Original Research

Rutin promotes osteogenic differentiation of periodontal ligament stem cells through the GPR30-mediated PI3K/AKT/mTOR signaling pathway

Bin Zhao[1,2], Yixuan Xiong[1,2], Yunpeng Zhang[1,2,3], Linglu Jia[1,2], Wenjing Zhang[1,2] and Xin Xu[1,2]

[1]School of Stomatology, Shandong University, Jinan 250012, P.R. China; [2]Shandong Provincial Key Laboratory of Oral Tissue Regeneration, Jinan 250012, P.R. China; [3]Department of Oral Implantology, the Affiliated Stomatology Hospital of Kunming Medical University, Kunming 100191, P.R. China

Corresponding author: Xin Xu. Email: xinxu@sdu.edu.cn

Impact statement

In our study, the effects and mechanisms of rutin on the osteogenic differentiation and proliferation of PDLSCs were investigated. Our findings might provide basic knowledge and guidance to understand and use rutin in the bioengineering of the periodontal tissues and regeneration of bones. The following is a short description of the main findings: rutin promotes the osteogenic differentiation and proliferation of PDLSCs; PI3K/AKT/mTOR signal pathway mediates the effects of rutin on PDLSCs; rutin activates PI3K/AKT/mTOR signal pathway via GPR30.

Abstract

Rutin is one of the flavonoids found in fruits and vegetables. Recent reports have revealed that rutin is a major player in proliferation and bone development. However, data on how rutin regulates the proliferation of periodontal ligament stem cells (PDLSCs), as well as the differentiation of osteogenic cells are scanty. Here, our findings showed that rutin enhanced PDLSCs proliferation, increased ALP activity, and matrix mineralization. Moreover, rutin significantly promoted the expression of osteogenic genes and elevated phosphorylated AKT and mTOR. Treatment with LY294002 reversed these effects by inhibiting PI3K. We also found that the expression levels of GPR30 were increased by rutin. Interestingly, this upregulation was not altered after the addition of LY294002. In addition, G15, a selective antagonist of GPR30, could reduce the beneficial effects induced by rutin and interfere with the modulation of PI3K/AKT/mTOR signal transduction. Collectively, our findings revealed that rutin increased proliferation and osteogenic differentiation of PDLSCs through GPR30-mediated PI3K/AKT/mTOR signal transduction. Therefore, it could be deduced that rutin as a certain flavonoid possesses therapeutic value for periodontal bone regeneration and tissue engineering.

Keywords: Rutin, periodontal ligament stem cells, proliferation, osteogenic differentiation, GPR30, PI3K/AKT/mTOR signaling pathway

***Experimental Biology and Medicine* 2020; 245: 552–561. DOI: 10.1177/1535370220903463**

Introduction

Local stimulations of the periodontal supporting tissues are known to cause chronic inflammation which triggers periodontitis, a condition that induces damage to the periodontal-supporting tissues.[1] Regeneration of lost periodontal tissue is the ideal outcome and ultimate goal of periodontal therapy.[2] In recent years, periodontal tissue engineering has become a mainstay treatment of periodontal tissue regeneration, which makes the functional periodontal tissue and bone tissue regenerated and reconstructed. The periodontal ligament is rich in periodontal ligament stem cells (PDLSCs), which are classified as undifferentiated mesenchymal stem cells (MSCs).[3] These stem cells exhibit self-renewal potential and can differentiate in a multidirectional manner.[4] The prevailing evidence suggested that PDLSCs could repair periodontal tissue defects and maintain periodontal dynamic balance and regulate periodontal tissue regeneration.[5] It was proved that PDLSCs was one of the most promising cell type which can promote the regeneration of damaged periodontal tissue.[6] When the periodontal tissue is damaged, stem cells of the periodontal ligament differentiate and

proliferate continuously, secrete growth factors, improve local micro-environment, and regenerate damaged tissue.[7]

Flavonoids for a class of polyphenolic compounds were the most abundant in the human diet, which is widely found in fruits, vegetables, and beans. In a study investigating the bone health in postmenopausal women after dietary intake of flavonoids, it was confirmed that bone mineral density and resorption were related to the intake of flavonoids.[8] Moreover, they are thought to increase osteoblast activity.[9] Flavonoids are widely distributed in nature, and more than 5000 flavonoids have been found.[10] Rutin, as one of the common flavonoids in fruits and vegetables, has many cellular functions e.g. anti-inflammatory, anti-tumor, anti-virus, and so on.[11] A previous study indicated that rutin is a therapeutically important flavonoid. According to the study, it can induce proliferation while having minimal influence on the distribution of the cell cycle. Moreover, it also possesses bone tissue formation functions.[12] Rutin was also found to stimulate proliferation and osteoblast differentiation in bone marrow MSCs.[13] However, following a literature review, we found that only a few studies have explored the effects of rutin on the osteogenic differentiation and proliferation of PDLSCs as well as associated mechanisms.

An essential pathway that modulates many physiological and pathological processes is the PI3K/AKT/mTOR, which has been implicated in cell differentiation, proliferation, metabolism, and survival.[14] Rutin is a natural flavonoid, when exert its function, related to the PI3K/AKT/mTOR signaling pathway. Previously, it was showed that, in A549 human lung carcinoma cells, rutin modulated COX-2, PGE2, and MMP-9 through PI3K/AKT signaling and ERK pathways.[15] Another study showed that rutin protects PC12 cells by triggering the activation of the PI3K/AKT/mTOR pathway following neurotoxicity induced by nitroprusside.[16] Other studies have found that rutin could be used to alleviate the apoptosis of Leydig cells and oxidative damage induced by hydrogen peroxide by triggering PI3K/AKT signaling pathway, which provided a novel strategy for reducing oxidative stress.[17] However, whether PI3K/AKT/mTOR modulates the osteogenic differentiation and proliferation of PDLSCs induced by rutin remains unclear. Previously, some researchers reported that estrogen receptors interact with some flavonoids and phytochemicals.[18,19]

Rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rhamnoglucoside) has a planar double benzene ring structure similar to endogenous estrogen 17-β-estradiol (E2). This structure can bind to the estrogen receptor occupied by E2, and thus play an estrogen-like role.[20] Moreover, the G protein-coupled receptor 30 (GPR30) is a G protein-coupled receptor that binds estrogen and participates in the transmission of classical signaling pathways.[21,22] As an estrogen receptor, GPR30 can transduce rapid effects of E2. Reports indicate that GPR30 confers protection on neurons and endothelial cells.[23,24] In addition, GPR30 is closely associated with the formation and metabolism of bones.[25,26]

In the present study, the effects of rutin on the osteogenic differentiation and proliferation of PDLSCs were investigated together with the mechanisms involved. Our findings might provide a basic knowledge and guidance to understand and use rutin in the bioengineering of periodontal tissues and regeneration of bones.

Materials and methods

PDLSCs isolation

The preparation and culture of PDLSCs were performed according to a previous study.[4] All experimental protocols and procedures were in line with the guideline of the Medical Ethical Committee of Shandong University. In brief, human premolars designed for orthodontic purposes were utilized in cell extraction procedures. The premolars were collected in precooled α-MEM (Gibco, USA) with 400 mg/mL streptomycin (Beyotime, China) and 400 U/mL penicillin (Beyotime, China), which were immediately transported to the laboratory. Next, a sharp scalpel was used to scrap the periodontal tissue of the middle one-third of the tooth root after washing three times with PBS, and then cut into sections (1 mm × 1 mm × 1 mm) under sterile conditions. Then, after mincing the PDL tissues blocks, they were incubated with medium containing the mixed digestive liquid of dispase (4 mg/mL, Sigma) and collagenase type I (3 mg/mL, Sigma) at 37°C for 60 min. After being subjected to a 70 μm strainer, single cells in suspension were obtained. The obtained cells were seeded into 25 cm^2 flasks, followed by culturing in 5% CO_2 at 37°C in α-minimal essential medium (α-MEM, Gibco) containing streptomycin (100 mg/mL) (Beyotime, China), penicillin (100 U/mL) (Beyotime, China), and 10% fetal bovine serum (Gibco). Media were replaced every three days. Cells were passaged to 3–5 and then used in the subsequent study.

Flow cytometry measurement of cell surface markers

Cell surface markers of the PDLSCs were detected using flow cytometry analysis. Similarly, at passage 3, the immunophenotypes of the cells were determined using flow cytometry analysis following the manufacturer's instructions (BD Bioscience, USA). Once trypsinized, the cells were washed with PBS and then cultured with monoclonal antibodies conjugated with fluorescent dyes in darkness for 20 min at 4°C. Antibodies against MSC-positive cocktail were CD44 PE, CD105 PerCP-Cy5.5, CD90 FITC, and CD73 APC. While those against MSC-negative cocktail were CD34 PE, HLA-DR PE, CD45 PE, CD19 PE, and CD11b PE. The FlowJo software was used to analyze the results.

Multipotent differentiation assays

Cells were incubated in a medium containing α-MEM plus 10% fetal bovine serum (FBS, Gibco), 10 mM β-glycerophosphate, 50 mg/L ascorbic acid, and 10 nM dexamethasone for osteogenic induction. Media were replaced every three days. Four weeks later, cells were subjected to Alizarin Red staining (Sigma) to detect the mineralized nodules. For the adipogenic differentiation assay, the cells were treated with α-MEM induction media comprising 10%

FBS, 0.2 mM indomethacin, 10 mg/L insulin, 2 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine. At the end of four weeks, Oil Red O staining was performed to detect lipid droplets for 30 min after which they were examined under an inverted microscope (Olympus IX73).

Cell proliferation test

The proliferation of the PDLSCs was determined using the cell counting kit-8 (CCK-8) (Dojindo Laboratories, Japan) assay. Briefly, 3000 cells PDLSCs were added into each well of a 96-well plate and then treated with various concentrations of rutin (Solarbio Science & Technology Co., Ltd., Beijing, China) (0, 0.01, 0.1, 1, 10, and 100 μM) for six days. The culture medium was refreshed every day. One hour before each test time point, 10 μL of CCK-8 reagent was added into the wells and cultured at 37°C for 1 h. The absorbance at 450 nm was then obtained using the SPECTROstar plate reader (Germany).

ALP activity assay

The ALP activity kit (Nanjing Jiancheng Bioengineering Institute, China) was used to assess the activity of ALP in PDLSCs as per the manufacturer's protocols. In summary, the cells were washed thrice using PBS, and RIPA buffer was used to lyse them. Centrifugation was then done at 4°C at a speed of 12,000*g* to separate the liquid and solid phase. The liquid phase (supernatant) was carefully transferred into a 96-well plate and treated with a substrate for ALP at 37°C for a period of 15 min. The optical densities of the samples were read by a spectrophotometer asset at a wavelength of 520 nm.

ALP staining

PDLSCs were cultured in six-well plates. After one week of induction with osteogenic inductive medium, the cells were washed thrice and then fixed in 4% paraformaldehyde. Then, ALP staining was performed using an ALP-specific staining kit (Beyotime Institute of Biotechnology, China), and the cells were viewed under an inverted microscope (Olympus Corporation, Tokyo, Japan).

RNA preparation and qRT-PCR assay

RNAios Plus reagent protocol (Takara Bio, Inc., Otsu, Japan) was used to isolate total cellular RNA. To prepare cDNA, one microgram of RNA was used as the template. This was done by a Super Script™ II Reverse Transcriptase kit (Thermo Fisher Scientific, Inc., MA, USA). The Roche Light Cycler®480II was used to perform the qRT-PCR. PCR conditions used were: initial denaturation at 95°C for 30 s followed by 40 cycles of denaturation and annealing at 95°C for 5 s and 60°C for 35 s, respectively. mRNA levels of the target genes were normalized using the GAPDH mRNA. Table 1 shows the primers used for this assay.

Western blot analysis

The collected treated cells were lysed for 30 min in RIPA buffer solution containing phosphate and protease inhibitors on ice. The amount of protein was determined using the BCA assay kit (Solarbio, Beijing, China). The proteins were separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was blocked with 5% non-fat milk solution for 1 h and then incubated with anti-GAPDH (1:20,000, Proteintech, USA), anti-ALP (1:10,000, Abcam, Cambridge, UK), anti-OCN (1:500, Abcam, Cambridge, UK), anti-RUNX2 (1:1000, Cell Signaling Technology, Inc., Danvers, USA), anti-COL1 (1:500, Wanleibio, Liaoning, China), anti-p-AKT (1:1000, Cell Signaling Technology, Inc., Danvers, USA), anti-AKT (1:1000, Cell Signaling Technology, Inc., Danvers, USA), anti-p-mTOR (1:1000, Cell Signaling Technology, Inc., Danvers, USA), anti-mTOR (1:1000, Cell Signaling Technology, Inc., Danvers, USA), and anti-GPR30 (1:500, Cusabio, Wuhan, China) at 4°C for 24 h. The protein bands were analyzed using the chemiluminescence system. The expression levels of target protein were normalized to that of GAPDH.

Statistical analysis

The analysis of variance (ANOVA) function in the SPSS17.0 software was used to determine the group differences. All tests were repeated more than thrice, and data presented as the mean ± S.D. Statistical significance was said to be present between variables when the *P*-value was less than 0.05.

Results

PDLSCs isolation and identification

The isolation of PDLSCs from periodontal ligament tissues was successful. The PDLSCs were fusiform and formed spiral arrangements (Figure 1(a)). Analysis done using flow cytometry indicated that the cells positively expressed cell-specific surface markers CD73C, CD105, CD44, and CD90 of the mesenchymal cells but negatively expressed the hematopoietic- or endothelial-specific antigens (HLA-DR, CD45, CD19, CD11b, and CD34) (Figure 1(d)). Osteogenic differentiation results revealed that after osteogenic induction, the mineralized nodules appeared as red nodules after staining with Alizarin Red (Figure 1(b)).

Table 1. Primer sequences.

GAPDH	F	5′-GGAGCGAGATCCCTCCAAAAT-3′	R	5′-GGCTGTTGTCATACTTCTCATGG-3′
ALP	F	5′-GTGAACCGCAACTGGTACTC-3′	R	5′-GAGCTGCGTAGCGATGTCC-3′
COL I	F	5′-GCTGATGATGCCAATGTGGTT-3′	R	5′-CCAGTCAGAGTGGCACATCTTG-3′
RUNX2	F	5′-GTTTCACCTTGACCATAACCGT-3′	R	5′-GGGACACCTACTCTCATACTGG-3′
OCN	F	5′-AATCCGGACTGTGACGAGTTG-3′	R	5′-CAGCAGAGCGACACCCTAGAC-3′

R: reverse primer; F: forward primer.

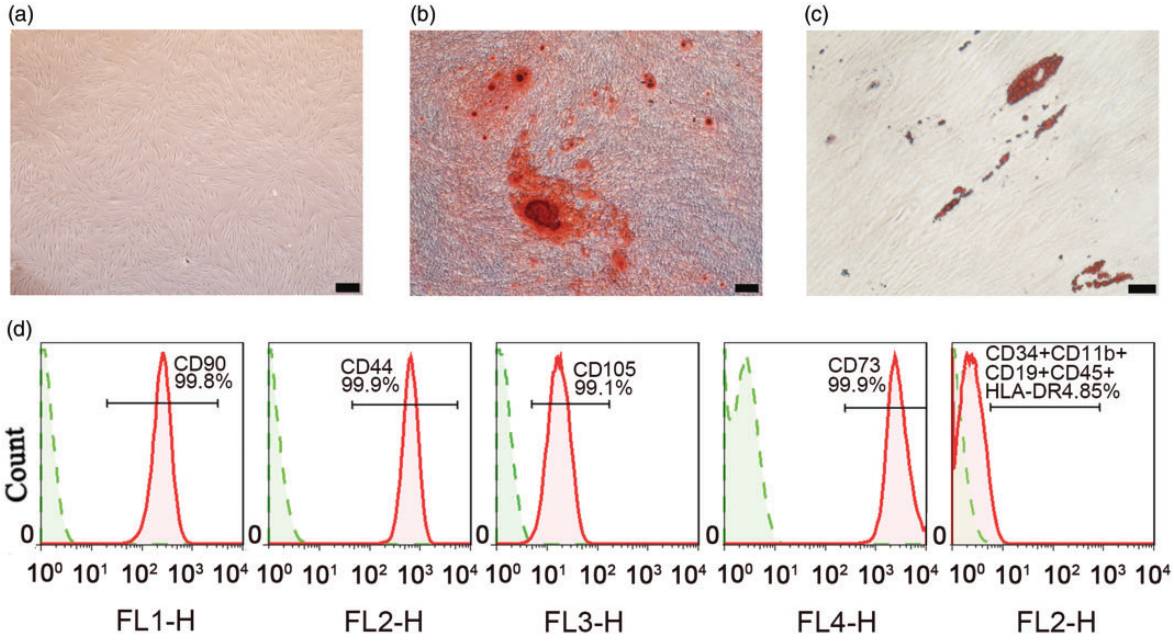

Figure 1. Isolation and identification of PDLSCs. Cells were observed under a phase contrast microscope. (a) The PDLSCs (P3) formed spiral arrangement and displayed spindle-shaped morphology (scale bar 200 μm). (b) Alizarin Red staining revealed that osteogenic differentiation of PDLSCs appeared as red mineralized nodules (scale bar 100 μm). (c) Oil red O staining of PDLSCs to assess adipogenic differentiation which appeared as red oil drops (scale bar 50 μm). (d) PDLSCs expressed the MSCs-positive cocktail (CD90, CD44, CD105, CD73) and negative for the MSCs negative cocktail (HLA-DR, CD45, CD11b, CD19, CD34). (A color version of this figure is available in the online journal.)

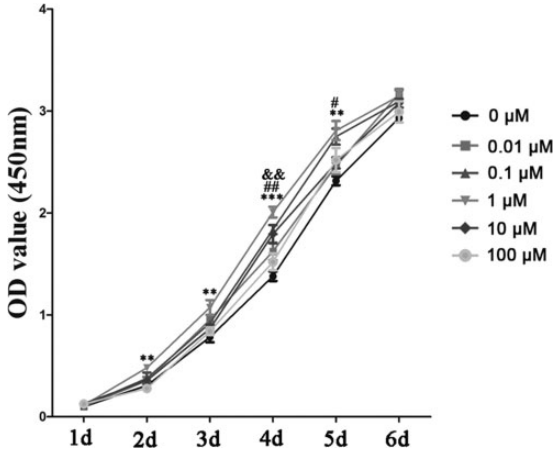

Figure 2. Effects of rutin on the proliferation of PDLSCs. Cell proliferation as determined by CCK-8 assay after treatment with various doses (0, 0.01, 0.1, 1, 10, and 100 μM) of rutin for six days. Rutin promoted the proliferation of PDLSCs in a dose-dependent pattern. Rutin at 0.01, 1, and 10 μM markedly increased the proliferation of PDLSCs as compared to the control groups, and rutin at 1 μM produced the highest proliferation activity among the groups (**$P < 0.01$, ***$P < 0.001$) rutin group (1 μM) vs. control group (0 μM); (#$P < 0.05$, ##$P < 0.01$) rutin group (0.1 μM) vs. control group (0 μM); (&&$P < 0.01$), rutin group (10 μM) vs. control group (0 μM).

For adipogenic differentiation assay, droplets appeared as red drops after staining with Oil Red O (Figure 1(c)).

Effects of rutin on the proliferation of PDLSCs

To assess the effects that rutin had on PDLSCs proliferation, the CCK-8 assay was used (Figure 2). When treated with different concentrations of rutin, PDLSCs showed different responses to cell proliferation. Rutin caused the PDLSCs to proliferate when compared to that of the control group. The promotion effect reached its peak when the concentration of rutin was 1 μM ($P < 0.001$) on day four. Therefore, a rutin concentration of 1 μM was used in the subsequent experiments.

Effects of rutin on osteogenic differentiation of PDLSCs

To determine the effect of rutin on osteogenic differentiation of PDLSCs, we performed Alizarin Red staining and measured ALP activity via ALP staining. These assays (Figure 3(a) and (b)) showed that after seven days of treatment with rutin, ALP activity was significantly higher relative to the control group. Moreover, the Alizarin Red staining demonstrated that rutin caused a significant increase in mineralization. We found that culture dishes in both the control group and the rutin group contained Alizarin Red-stained nodules after culturing, but more calcium nodules were observed in the rutin group (Figure 3 (c)). We also measured protein and gene expression levels in the cells after 14 days by Western blot and qRT-PCR, in order to confirm the rutin-induced osteogenesis acceleration on a molecular level. The p-mTOR, mTOR, p-AKT, and AKT levels were also detected in the control and rutin groups. Generally, the rutin group displayed higher expression levels of COL1, ALP, RUNX2, and OCN both at the mRNA (Figure 3(d)) and at the protein level (Figure 3(e) and (f)). The protein levels of p-mTOR and p-AKT were also elevated in the rutin group (Figure 3(g) and (h)).

Rutin induces proliferation and osteogenic differentiation through the PI3K/AKT/mTOR signaling pathway

Based on our findings that AKT and mTOR signaling pathways are activated by rutin, we further examined

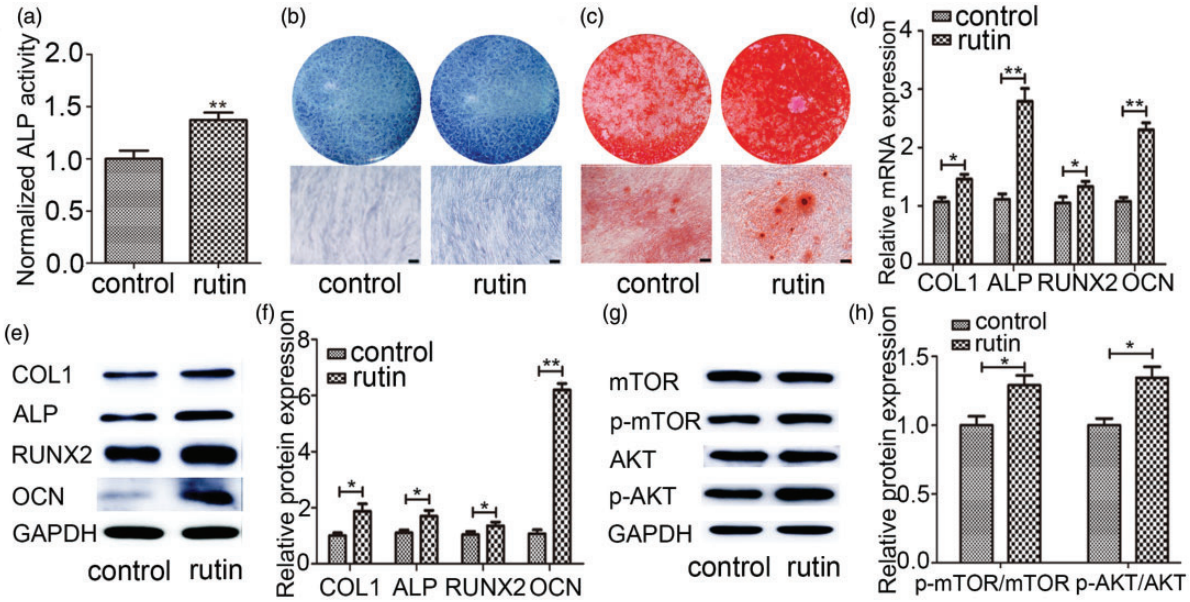

Figure 3. Effects of rutin on osteogenic differentiation of PDLSCs. Rutin enhanced the osteogenic differentiation of PDLSCs. (a) The result of ALP activity assay after osteogenic induction for seven days. (**$P < 0.01$) (b) Representative images of ALP staining after osteogenic induction for seven days (scale bar 100 μm). (c) Representative images of Alizarin Red staining after osteogenic induction for 28 days (scale bar 100 μm). (d) The mRNA level of COL1, ALP, OCN, and RUNX2 in the rutin and control groups after osteogenic induction for 14 days (*$P < 0.05$, **$P < 0.01$). (e) The protein levels of COL1, ALP, OCN, and RUNX2 proteins in the rutin and control groups after osteogenic induction for 14 days. (f) Relative quantification of COL1, ALP, RUNX2, and OCN protein levels. GAPDH was used as a loading control. (*$P < 0.05$, **$P < 0.01$) (g) After osteogenic induction for 14 days, the expression of p-mTOR, mTOR, p-AKT, and AKT proteins was measured in PDLSCs. (h) Relative quantification of p-AKT/AKT, p-mTOR/mTOR. GAPDH was used as a loading control (*$P < 0.05$). (A color version of this figure is available in the online journal.)

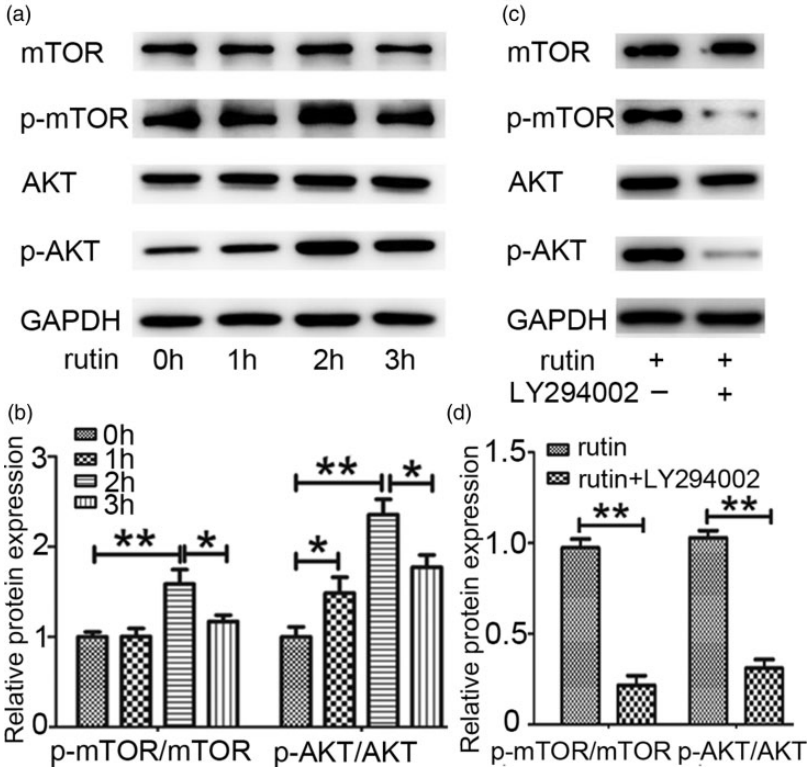

Figure 4. Rutin activated AKT and mTOR in PDLSCs. Attached PDLSCs were cultured with serum-free medium for 1 h in the absence or presence of LY294002 (10 μM). After deprivation, they were then stimulated with or without rutin at 1 μM for 1 h, 2 h, and 3 h. To assess the phosphorylation level of AKT and mTOR, total AKT and total mTOR by Western blot assay, we prepared total cell lysates. (a) Rutin increased mTOR and AKT phosphorylation in a time-dependent pattern. The phosphorylation level of AKT and mTOR reached the peak at 2 h. (b) Relative quantification of p-AKT/AKT, p-mTOR/mTOR. GAPDH was used as a loading control. (*$P < 0.05$, **$P < 0.01$) (c) Rutin group with LY294002 displayed suppressed p-AKT and p-mTOR. (d) Relative quantification of p-AKT/AKT, p-mTOR/mTOR. GAPDH was used as a loading control. (**$P < 0.01$).

the activation patterns. Upon treatment with rutin, the phosphorylation level of AKT was higher after 1 h, reached a peak at 2 h, and then declined noticeably after 3 h. Similar changes were also observed in the phosphorylation of mTOR (Figure 4(a) and (b)). The results clearly demonstrated that rutin activated the AKT and mTOR signaling pathways. Moreover, 10 μM of LY294002 (an inhibitor of PI3K) (Cell Signaling Technology, Inc., Danvers, USA) was found to largely inhibit the activation of AKT and mTOR induced by rutin (Figure 4(c) and (d)). To characterize the ways in which rutin affects the proliferation and osteogenic differentiation through PI3K/AKT/mTOR signaling pathway, we used LY294002 in rutin-treated PDLSCs during proliferation and osteogenic induction. After adding LY294002, the PDLSCs were treated with rutin for six days to assess its effect on the growth rate relative to PDLSCs not treated with LY294002. The results showed that LY294002 decreased the proliferation of PDLSCs promoted by rutin (Figure 5(a)). In addition, PDLSCs were incubated with osteogenic induction media for 7 days, 14 days, and 28 days to evaluate the effect of LY294002 on PDLSCs osteogenic differentiation induced by rutin. The findings of the ALP activity and staining after seven days (Figure 5(b) and (c)) revealed that the ALP activity of the rutin group with LY294002 was remarkably decreased compared with the rutin group without LY294002. Alizarin Red staining on day 28 revealed a significant decrease in mineralization in the rutin group upon LY294002 treatment (Figure 5(d)).

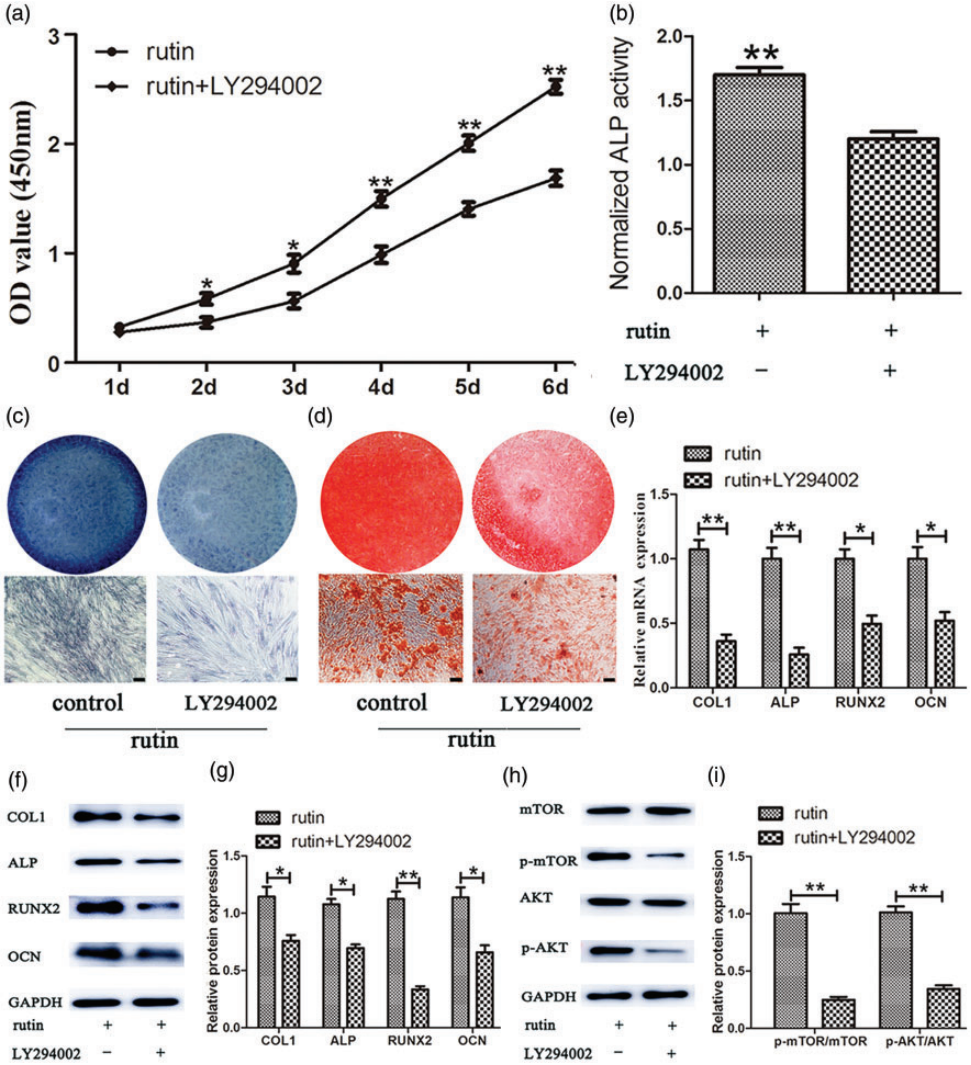

Figure 5. Rutin induces proliferation and osteogenic differentiation through PI3K/AKT/mTOR signaling pathway. For proliferation, the common culture medium supplemented with rutin (1 μM) or vehicle in with or without LY294002 (10 μM) was used to culture PDLSCs for six successive days. For osteogenic differentiation, osteogenic differentiation medium supplemented with rutin (1 μM) or vehicle in with or without LY294002 (10 μM) was used to culture PDLSCs. (a) Effects of LY294002 on proliferation of PDLSCs induced by rutin (*$P < 0.05$, **$P < 0.01$). (b) The ALP activity following osteogenic induction for seven days of rutin and LY294002 groups (**$P < 0.01$). (c) ALP staining images following osteogenic induction for seven days of rutin and LY294002 groups (scale bar 100 μm). (d) Representative images of Alizarin Red staining after osteogenic induction for 28 days of rutin and LY294002 groups (scale bar 100 μm). (e) The mRNA level of COL1, ALP, OCN, and RUNX2 in the rutin and LY294002 groups after osteogenic induction for 14 days (*$P < 0.05$, **$P < 0.01$). (F) The expression of COL1, ALP, RUNX2, and OCN proteins in the rutin and LY294002 groups after osteogenic induction for 14 days. GAPDH served as the loading control. (g) Relative quantification of COL1, ALP, RUNX2, and OCN protein levels. GAPDH was used as a loading control (*$P < 0.05$, **$P < 0.01$). (h) After osteogenic induction with or without LY294002 for 14 days, the protein levels of mTOR, p-mTOR, p-AKT, and AKT proteins in PDLSCs were measured. (i) Relative quantification of p-AKT/AKT, p-mTOR/mTOR. GAPDH was used as a loading control (**$P < 0.01$). (A color version of this figure is available in the online journal.)

Moreover, transcription of osteogenic genes COL1, OCN, RUNX2, and ALP was detected on day 14; it was reduced both at the mRNA level and protein level (Figure 5(e) to (g)). Similarly, the level of proteins of both the p-mTOR and p-AKT was reduced (Figure 5(h) and (i)).

GPR30 mediates the PI3K/AKT/mTOR signal transduction

The expression levels of GPR30 in PDLSCs were increased by rutin, and interestingly, this upregulation was not altered after the addition of LY294002 (Figure 6(a) and (b)). To explore the question of whether GPR30 mediates PI3K/AKT/mTOR pathway activation, PDLSCs were treated with G15 (a selective antagonist of GPR30) (30 μM) (APExBIO, USA) and rutin. In terms of proliferation, after adding G15, the OD value of cells was measured by CCK-8 for six consecutive days. The results revealed that G15 decreased PDLSCs proliferation promoted by rutin (Figure 6(c)). In terms of osteogenic differentiation, on day 7, both the ALP activity assay and staining (Figure 6(d) and (e)) revealed that the activity of ALP in the G15 group with rutin was remarkably reduced relative to the rutin group, and fewer mineralized nodules were formed (Figure 6(f)). On day 14, the levels of osteogenic genes RUNX2, OCN, COL1, and ALP were reduced at both the mRNA and at the protein level (Figure 6(g) to (i)) after treatment with G15. The protein levels of GPR30, mTOR and p-mTOR, AKT and p-AKT were measured using Western blotting assays. Western blotting revealed that G15 reduced the expression of GPR30 and inhibited the effects of rutin. Meanwhile, the PI3K/AKT/mTOR

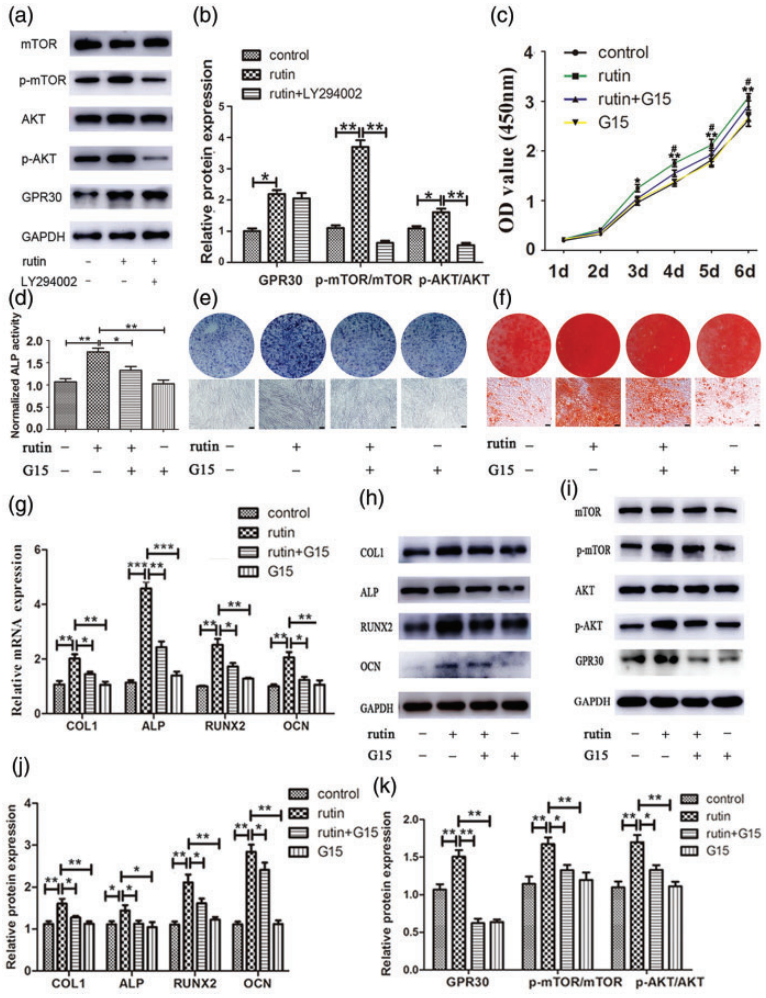

Figure 6. GPR30 mediates the activation of PI3K/AKT/mTOR signaling pathway induced by rutin. G15 (a selective antagonist of GPR30) decreased rutin-induced PI3K/AKT/mTOR pathway activation of PDLSCs. For proliferation, PDLSCs were grown in the common culture medium or osteogenic differentiation medium with or without rutin (1 μM) or vehicle in the absence or presence of G15 (30 μM). (a) The expression of GPR30 proteins in the absence or presence of rutin or LY294002 group after osteogenic induction was detected. (b) Relative quantification of GPR30 protein level, p-AKT/AKT, and p-mTOR/mTOR. GAPDH was used as a loading control (*$P < 0.05$, **$P < 0.01$). (c) Effects of G15 on proliferation of PDLSCs induced by rutin. (d) Data analysis of the ALP activity after osteogenic induction for seven days (*$P < 0.05$, **$P < 0.01$, vs. control group). (e) Representative images of ALP staining after osteogenic induction for seven days (scale bar 100 μm). (f) Representative images of Alizarin Red staining following osteogenic induction for 28 days (scale bar 100 μm). (g) qRT-PCR assay for COL1, RUNX2, ALP, and OCN mRNA after osteogenic induction for 14 days (*$P < 0.05$, **$P < 0.01$, vs. control group). (h) The expression of COL1, ALP, RUNX2, and OCN proteins after osteogenic induction for 14 days. GAPDH was used as a loading control. (i) Relative quantification of COL1, ALP, RUNX2, and OCN protein levels. GAPDH was used as a loading control (*$P < 0.05$, **$P < 0.01$,***$P < 0.001$). (g) Western blotting analysis of the expression of AKT, p-AKT, mTOR, p-mTOR and GPR30 proteins after osteogenic induction for 14 days. (k) Relative quantification of GPR30 protein level, p-AKT/AKT, and p-mTOR/mTOR. GAPDH was used as a loading control (*$P < 0.05$, **$P < 0.01$). (A color version of this figure is available in the online journal.)

signaling was not activated (Figure 6(j) and (k)). In other words, we found that G15 interfered with the activation of the PI3K/AKT/mTOR signal pathway, indicating that GPR30 acts as a protein mediating the activation of this pathway.

Discussion

Periodontal tissue defect is a common feature of periodontitis. Reconstructing the lost periodontal tissue is a serious challenge. The application of periodontal surgery and anti-inflammation are common methods to repair periodontal tissue,[27] but these methods cannot effectively improve the regeneration potential of periodontal tissue.[28] In the recent past, periodontal tissue engineering has gradually become a new therapeutic strategy for reconstructing lost tissue. Owing to the strong ability of PDLSCs to undergo self-differentiation and renewal, they are ideal for tissue engineering research.[29] Improving the regeneration ability of PDLSCs is a key research direction. Flavonoids are natural compounds with many medicinal values, which have certain effects on cell proliferation and bone metabolism.[30] Rutin is a common flavonoid, which may promote the regeneration of periodontal tissue. Here, we studied the impacts of rutin on PDLSCs, with particular attention to proliferation, osteogenic differentiation, and potential mechanisms, in order to provide some novel ideas for application of flavonoids in promoting stem cell regeneration.

In periodontal tissue engineering, stem cell acquisition is a key step. We assessed the proliferation of PDLSCs and found that the proliferation was enhanced by treatment with rutin in a concentration-dependent pattern. The proliferative capacity of PDLSCs was significantly enhanced following rutin treatment at a concentration of 1 μM. To explore the potential of PDLSCs in the stimulation of osteogenesis, the transcription of osteogenesis-related genes, e.g. COL1, RUNX2, ALP, and OCN, was detected. The ALP protein can promote bone matrix mineralization and has a positive effect on bone formation.[31] ALP and COL1 are also considered early markers in osteogenic differentiation.[32,33] RUNX2 is a key gene of bone formation and a specific transcription factor required for osteogenic differentiation and development of bone marrow MSCs.[34–36] OCN is a biochemical marker reflecting the function of osteoblasts and a late indicator of osteogenic differentiation. And the expression of osteocalcin during matrix mineralization is the main manifestation of osteoblasts differentiation.[37,38] Fourteen days after PDLSCs were treated with 1 μM rutin, the transcription of COL1, OCN, RUNX2, and ALP was relatively elevated compared to that of the control group, thus strongly suggesting that rutin improved the osteogenic differentiation of PDLSCs. In fact, some researches have demonstrated the correlation between flavonoids and multiple differentiation potential of stem cells. For instance, *Herba epimedii* flavonoids improve bone health by mediating the effects of RUNX2 on osteogenesis, strongly suggesting that it could be a novel agent for treating osteoporosis.[39] Similarly, Drynaria fortune can improve the proliferation and osteogenic differentiation of stem cells in the dental pulp in a dose-dependent manner.[40] At the same time, Icariin is a flavonoid with potent effects on bone marrow MSCs' activity.[41] All these studies are consistent with our results.

The PI3K/AKT/mTOR pathways has key regulatory functions in cell proliferation[42,43] and differentiation[44] in cells. AKT is a crucial element in differentiation[45] and is involved in bone development.[46] mTOR signaling possesses key physiological roles in the modulation of osteogenic differentiation and proliferation.[47,48] The mechanisms by which rutin promotes PDLSCs to proliferate and differentiate could be complicated, and many details remain to be investigated. This study discovered that the phosphorylation of mTOR and AKT was enhanced in PDLSCs cultured in rutin; the effects were time-dependent and reached a peak at 2 h. We hypothesize that the rutin-induced changes in the AKT/mTOR signaling pathway are related to the enhancement of PDLSCs proliferation as well as their differentiation. Moreover, LY294002 decreased the proliferation of PDLSCs enhanced by rutin, reduced the levels of p-mTOR and p-AKT, and inhibited the expression of genes related to osteogenic differentiation during osteogenic induction. Collectively, these findings prove that the PI3K/AKT/mTOR signal pathway is involved in rutin-mediated proliferation and osteogenic differentiation of the PDLSCs. However, other regulators affecting the PI3K/AKT/mTOR signal pathway require further study.

GPR30 has been postulated to promote some cellular and physiological effects of estrogen.[49] GPR30-mediated estrogenic effect involves many signal transductions, including ERK, MAPK, and the PI3K/AKT signal pathway.[50] Some studies have shown that GPR30 can specifically bind to estrogen, enhance the proliferative capacity of cancer cells, and rapidly activate PI3K/AKT signaling in

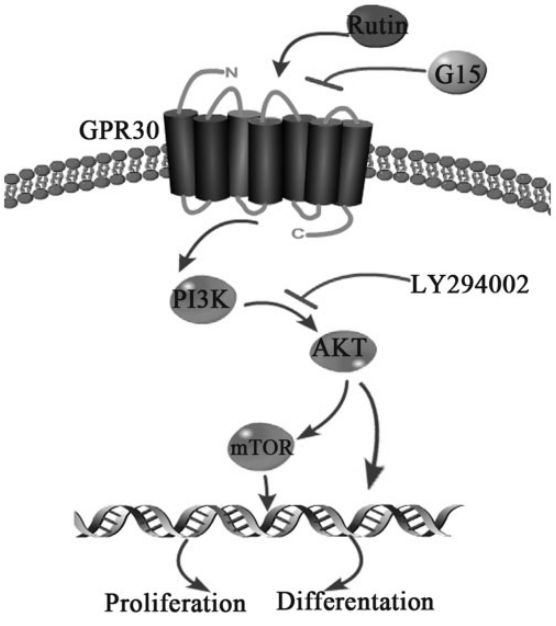

Figure 7. Model for GPR30/PI3K/AKT/mTOR signaling in rutin-induced proliferation and osteogenic differentiation of PDLSCs. Through the GPR30, rutin activates AKT, subsequently activating the activation of mTOR, finally resulting in proliferation and osteogenic differentiation.

endometrial tumor cells.[51,52] In this study, rutin increased the expression level of GPR30 but LY294002 treatment failed to do so, suggesting that GPR30 acts upstream of the PI3K/AKT/mTOR signal pathway. To further explore whether GPR30 mediates the effects of rutin on PI3K/AKT/mTOR pathway, PDLSCs were treated with rutin in the presence or absence of G15. The PI3K/AKT/mTOR pathway was inhibited by G15, indicating the importance of GPR30 in the activation of this pathway. Previous studies revealed that rutin could play an estrogen-like role because of its similar structure to endogenous and phytoestrogens and its plane diphenyl ring structure similar to estrogen.[53] Similarly, our results confirmed that GPR30 could be activated by rutin. Although the G15 used in this experiment is a highly specific antagonist with no affinity for other receptors of estrogen,[54] the specific binding mode of rutin to GPR30 needs further study in the future. Besides, to further develop the understanding of the underlying mechanisms, more researches are also necessary.

In conclusion, our study proved that rutin, a common flavonoid, promoted the proliferation and osteogenic differentiation of PDLSCs via the GPR30-mediated PI3K/AKT/mTOR signal pathway (Figure 7). All of our findings indicate that rutin is a novel and promising candidate drug for bone regeneration and periodontal tissue engineering.

Authors' contributions: The experiments were conceived and designed by Xu X and carried out by Zhao B and Xiong YX. Zhang YP and Jia LL assisted in carrying out experiments. The data was analyzed by Zhao B, Zhang WJ, and Jia LL. The article was written by Zhao B and revised by Xu X. The article was read and approved by all the authors.

ACKNOWLEDGMENTS

We thank the Director of Shandong Provincial Key Laboratory of Oral Tissue Regeneration for their technical support.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Construction Engineering Special Fund of Taishan Scholars (grant number TS201511106).

ORCID iD

Xin Xu https://orcid.org/0000-0002-5385-6115

REFERENCES

1. Chapple IL, Van dWF, Dorfer C, Herrera D, Shapira L, Polak D, Madianos P, Louropoulou A, Machtei E, Donos N. Primary prevention of periodontitis: managing gingivitis. *J Clin Periodontol* 2015;**42**:S71–6
2. Pihlstrom BL, Michalowicz BS, Johnson NW. Periodontal diseases. *Lancet* 2005;**366**:1809–20
3. Mrozik KM, Zilm PS, Bagley CJ, Hack S, Hoffmann P, Gronthos S, Bartold PM. Proteomic characterization of mesenchymal stem cell-like populations derived from ovine periodontal ligament, dental pulp, and bone marrow: analysis of differentially expressed proteins. *Stem Cells Dev* 2010;**19**:1485–99
4. Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, Young M, Robey PG, Wang CY, Shi S. Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* 2004;**364**:149–55
5. Mrozik KM, Wada N, Marino V, Richter W, Shi S, Wheeler DL, Gronthos S, Bartold PM. Regeneration of periodontal tissues using allogeneic periodontal ligament stem cells in an ovine model. *Regen Med* 2013;**8**:711–23
6. Gronthos S, Mrozik K, Shi S, Bartold PM. Ovine periodontal ligament stem cells: isolation, characterization, and differentiation potential. *Calcif Tissue Int* 2006;**79**:310–7
7. Ding G, Liu Y, Wang W, Wei F, Liu D, Fan Z, Zhang C, Wang S. Allogeneic periodontal ligament stem cell therapy for periodontitis in swine. *Stem Cells* 2010;**28**:1829–38
8. Hardcastle AC, Aucott L, Reid DM, Macdonald HM. Associations between dietary flavonoid intakes and bone health in a Scottish population. *J Bone Miner Res* 2011;**26**:941–7
9. Welch AA, Hardcastle AC. The effects of flavonoids on bone. *Curr Osteoporos Rep* 2014;**12**:205–10
10. Ross JA, Kasum CM. Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annu Rev Nutr* 2002;**22**:19–34
11. Araújo JR, Gonçalves P, Martel F. Chemopreventive effect of dietary polyphenols in colorectal cancer cell lines. *Nutr Res* 2011;**31**:77–87
12. Procházková D, Boušová I, Wilhelmová N. Antioxidant and prooxidant properties of flavonoids. *Fitoterapia* 2011;**82**:513–23
13. Srivastava S, Bankar R, Roy P. Assessment of the role of flavonoids for inducing osteoblast differentiation in isolated mouse bone marrow derived mesenchymal stem cells. *Phytomedicine* 2013;**20**:683–90
14. Porta C, Paglino C, Mosca A. Targeting PI3K/akt/mTOR signaling in cancer. *Front Oncol* 2014;**4**:64
15. Eo SH, Kim SJ. Rutin induces cyclooxygenase-2 (COX-2) and matrix metalloproteinase (MMP)-9 expression via the ERK and PI3K/akt pathways in A549 human lung cancer cells. *J Converg Inform Technol* 2013;**8**:414–21
16. Wang R, Sun Y, Huang H, Lan W, Chen J, Wei S. Rutin, a natural flavonoid protects PC12 cells against sodium Nitroprusside-Induced neurotoxicity through activating PI3K/akt/mTOR and ERK1/2 pathway. *Neurochem Res* 2015;**40**:1945–53
17. Sun J, Wang H, Liu B, Shi W, Shi J, Zhang Z, Xing J. Rutin attenuates H_2O_2-induced oxidation damage and apoptosis in Leydig cells by activating PI3K/akt signal pathways. *Biomed Pharmacother* 2017;**88**:500–6
18. Lecomte S, Lelong M, Bourgine G, Efstathiou T, Saligaut C, Pakdel F. Assessment of the potential activity of major dietary compounds as selective estrogen receptor modulators in two distinct cell models for proliferation and differentiation. *Toxicol Appl Pharmacol* 2017;**325**:61–70
19. Wang X, Zheng N, Dong J, Wang X, Liu L, Huang J. Estrogen receptor-α36 is involved in icaritin induced growth inhibition of triple-negative breast cancer cells. *J Steroid Biochem Mol Biol* 2017;**171**:318–27
20. Tham DM, Gardner CD, Haskell WL. Clinical review 97: potential health benefits of dietary phytoestrogens: a review of the clinical, epidemiological, and mechanistic evidence. *J Clin Endocrinol Metab* 1998;**83**:2223–35
21. Pietras RJ, Levin ER, Szego CM, Prossnitz ER, Revankar CM, Arterburn JB, Sklar LA. Estrogen receptors and cell signaling. *Science* 2005;**310**:51–3
22. Revankar CM, Cimino DF, Sklar LA, Arterburn JB, Prossnitz ER. A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science* 2005;**307**:1625–30
23. Chen JY, Hu R, Ge HF, Duanmu WS, Li YH, Xue XS, Hu SL, Feng H. G-protein-coupled receptor 30-mediated antiapoptotic effect of estrogen on spinal motor neurons following injury and its underlying mechanisms. *Mol Med Rep* 2015;**12**:1733–40

24. Ding Q, Hussain Y, Chorazyczewski J, Gros R, Feldman RD. GPER-Independent effects of estrogen in rat aortic vascular endothelial cells. *Mol Cell Endocrinol* 2014;**399**:60–8
25. Windahl SH, Andersson N, Chagin AS, Mårtensson UE, Carlsten H, Olde B, Swanson C, Movérare-Skrtic S, Sävendahl L, Lagerquist MK. The role of the G protein-coupled receptor GPR30 in the effects of estrogen in ovariectomized mice. *Am J Physiol Endocrinol Metab* 2009;**296**:E490–6
26. Oz OK, Hajibeigi A, Ford JJ. Emerging role of GPR30/GPER1 in skeletal metabolism. *Immunology* 2011;**11**:275–81
27. Xiong X, Buekens P, Goldenberg RL, Offenbacher S, Qian X. Optimal timing of periodontal disease treatment for prevention of adverse pregnancy outcomes: before or during pregnancy? *Am J Obstet Gynecol* 2011;**205**:111e1–6
28. Chen FM, Zhang M, Wu ZF. Toward delivery of multiple growth factors in tissue engineering. *Biomaterials* 2010;**31**:6279–308
29. Ji K, Liu Y, Lu W, Yang F, Yu J, Wang X, Ma Q, Yang Z, Wen L, Xuan X. Periodontal tissue engineering with stem cells from the periodontal ligament of human retained deciduous teeth. *J Periodont Res* 2013;**48**:105–16
30. Zhang DW, Cheng Y, Wang NL, Zhang JC, Yang MS, Yao XS. Effects of total flavonoids and flavonol glycosides from epimedium koreanum nakai on the proliferation and differentiation of primary osteoblasts. *Phytomedicine* 2008;**15**:55–61
31. Hessle L, Johnson KA, Anderson HC, Narisawa S, Sali A, Goding JW, Terkeltaub R, Millan JL. Tissue-nonspecific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization. *Proc Natl Acad Sci Usa* 2002;**99**:9445–9
32. Anderson HC, Sipe JB, Hessle L, Dhanyamraju R, Atti E, Camacho NP, MillÃ N JL, Dhamyamraju R. Impaired calcification around matrix vesicles of growth plate and bone in alkaline phosphatase-deficient mice. *Am J Pathol* 2004;**164**:841–7
33. Franceschi RT, Iyer BS. Relationship between collagen synthesis and expression of the osteoblast phenotype in M. C3T3-E1 cells. *J Bone Miner Res* 1992;**7**:235–46
34. Fei C, Guo J, Zhao Y, Gu S, Zhao S, Li X, Chang C. Notch-Hes pathway mediates the impaired osteogenic differentiation of bone marrow mesenchymal stromal cells from myelodysplastic syndromes patients through the down-regulation of Runx2. *Am J Transl Res* 2015;**7**:1939–51
35. Xu J, Li Z, Hou Y, Fang W. Potential mechanisms underlying the Runx2 induced osteogenesis of bone marrow mesenchymal stem cells. *Am J Transl Res* 2015;**7**:2527–35
36. Chen Y, Hu Y, Yang L, Zhou J, Tang Y, Zheng L, Qin P. Runx2 alleviates high glucose-suppressed osteogenic differentiation via PI3K/AKT/GSK3β/β-catenin pathway. *Cell Biol Int* 2017;**41**:822–32
37. Neve A, Corrado A, Cantatore FP. Osteocalcin: skeletal and extra-skeletal effects. *J Cell Physiol* 2013;**228**:1149–53
38. Frenkel B, Capparelli C, Van AM, Baran D, Bryan J, Stein JL, Stein GS, Lian JB. Activity of the osteocalcin promoter in skeletal sites of transgenic mice and during osteoblast differentiation in bone marrow-derived stromal cell cultures: effects of age and sex. *Endocrinology* 1997;**138**:2109–16
39. Zhang D, Liu L, Jia Z, Yao X, Yang M. Flavonoids of *Herba epimedii* stimulate osteogenic differentiation and suppress adipogenic differentiation of primary mesenchymal stem cells via estrogen receptor pathway. *Pharm Biol* 2016;**54**:954–63
40. Huang XF, Yuan SJ, Yang C. Effects of total flavonoids from Drynaria fortunei on the proliferation and osteogenic differentiation of rat dental pulp stem cells. *Mol Med Rep* 2012;**6**:547–52
41. Chen KM, Ge BF, Ma HP, Liu XY, Bai MH, Wang Y. Icariin, a flavonoid from the herb epimedium enhances the osteogenic differentiation of rat primary bone marrow stromal cells. *Pharmazie* 2005;**60**:939–42
42. Lim W, Yang C, Bazer FW, Song G. Luteolin inhibits proliferation and induces apoptosis of human placental choriocarcinoma cells by blocking the PI3K/AKT pathway and regulating sterol regulatory element binding protein activity. *Biol Reprod* 2016;**95**:82
43. Yu JS, Cui W. Proliferation, survival and metabolism: the role of PI3K/AKT/mTOR signalling in pluripotency and cell fate determination. *Development* 2016;**143**:3050–60
44. Follo MY, Manzoli L, Poli A, Mccubrey JA, Cocco L. PLC and PI3K/akt/mTOR signalling in disease and cancer. *Adv Biol Regul* 2015;**57**:10–6
45. Sugatani T, Hruska KA. Akt1/Akt2 and mammalian target of rapamycin/bim play critical roles in osteoclast differentiation and survival, respectively, whereas akt is dispensable for cell survival in isolated osteoclast precursors. *J Biol Chem* 2005;**280**:3583–9
46. Peng XD, Xu PZ, Chen ML, Hahnwindgassen A, Skeen J, Jacobs J, Sundararajan D, Chen WS, Crawford SE, Coleman KG. Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2. *Genes Dev* 2003;**17**:1352–65
47. Gu X, Fu X, Lu J, Saijilafu Li B, Luo ZP, Chen JQ. Pharmacological inhibition of S6K1 impairs self-renewal and osteogenic differentiation of bone marrow stromal cells. *J Cell Biochem* 2018;**119**:1041–9
48. Luo G, Xu B, Huang Y. Icariside II promotes the osteogenic differentiation of canine bone marrow mesenchymal stem cells via the PI3K/AKT/mTOR/S6K1 signaling pathways. *Am J Transl Res* 2017;**9**:2077–87
49. Prossnitz ER, Maggiolini M. Mechanisms of estrogen signaling and gene expression via GPR30. *Mol Cell Endocrinol* 2009;**308**:32–8
50. Prossnitz ER. GPER modulators: opportunity nox on the heels of a class akt. *J Steroid Biochem Mol Biol* 2018;**176**:73–81
51. Wei Y, Zhang Z, Liao H, Wu L, Wu X, Zhou D, Xi X, Zhu Y, Feng Y. Nuclear estrogen receptor-mediated notch signaling and GPR30-mediated PI3K/AKT signaling in the regulation of endometrial cancer cell proliferation. *Oncol Rep* 2012;**27**:504–10
52. Ge X, Guo R, Qiao Y, Zhang Y, Lei J, Wang X, Li L, Lu D. The G protein-coupled receptor GPR30 mediates the nontranscriptional effect of estrogen on the activation of PI3K/akt pathway in endometrial cancer cells. *Int J Gynecol Cancer* 2013;**23**:52–9
53. Liu H, Zhong L, Zhang Y, Liu X, Li J. Rutin attenuates cerebral ischemia/reperfusion injury in ovariectomized rats via estrogen receptor-mediated BDNF-TrkB and NGF-TrkA signaling. *Biochem Cell Biol* 2018;**96**:672–81
54. Masuhara M, Tsukahara T, Tomita K, Furukawa M, Miyawaki S, Sato T. A relation between osteoclastogenesis inhibition and membrane-type estrogen receptor GPR30. *Biochem Biophys Rep* 2016;**8**:389–94

(Received October 3, 2019, Accepted December 23, 2019)