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8-prenylgenistein exerts osteogenic effects via ERα and Wnt-dependent signaling pathway

Running title: Osteogenic mechanism of 8-prenylgenstein

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ABSTRACT

8-prenylgenistein (8PG) was previously reported to exert stronger osteogenic activity than genistein, a well-known soy phytoestrogen. However, the molecular mechanism underlying the actions of 8PG on osteoblasts was far from clear. In the present study, the osteogenic effects and mechanisms of 8PG and genistein were studied using human BMSC and murine pre-osteoblast MC3T3-E1 cells. Our results indicated that the stimulatory effects of 8PG and genistein on osteoblast differentiation were abolished by co-incubation with MPP (10^{-6} M, an ERα antagonist), but not PHTPP (10^{-6} M, an ERβ antagonist). Molecular docking indicated that the binding mode of 8PG toward ERα was similar to that of genistein and therefore could not account for their differential osteogenic actions. *In silico* target profiling identified the involvement of glycogen synthase kinase-3β (GSK-3β), a key mediator of Wnt/β-catenin pathway, in the actions of 8PG. However, instead of directly inhibiting GSK-3β enzymatic activities, 8PG and genistein were found to induce GSK-3β phosphorylation at Serine-9 in osteoblastic MC3T3-E1 cells. 8PG exerted more potent effects than genistein in stimulating expressions of LRP5, β-catenin, Runx2, osteocalcin, alp, opg, major protein and gene markers involved in Wnt signaling pathway in MC3T3-E1 cells. Moreover, the inhibition of Wnt signaling by DKK1 could be restored by treatment with 8PG and genistein. However, 8PG, but not genistein, stimulated ERα-dependent β-catenin protein expression in MC3T3-E1 cells. Furthermore, the increase in ALP activity, LRP5 and phospho-Akt/Akt expression by 8PG and genistein were abolished by co-treatment with LY294002

(10^{-5} M, a PI3K pathway inhibitor). Collectively, our results suggested that the osteogenic activities of 8PG was mediated by GSK-3β phosphorylation through the induction of Wnt/β-catenin and ERα-associated PI3K/Akt signaling.

Keywords: genistein; 8-prenylgenistein; osteoblast; Estrogen receptor α; GSK-3β; Wnt/β-catenin signaling pathway; PI3K/Akt signaling pathway;

Abbreviations: 8PG, 8-prenylgenistein; Wnt, The wingless-type MMTV integration site; ER, Estrogen receptor; GSK-3β, Glycogen synthase kinase 3 β; p-GSK-3β, phospho-Glycogen synthase kinase 3 β;α-MEM, α-Minimum essential medium; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carb-oxy-methoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium); ALP activity, Alkaline phosphatase activity; E2, 17-β estradiol; OPG: Osteoprotegerin; TBST, Tris-buffered saline containing 0.1% tween 20; FBS, Fetal bovine serum; MPP, 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride; AZD2858, 3-Amino-6-[4-[(1-methyl-1-piperazinyl)sulfonyl]phenyl]-N-3-pyridinyl-2-pyrazinecarboxamide; LY294002, 2-(4-Morpho linyl) -8-phenyl-4H-1-benzopyran-4-one hydrochlorid; PHTPP, 4-[2-Phenyl -5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol;

1. Introduction

Phytoestrogens are widely regarded as a potential alternative therapy for postmenopausal bone loss due to their lower risks of inducing adverse side effects than hormone replacement therapy [1-3]. Indeed, the beneficial effects of genistein, a soy-derived phytoestrogen, on bone have been extensively studied for more than 60 years [2]. Genistein has been shown *in vitro* to promote osteoblastogenesis, suppress osteoclastogenesis [4, 5] as well as prevent bone loss in animal and clinical studies [2, 6].

Recently, the bioactivities of prenylflavonoids, which naturally exist in many Chinese medicinal herbs belonging to the legume family, such as *Erythrina Variegate* (EV), *Herba Epimedii* [2, 7], and *Rizhoma Drynariae* [8], have gained

much attention [9]. Numerous studies have reported the effect of parent compounds modified with prenyl chemical group on the osteogenic activity of parent compounds. Numerous studies have reported modification of the prenyl group in flavonoids could enhance their osteogenic activity [2]. The osteogenic activity of 8-prenylnaringenin, which is highly abundant in hops and beers, was found to be more potent than that of naringenin [10]. Similarly, the osteogenic activity of icariin, an 8-prenylated flavonol glycoside derived from *Herba Epimedii*, was discovered to be stronger than that of genistein [2]. These *in vitro* studies indicated that the introduction of prenyl groups may strengthen the osteogenic activity of parent compounds.

Our previous studies demonstrated that extracts of EV could significantly prevent ovariectomy-induced osteoporosis [11] and discovered that the major bioactive ingredients in EV were prenylated derivatives from genistein [7]. A study on the structure-function relationship indicated that the addition of a prenyl group at C-8 (8-prenylgenistein, 8PG), but not C-6, on the genistein skeleton could potently increase osteoblastic proliferation, differentiation and mineralization of UMR106 osteosarcoma cells [7]. However, the molecular mechanisms underlying the mediating of the osteogenic effects of genistein prenylated at C-8 are yet to be determined.

Estrogen receptor (ER)-dependent genomic and non-genomic pathways have been widely reported to play a critical role in osteoblastogenesis [2]. The genomic pathway involves the ligands binding to ERs in the nucleus, after which the ERs dimerize and bind to estrogen response elements (ERE) or other transcription factor complexes, such as AP-1, STATs and ATF-2, finally lead to the gene transcription [12]. The non-genomic pathway is rapidly mediated by activating

membrane-associated ER, an initiation that subsequently alters signal transduction pathways such as extracellular signal regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K) [13]. The Wnt/β-catenin is another important signaling pathway for osteoblast differentiation [14]. It is initiated by the ligands (such as Wnt3a) interacting with Frizzled receptors and low-density lipoprotein receptor-related protein (LRP) 5/6, and forming a ternary complex, then led to the inhibition of Glycogen synthase kinase-3β（GSK-3β), finally reduced degradation of β-catenin. The accumulation of β-catenin is translocated to the nucleus, which leads to the activation of osteoblastogenesis by modulating the expression of specific transcription factors such as Runx2 [15-17]. Indeed, the ER genomic signaling pathway and the canonical Wnt/β-catenin signaling pathway have been shown to synergistically promote osteogenic differentiation of mesenchymal progenitor cells [18]. When activated by Wnt signaling, Akt, the downstream signaling molecule of ERα/PI3K, would enhance the inactivation of GSK-3β by phosphorylation at serine-9. The phosphorylation of GSK-3β would lead to the accumulation of free β-catenin, and subsequently stimulate the gene expression of osteoblastogenesis markers. [19]. It is of interest to determine if the ERα-associated PI3K/Akt pathway and Wnt/β-catenin pathway participate in stimulating the osteogenic activity of genistein and its prenylated derivatives (8PG).

The present study characterized the osteogenic effect of 8PG and genistein and investigated if 8PG and genistein exerted any differential actions in the regulation of GSK-3β via simultaneously activating Wnt/β-catenin and ERα-associated PI3K/Akt signaling using human BMSCs and pre-osteoblastic MC3T3-E1 cells.

2. Materials and methods

2.1 Reagents

Genistein and 17β-Estradiol (E2) were obtained from Sigma-Aldrich (St. Louis, MO, USA). ERα selective antagonist 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy) phenol]-1H–pyrazole dihydrochloride (MPP) (purity ≥ 98%) and ERβ selective antagonist 4-[2-Phenyl-5,7-bis(trifluoromethyl) pyrazolo[1,5-a] pyrimidin-3-yl] phenol (PHTPP) were the products of TOCRIS Bioscience (Bristol, UK). AZD2858 was purchased from StemRD (Burlingame, CA, USA). Dulbecco's modified Eagle medium (DMEM), Minimum Essential Medium α (α-MEM), Fetal bovine serum (FBS), 0.5% Trypsin-EDTA and penicillin-streptomycin(P/S) were purchased from Gibco (Gaithersburg, MD, USA). 8-prenylgenistein was synthesized starting from genistein according to literature [20], the procedures were shown in Supplementary Materials and Methods in detail.

2.2 Cell culture

Human bone marrow stromal cells (hBMSC) were kindly provided by Prof. LEE Yuk-wai, Wayne (The Chinese University of Hong Kong, HK, PRC). Murine osteoblastic MC3T3-E1 cell line was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). hBMSC and MC3T3-E1 cells were routinely cultured in α-MEM, supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified incubator with 95% air and 5% CO_2 at 37 °C. To induce osteoblast differentiation of MC3T3-E1, an osteogenic differentiation medium (OM) that consisted of MEM-α medium, 10% FBS, 10 mM β-glycerophosphate and 50 μg/mL L-ascorbic acid was used. To induce osteoblast differentiation of hBMSC, the medium was replaced with a stem cell osteogenic differentiation (OD) medium (StemPro® osteogenesis differentiation basal medium and StemPro® osteogenesis supplement). The OD medium was replaced every 3 days.

2.3 Alkaline phosphatase (ALP) activity assay

Cells were seeded at 24-well plates with a cell density of 1.2×10^4 cells/well. When the cells reach 80~90% confluence in the well, the medium was changed with OM with or without E2 (10^{-8} M), 8PG (10^{-8} to 10^{-5} M), or genistein (10^{-8} to 10^{-5} M).

Upon treatment for 7 days, the cells were collected and lysed with passive lysis buffer (Promega, USA). ALP activity was measured by Wako lab assay ALP (Wako, Japan). The absorbance at 405 nm was determined by using a spectrophotometric plate reader (Bio-Rad model 550, Japan). ALP enzymatic activity was normalized with total protein content of samples measured by Bradford protein assay.

2.4 Bone nodule formation assay

When the cultured cells reached 80%-90% confluence, the medium was changed to OM with Ctrl (1% ethanol), E2 (10^{-8} M) or different concentration of 8PG (10^{-8} M to 10^{-5} M) or genistein (10^{-8} M to 10^{-5} M). Upon treatment for 18-21 days, the cells were fixed with 4% formaldehyde and stained with 1% Alizarin Red S. The stained images were captured under a light microscope (Olympus, Japan). To quantify bone calcified nodules, 0.5 M HCl and 5 % SDS were injected in the stained well to dissolve the stained bone nodules. The absorbance was detected at 415 nm by using a spectrophotometric plate reader (Bio-Rad model 550, Japan). The OD value was used to quantify the degree of mineralization.

2.5 *In silico* target profiling of 8PG and genistein

A similarity searching-based tool PredictFX 1.1 (Tripos Inc., St. Louis, USA) was applied to predict targets of 8PG and genistein that were involved in their osteogenic activity. This tool relies on data sources (such as ChEMBL and Pubchem) that contain information of compounds with binding or functional activity to target proteins. In order to perform similarity searching, this tool adopt three types of two-dimensional descriptors, namely pharmacophoric fragments (PHRAG), shannon entropy distributions (SHED) and feature-pair distribution (FPD) to encode chemical structures. The details of this method could be found in a previous study [21].

2.6 Docking experiment

Molecular modelling was carried out by using Surflex-Dock module of SYBYL 8.0 (Tripos Inc., St. Louis, USA). All structures were built, hydrogen atoms were added, energies were minimized under Tripos Force field with 0.05 kcal/mol,

Gasteiger-Hückel charges were assigned, and energy minimization was run using the Powell method with the maximum iterations set at 2000. The crystal structure of Estrogen Receptor (ER) α (PDB: 1x7r) complexed with genistein was obtained from PDB (Protein Data Bank-http://www.pdb.org). Docking mode was set to Surflex-Dock GeomX, all waters were removed from the complex structure, hydrogen atoms were added to ERα, and the protomol of ligand binding site was generated using genistein as reference. The virtual binding modes were visualized in the PyMol software (Schrödinger LLC, NY, USA). Amino acid residues within the distance of 5Å around the docked ligand were labeled.

2.7 GSK-3β inhibition activity assay

The GSK-3β kinase activity was measured using the ADP-Glo™ Kinase Assay System combined with GSK-3β Enzyme System (Promega, USA) in the absence or presence of compounds. Recombinant human GSK-3β was used as the kinase. The kinase substrate was a peptide derived from human muscle glycogen synthase 1 (YRRAAVPPSPSLSRH-SSPHQ(pS)EDEEE), which is related to a domain of glycogen synthase that is phosphorylated by GSK-3β [22]. Kinase activities were calculated as relative light units that directly correlated with the content of ADP produced. AZD2858, 8PG and genistein were dissolved in DMSO and diluted in kinase buffer to the tested concentrations (Final concentration: 0.1 nM-100000 nM). GSK-3β (10 ng) was first mixed with the tested compound, followed by the addition of the ATP/substrate mixture according to the manufacturer's instruction (Promega, USA). The reaction was terminated by the addition of ADP-Glo™ Reagent. Luminescence (RLU) was recorded with a microplate reader (BioTek Synergy H1, USA) upon incubation with Kinase Detection Reagent. The IC50 of GSK-3β kinase activity was calculated using Prism 5.0.

2.8 Western blotting analysis

MC3T3-E1 cells were treated with E2 (10^{-8} M), AZD2858 (a GSK-3 inhibitor

[23], 10^{-10} M), and different concentrations of 8PG and genistein in the presence or absence of different signaling pathway blockers. Treated cells were homogenized in RIPA lysis buffer containing 1% protease inhibitor cocktail (Roche, USA) and 1% phosphatase inhibitor cocktail (Roche, USA). Total protein of cell lysates was quantified with BCA protein assay kit (Beyotime Biotechnology, China). Equal amounts of protein (60-80 μg) in each sample were separated by 8.5 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidine fluoride (PVDF) membranes and probed with primary antibodies against β-catenin (1:1000 dilution, CST, USA), Phospho-β-catenin (Ser33/37/Thr41) (1:1000 dilution, CST, USA), LRP5 (1:1000 dilution，CST, USA), GSK-3β (1:500 dilution，CST, USA)， Phospho-GSK-3β (Ser9) (1:500 dilution, CST, USA), RUNX2 (1:1000 dilution，abcam, USA), β-tubulin (1:2000 dilution, CST, USA)，β-actin (1:2000 dilution, CST, USA), Akt (1:1000, CST, USA), Phospho-β-Akt (Ser473)(1:1000, CST, USA) and subsequently HRP AffiniPure Goat Anti-rabbit lgG (H+L) (1:5000 dilution, EarthOx, USA) as secondary antibodies. Proteins were detected with the enhanced chemiluminescence (ECL) detection kit (Bio-Rad, USA) and visualized by a Bio-Rad imaging system with Quantity One analysis software. Relative protein expressions were normalized to the β-tubulin or β-actin expression level.

2.9 Quantitative Real Time-PCR

Total RNA was extracted using an RNeasy kit (Qiagen, USA) according to the manufacturer's instructions. Reverse transcription was performed using PrimeScriptTM RT Master Mix (TaKaRa, Japan). The primer sequences used in the assay were listed in Supplementary Table 1. 10 μL of the final reaction solution contained 1 μL of the diluted cDNA product, 5 μL of 2×TB Green Premix Ex Taq II (TaKaRa, Japan), 0.5 μL each of forward and reverse primers and 4μl nuclease free water. The amplification conditions and procedures were as follows: 50℃ for 2 min, 95℃ for 10 min, 40 cycles of 95℃ for 15 sec, 60℃ for 1 min. Fluorescence signals were recorded by Roche Light Cycler 480 Detection System and then the signals were converted into numerical values. Relative gene expression was determined by

214 employing the Comparative CT-method. The mRNA levels of all genes were
215 normalized by β-actin as internal control. These analyses were performed in duplicate
216 for each sample using cells from two different cultured wells, and each experiment
217 was repeated two times.

218 **2.10 Statistical analysis**

219 The data were stated as mean ± SEM. Comparisons between groups were
220 performed by One-way analysis of variance (ANOVA), followed by post hoc Tukey's
221 test for multiple comparisons in GraphPad PRISM (San Diego, CA, USA) with
222 *P*-value < 0.05 considered statistically significant.
223

224 **3. Results**

225 **3.1 8PG significantly enhanced osteogenic activity of hBMSC and MC3T3-E1**
226 **cells**

227 The osteogenic activities of 8PG and genistein were determined using primary
228 hBMSC and MC3T3-E1 cells. Treatment of hBMSC with E2 (10^{-8} M) significantly
229 increased osteoblast differentiation (ALP activity, a crucial osteoblast differentiation
230 indicator) and mineralization (formation of calcified nodules) by nearly 1.5 to 2 folds.
231 Similarly, 8PG and genistein ($5*10^{-7}$ to 10^{-5} M) significantly increased ALP activity
232 by nearly 1.2 to 1.5 folds, and increased mineralization by nearly 2 to 4 folds. Overall,
233 8PG was more potent in enhancing osteogenic activity than genistein (Figs. 1A & 1B
234 & 1C). The effects of 8PG and genistein on ALP activity were also measured in
235 MC3T3-E1 cells (Fig. 1C). The results suggested that treatment with E2 (10^{-8} M) for
236 7 days significantly increased ALP activity in MC3T3-E1 cells by nearly 22%. 8PG at
237 10^{-6} M to 10^{-5} M had increased ALP activity in a dose-dependent manner by 17-61%;
238 while genistein at $5*10^{-6}$ to 10^{-5} M only had significantly increased ALP activity by

17-23% in MC3T3-E1 cells. The effects of 8PG and genistein on mineralization were determined in MC3T3-E1 cells and quantified by Alizarin Red S staining (Figs. 1D & E). E2 (10^{-8} M) significantly increased mineralization by 65% while 8PG and genistein at 10^{-6} M and 5×10^{-6} M significantly increased mineralization in MC3T3-E1 cells by 48-131%. Overall, the effects of 8PG at 5×10^{-6} M on mineralization was more potent than those of genistein in MC3T3-E1 cells.

3.2 The effects of 8PG on osteoblast differentiation were only mediated by ERα

ERs are known to mediate estrogenic actions in the skeletal system. To determine the selectivity of 8PG and genistein towards ERα and β in osteoblasts, the responses of MC3T3-E1 cells to treatment with 8PG or genistein were determined in the presence of ERα selective antagonist MPP (10^{-6} M) or ERβ selective antagonist PHTPP (10^{-6} M). The results showed that MPP, but not PHTPP, abolished the stimulatory effects of 8PG and genistein on ALP activity (Fig. 2A) and bone nodule formation (Figures 2B & C) in MC3T3-E1 cells. The results suggested that ERα, but not ERβ, was involved in the mediating of the effects of 8PG and genistein in osteoblastic cells. Our previous study demonstrated that 8PG and genistein could directly bind to ERα[24]. In order to determine the possible contributing molecular properties that could account for the differential binding affinities of 8PG and genistein towards ERα, molecular docking study was performed. As indicated in Fig. 2D, 8PG formed H-bonds with GLU353, ARG 394 and HIS 524 of ERα (1x7j), and the core scaffold of 8PG filled the remainder of the primary hydrophobic pocket. On the whole, the binding mode of 8PG to ERα was similar to that of genistein.

3.3 8PG did not directly inhibit GSK-3β activity but promoted the

phosphorylation of GSK-3β at Serine-9

To identify the possible targets of 8PG, *in silico* target profiling method based on similarity-searching in databases containing target-known ligands was employed [25]. GSK-3β, a key regulator of Wnt/β-catenin pathway, was one of the predicted targets that might mediate the actions of 8PG and genistein. To validate whether GSK-3β could be directly inhibited by 8PG and by genistein, the effects of 8PG and genistein on GSK-3β enzyme activities were determined. As shown in Fig. 3A, AZD2858, a GSK-3β inhibitor, suppressed GSK-3β enzyme activities in a dose-dependent manner and with an IC50 value of 106 nM. However, both 8PG and genistein failed to alter GSK-3β enzyme activities, suggesting that GSK-3β might not be the direct target of 8PG or genistein. As phosphorylation of GSK-3β at Serine 9 could also lead to activation of Wnt/β-catenin signaling pathways [19], the effects of 8PG and genistein on protein expression of p-GSK-3β (Ser 9) in MC3T3-E1 cells were determined (Figs. 3 B& C). 17-β-estradiol (E2, 10^{-8} M), 8PG (5×10^{-6} M) and genistein (5×10^{-6} M) significantly up-regulated the expression ratio of p-GSK-3β/GSK-3β. Most importantly, the induction of p-GSK-3β by 8PG appeared to be much higher than that by genistein.

3.4 8PG was more potent than genistein in activating Wnt/β-catenin pathway in MC3T3-E1 cells

Increased GSK-3β phosphorylation would lead to the activation of Wnt/β-catenin pathway. To investigate if Wnt/β-catenin signaling pathway participated in the mediating of the actions of 8PG and genistein on osteoblast differentiation, the expressions of the major proteins involved in this pathway, including LRP5, β-catenin,

phospho-β-catenin, Runx2, and target genes including osteocalcin, alp, and opg were determined (Fig. 4A). AZD2858, which inhibited GSK-3β, was used as positive control for the activation of Wnt/β-catenin signaling. The protein expressions of LRP5 and Runx2 were significantly up-regulated, while the phospho-β-catenin/β-catenin ratio was significantly down-regulated by AZD2858 (10^{-10} M) after treatment for 5 days in MC3T3-E1 cells (Fig. 4B). 8PG, but not genistein, significantly induced the protein expressions of LRP5 and Runx2 in MC3T3-E1 cells (Fig. 4B). Furthermore, expressions of Wnt signaling target genes, including osteocalcin, opg, and alp, were more significantly up-regulated by 8PG than by genistein after treatment for 3 and 6 days (Fig. 4C). Overall, 8PG significantly suppressed the phosphorylation of β-catenin, a wnt signaling-related downstream protein, and the wnt targeted gene expressions of osteocalcin, alp, and opg, which was more potent than that of genistein in MC3T3-E1 cells (Fig. 4B).

3.5 8PG activated Wnt signaling in a DKK1-dependent manner in MC3T3-E1 cells

DKK1 (Dickkopf-related protein-1), as a potent Wnt antagonist, can form ternary complex with LRP5/6 and Kremen, which specifically block the down-stream Wnt/β-catenin signaling pathway (22). In fact, Wnt signaling can be activated by inhibiting either Kremen or LRP5/6 (23). To further determine whether canonical Wnt signaling pathway is activated by 8PG and genistein via inducing the dissociation of this ternary complex, their effects in MC3T3-E1 cells in the presence of DKK1 were studied. As expected (Figs. 5A & B), DKK1 (150 ng/ml) significantly down-regulated

the protein expression of LRP5 and Runx2, and up-regulated the ratio of phospho-β-catenin/β-catenin in MC3T3-E1 cells. Both 8PG and genistein could restore or even further increase the expression of LRP5 and Runx2 in MC3T3-E1 cells pre-treated with DKK1. Moreover, the effect of 8PG on restoring the expression of LRP5 and RUNX2 was more potent than that of genistein. These results suggested that 8PG and genistein might activate Wnt signaling pathway which was associated with Dicckopf/Kremen/LRP5 complex in MC3T3-E1 cells.

3.6 Osteoblast differentiation induced by 8PG involved the cross-talk of Wnt/β-catenin and PI3K/Akt signaling in inducing GSK-3β phosphorylation

To further determine if Wnt/β-catenin pathway could cross-talk with ERα-associated PI3K/Akt/GSK-3β signaling, the protein expressions of LRP5, β-catenin, phospho-GSK-3β, GSK-3β, Akt and phospho-Akt were studied upon treatment with 8PG and genistein (at 5 x 10^{-6} M) in MC3T3-E1 cells pre-treated with either ERα blocker (MPP, 10^{-6} M) or PI3K pathway inhibitor (LY294002, 10^{-5} M). As indicated in Fig. 6A, 8PG at 5 x 10^{-6} M significantly increased β-catenin protein expression in MC3T3-E1 cells and the increase was abolished by co-treatment with MPP in which the expression of ERα was also suppressed. In contrast, neither ERα nor β-catenin protein expression were altered in MC3T3-E1 cells by treatment with genistein at 5 x 10^{-6} M in the presence or absence of MPP. The results suggested that the stimulatory effects of 8PG, but not those of genistein, on β-catenin expression were ERα–dependent. The stimulatory effects of 8PG and genistein on ALP activities were also abolished by co-treatment with LY294002 in MC3T3-E1 cells (Fig. 6B). Moreover, 8PG (5 x 10^{-6} M), genistein (5 x 10^{-6} M) and E2 (10^{-8} M) significantly

increased the protein expression of LRP5, the phospho-Akt/Akt ratio and the phospho-GSK-3β/GSK-3β ratio in MC3T3-E1 cells (Figs. 5C & D). These stimulatory effects were completely abolished by co-treatment with LY294002 in MC3T3-E1 cells (Figs. 6 C & D). The results suggested the involvement of PI3K pathway in mediating the effects of 8PG and genistein on ALP activities and Wnt signaling pathways in MC3T3-E1 cells.

4. Discussion

The osteoprotective effect of genistein as a nonsteroidal soy-derived phytoestrogen has been widely studied [2], but study on its prenylated derivative is limited. Our previous study demonstrated that 8PG prevented ovariectomy-induced bone loss in trabecular bone without any side effects on the uterus, and interestingly, its estrogenic actions were different from those of genistein [24]. However, interferences from other systemic regulation *in vivo* prevented us from investigating the signaling pathways mediated by 8PG in osteoblast differentiation independently. Therefore, the present study employed an *in vitro* platform in order to delineate the mechanism that might be involved.

The present study systematically characterized the effects of the 8-prenylated derivative of genistein (8-prenylgenistein, 8PG) on stimulating osteogenic activity of human BMSC and murine pre-osteoblast MC3T3-E1 cells. Our results indicated that 8PG appeared to be more potent than genistein in stimulating osteoblast differentiation and mineral nodule formation in osteoblastic cells. Numerous studies indicated that phytoestrogens could interfere with estrogen-dependent signal transduction cascades via direct binding to ERs, owing to their structural similarities to the mammalian estrogens [26]. Genistein, with the highest ER binding affinity

among isoflavones present in soybean, was shown to prevent ovariectomy-induced bone loss in rats and suppress osteoclastic differentiation via ER [2]. Although our previous publication demonstrated that 8PG had higher affinity than genistein towards ERβ [24], such property was unable to account for the potent osteogenic stimulatory effect of 8PG as bone protective effect was widely reported to be mediated by ERα [2]. In line with this notion, our present results confirmed that the ALP activity and mineral nodule formation stimulated by 8PG and genistein were abolished by ERα, but not by ERβ, antagonist. However, our previous study suggested that the binding affinity of 8PG and genistein toward ERα as well as their abilities to activate ERE-dependent transcription in human osteoscaroma MG63 cells were comparable [24]. The above results suggested there might be the participation of other non-ER dependent pathways in the regulating of the actions of 8PG that were responsible for its higher potency than genistein in exerting bone protective effects.

Wnt/β-catenin signaling pathway was found to be a key regulator of bone formation [15]. Indeed, GSK-3β, as a key mediator of Wnt/β-catenin signaling pathway, was predicted by *in silico* target profiling to be a target of 8PG and genistein. A previous study reported that targeted inactivation of GSK-3β increased bone mass in mice [27] and a variety of citrus flavonoids including luteolin, apigenin, and quercetin could inhibit GSK-3β activity via direct binding to the active site of the enzyme [28]. However, our study clearly showed that neither GSK-3β enzymatic activity nor expression in MC3T3-E1 cells were altered by treatment with 8PG or genistein. In contrast, our study showed that 8PG was more potent than genistein in inducing the expression of phospho-GSK-3β (Ser9) in MC3T3-E1 cells. Indeed, the induction of phosphorylation on GSK-3β at serine 9 residue is known to increase in the stabilization, accumulation, and nuclear translocation of β-catenin, which in turn

lead to the induction of osteoblast-related gene expression (including runx2 and alp), thereby increasing bone mass [29, 30]. Thus, our results suggested that 8PG and genistein could increase bone mass via the increase in GSK-3β phosphorylation, instead of inhibiting its enzymatic activities through direct binding to the active site. Moreover, our present study clearly showed that Wnt/β-catenin pathway was activated in the presence of 8PG and genistein in bone cells. 8PG was more potent than genistein in promoting the expression of upstream protein (LRP5), downstream proteins (β-catenin, Runx2) and osteoblastogenesis-related target genes (alp, opg, osteocalcin) in the Wnt signaling pathway in MC3T3-E1 cells. Indeed, Wnt/β-catenin signaling pathway has been reported to mediate the effects of soybean isoflavones (mixing with genistein, glycetin and daidzein) on osteoblast differentiation and proliferation[31]. Thus, it was not surprising to find that 8PG as well as genistein could induce osteoblast differentiation via the activation of Wnt/β-catenin signaling pathway. According to a previous literature, LRP5 was a co-receptor for Wnts, while Dkk1, as a wnt signaling antagonist, could inhibit Wnt signaling by acting in concert with its receptor Kremen to form a ternary complex with LRP5/6[32]. Our study showed that the inhibition of Wnt signaling by DKK1 could be restored by treatment with 8PG or genistein in MC3T3-E1 cells. More importantly, LRP5 in the cell membrane was down-regulated by 8PG. Although there were no published studies that directly demonstrated that expression of LRP5/6 was a target of Wnt3a, there were two published studies that indicated the protein level of LRP 5/6 was decreased with the treatment of DKK1[33, 34]. Thus, 8PG and genistein might activate Wnt/β-catenin pathway by dissociating the up-stream Dicckopf/Kremen/LRP5 complex, which promoted the accumulation of LRP5 and inhibited the formation of axin/APC/GSK-3β destruction complex, and subsequently led to phosphorylation of

GSK-3β at serine 9 residues. Such a mechanism of action was similar to that of isobavachin and bakuchiol, two reported compounds with prenylated group, which also exhibited bone protective activity[34]. However, 8PG was different from Lithium choloride (a known GSK-3β inhibitor). The latter had been reported to increase bone formation via activation of Wnt pathway independent of LRP5.[35]

The interaction between Wnt/β-catenin and PI3K/Akt signals was a novel mechanism reported to be involved in the regulation of osteoblast differentiation [36]. Indeed, the phosphorylation of GSK-3β was simultaneously regulated by signaling events of Wnt/β-catenin and PI3K/Akt pathway [37]. Wnt signaling could stimulate Akt and that activated Akt, in association with Dishevelled, enhance the phosphorylation of GSK3β in the Axin complex[19]. Our current results indicated that the stimulatory effect of 8PG on β-catenin protein expression in MC-3T3-E1 cells was significantly abrogated by co-treatment with an ERα selective antagonist. Furthermore, the increased expressions of LRP5, p-Akt/Akt and ALP activity by 8PG and genistein were completely abolished by co-treatment with a PI3K/Akt pathway inhibitor. Based on these findings, we postulated that 8PG regulated osteoblast differentiation through inducing Wnt/β-catenin signaling and ERα-associated non-genomic PI3K/Akt signaling simultaneously, which led to the increase in phosphorylation of GSK-3β at Serine-9 and subsequently led to stabilization, accumulation, and translocation of β-catenin from cytoplasm to the nucleus, the β-catenin binds with T-cell-specific transcription factor/lymphoid enhancer binding factor (LCF/LEF), and activates expression of osteoblast-related marker Runx2 and ALP. Indeed, the interactions among GSK-3β phosphorylation, ERα and β-catenin were previously reported in human breast cancer MCF-7 cells [38]. As reported, GSK-3β phosphorylation could lead to the release of ERα and translocation of ER

receptor into the nucleus, while β-catenin could promote the mRNA and protein expressions of ERα [38]. In addition, ERα was shown to interact directly with β-catenin in a ligand-independent manner but the interaction could be enhanced in the presence of estradiol [39]. In contrast to 8PG, genistein could only increase the PI3K-dependent expression of LRP5, p-Akt/Akt and GSK-3β phosphorylation, but not ERα-dependent β-catenin expression in MC3T3-E1 cells. Thus, the stronger effect of 8PG in comparison to genistein in inducing osteoblastic differentiation might be due to the stronger ability of 8PG to simultaneously induce Wnt/β-catenin and ERα-associated PI3K/Akt signaling pathways and to induce ERα-dependent expression and stabilization of β-catenin in osteoblastic cells (Figure 7).

5. Conclusion

The present study has clearly demonstrated that both 8PG and genistein could stimulate ERα-dependent osteoblastic differentiation as well as GSK-3β phosphorylation in osteoblastic cells. This study increases our understanding of the molecular actions of genistein derivatives in exerting bone sparing function and provides evidence to support their use in the management of bone health.

Acknowledgements

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Conflict of interests

The authors declare that there are no conflicts of interests.

Author contributions

Zuocheng Qiu: Conceptualization, Methodology, Investigation, Data Curation, Visualization, Writing-Original draft preparation. Mansau Wong: Conceptualization, Writing-Reviewing and Editing, Funding acquisition. Yang Zhang: Conceptualization, Writing-Reviewing and Editing. Huihui Xiao: Data Curation, Resources. Xiaoli li: Data Curation. Christina Chui-Wa Poon: Resources, Project administration. Jian-fang Cui: Resources, Investigation. Man-kin Wong: Resources, Methodology. Xin-sheng Yao: Supervision, Funding acquisition.

REFERENCES

[1] R. C. Poulsen, M. C. Kruger, Soy phytoestrogens: impact on postmenopausal bone loss and mechanisms of action, Nutrition reviews 66 (2008) 359-374.

[2] L. G. Ming, K. M. Chen, C. J. Xian, Functions and action mechanisms of flavonoids genistein and icariin in regulating bone remodeling, Journal of cellular physiology 228 (2013) 513-521.

[3] A. Atmaca, M. Kleerekoper, M. Bayraktar, O. Kucuk, Soy isoflavones in the management of postmenopausal osteoporosis, Menopause: The Journal of The North American Menopause Society 15 (2008) 748-757.

[4] S. Kanno, S. Hirano, F. Kayama, Effects of phytoestrogens and environmental estrogens on osteoblastic differentiation in MC3T3-E1 cells, Toxicology 196 (2004) 137-145.

[5] M. Heim, O. Frank, G. Kampmann, N. Sochocky, T. Pennimpede, P. Fuchs, W. Hunziker, P. Weber, I. Martin, I. Bendik, The phytoestrogen genistein enhances osteogenesis and represses adipogenic differentiation of human primary bone marrow stromal cells, Endocrinology 145 (2004) 848-859.

[6] P. Fanti, M. C. Monier-Faugere, Z. Geng, J. Schmidt, P. E. Morris, D. Cohen, H. H. Malluche, The Phytoestrogen Genistein Reduces Bone Loss in Short-Term Ovariectomized Rats, Osteoporos Int 8 (1998) 274-281.

[7] Y. Zhang, X. L. Li, X. S. Yao, M. S. Wong, Osteogenic activities of genistein derivatives were influenced by the presence of prenyl group at ring A, Archives of pharmacal research 31 (2008) 1534-1539.

[8] X. L. Wang, N. L. Wang, H. Gao, G. Zhang, L. Qin, M. S. Wong, X. S. Yao, Phenylpropanoid and flavonoids from osteoprotective fraction of Drynaria fortunei, Natural product research 24 (2010) 1206-1213.

[9] X. Chen, E. Mukwaya, M. S. Wong, Y. Zhang, A systematic review on biological activities of prenylated flavonoids, Pharmaceutical biology 52 (2014) 655-660.

[10] L. G. Ming, X. Lv, X. N. Ma, B. F. Ge, P. Zhen, P. Song, J. Zhou, H. P. Ma, C. J. Xian, K. M.

Chen, The prenyl group contributes to activities of phytoestrogen 8-prenynaringenin in enhancing bone formation and inhibiting bone resorption in vitro, Endocrinology 154 (2013) 1202-1214.
[11] Y. Zhang, X. L. Li, W. P. Lai, B. Chen, H. K. Chow, C. F. Wu, N. L. Wang, X. S. Yao, M. S. Wong, Anti-osteoporotic effect of Erythrina variegata L. in ovariectomized rats, Journal of ethnopharmacology 109 (2007) 165-169.
[12] M. X. Ho, C. C. Poon, K. C. Wong, Z. C. Qiu, M. S. Wong, Icariin, but Not Genistein, Exerts Osteogenic and Anti-apoptotic Effects in Osteoblastic Cells by Selective Activation of Non-genomic ERalpha Signaling, Frontiers in pharmacology 9 (2018) 474.
[13] M. M. Roforth, E. J. Atkinson, E. R. Levin, S. Khosla, D. G. Monroe, Dissection of estrogen receptor alpha signaling pathways in osteoblasts using RNA-sequencing, PloS one 9 (2014) e95987.
[14] J. G. Y. Maria P. Yavropoulou, The role of the wnt signaling pathway in osteoblast commitment and differentiation, HORMONES 6 (2007) 279-294.
[15] V. Krishnan, H. U. Bryant, O. A. Macdougald, Regulation of bone mass by Wnt signaling, The Journal of clinical investigation 116 (2006) 1202-1209.
[16] M. B. Kim, Y. Song, J. K. Hwang, Kirenol stimulates osteoblast differentiation through activation of the BMP and Wnt/beta-catenin signaling pathways in MC3T3-E1 cells, Fitoterapia 98 (2014) 59-65.
[17] S. E. Ross, N. Hemati, K. A. Longo, C. N. Bennett, P. C. Lucas, R. L. Erickson, O. A. MacDougal, Inhibition of Adipogenesis by Wnt Signaling, Science 289 (2000) 950-953.
[18] Y. Gao, E. Huang, H. Zhang, J. Wang, N. Wu, X. Chen, N. Wang, S. Wen, G. Nan, F. Deng, Z. Liao, D. Wu, B. Zhang, J. Zhang, R. C. Haydon, H. H. Luu, L. L. Shi, T. C. He, Crosstalk between Wnt/beta-catenin and estrogen receptor signaling synergistically promotes osteogenic differentiation of mesenchymal progenitor cells, PloS one 8 (2013) e82436.
[19] S. Fukumoto, C. M. Hsieh, K. Maemura, M. D. Layne, S. F. Yet, K. H. Lee, T. Matsui, A. Rosenzweig, W. G. Taylor, J. S. Rubin, M. A. Perrella , M. E. Lee, Akt Participation in the Wnt Signaling Pathway through Dishevelled, The Journal of biological chemistry 276 (2001) 17479-17483.
[20] N. Al-Maharik, N. P. Botting, Synthesis of lupiwighteone via a para-Claisen–Cope rearrangement, Tetrahedron 59 (2003) 4177-4181.
[21] Z. C. Qiu, X. L. Dong, Y. Dai, G. K. Xiao, X. L. Wang, K. C. Wong, M. S. Wong, X. S. Yao, Discovery of a New Class of Cathepsin K Inhibitors in Rhizoma Drynariae as Potential Candidates for the Treatment of Osteoporosis, International journal of molecular sciences 17 (2016).
[22] C. H. Lin , Y. S. Hsieh, Y. R. Wu, C. J. Hsu, H. C. Chen, W. H. Huang, K. H. Chang , H. M. Hsieh-Li , M. T. Su, Y. C. Sun, G. C. Lee, G. J. Lee-Chen, Identifying GSK-3beta kinase inhibitors of Alzheimer's disease: Virtual screening, enzyme, and cell assays, European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences 89 (2016) 11-19.
[23] G. Sisask, R. Marsell, A. Sundgren-Andersson, S. Larsson, O. Nilsson, O. Ljunggren, K. B. Jonsson, Rats treated with AZD2858, a GSK3 inhibitor, heal fractures rapidly without endochondral bone formation, Bone 54 (2013) 126-132.
[24] Y. Zhang, L. P. Zhou, X. L. Li, Y. J. Zhao, M. X. Ho, Z. C. Qiu, D. F. Zhao, D. K. W. Mok , Q. Shi, Y. J. Wang, M. S. Wong, 8-Prenylgenistein, a prenylated genistein derivative, exerted tissue selective osteoprotective effects in ovariectomized mice, Oncotarget 9 (2018) 24221-24236.
[25] E. Lounkine, M. J. Keiser, S. Whitebread, D. Mikhailov, J. Hamon, J. L. Jenkins, P. Lavan, E. Weber, A. K. Doak, S. Cote, B. K. Shoichet, L. Urban, Large-scale prediction and testing of drug activity on side-effect targets, Nature 486 (2012) 361-367.
[26] G. Kretzschmar, O. Zierau, J. Wober, S. Tischer, P. Metz, G. Vollmer, Prenylation has a compound

specific effect on the estrogenicity of naringenin and genistein, The Journal of steroid biochemistry and molecular biology 118 (2010) 1-6.
[27] R. Marsell, G. Sisask, Y. Nilsson, A. K. Sundgren-Andersson, U. Andersson, S. Larsson, O. Nilsson, O. Ljunggren, K. B. Jonsson, GSK-3 inhibition by an orally active small molecule increases bone mass in rats, Bone 50 (2012) 619-627.
[28] J. L. Johnson, S. G. Rupasinghe, F. Stefani, M. A. Schuler, E. Gonzalez de Mejia, Citrus flavonoids luteolin, apigenin, and quercetin inhibit glycogen synthase kinase-3beta enzymatic activity by lowering the interaction energy within the binding cavity, Journal of medicinal food 14 (2011) 325-333.
[29] N. H. Kulkarni, J. E. Onyia, Q. Zeng, X. Tian, M. Liu, D. L. Halladay, C. A. Frolik, T. Engler, T. Wei, A. Kriauciunas, T. J. Martin, M. Sato, H. U. Bryant, Y. L. Ma, Orally bioavailable GSK-3alpha/beta dual inhibitor increases markers of cellular differentiation in vitro and bone mass in vivo, Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 21 (2006) 910-920.
[30] S. C. Manolagas, Wnt signaling and osteoporosis, Maturitas 78 (2014) 233-237.
[31] F. Yu, Z. Liu, Z. Tong, Z. Zhao, H. Liang, Soybean isoflavone treatment induces osteoblast differentiation and proliferation by regulating analysis of Wnt/beta-catenin pathway, Gene 573 (2015) 273-277.
[32] R. Baron, M. Kneissel, WNT signaling in bone homeostasis and disease: from human mutations to treatments, Nature medicine 19 (2013) 179-192.
[33] B. Y. Mao, W. Wu, G. Davidson, J. Marhold, M. F. Li, B. M. Mechler, H. Delius, D. Hoppe, P. Stannek, C. Walter, A. Glink, C. Niehrs, Kremen proteins are Dickkopf receptors that regulate Wnt/b-catenin signalling, Nature 417 (2002) 664-667.
[34] Z. B. Weng, Q. Q. Gao, F. Wang, G. H. Zhao, F. Z. Yin, B. C. Cai, Z. P. Chen, W. D. Li, Positive skeletal effect of two ingredients of Psoralea corylifolia L. on estrogen deficiency-induced osteoporosis and the possible mechanisms of action, Molecular and cellular endocrinology 417 (2015) 103-113.
[35] P. Cle´ ment-Lacroix, M. Ai, F. Morvan, S. Roman-Roman, B. Vayssie` re, C. Belleville, K. Estrera, M. L. Warman, R. Baron, G. Rawadi, Lrp5-independent activation of Wnt signaling by lithium chloride increases bone formation and bone mass in mice, PNAS 102 (2005) 17406–17411.
[36] X. Yin, X. Wang, X. Hu, Y. Chen, K. Zeng, H. Zhang, ERbeta induces the differentiation of cultured osteoblasts by both Wnt/beta-catenin signaling pathway and estrogen signaling pathways, Experimental cell research 335 (2015) 107-114.
[37] K. Bhukhai, K. Suksen, N. Bhummaphan, K. Janjorn, N. Thongon, D. Tantikanlayaporn, P. Piyachaturawat, A. Suksamrarn, A. Chairoungdua, A phytoestrogen diarylheptanoid mediates estrogen receptor/Akt/glycogen synthase kinase 3beta protein-dependent activation of the Wnt/beta-catenin signaling pathway, The Journal of biological chemistry 287 (2012) 36168-36178.
[38] N. Gupta, F. Schmitt, S. Grebhardt, D. Mayer, beta-Catenin Is a Positive Regulator of Estrogen Receptor-alpha Function in Breast Cancer Cells, Cancers 3 (2011) 2990-3001.
[39] A. P. Kouzmenko, K. Takeyama, S. Ito, T. Furutani, S. Sawatsubashi, A. Maki, E. Suzuki, Y. Kawasaki, T. Akiyama, T. Tabata, S. Kato, Wnt/beta-catenin and estrogen signaling converge in vivo, The Journal of biological chemistry 279 (2004) 40255-40258.

Figure legends

Figure 1. The effects of 8PG and genistein on osteogenic activity of hBMSC and pre-osteoblast MC3T3-E1 cell. (A) The effects of 8PG and genistein on ALP activity of MC3T3-E1 cells; (B) Representative pictures and (C) the quantitation of calcified nodule of hBMSC. (D) The effects of 8PG and genistein on ALP activity of MC3T3-E1 cells; (E) Representative morphological images and (F) the quantitation of calcified nodule formation in MC3T3-E1 cells. The results were expressed as mean ± SEM of at least three independent experiments in triplicate. **P*<0.05, ***P*<0.01, ****P* < 0.001 vs. Ctrl. $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001 vs. genistein-treated group with the same concentration. Ctrl: control group.

Figure 2. The ER selectivity of 8PG and genistein for osteogenic activity and their binding modes toward ER α. (A-C) The effects of 8PG and genistein on stimulating osteogenic activity of MC3T3-E1 cells in the absence or presence of ERα-selective (MPP, 10^{-6} M), ERβ-selective (PHTPP, 10^{-6} M) antagonist, respectively. (A) ALP activity was measured upon treatment for 7 days; (B) Representative morphological pictures and (C) quantification of bone red-stained calcified nodules upon treatment for 21 days. Results were mean ± SEM (n=3), **P*<0.05, ***P*<0.01, ****P*<0.001 vs. Ctrl. $^{\#\#}P$<0.01, $^{\#\#\#}P$ <0.001 vs. no ER antagonist-treated group. (D) The binding mode of 8PG (yellow) and genistein (red) with the active site of the ERα crystal structure (PDB ID: 1x7r). The structures of compounds were represented in stick model with color. The protein was shown in the cartoon representation, the key residues in the active site were also represented as stick model with color (blue, red and gray), yellow dashed lines denote protein-ligand H-bonds.

Figure 3. (A) Inhibition curves of AZD2858, 8PG and genistein on GSK-3β kinase activity. Data are mean ± SEM. from five independent experiments in which each point was measured in quadruplicate. (B) Effect of 8PG and genistein on protein expression of GSK-3β and its phosphorylation form (Ser 9). MC3T3-E1 cells were treated with either E2 (10^{-8} M), 8PG (5×10^{-6} M) or genistein (5×10^{-6} M) for additional 24 h. Cells were collected and immunoblotted for the ratio of protein expression of p-GSK-3(Ser9)/GSK-3β using anti-p-GSK-3 (Ser9), anti-GSK-3β, and anti-β-actin antibodies. (B) Representative blots of three independent experiments and (C) the bar graph that quantified the protein bands used β-actin as a reference and normalized to

Ctrl group. The data were expressed as mean ± SEM. *P<0.05, **P<0.01, ***P< 0.001 vs. Ctrl. $^{\#}P$< 0.05 vs. genistein-treated group.

Figure 4. The effects of 8PG and genistein (G) on expression of proteins and genes involved in Wnt/β-catenin pathway of osteoblast differentiation. (A) Representative blots of Wnt signaling-related proteins after treatment for 5 days and (B) the bar graph that quantified used β-tubulin as a reference and normalized to Ctrl group (n=3). (C) mRNA expression levels of osteocalcin, alp, and opg were detected after treatment for 3 days and 6 days; target gene was normalized to Ctrl group using β-actin as a reference (n=4 from 2 repeated experiments). MC3T3-E1 cells were cultured and treated with AZD2858 (10^{-10} M), 8PG (10^{-6} M to 10^{-5} M) or genistein (10^{-6} M to 10^{-5} M). Data were expressed as mean ± SEM, *P<0.05, **P<0.01, ***P<0.001 vs. Ctrl. $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001 vs. Genistein-treated group with the same concentration.

Figure 5. The effects of 8PG and genistein (G) on restoring the inhibitory effects of DKK1 on Wnt signaling pathways in MC3T3-E1 cells. MC3T3-E1 cells were cultured in the presence or absence of DKK1 (0.15 μg/mL) and treated with either 8PG or genistein (10^{-6} M to 10^{-5} M) for 5 days. Proteins were extracted from cell lysates and probed with anti-LRP5, anti-β-catenin, anti-phospho-β-catenin, anti-Runx2 and anti-β-tubulin antibodies by immunoblotting. (A) Representative blots of three independent experiments and (B) the bar graph that quantified the protein bands using β-tubulin as a reference and normalized to Ctrl group. The data were expressed as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs. DKK1-alone treated group. $^{\#}P$ < 0.05 vs. genistein-treated group with the same concentration.

Figure 6. The differentiation effect of 8PG on osteoblast involving PI3K/Akt and Wnt/β-catenin signaling pathways. (A) Effect of 8PG and genistein on protein expression of ERα and β-catenin in MC3T3-E1 cells treated for 5 days with or without pretreatment with MPP (ERα-selective blocker, 10^{-6} M) for 2 h. (B) The intensities of the protein bands were quantified using β-tubulin as a reference and normalized to Ctrl group. (C) Effects of 8PG and genistein on ALP activity in MC3T3-E1 cells co-treated with LY294002 (selective PI3K inhibitor, 10^{-5} M). Cells were treated with Ctrl (1% ethanol as vehicle), E2 (10^{-8} M), 8PG (5×10^{-6} M) and G

(5×10^{-6} M) for 7 days with or without pretreatment of LY294002 (10^{-5} M) for 3 h. (D) Effect of 8PG and genistein on the protein expression ratio of LRP5/β-actin, phospho-β-Akt (ser473)/Akt, p-GSK-3β (Ser 9)/GSK3β after treatment for 24 h with or without pretreatment of LY294002(10^{-5} M) for 3 h. (E) The intensities of the protein bands were quantified using β-actin as a reference and normalized to Ctrl group. The blots were representatives of three independent experiments. The value bars represented mean ± SEM (n=3), $*P<0.05$, $**P<0.01$, $***P < 0.001$ vs. Ctrl. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. MPP or LY294002-treated group.

Figure 7. The schematic diagram of the postulated mechanism involved in the regulation of 8PG on osteoblast differentiation. 8PG induced phosphorylation of GSK-3β via activating ERα-associated PI3K/Akt and Wnt-dependent signaling pathways, then led to the stabilization, accumulation and translocation of β-catenin from cytoplasm to the nucleus, after that β-catenin binds to T-cell-specific transcription factor/lymphoid enhancer binding factor (LCF/LEF), finally activates the expression of osteoblast-related markers: Runx2, ALP, et al.

Figure 1

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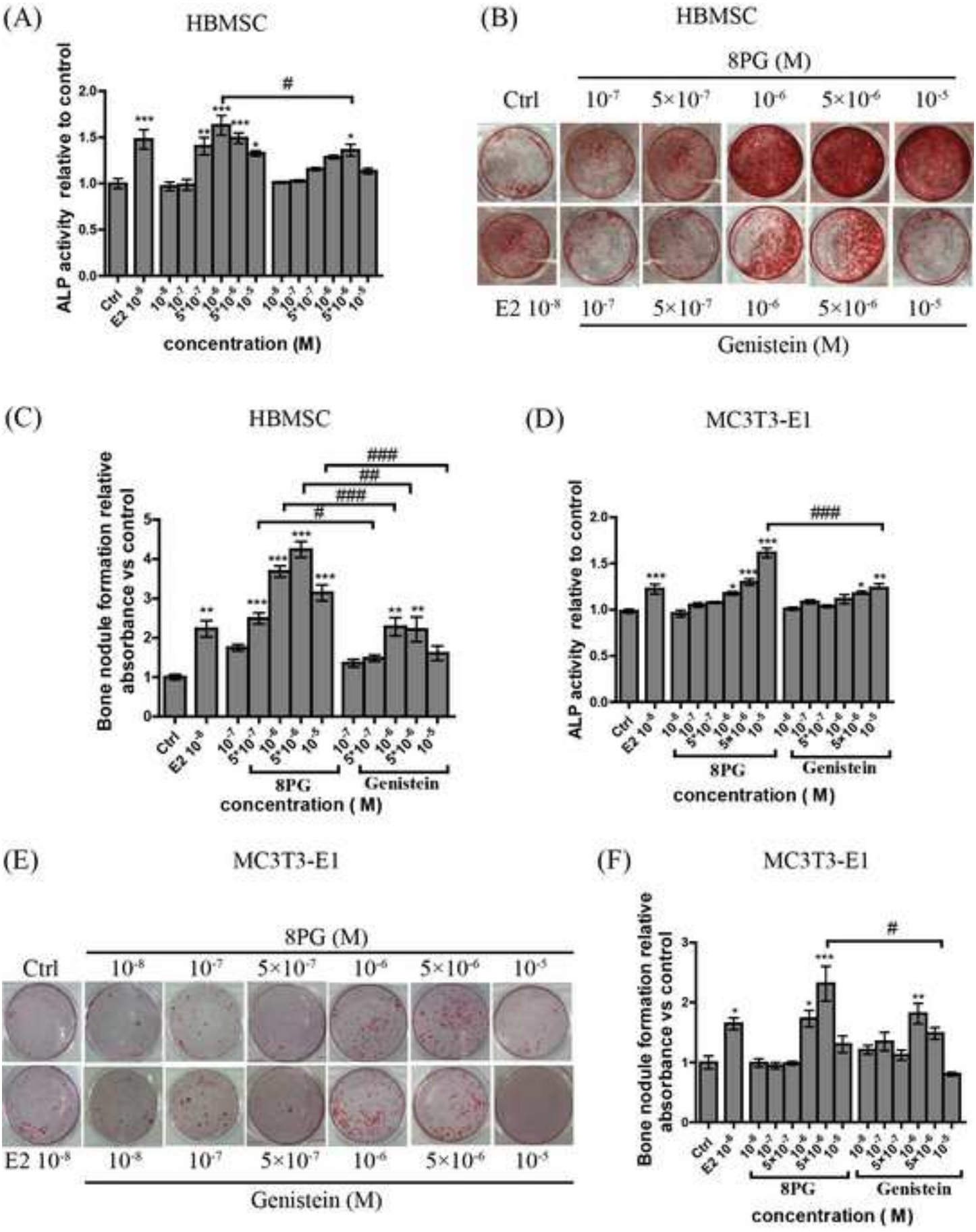

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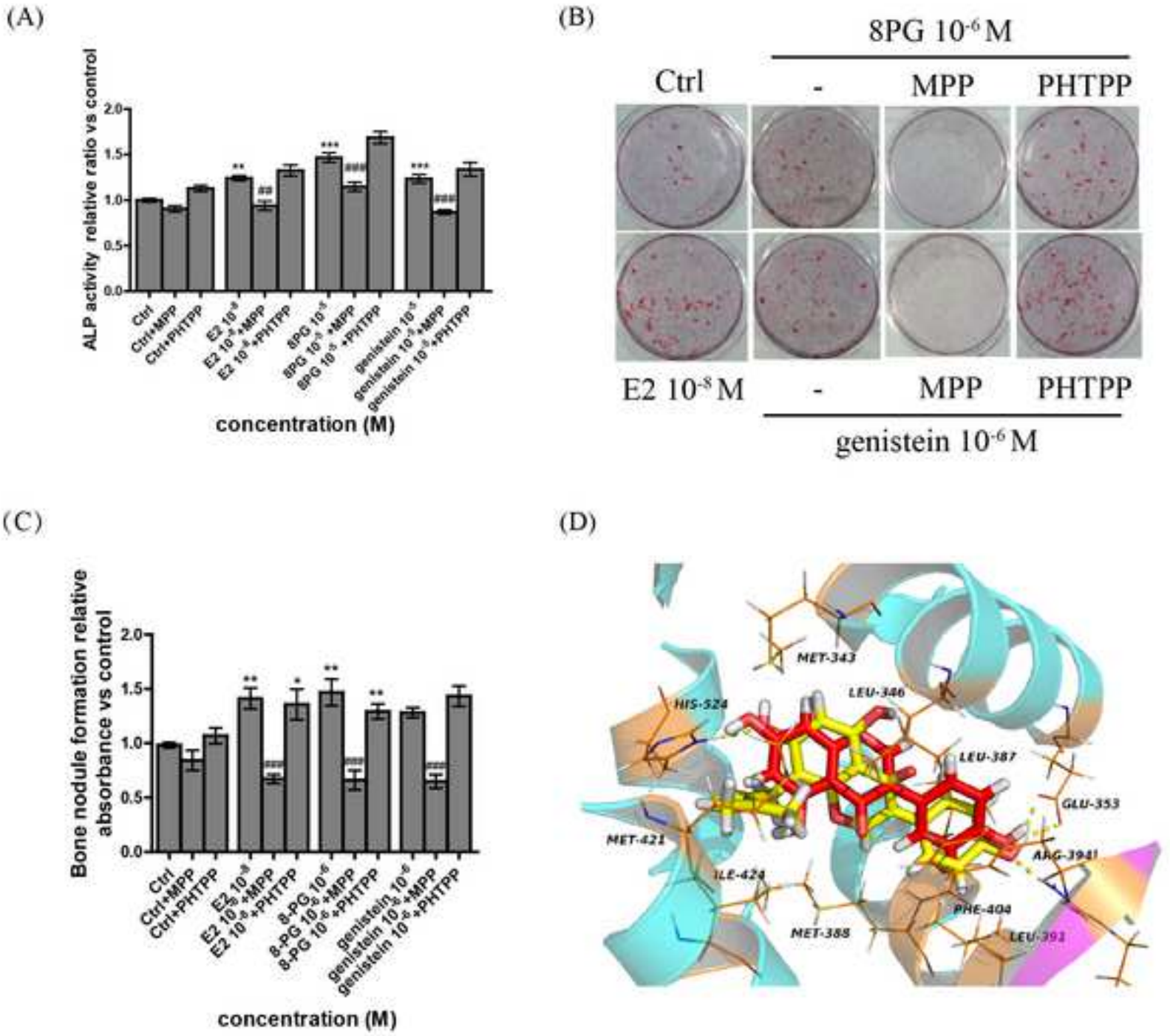

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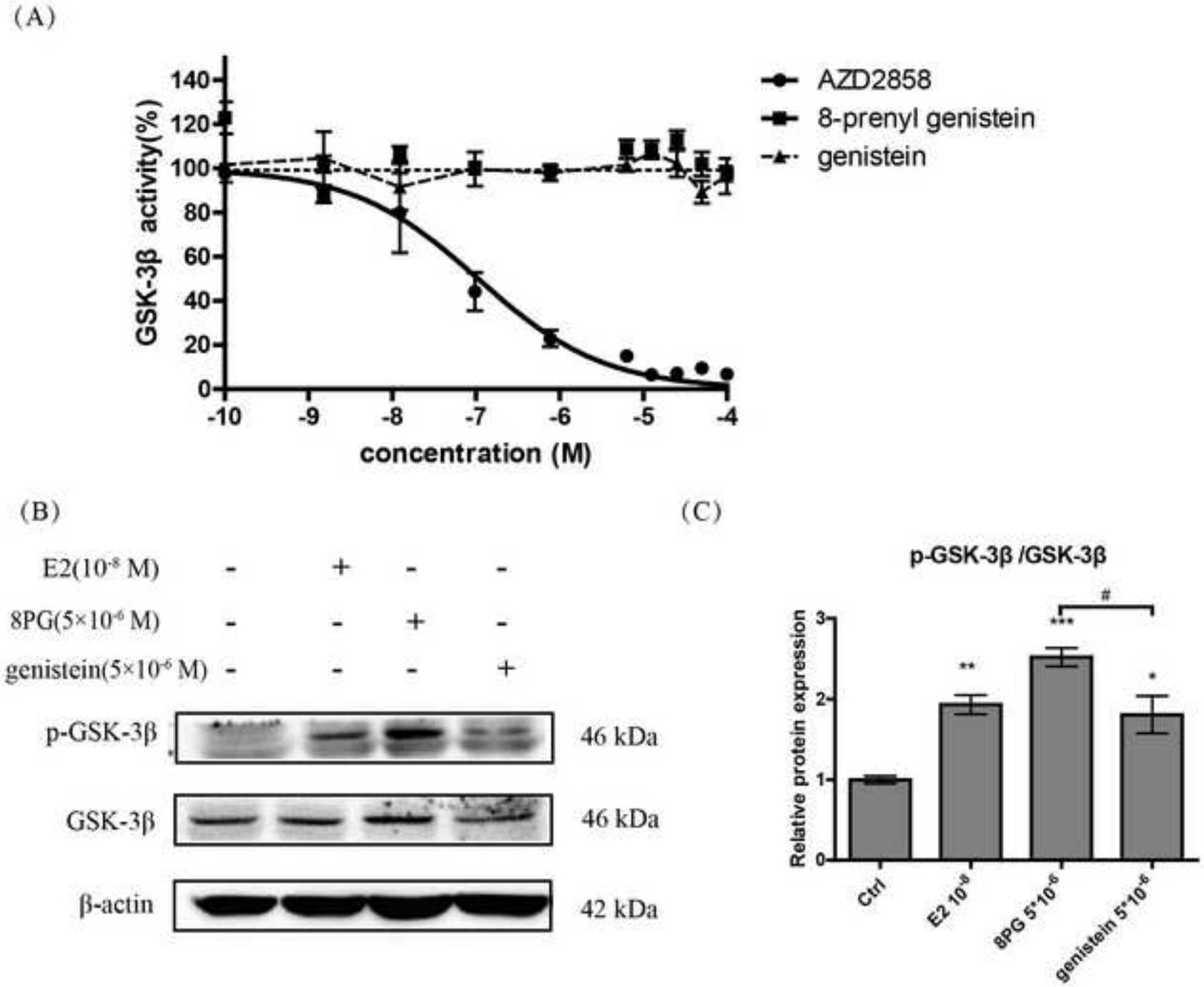

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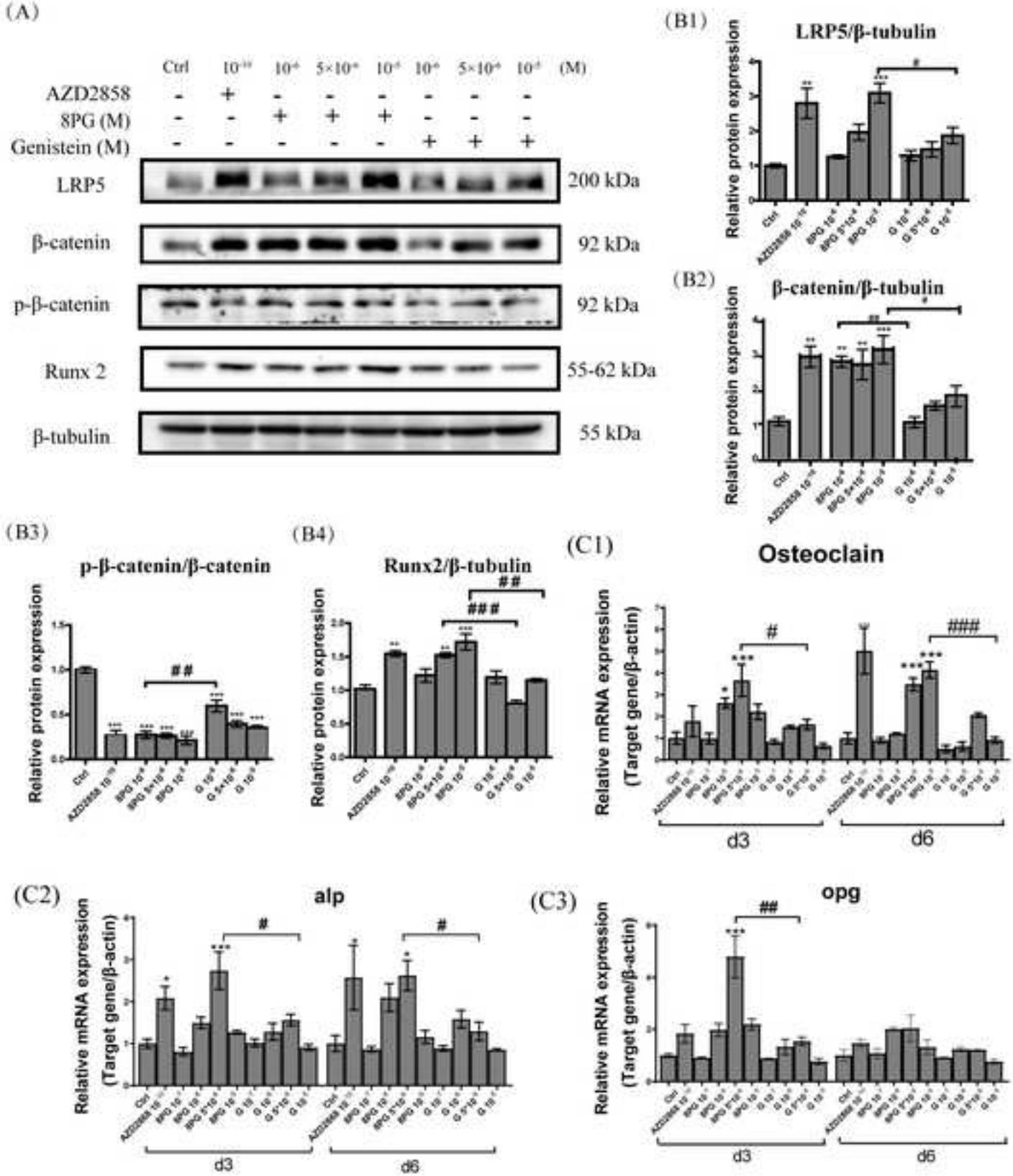

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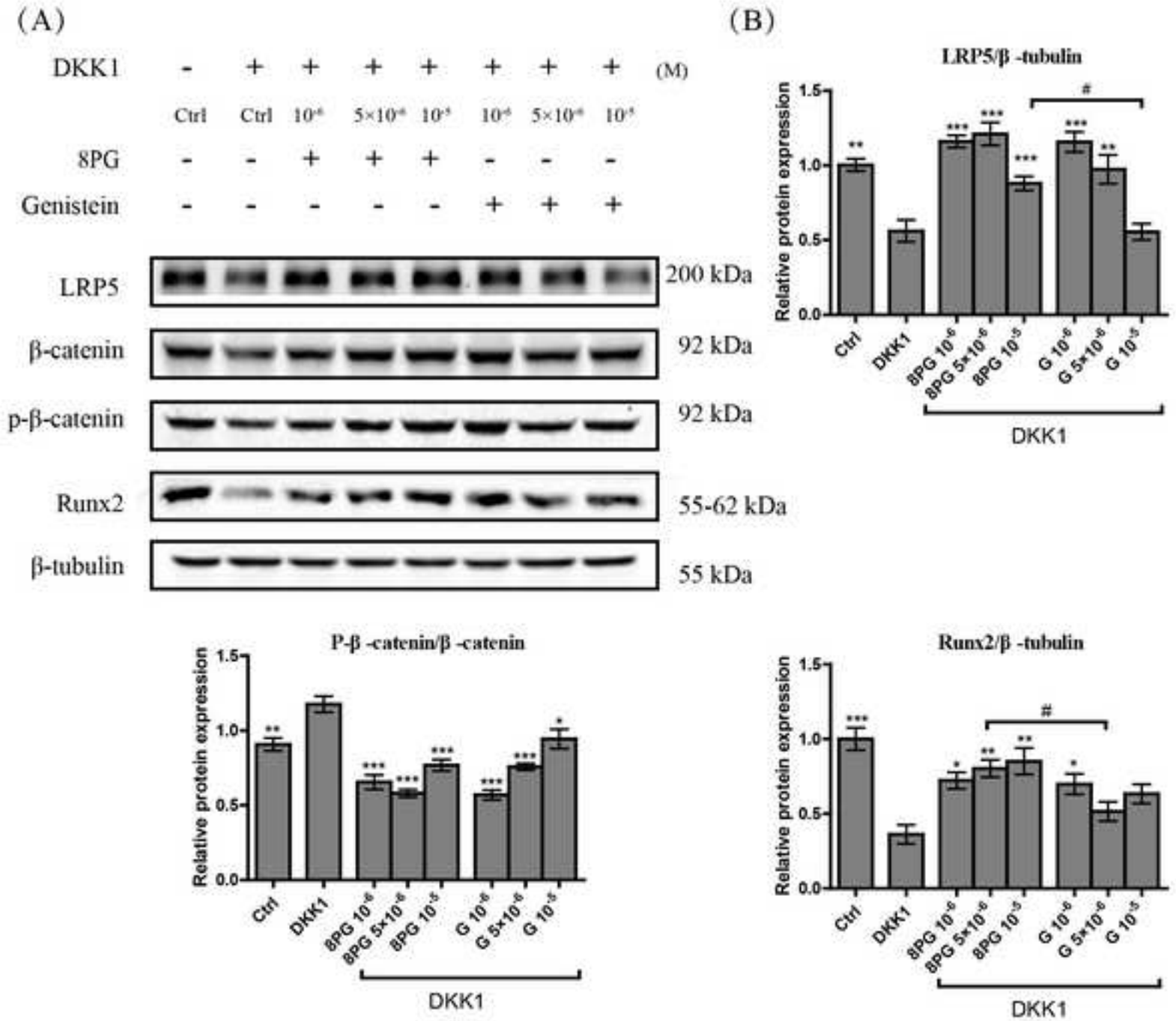

Figure 6

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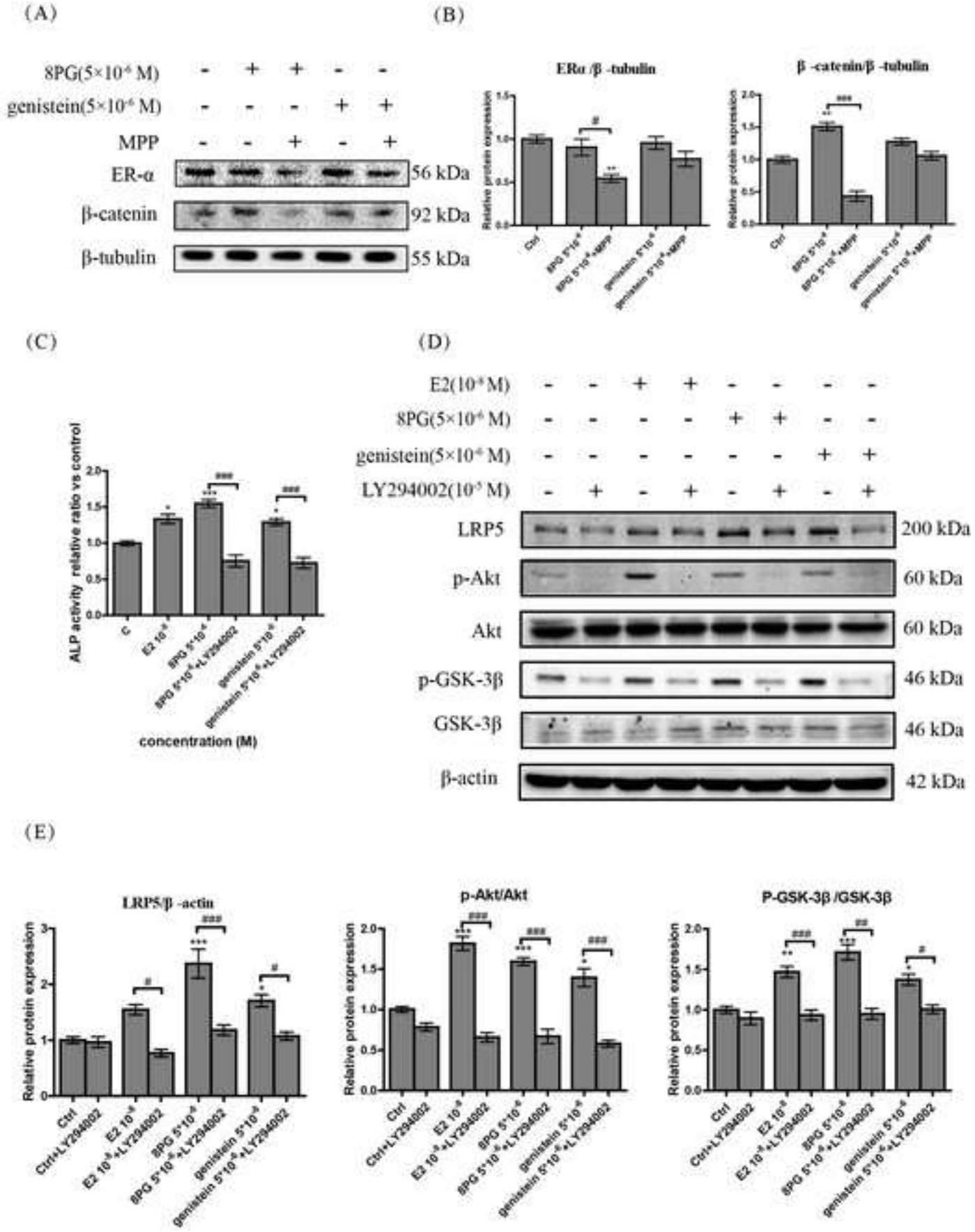

Figure 7

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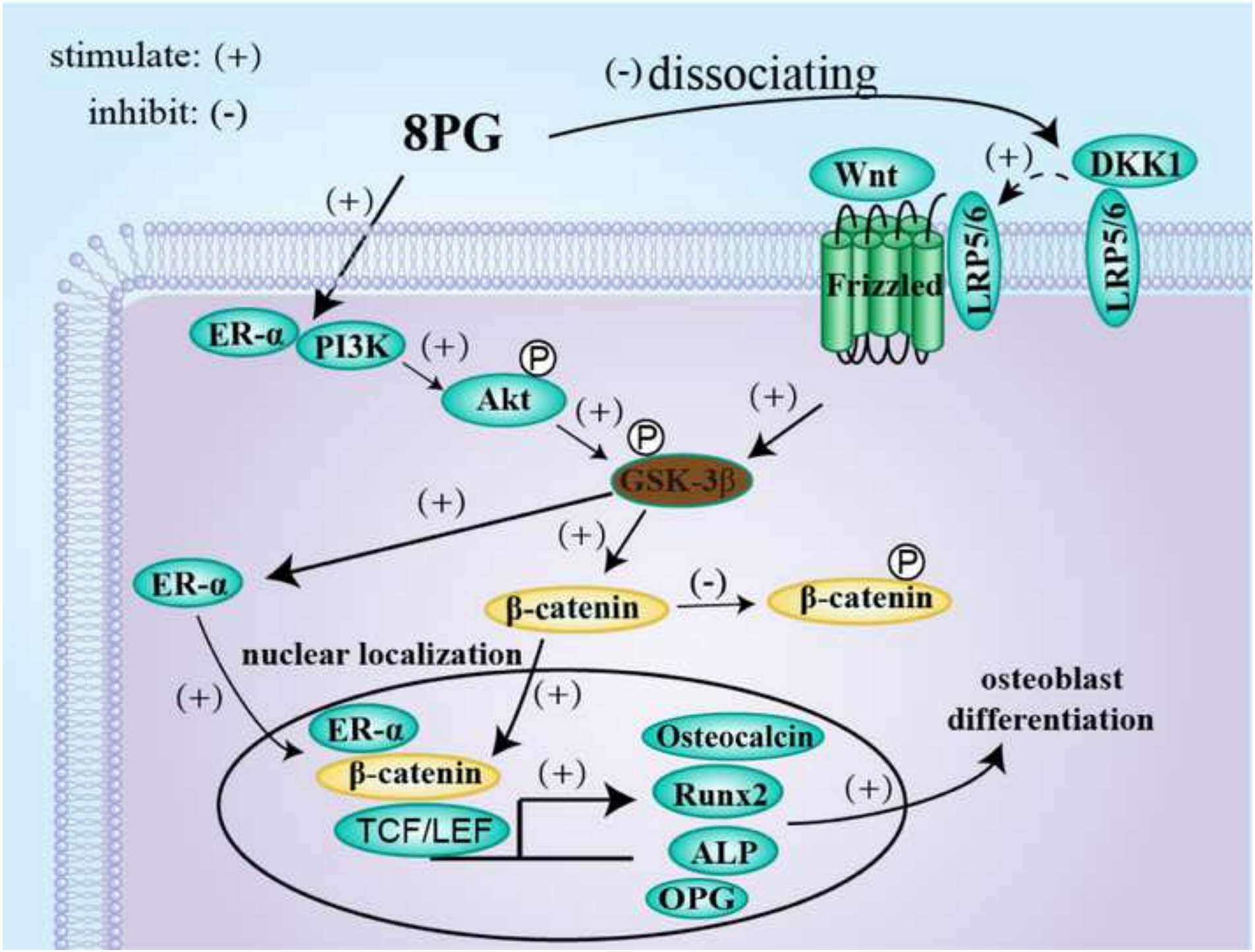

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Conflict of interest statement

Declarations of interest: none

Credit Author Statement

Zuocheng Qiu: Conceptualization, Methodology, Investigation, Data Curation, Visualization, Writing-Original draft preparation.
Mansau Wong: Conceptualization, Writing-Reviewing and Editing, Funding acquisition.
Yang Zhang: Conceptualization, Writing-Reviewing and Editing.
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Jian-fang Cui: Resources, Investigation.
Man-kin Wong: Resources, Methodology
Xin-sheng Yao: Supervision, Funding acquisition.