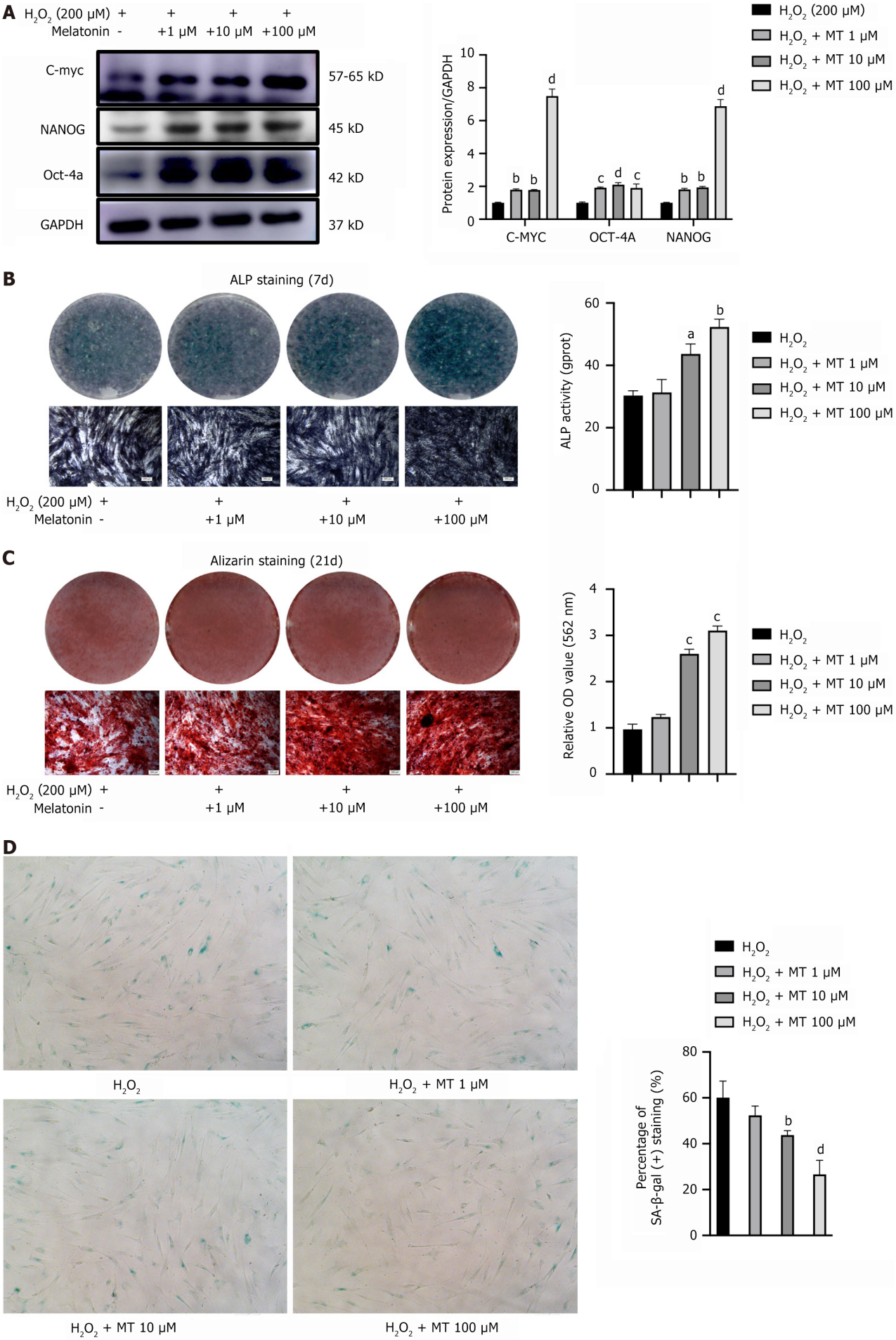


Figure 4 Melatonin improved proliferative capacity and reduced oxidative stress levels in hydrogen peroxide-induced periodontal ligament stem cells. A: Melatonin (MT) restored the growth state and enhanced the proliferative capacity of hydrogen peroxide (H_2O_2)-induced periodontal ligament stem cells (PDLSCs); B: MT reduced the proportion of G0/G1 phase and increased the proportion of G2/M phase in H_2O_2-induced PDLSCs; C: MT decreased the reactive oxygen species fluorescence intensity of H_2O_2-induced PDLSCs; D: MT decreased the proportion of reactive oxygen species-positive cells in H_2O_2-induced PDLSCs. Data are presented as mean ± SD, n = 3. $^aP < 0.05$; $^bP < 0.01$; $^dP < 0.0001$. NS: No significance; H_2O_2: Hydrogen peroxide; MT: Melatonin; NC: Negative control.

in rats with chronic kidney disease and promote cell proliferation and adhesion by maintaining mitochondrial I and IV complex activity[20]. MT has also been found to prevent senescence and restore the impaired osteogenic differentiation potential in bone marrow MSCs in ovariectomized rats by activating the AMP-activated protein kinase-sirtuin 1 signaling pathway through the MT receptor[21]. In addition, MT can also promote osteogenic differentiation of dental pulp stem cells *in vivo*[22]. Our results indicate that MT significantly delays the oxidative stress-induced senescence of PDLSCs and enhances their stemness and osteogenic differentiation potential. This finding is generally consistent with those of prior studies; however, the molecular mechanisms by which MT regulates the biological functions of PDLSCs under oxidative stress remain unclear.

A growing number of studies have shown that the Hippo-YAP signaling pathway is associated with cellular oxidative stress. YAP1 protects cardiomyocytes from ROS-induced cell death and mediates cellular defense against oxidative stress [23]. Furthermore, in neurofibromatosis type 2 tumor cells, interference with YAP/TAZ expression has been found to enhance mitochondrial respiratory function and lead to ROS accumulation, which causes oxidative stress and cell death [24]. In the present study, we found that YAP expression in PDLSCs was significantly downregulated under oxidative stress conditions and significantly upregulated by MT. This finding is consistent with the results of previous studies and suggests that YAP signaling regulates oxidative stress-induced changes in PDLSCs. Verteporfin, an inhibitor of TEAD-YAP association[25], mimics a YAP knockdown phenotype and has been used to inhibit YAP in related studies[26,27]. Our results showed that MT-upregulated YAP levels were suppressed after YAP antagonism by verteporfin. Furthermore, previous studies have shown that YAP promotes the proliferation of PDLSCs and delays cellular senescence [28,29]. Our results showed that verteporfin significantly inhibited PDLSCs senescence under oxidative stress conditions,


A
H2O2 (200 μM) + + + +
Melatonin - +1 μM +10 μM +100 μM
C-myc
NANOG
Oct-4a
GAPDH
57-65 kD
45 kD
42 kD
37 kD
Protein expression/GAPDH
C-MYC
OCT-4A
NANOG
H2O2 (200 μM)
H2O2 + MT 1 μM
H2O2 + MT 10 μM
H2O2 + MT 100 μM
B
ALP staining (7d)
ALP activity (gprot)
C
Alizarin staining (21d)
Relative OD value (562 nm)
D
Percentage of SA-β-gal (+) staining (%)

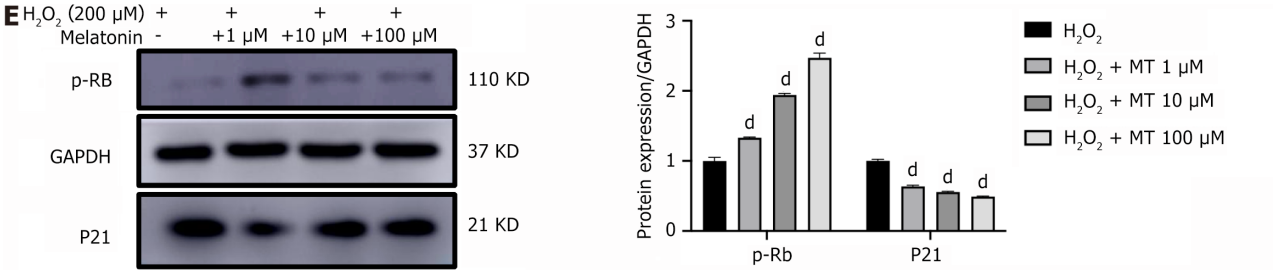
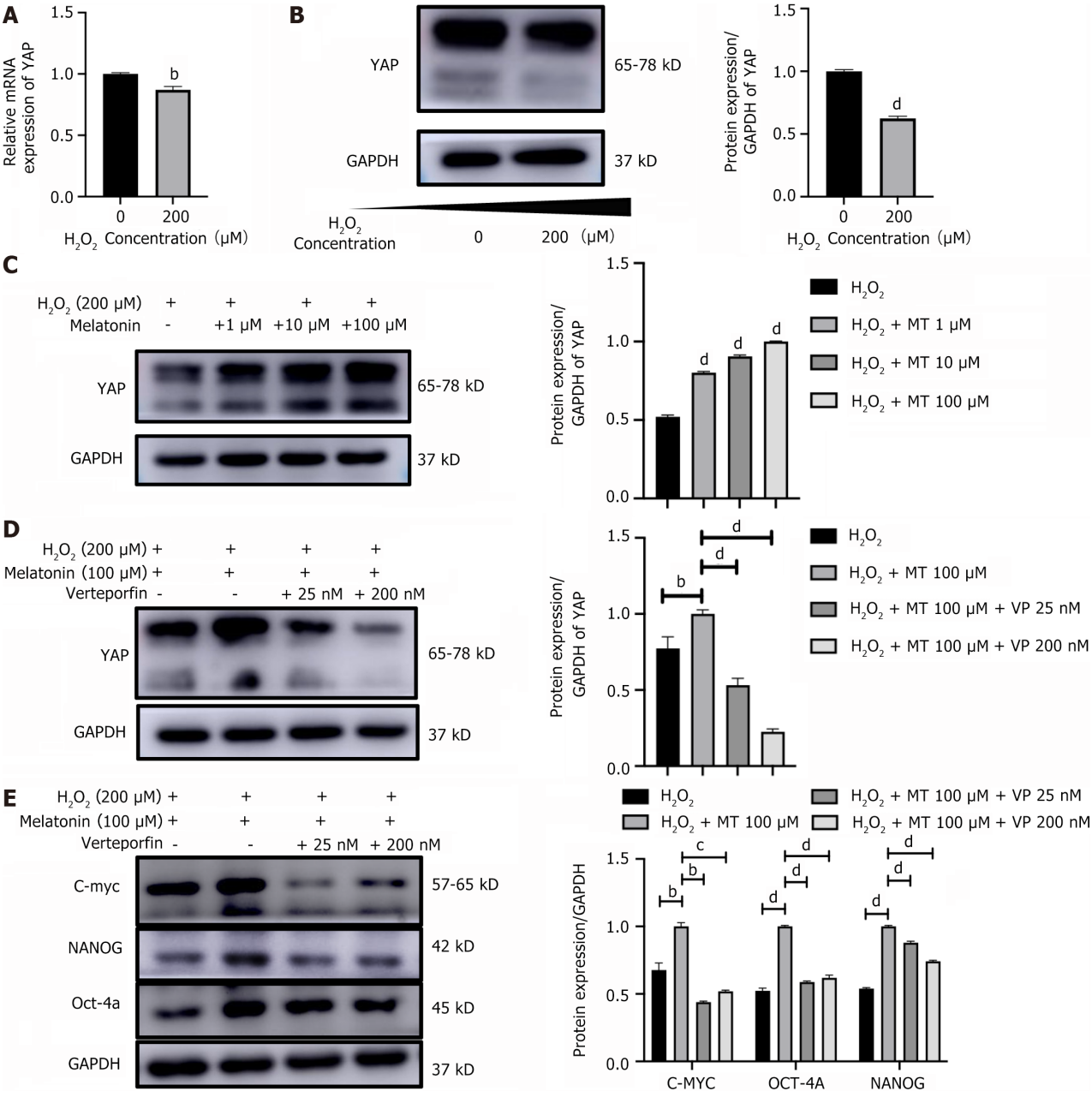


Figure 5 Melatonin increased stemness and delayed senescence in hydrogen peroxide-induced periodontal ligament stem cells. A: Melatonin (MT) restored the expression levels of stemness-related proteins C-MYC, OCT-4, and NANOG in hydrogen peroxide (H_2O_2)-induced periodontal ligament stem cells (PDLSCs); B: Alkaline phosphatase staining and alkaline phosphatase activity showed that MT enhanced the osteogenic differentiation ability of the H_2O_2-induced PDLSCs; C: Alizarin Red staining and quantitative analysis showed that MT enhanced the osteogenic differentiation ability of the H_2O_2-induced PDLSCs; D: Senescence-associated β-galactosidase staining indicated MT decreased senescence-associated β-galactosidase-positive cells in H_2O_2-induced PDLSCs; E: Western blot results showed that MT increased the expression of p-RB but inhibited the expression of p21 in H_2O_2-induced PDLSCs. Data are presented as mean ± SD, n = 3. $^aP < 0.05$; $^bP < 0.01$; $^cP < 0.001$; $^dP < 0.0001$. H_2O_2: Hydrogen peroxide; ALP: Alkaline phosphatase; ARS: Alizarin Red staining; MT: Melatonin; SA β-gal: Senescence-associated β-galactosidase.



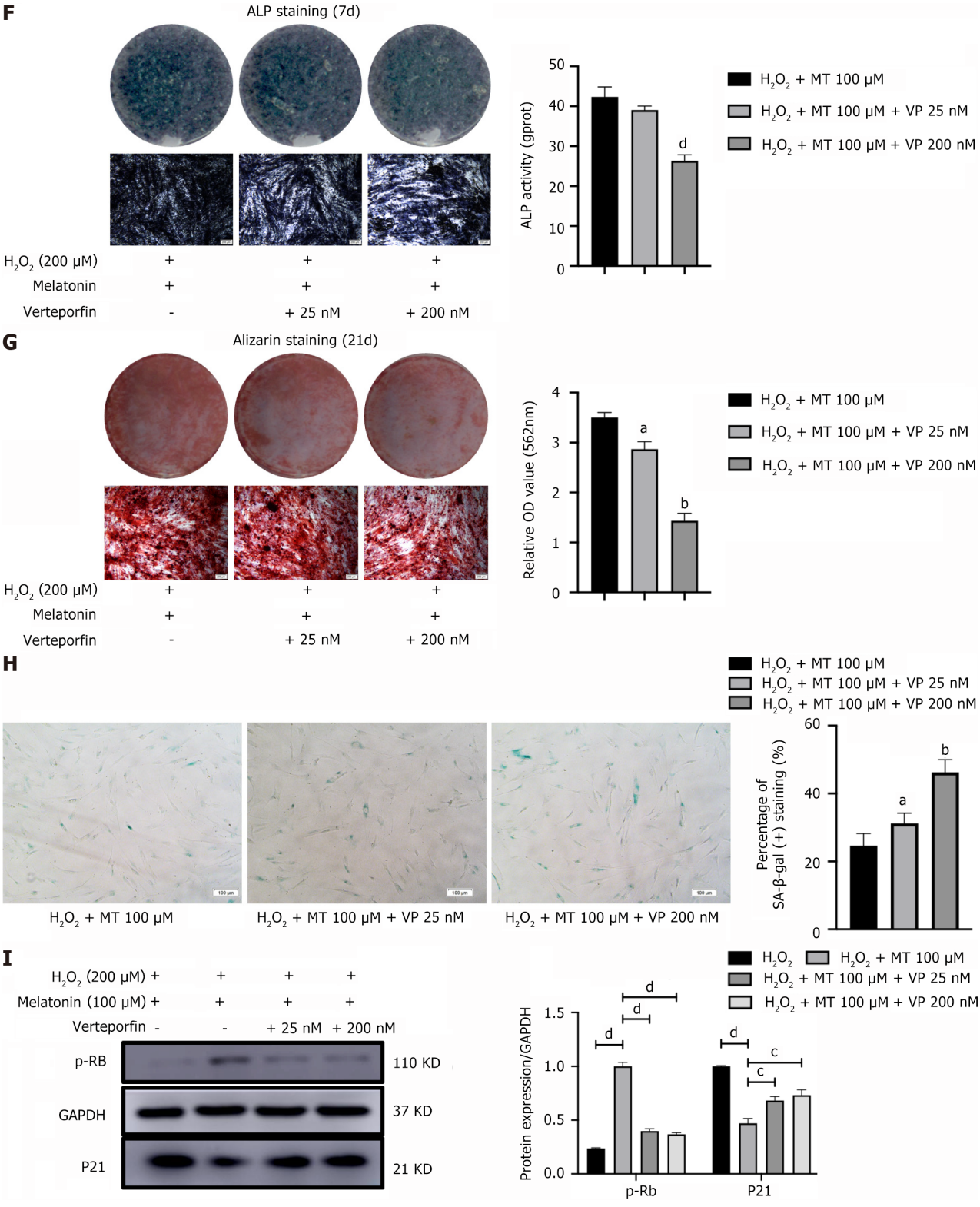


Figure 6 Yes-associated protein mediated melatonin to enhance stemness and reduce senescence in hydrogen peroxide-induced periodontal ligament stem cells. A: Hydrogen peroxide (H_2O_2) reduced the mRNA expression level of Yes-associated protein (YAP) in periodontal ligament stem cells (PDLSCs); B: H_2O_2 reduced the protein expression level of YAP in PDLSCs; C: Melatonin (MT) increased the protein expression level of YAP in H_2O_2-induced PDLSCs; D: Verteporfin reduced the protein expression level of YAP in MT-increased PDLSCs; E: Verteporfin decreased the protein expression levels of stemness-related proteins C-MYC, OCT-4 and NANOG in MT-increased PDLSCs; F: Alkaline phosphatase staining and alkaline phosphatase activity showed that verteporfin reduced the osteogenic differentiation ability in MT-increased PDLSCs; G: Alizarin Red staining and quantitative analysis showed that verteporfin reduced the osteogenic differentiation ability in MT-increased PDLSCs; H: Senescence-associated β-galactosidase staining indicated verteporfin increased senescence-associated β-galactosidase-positive cells in MT-increased PDLSCs; I: Western blot results showed that verteporfin decreased the expression of p-RB but increased the expression of p21 in MT-treated PDLSCs. Data are presented as mean ± SD, $n = 3$. $^aP < 0.05$; $^bP < 0.01$; $^cP < 0.001$; $^dP < 0.0001$. H_2O_2: Hydrogen peroxide; MT: Melatonin; YAP: Yes-associated protein; VP: Verteporfin; ALP: Alkaline phosphatase; ARS: Alizarin Red staining.

which confirms that YAP mediates the delaying effect of MT. Another study found that the maintenance of stemness in senescent PDLSCs was closely related to the Hippo-YAP signaling pathway[30]. Verteporfin also significantly inhibited the effect of MT on the stemness and osteogenic differentiation potential of PDLSCs, which further suggests that YAP mediates the effect of MT on these processes. In conclusion, we discovered for the first time that YAP regulates MT in the biological functions of PDLSCs under oxidative stress, providing a key target for improving MSC function and regenerative medicine efficiency.

Although the use of MSCs in preclinical and clinical studies for the treatment of various diseases has provided promising results, the oxidative stress microenvironment inhibits the efficacy of MSC-mediated tissue regeneration by suppressing endogenous and exogenous MSC physiological functions. This is currently a major challenge that limits the clinical application of MSCs. Our findings provide new insights into the biological functions of MT-regulated oxidative stress-induced PDLSCs and elucidate the mechanisms of YAP-mediated MT activity. These findings not only strengthen our understanding of the role of MT in processes related to cellular oxidative stress but also present potential targets for developing new therapeutic strategies for promoting tissue regeneration.

CONCLUSION

While the use of MSCs in preclinical and clinical studies for the treatment of various diseases has provided promising results, the microenvironment of oxidative stress inhibits the efficacy of MSC-mediated tissue regeneration by suppressing endogenous and exogenous MSC physiological functions and transmigration, which currently remains a major challenge that limits the clinical application of MSCs. Our findings provide new insights into the biological functions of MT-regulated oxidative stress-induced PDLSCs and elucidate the potential mechanisms by which YAP-mediated MT acts. These findings not only strengthen our comprehension of the role of MT in the process of cellular oxidative stress, but also present potential targets to develop new therapeutic strategies for promoting tissue regeneration.

FOOTNOTES

Author contributions: Gu K and Feng XM contributed equally to the manuscript, they are the co-first authors of this manuscript; Feng XM and Wen Y conceived and designed the research; Gu K, Sun SQ, and Hao XY performed the research and acquired the data; Gu K assembled the figures. All authors contributed to the analysis and interpretation of the data and were involved in drafting and revising the manuscript. All authors have read and approved the final manuscript.

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