Glucagon-like peptide 2 decreases osteoclasts by stimulating apoptosis dependent on nitric oxide synthase

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Abstract

Objectives: Glucagon-like peptide 2 (GLP2) is involved in the regulation of energy absorption and metabolism. Despite the importance of the GLP2 signalling mechanisms on osteoclast, little has been studied on how GLP2 works during osteoclastogenesis.

Materials and Methods: RAW264.7 cells were infected with rLV-Green-GLP2. The induction of osteoclasts was performed by RANKL. TRAP were detected by RT-PCR, Western blotting and staining. Total nitric oxide and total NOS activity were measured. Cells apoptosis was detected by Hoechst33258 and Annex V staining. Animal test, chromatin immunoprecipitation (CHIP), co-immunoprecipitation (IP) and luciferase reporter assay were also performed.

Results: We indicate that GLP2 is associated with osteoporosis-related factors in aged rats, including BALP, TRAP, IL6, TNF α , Nitric Oxide (NO), iNOS, calcitonin and occludin. Moreover, GLP2 is demonstrated to result in negative action during proliferation of tartrate-resistant acid phosphatase-positive (TRAP+) osteoclasts. Furthermore, GLP2 decreases osteoclasts induced from monocyte/macrophage cells RAW264.7 as well as the serum TRAP activity in aged rats. Mechanistic investigations reveal GLP2 enhances the expression of iNOS through stimulating the activity of TGF β -Smad2/3 signalling in osteoclasts. In particular, inhibition of TGF β fully abrogates this function of GLP2 in osteoclasts. Strikingly, overexpression of GLP2 significantly increases the product of nitric oxide via iNOS which promotes apoptosis of osteoclasts by decreasing bcl2 or increasing caspase3. Thereby, the ability of GLP2 to regulate apoptosis depends on TGF β -Smad2/3-iNOS-NO signalling pathway since total NOS inhibitor L-NMMA specifically inhibits the actions by GLP2.

Conclusions: GLP2 induces apoptosis via TGF β -Smad2/3 signalling, which contributes to the inhibition of the proliferation of osteoclasts and which may provide potential therapeutic targets for the treatment of osteoporosis.

1 | INTRODUCTION

The osteoclast is a type of bone cell that is a large multinucleated cell and breaks down bone tissue which function is critical in the maintenance, repair and remodelling of bones.¹⁻³ Osteoclasts are characterized by a cytoplasm with a homogeneous, “foamy”

appearance with high expression of tartrate-resistant acid phosphatase (TRAP).⁴ Furthermore, osteoclast formation requires the presence of RANKL (receptor activator of nuclear factor κ B ligand) and M-CSF (Macrophage colony-stimulating factor).⁵ A report indicates that the number and activity of osteoclasts are strongly enhanced by myeloma cells, leading to significant bone lesions in patients with

multiple myeloma (MM).⁶ Two reports also indicate that Smad4 regulates apoptosis in osteocytes through a mitochondrial pathway⁷ and Musashi2 is required for cell survival and optimal osteoclastogenesis by affecting Notch signalling and NF- κ B activation.⁸ Thus, osteoclasts are regulated by many factors.

Glucagon-like peptide 2 (GLP2) is a peptide hormone of the glucagon family produced by the L cells of the intestinal mucosa and originates from the same prohormone as glucagon. GLP2 acts as a beneficial factor for glucose metabolism in mice with high-fat diet-induced obesity.⁹ Moreover, GLP2 agonists decrease the need for parenteral nutrition (PN) in short bowel syndrome (SBS)¹⁰ and the absence of a motif in GLP2 could be the reason for a significantly lower strength of interaction between GLP2 and heparin in inducing protein aggregation.¹¹ In particular, alteration of the intestinal barrier and GLP2 secretion was found in Berberine-treated type 2 diabetic rats.¹² On the other hand, the evidence that suppressor of cytokine signalling protein is induced by GLP2 in normal or inflamed intestine may limit IGF1-induced growth, but protect against risk of dysplasia or fibrosis.¹³ However, little has been studied the functions of glucagon-like peptide 2 (GLP2) during osteoclastogenesis.

The transforming growth factor (TGF- β) signalling plays a key role in the temporal and spatial regulation of bone remodelling and disruptions to the TGF- β signalling pathway lead to loss of skeletal integrity.¹⁴ Studies have shown that TGF- β promotes receptor activator of nuclear factor- κ B ligand (RANKL)-induced osteoclastogenesis and TGF- β is indispensable in RANKL-induced osteoclastogenesis.¹⁵ Recent studies have shown that the TGF- β superfamily members TGF- β 1, myostatin and activin A directly regulate osteoclast differentiation through mechanisms that depend on the RANKL-RANK interplay and transduce their signals through type I and II receptor serine/threonine kinases, thereby activating the Smad pathway.¹⁶ Therefore, we speculate that GLP2 may play a role during osteoclastogenesis through the transforming growth factor (TGF- β) signalling. The aim of this study was to explore the role of GLP2 in the control of proliferation and apoptosis of osteoclast.

2 | MATERIAL AND METHODS

2.1 | Cell lines and lentivirus

Mouse RAW264.7 cell line was obtained from the Cell Bank of FuDan IBS Cell Center (Shanghai, China). These cell lines were maintained in α -Minimum Essential Medium (α -MEM) medium supplemented with 10% foetal bovine serum (Gibco BRL Life Technologies) in a humidified atmosphere of 5% CO₂ incubator at 37°C. Lentivirus rLV-Green and rLV-Green-GLP2 was purchased from Wu Han viral therapy Technologies Co. Ltd.

2.2 | Cell infection and stable cell lines

RAW264.7 cells were infected with rLV-Green and rLV-Green-GLP2, respectively, according to manufacturer's instructions. We selected

the single cell clone with overexpressing GLP2 to establish the stable cell lines. Transfection efficiency was observed by Green imaging and measured by Western blotting.

2.3 | Differentiation from RAW264.7 to osteoclast cells

The differentiation from RAW264.7 to osteoclast cells were preformed by 2-10 ng/mL RANKL according to the manufacturer instruction (R&D). TRAP were detected by RT-PCR, Western blotting and staining in these cells.

2.4 | Glucagon-like peptide-2 (GLP2) and Total NOS inhibitor

Total NOS inhibitor (L-NMMA) was purchased from Beyotime and was preformed according to the manufacturer's instructions. L-NMMA inhibits nNOS, eNOS and iNOS. The cells were treated with 4 μ mol L⁻¹ L-NMMA for 72 hours at least. Glucagon-like peptide-2 (GLP2) was purchased from ProSpec and preformed according to the manufacturer's instructions. The cells were treated with 1 μ mol L⁻¹ GLP2 for 72 hours at least.

2.5 | TRAP staining

The TRAP staining kit (Sigma) was adopted according to the manufacturer's instructions. Slide is fixed by immersing in Fixative solution for 30 seconds and rinse thoroughly in deionized water. Then, to each of 2 test tubes add 0.5 mL fast Garnet GBC base solution and add 0.5 mL sodium nitrite solution and mix by gentle inversion for 30 seconds (Let stand 2 minutes). Next, labelling two 100 mL beakers A and B, and add the related reagent while mixing. Labelling Coplin jars A and B, and then transfer solutions from beakers to appropriate Coplin jar. Then, slide is added to Coplin jars and incubate 1 hour in 37°C water bath protected from light. After 1 hour, slide is thoroughly rinsed in deionized water. Ultimately, air dry and evaluate microscopically.

2.6 | Total nitric oxide assay

Total nitric oxide assay kit was purchased from Beyotime and was preformed according to the manufacturer's instructions. The standard and sample were added to 96-well plates at 50 μ L/well. Griess Reagent I was added to each well at 50 μ L/well at 50 μ L/well in 96-well plates at room temperature, and then Griess Reagent II was added to each well at 50 μ L/well at room temperature. Ultimately, absorbance was measured at 540 nm.

2.7 | Nitric oxide synthase assay

Total synthase (nNOS, eNOS, iNOS) assay kit was purchased from Beyotime and was preformed according to the manufacturer's instructions.

2.8 | Reverse-transcriptase polymerase chain reaction

Total RNA was purified using Trizol (Invitrogen) according to manufacturer's instructions. cDNA was prepared by using oligonucleotide (dT)₁₈ and a SuperScript First-Strand Synthesis System (Invitrogen). β -actin as internal control. The PCR amplification kit (TaKaRa) was adopted according to the manufacturer's instructions.

2.9 | Western blotting

Cells lysates were centrifuged at 12,000 g for 20 minutes at 4°C after sonication on ice, and supernatants were separated. After being boiled for 10 minutes in the presence of 2-mercaptoethanol, samples containing cells or tissue lysate proteins were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membranes were stained and then blocked in 10% dry milk-TBST for 1 hour at 37°C. Following 3 washes in TBST, the blots were incubated with antibody (appropriate dilution) overnight at 4°C. Following 3 washes, membranes were then incubated with secondary antibody for 60 minutes at 37°C. Signals were visualized by ECL. β -actin as internal control.

2.10 | Chromatin immunoprecipitation(CHIP)

Cells were cross-linked with 1% (v/v) formaldehyde for 10 minutes at room temperature and stopped with 125 mmol L⁻¹ glycine for 10 min. Crossed-linked cells were washed with phosphate-buffered saline, resuspended in lysis buffer and sonicated. Chromatin extracts were pre-cleared with Protein-A/G-Sepharose beads, and immunoprecipitated with specific antibody on Protein-A/G-Sepharose beads. After washing, elution and de-cross-linking, the CHIP DNA was detected by PCR.

2.11 | Co-immunoprecipitation (IP)

Protein was pre-cleared with 30 μ L protein G/A-plus agarose beads (Santa Cruz, Biotechnology, Inc., CA, USA) for 1 hour at 4°C and the supernatant was obtained after centrifugation (3000 g) at 4°C. Pre-cleared homogenates (supernatant) were incubated with 2 μ g of antibody and/or normal mouse/rabbit IgG with rotation for 4 hours at 4°C. The immunoprecipitates were incubated with 30 μ L protein G/A-plus agarose beads by rotation overnight at 4°C, and then centrifuged at 3000 g for 5 minutes at 4°C. The precipitates were washed 5 times for 10 minutes with beads wash solution, resuspended in 80 μ L 2 $\times$ SDS-PAGE sample loading buffer. Western blotting was performed with related antibodies.

2.12 | Luciferase reporter assay

Cells were transfected with luciferase construct plasmids and pRL-tk. After incubation for 48 hours, the cells were harvested with Passive Lysis Buffer (Promega), and luciferase activities of cell extracts were

measured with the use of the Dual luciferase assay system (Promega) according to manufacturer's instructions.

2.13 | Cells apoptosis assay

Hoest 33258 staining, Annex V-PI staining and DNA ladder electrophoresis according to manufacturer's instructions.

2.14 | Rats treated with GLP2 in vivo

The 16 cases of 22- to 26-month-old female Sprague Dawley rats were injected at abdominal cavity with GLP2 dissolved in PBS (100 μ g/kg) or PBS twice a week for 1 month. Then the rats were observed 2 weeks, and then sacrificed to detect the tissues and blood, including serum BALP, TRAP, TNF α , IL6, TGF β , NO, iNOS and calcitonin and occludin. The use of mice for this work was reviewed and approved by the institutional animal care and use committee in accordance with China national institutes of health guidelines. Histopathology analysis of cartilage was performed by HE staining.

2.15 | Statistical analysis

The significant differences between mean values obtained from at least 3 independent experiments. Each value was presented as mean $\pm$ standard error of the mean (SEM) unless otherwise noted, with a minimum of 3 replicates. Student's *t* test was used for comparisons, with *P* < .05 considered significant.

3 | RESULTS

3.1 | GLP2 is associated with osteoporosis-related factors in aged rats

To validate whether GLP2 affects on physiological functions of aged SD rats, the 8 rats were treated with GLP2 (100 μ g/kg) (GLP2 group) and the other 8 rats were treated with PBS (control). As shown in Figure 1A, the differentiation of cartilage was well grade and cartilage is composed of more chondrocytes which are closer together creating less intercellular space in GLP2 group compared to control ($28.17 \pm 5.11\%$ vs $53.96 \pm 8.62\%$, *n* = 8, *P* = .000007 < .01). Moreover, the body weight of rat was significantly increased in GLP2 group compared to control (566.38 ± 76.98 g vs 796 ± 142.50 g, *n* = 8, *P* = .00617 < .01) (Figure 1B). The serum total proteins of the rats was significantly higher in GLP2 group compared to control (37.71 ± 6.92 vs 64.38 ± 12.34 , *P* = .0006 < .01) (Figure 1C). Intriguingly, serum BALP of the rats was significantly increased in GLP2 group compared to control (1.08 ± 0.283 vs 2.77 ± 0.512 , *P* = .000086 < .01) (Figure 1D). And serum TRAP of the rats was significantly decreased in GLP2 group compared to control (1.24 ± 0.46 vs 0.41 ± 0.11 , *P* = .00064 < .01) (Figure 1E). In addition, serum TGF β of the rats was no significantly altered in the 2 groups (0.001445 ± 0.00053 nmol/L vs 0.00154 ± 0.000415 nmol/L, *P* = .3331 > .05) (Figure 1F). However, serum IL6 of the rats were significantly decreased in

FIGURE 1 The analysis of GLP2 action in aged rats. **A.** The histopathology analysis of cartilage. Chondrocytes were measured in cartilage. Each value was presented as mean $\pm$ standard error of the mean (SEM) (Student's *t* test). **B.** Body weight (gram) of the rats (control group and GLP2 group). **C.** The total serum proteins of the rats (control group and GLP2 group). **D.** The assay of serum BALP of the rats (control group and GLP2 group). **E.** The assay of serum TRAP of the rats (control group and GLP2 group). **F.** The assay of serum TGF β of the rats (control group and GLP2 group). **G.** The assay of activity of serum IL6 of the rats (control group and GLP2 group). **H.** The assay of activity of serum TNF α of the rats (control group and GLP2 group). **I.** The assay of serum total Nitric Oxide (NO) of the rats (control group and GLP2 group). **J.** RT-PCR analysis of iNOS mRNA, calcitonin mRNA and occludin mRNA in the rats liver (control group and GLP2 group). β -actin as internal control. **K.** Western blotting with anti-iNOS, anti-calcitonin and anti-occludin in the rats liver (control group and GLP2 group). β -actin as internal control. Each value was presented as mean $\pm$ standard error of the mean (SEM). Bar $\pm$ SEM. ***P* < .01; **P* < .05. For all assays, eg, Western blotting, RT-PCR, apoptosis assay, CHIP, IP, luciferase assay, we repeated the experiments for 3 times at least. We measured gray value of the bands for quantification. Each value was presented as mean $\pm$ standard error of the mean (SEM) (Student's *t*-test)

GLP2 group compared to control (0.0605 ± 0.0153 pmol/L vs 0.01398 ± 0.0035 pmol/L, *P* = .00016 < .01) (Figure 1G). Moreover, serum TNF α of the rats were significantly decreased in GLP2 group compared to control (0.0021375 ± 0.00086 pmol/L vs 0.000295 ± 0.000089 pmol/L, *P* = .00031 < .01) (Figure 1H). In particular, serum Nitric Oxide(NO) of the rats was significantly increased in GLP2 group compared to control (0.006875 ± 0.00156 vs 0.01521 ± 0.0045 , *P* = .044198 < .05) (Figure 1I). On the other hand, mRNAs of iNOS, calcitonin and occludin in the rats liver was significantly increased in GLP2 group compared to control (Figure 1J) and the translational level of iNOS, calcitonin and occludin in the rats was significantly increased in GLP2 group compared to control (Figure 1K). These observations suggest that GLP2 is associated with osteoporosis-related factors in aged rats.

3.2 | GLP2 could decrease the osteoclasts induced from RAW264.7 cells

To explore whether the GLP2 impacts on the growth of osteoclasts, we prepared the rLV-GLP2 lentivirus and then carried out related experiments according to the schematic diagram illustrates a model of osteoclasts induced from mouse RAW264.7 cells with RANKL (Figure 2Aa). At the first time, we established the 2 stable RAW264.7 cell lines infected with rLV, rLV-GLP2 respectively. The Green expression was positive in 2 stable RAW264.7 cell lines (Figure 2Ab). On the level of both transcription and translation, GLP2 was significantly overexpressed in RAW264.7 infected with rLV-GLP2 compared to the control (Figure 2Ac&d). Then, we detected TRAP activity in the 2 stable cell lines. As shown in Figure 2Ba, At the fourth and fifth days of differentiation induced by RANKL, the TRAP activity was significantly decreased in rLV-GLP2 group compared to control (the fourth day: 1.68 ± 0.3027 vs 0.2767 ± 0.085 , *P* = .01; the fifth days: 3.573 ± 0.5301 vs 0.71 ± 0.15 , *P* = .004434 < .01). On the other hand, the results of the RT-PCR and Western blotting showed that both TRAP mRNA and TRAP protein were significantly decreased in rLV-GLP2 group compared to control (Figure 2Bb). The formation rate of osteoclast was significantly decreased in rLV-GLP2 group compared to rLV-Green group (the fourth day: $33.12 \pm 5.58\%$ vs $7.79 \pm 1.61\%$, *P* = .0129 < .05; the fifth day: 75.27 ± 8.78 vs 1.70 ± 0.57 , *P* = .0024 < .01) (Figure 2C). In particular, the TRAP staining positive rate was $80.57 \pm 8.14\%$ in rLV-Green group, as

well as the TRAP staining positive rate was $4.32 \pm 1.14\%$ in rLV-GLP2 group (*P* = .0022 < .01) (Figure 2D). Furthermore, RAW264.7 was differentiated into osteoclasts and then the osteoclasts were treated with GLP2 ($1 \mu\text{mol L}^{-1}$) for 3 days. The results showed that osteoclasts were significantly decreased in GLP2 group compared to control after 3 days (Figure 2E). Together, these findings suggest that GLP2 decreased the growth ability of osteoclasts.

3.3 | GLP2 aggravates the apoptosis of osteoclasts

Given that GLP2 decreases the osteoclasts induced from RAW264.7 cells, we consider whether GLP2 triggers the apoptosis of osteoclasts. As expected, by staining of Hoechst33258 in OC cells lines infected with rLV-Green or rLV-Green-GLP2, respectively, we found that excessive GLP2 significantly promoted the apoptosis of osteoclasts (Figure 3Aa). The apoptosis rates was $55.89 \pm 9.14\%$ in rLV-Green-GLP2 group, as well as the apoptosis rates was $6.65 \pm 1.76\%$ in rLV -GLP2 group (*P* = .0037 < .01) (Figure 3Ab). On the other hand, the apoptosis assay via Ladder electrophoresis in osteoclasts showed that excessive GLP2 significantly promoted the apoptosis of osteoclasts (Figure 3B). Furthermore, RAW264.7 was differentiated into osteoclasts using RANKL (10 ng/mL) and then the osteoclasts were treated with GLP2 ($1 \mu\text{mol L}^{-1}$) for 3 days. As shown in Figure 3C(a,b,c), GLP2 promoted the apoptosis of osteoclasts either in the secondary days after CLP2 treatment ($4.98 \pm 1.71\%$ vs $59.2 \pm 9.53\%$, *P* = .0059 < .01) or in the third days after CLP2 treatment ($23.6 \pm 4.96\%$ vs $51.5 \pm 10.41\%$, *P* = .0081 < .01). Collectively, these observations suggest that GLP2 promotes the apoptosis of osteoclasts.

3.4 | GLP2 activates the TGF β -Smad2/3 signalling

To demonstrate whether GLP2 induced apoptosis was associated with TGF β -Smad2/3 signalling, we performed related analysis in osteoclasts cell lines, including rLV-Green group and rLV-Green-GLP2 group, respectively. As shown in Figure 4A, the results of Co-Immunoprecipitation(IP) showed that interaction between TGF β RII and SARA was significantly enhanced in rLV-Green-GLP2 group compared to rLV-Green group. Moreover, TGF β RII and GLP2 was significantly enhanced in rLV-Green-GLP2 group compared to rLV-Green group. Furthermore, p3TP-Luc reporter gene (a TGF β

FIGURE 2 GLP2 could decrease the osteoclasts induced from RAW264.7 cells **A. a.** The schematic diagram illustrates a model of osteoclast induced from mouse RAW264.7 cells with RANKL. **b.** The photography of the RAW264.7 cell lines infected with rLV-Green or rLV-Green-GLP2. **c.** Western blotting with anti-GLP2 in RAW264.7 infected with rLV-GLP2 or rLV. β -actin as internal control. **d.** Western blotting with anti-GLP2 in induced OC. β -actin as internal control. **B. a.** Cellular TRAP activity assay. Data are means of value from 3 independent experiment, bar $\pm$ SEM. $**P < .01$; $*P < .05$. **b.** Western blotting with anti-TRAP and RT-PCR with TRAP primers in OC. β -actin as internal control. **C. a.** The photography of OC in control group and GLP2 overexpressing group respectively. 4d: the fourth day after RANKL(10 ng mL) induction; 5d: the fifth day after RANKL induction. **b.** The analysis of OC positive rate. **D. a.** The photography of TRAP staining in control group and GLP2 overexpressing group in the fifth day after RANKL (10 ng mL) induction, respectively. **b.** The analysis of TRAP positive rate(%). **E.** RAW264.7 was differentiated into osteoclasts using RANKL (10 ng mL) and then the osteoclasts were treated with GLP2 ($1 \mu\text{mol L}^{-1}$) or PBS for 3 days. The osteoclasts were significantly measured after 3 days. For further details, see caption to Figure 1

FIGURE 3 GLP2 aggravates the apoptosis of osteoclasts (OC). **(A) a.** The staining of Hoechst33258 in OC cells lines infected with rLV-Green or rLV-Green-GLP2 respectively. The nucleus of apoptotic cells is blue. **b.** the cell apoptosis rate in OC cells lines infected with rLV-Green or rLV-Green-GLP2 respectively. Data are means of value from 3 independent experiment, bar $\pm$ SEM. $**P < .01$; $*P < .05$. **(B)** The apoptosis assay via Ladder electrophoresis in OC cells lines infected with rLV-Green or rLV-Green-GLP2, respectively. **(C)** RAW264.7 was differentiated into osteoclasts using RANKL(10 ng mL) and then the osteoclasts were treated with GLP2 ($1 \mu\text{mol L}^{-1}$) or PBS. **a.** The staining Annex V-PI was performed in osteoclasts treated with GLP2 ($1 \mu\text{mol L}^{-1}$) for 2 days. The nucleus of apoptotic cells is red and the membrane of apoptotic cells. **b.** The staining Annex V-PI was performed in osteoclasts treated with GLP2 ($1 \mu\text{mol L}^{-1}$) for 3 days. The nucleus of apoptotic cells is red and the membrane of apoptotic cells. **c.** The cell apoptosis rate in osteoclasts treated with GLP2 ($1 \mu\text{mol L}^{-1}$) for 2 or 3 days. Data are means of value from 3 independent experiment, bar $\pm$ SEM. $**P < .01$; $*P < .05$. For further details, see caption to Figure 1

signalling element) activity was significantly enhanced in rLV-Green-GLP2 group compared to rLV-Green group (4177.67 ± 816.73 vs 81225.67 ± 11290 , $P = .004 < .01$) (Figure 4B). Thereby, both

pSmad2 and pSmad3 were significantly increased in rLV-Green-GLP2 group compared to rLV-Green group (Figure 4Ca), and both nuclear pSmad2 and nuclear pSmad3 were also significantly

FIGURE 4 GLP2 activates the TGFβ signalling pathway in OC. (A) Co-Immunoprecipitation (IP) with anti-TGFβRII followed by Western blotting with anti-SARA, anti-GLP2 and anti-TGFβRI in OC cells (rLV group and rLV-GLP2 group). IgG IP as negative control. INPUT refers to Western blotting with anti-TGFβRII, anti-SARA and anti-GLP2. (B) The assay of p3TP-Luc reporter gene activity in OC cells (rLV group and rLV-GLP2 group). Data are means of value from 3 independent experiment, bar $\pm$ SEM. ** $P < .01$; * $P < .05$. (C) Western blotting with anti-Smad2, anti-pSmad2, anti-Smad3 and anti-pSmad3 in OC cells (rLV group and rLV-GLP2 group). β-actin as internal control. (D) Co-Immunoprecipitation (IP) with anti-pSmad2 followed by Western blotting with anti-pSmad3 in OC cells (rLV group and rLV-GLP2 group). IgG IP as negative control. INPUT refers to Western blotting with anti-pSmad2. For further details, see caption to Figure 1

increased in rLV-Green-GLP2 group compared to rLV-Green group (Figure 4Ca). In particular, the interaction between pSmad2 and pSmad3 was significantly increased in rLV-Green-GLP2 group compared to rLV-Green group (Figure 4D). Together, these observations suggest GLP2 enhances the interaction between pSmad2 and pSmad3 by activating the TGFβ signalling pathway.

3.5 | GLP2 stimulates nitric oxide (NO) in osteoclasts

To address whether GLP2 could stimulate nitric oxide mediated by TGFβ-Smad2/3 signalling pathway, we detected the iNOS and NO in osteoclasts cell lines, including rLV-Green group, rLV-Green-GLP2 group and rLV-Green-GLP2 plus anti-TGFβ group, respectively. The results of CHIP assay showed that the loading of pSmad2 or pSmad3 on the promoter region of iNOS was significantly enhanced in rLV-Green-GLP2 group compared to rLV-Green group (Figure 5A). The iNOS promoter luciferase reporter gene activity was significantly increased in rLV-Green-GLP2 group compared to rLV-Green group ($P < .01$) (Figure 5B). Moreover, excessive GLP2 increased the level of iNOS mRNA and iNOS protein compared to rLV-Green. However, anti-TGFβ abrogated this action of GLP2 (Figure 5C). Intriguingly, the interaction between iNOS and GLP2 was found in the rLV-Green-GLP2 group (Figure 5D). Furthermore, excessive GLP2 increased the activity of total NOS compared to rLV-Green (0.98 ± 0.12 vs 8.62 ± 0.84 , $P = .00173 < .01$). However, anti-TGFβ abrogated this action of GLP2 (0.98 ± 0.12 vs

0.86 ± 0.29 , $P = .1654 > .05$) (Figure 5E). In addition, RAW264.7 was differentiated into osteoclasts using RANKL (10 ng/mL) and then the osteoclasts were treated with GLP2 ($1 \mu\text{mol L}^{-1}$) for 3 days. As shown in Figure 5F, GLP2 increased the activity of total NOS compared to control (1.12 ± 0.397 vs 4.1 ± 0.96 , $P = .0076 < .01$). Ultimately, as shown in Figure 5G, the results of the assay of nitric oxide (NO) showed that NO was significantly increased in the rLV-Green-GLP2 group compared to rLV-Green group (0.00038 ± 0.00013 vs 0.004345 ± 0.00059 , $P = .0045 < .01$). However, anti-TGFβ fully abrogated the GLP2 function (0.00038 ± 0.00013 vs 0.0004733 ± 0.00014 , $P = .2399 > .05$). Together, these observations suggest that GLP2 stimulates nitric oxide mediated by TGFβ signalling pathway.

3.6 | GLP2-induced apoptosis dependent on nitric oxide synthase

To investigate whether GLP2 induced apoptosis via nitric oxide (NO), we carried out related experiments in OC, including rLV group, rLV-GLP2 group and rLV-GLP2 plus L-NMMA (a total NOS inhibitor which inhibits nNOS, eNOS, iNOS) group. At the first, we performed the CHIP assay and the results showed that the loading of AP1 (a transcription factor) on the promoter region of bcl2 was significantly decreased in rLV-Green-GLP2 group compared to rLV-Green group and the loading of AP1 on the promoter region of Caspase3 was significantly increased in rLV-Green-GLP2 group compared to rLV-Green group. However, NOS inhibitor L-NMMA blocked this GLP2 action (Figure 6A).

FIGURE 5 GLP2 stimulates nitric oxide (ON) in OC. (A) CHIP assay with anti-pSmad2/3 followed by PCR with DNA primers (promoter region) of iNOS2 in OC cells. IgG CHIP was the negative control. The promoters of iNOS2 as INPUT. (B) The assay of iNOS2 promoter luciferase reporter gene activity in OC cells (rLV group and rLV-GLP2 group). Data are means of value from 3 independent experiment, bar $\pm$ SEM. ** $P < .01$; * $P < .05$. (C) The Western blotting with anti-iNOS2 and RT-PCR with primers of iNOS2 in OC (rLV group, rLV-GLP2 group and rLV-GLP2 plus anti-TGFβ group). β-actin as internal control. (D) Co-Immunoprecipitation (IP) with iNOS2 followed by Western blotting with anti-GLP2 in OC cells (rLV group, rLV-GLP2 group and rLV-GLP2 plus anti-TGFβ group). IgG IP as negative control. INPUT refers to Western blotting with anti-GLP2. (E) The assay of activity of NOS in OC cells (rLV group, rLV-GLP2 group and rLV-GLP2 plus anti-TGFβ group). Data are means of value from 3 independent experiment, bar $\pm$ SEM. ** $P < .01$; * $P < .05$. F. RAW264.7 was differentiated into osteoclasts using RANKL (10 ng/mL) and then the osteoclasts were treated with GLP2 ($1 \mu\text{mol L}^{-1}$) for 3 days. The assay of activity of NOS was performed in the osteoclasts. Data are means of value from 3 independent experiment, bar $\pm$ SEM. ** $P < .01$; * $P < .05$. G. The assay of NO in OC cells. Each value was presented OC (rLV group, rLV-GLP2 group and rLV-GLP2 plus anti-TGFβ group). Data are means of value from 3 independent experiment, bar $\pm$ SEM. ** $P < .01$; * $P < .05$. For further details, see caption to Figure 1

Moreover, as shown in Figure 6Ba, the bcl2 promoter luciferase reporter gene activity was significantly decreased (12558.67 ± 2611.47 vs 1291 ± 204.92 , $P = .01$). However, L-NMMA abolished this function of GLP2 (12558.67 ± 2611.47 vs 12018.67 ± 1150.65 , $P = .393 > .05$). Moreover, as shown in Figure 6Bb, the caspase3 promoter luciferase reporter gene activity was significantly increased in rLV-Green-GLP2 group compared to rLV-Green group (4443 ± 1075.02 vs 36007 ± 5866.38 , $P = .0073 < .01$). However, L-NMMA fully abolished the functions of GLP2 (4443 ± 1075.02 vs 3455.67 ± 610.88 , $P = .205 > .05$). Furthermore, the both bcl2 mRNA and bcl-2 protein were significantly decreased in rLV-Green-GLP2 group compared to rLV-Green group, and both caspase3 mRNA and caspase3 protein were significantly increased in rLV-Green-GLP2 group compared to

rLV-Green group. L-NMMA fully abolished the functions of GLP2 (Figure 6C). On the other hand, RAW264.7 was differentiated into osteoclasts using RANKL (10 ng/mL) and then the osteoclasts were treated with GLP2 ($1 \mu\text{mol L}^{-1}$) for 3 days. As shown in Figure 6D, the expression bcl-2 was significantly decreased in GLP2 treated group compared to control group and the expression of caspase3 was significantly increased in GLP2 treated group compared to control group. As expected, by staining of Hoechst33258 in osteoclasts, we found that excessive GLP2 significantly promoted the apoptosis of osteoclasts. However, L-NMMA fully abolished the functions of GLP2 (Figure 6Ea). The apoptosis rates was increased in rLV-Green-GLP2 group compared to rLV-Green group ($3.25 \pm 1.23\%$ vs $44.12 \pm 5.76\%$, $P = .0029 < .01$). However, L-NMMA fully abrogated the function of

FIGURE 6 GLP2 induced apoptosis dependent on nitric oxide synthase (NOS) in OC. (A) CHIP assay with anti-AP1 followed by PCR with DNA primers (promoter region) of bcl2 and Caspase3 in OC cells. IgG CHIP was the negative control. The promoters of bcl2 and Caspase3 as INPUT. (B) The assay of the promoter luciferase activity of bcl2 (a) and Caspase3 (b) in OC cells. Data are means of value from 3 independent experiment, bar $\pm$ SEM. ** $P < .01$; * $P < .05$. (C) The Western blotting with anti-bcl2 and anti-Caspase3 and RT-PCR with primers of bcl2 and Caspase3 in OC (rLV group, rLV-GLP2 group and rLV-GLP2 plus L-NMMA group). Total NOS inhibitor, L-NMMA which inhibits nNOS, eNOS and iNOS. The $4 \mu\text{mol L}^{-1}$ L-NMMA was added in cells for 72 hours. β -actin as internal control. (D) RAW264.7 was differentiated into osteoclasts using RANKL (10 ng mL^{-1}) and then the osteoclasts were treated with GLP2 ($1 \mu\text{mol L}^{-1}$) for 3 days. The Western blotting with anti-bcl2 and anti-Caspase3 in these osteoclasts. β -actin as internal control. (E) a. The staining of Hoechst33258 in OC(rLV group, rLV-GLP2 group and rLV-GLP2 plus L-NMMA group). The nucleus of apoptotic cells is blue. b. the apoptosis rate in OC cells. Data are means of value from 3 independent experiment, bar $\pm$ SEM. ** $P < .01$; * $P < .05$. (F) The apoptosis assay via DNA Ladder electrophoresis in OC cell lines(rLV group, rLV-GLP2 group and rLV-GLP2 plus L-NMMA group). For further details, see caption to Figure 1

GLP2 ($3.25 \pm 1.23\%$ vs $4.57 \pm 0.92\%$, $P = .185 > .01$) (Figure 6Eb). On the other hand, by the apoptosis assay via Ladder electrophoresis in OC cells, our results also showed that excessive GLP2 significantly increased the apoptosis of osteoclasts. However, L-NMMA fully abrogated the functions of GLP2 (Figure 6F). Together, these observations suggest that GLP2 increases the apoptosis of osteoclasts dependent on nitric oxide synthase (NOS).

4 | DISCUSSION

It is well known that osteoclasts primarily responsible for bone resorption and is regulated by survival factors. For example Interleukin-21

promotes osteoclastogenesis in RAW264.7 cells through the PI3K/AKT signalling pathway.¹⁷ Possibly, implant-related inflammation might merely have an impact on osteoclast differentiation rather than on the regulation of osteoblast activity.¹⁸ A report indicates that insulin signalling increases the receptor activator of nuclear factor (NF)- κ B ligand (RANKL)-RANK signalling, thus enhancing osteoclast differentiation by RANKL.¹⁹ In addition, PPAR γ inhibits inflammation and RANKL expression in epoxy resin-based sealer-induced osteoblast precursor cells E1 cells.²⁰ A study shows glucocorticoids also enhance bone resorption and prolong the life span of osteoclasts.²¹ On the other hand, Ataxia-telangiectasia mutated (ATM) regulates bone metabolism by suppressing the lifespan of osteoclasts.²² In particular, at the present, the potential applications

FIGURE 7 The schematic illustrates a model of GLP2 inhibits osteoclast through stimulating apoptosis. GLP2 enhances the expression of iNOS through stimulating the activity of TGFβ-Smad2/3 signalling in osteoclasts. Overexpression of GLP2 significantly increases the product of nitric oxide via iNOS which promotes apoptosis of osteoclasts by decreasing bcl2 or increasing Caspase3. Thereby, the ability of GLP2 to regulate apoptosis depends on TGFβ-Smad2/3-iNOS-NO signalling pathway since NOS inhibitor specifically inhibits the actions by GLP2

for GLP2 in some diseases therapy has been shown. In this report, we provide the first evidence that GLP2 inhibits proliferation of osteoclasts by triggering apoptosis dependent on TGFβ-smad2/3-iNOS-NO-Caspase3/bcl-2 signalling (Figure 7). To our knowledge, this is the first report demonstrating GLP2 influences on the apoptosis of osteoclasts.

It is worth mentioning that our observations clearly demonstrate that GLP2 is crucial for inhibition of proliferation of osteoclasts. This assertion is based on several observations: (1) GLP2 is associated with osteoporosis-related factors in aged rats, including blood calcium, BALP, TRAP, IL6, TNFα, Nitric Oxide(NO), iNOS, calcitonin, occludin and chondrocytes intercellular space, etc.(2) Our findings suggest that GLP2 could decrease the osteoclasts induced

from RAW264.7.(3) GLP2 aggravates the apoptosis of osteoclasts. It consistent with some reports, for example, cartilage is composed of specialized cells called chondrocytes that produce a large amount of collagenous extracellular matrix and are closer together creating less intercellular space.²³ And TRAP produced by osteoclasts can alleviate the inhibitory effect of OPN on mineralization, suggesting a potential role for TRAP in skeletal mineralization.²⁴ Furthermore, human calcitonin (hCt) is a small peptide hormone that exerts its physiological effect on Ca(2+) metabolism by means of osteoclast-mediated bone resorption inhibition.²⁵

Furthermore, it is obvious that GLP2 activates the TGFβ-Smad2/3 signalling. Herein, the involvement of GLP2 is supported by results from 5 parallel sets of experiments: (i) GLP2 enhances the interaction between TGFβRII and SARA, and between TGFβRII and GLP2 significantly.(ii) GLP2 promotes the p3TP-Luc reporter gene activity (a TGFβ binding element). (iii) GLP2 increased the phosphorylation modification of Smad2 and Smad3. (iv) both nuclear pSmad2 and nuclear pSmad3 were significantly increased in rLV-Green-GLP2 group compared to rLV-Green group. (v) GLP2 enhances the interaction between pSmad2 and pSmad3 by activating the TGFβ signalling pathway. Studies show that TGF-β (a major bone-derived growth factor) plays an essential role in receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclastogenesis and TGF-β regulates RANKL-induced osteoclastogenesis through reciprocal cooperation between Smad2/3 and c-Fos.²⁶ Moreover, a report also indicates that TGF-βRI kinase activity is necessary to mediate in vitro effects of Emdogain on osteoclastogenesis.²⁷ Furthermore, TGF-β1 stimulates Wnt10b production in osteoclasts, which may enhance restoration of the bone lost during the resorptive phase of bone turnover.²⁸ Moreover, TGFβ1/Smad4 signalling affects osteoclast differentiation by regulation of miR-155 expression.²⁹ It is worth noting that our results show that GLP2 promotes apoptosis of osteoclast mediated by TGFβ, however, a report suggests that TGF-beta suppresses osteoclast apoptosis by altering the ratio of prosurvival Bcl2 family member Bcl-X(L) to proapoptotic family member Bcl-2 interacting domain, leading to prolonged osteoclast survival.³⁰ And a study also shows that TGF-β leads to mouse pre-osteoclastic cell line (RAW264.7) has a higher sensitivity to palmitine which caused apoptosis.³¹

Our results imply that GLP2 stimulates nitric oxide (NO) in osteoclasts dependent on TGFβ-smad2/3 pathway. This assertion is based on several observations: (i) the loading of pSmad2 or pSmad3 on the promoter region of iNOS was significantly enhanced in rLV-Green-GLP2 group.(ii) The iNOS2 promoter luciferase reporter gene activity was significantly increased in rLV-Green-GLP2 group. (iii) GLP2 overexpression increased the level of iNOS. However, anti-TGFβ abrogated the action of GLP2. (iv) the interaction between iNOS and GLP2 was also found in the rLV-Green-GLP2 group. (v) GLP2 increases the product of NO in Osteoclast. Researches indicate that inducible nitric oxide synthase (iNOS) plays a critical role in the pathogenesis of osteoporosis by generating nitric oxide (NO) which contributes to the imbalance between bone formation and resorption caused by apoptosis.³² A study indicates that iNOS-dependent

NO generation contributes to the survival-promoting function of TNF- α in osteoclasts.³³ Of significance, unloading has also been linked to an increase in apoptosis of osteocytes through production of nitric oxide (NO) and increased expression of NO synthases (NOS).³⁴ Furthermore, a novel, direct NO donor regulates osteoblast and osteoclast functions and increases bone mass in ovariectomized mice.³⁵

Importantly, our results showed that GLP2-induced apoptosis dependent on nitric oxide synthase (NOS). Total NOS inhibitor(L-NMMA) L-NMMA inhibits nNOS, eNOS and iNOS. Herein, the involvement of GLP2 is supported by results from 5 parallel sets of experiments: (i) the loading of AP1 on the promoter region of bcl2 was significantly decreased and the loading of AP1 on the promoter region of Caspase3 was significantly increased in rLV-Green-GLP2 group compared to control group. However, NOS inhibitor blocked this GLP2 action. (ii) the bcl2 promoter luciferase reporter gene activity was significantly decreased and the Caspase3 promoter luciferase reporter gene activity was significantly decreased in rLV-Green-GLP2 group. However, NOS inhibitor abolished the functions of GLP2. (iii) The bcl-2 was significantly decreased and Caspase3 was significantly increased in rLV-Green-GLP2 group compared to rLV-Green group. However, NOS inhibitor abolished the functions of GLP2. (iv) excessive GLP2 significantly promoted the apoptosis of osteoclasts. However, nitric NOS inhibitor abolished the functions of GLP2. Moreover, a study shows reactive oxygen species are required for zoledronic acid-induced apoptosis in osteoclast precursors and mature osteoclast-like cells.³⁶ Furthermore, a report indicates that protocatechuic acid prevents osteoclast differentiation through regulating oxidative stress, inflammation and inducing apoptosis in RAW264.7 murine macrophage cells.³⁷ In particular, avenanthramides (AVAs) with antioxidant properties increase osteoclast apoptosis³⁸ and mangiferin can also inhibit osteoclast formation and bone resorption through regulating oxidative stress, inflammation and the Bcl-2 and Bax pathway.³⁹ In addition, IL-15 stimulates apoptosis of osteoblasts via activation of NK cells by Caspase3.⁴⁰

Strikingly, in the report, we first proved that GLP2 exerts its effect in part through the upregulation of iNOS expression. Our present approaches provided an unequivocal evidence for critical suppressor roles of the GLP2 in the osteoclastogenesis and supported the notion that GLP2 may be an alternative bona fide inhibiting factor of osteoporosis. However, the exact mechanism underlying the role of GLP2 in the osteoclastogenesis remains to be elucidated. We further explore the different pathways of GLP2-signalling to suggest suitable GLP2-based therapeutic strategies in osteoporosis.

5 | CONCLUSIONS

Our results suggested that GLP2 inhibits the growth of osteoclasts, indicating that GLP2 therapy could be a valuable approach to promote bone regeneration. These findings have opened a new field for exploring mechanisms of osteoclast differentiation and proliferation.

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CONFLICTS OF INTEREST

The authors disclose no conflicts.

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