GIP reduces osteoclast activity and improves osteoblast survival in primary human bone cells

Hansen, Morten S; Søe, Kent; Christensen, Line L; Fernandez-Guerra, Paula; Hansen, Nina W; Wyatt, Rachael A.; Martin, Claire; Hardy, Rowan S; Andersen, Thomas L; Olesen, Jacob B; Hartmann, Bolette; Rosenkilde, Mette M; Kassem, Moustapha; Rauch, Alexander; Gorvin, Caroline; Frost, Morten

DOI:
10.1093/ejendo/lvac004

European Journal of Endocrinology, 2023, **188**, 1–14
https://doi.org/10.1093/ejendo/lvac004
Advance access publication 16 January 2023
Original Research

GIP reduces osteoclast activity and improves osteoblast survival in primary human bone cells

Morten S. Hansen,[1,2,3,4] Kent Søe,[2,5,6] Line L. Christensen,[1] Paula Fernandez-Guerra,[1] Nina W. Hansen,[1] Rachael A. Wyatt,[3,4] Claire Martin,[3] Rowan S. Hardy,[3] Thomas L. Andersen,[2,5,6] Jacob B. Olesen,[5] Bolette Hartmann,[7] Mette M. Rosenkilde,[7] Moustapha Kassem,[1,2] Alexander Rauch,[1,2,8] Caroline M. Gorvin,[3,4,*†] and Morten Frost[1,2,8,*†]

[1]Molecular Endocrinology Laboratory (KMEB), Department of Endocrinology, Odense University Hospital, Odense C DK-5000, Denmark
[2]Department of Clinical Research, Faculty of Health Sciences, University of Southern Denmark, Odense C DK-5000, Denmark
[3]Institute of Metabolism and Systems Research (IMSR) and Centre for Diabetes, Endocrinology and Metabolism (CEDAM), University of Birmingham, Birmingham B15 2TT, United Kingdom
[4]Centre for Membrane Proteins and Receptors (COMPARE), Universities of Birmingham and Nottingham, Birmingham B15 2TT, United Kingdom
[5]Clinical Cell Biology, Department of Pathology, Odense University Hospital, Odense C DK-5000, Denmark
[6]Department of Molecular Medicine, University of Southern Denmark, Odense C DK-5000, Denmark
[7]Department of Biomedical Sciences, University of Copenhagen, Copenhagen N DK-2200, Denmark
[8]Steno Diabetes Centre Odense, Odense University Hospital, Odense C DK-5000, Denmark

*Corresponding authors: Institute of Metabolism and Systems Research (IMSR) and Centre for Diabetes, Endocrinology and Metabolism (CEDAM), University of Birmingham, Birmingham B15 2TT, UK. Email: c.gorvin@bham.ac.uk (C.M.G.); Molecular Endocrinology Laboratory (KMEB), Department of Endocrinology, Odense University Hospital, Odense C DK-5000, Denmark. Email: mmfnielsen@health.sdu.dk (M.F.)

Abstract

Objective: Drugs targeting the glucose-dependent insulinotropic polypeptide (GIP) receptor (GIPR) are emerging as treatments for type-2 diabetes and obesity. GIP acutely decreases serum markers of bone resorption and transiently increases bone formation markers in short-term clinical investigations. However, it is unknown whether GIP acts directly on bone cells to mediate these effects. Using a GIPR-specific antagonist, we aimed to assess whether GIP acts directly on primary human osteoclasts and osteoblasts.

Methods: Osteoclasts were differentiated from human $CD14^+$ monocytes and osteoblasts from human bone. GIPR expression was determined using RNA-seq in primary human osteoclasts and in situ hybridization in human femoral bone. Osteoclastic resorptive activity was assessed using microscopy. GIPR signaling pathways in osteoclasts and osteoblasts were assessed using LANCE cAMP and AlphaLISA phosphorylation assays, intracellular calcium imaging and confocal microscopy. The bioenergetic profile of osteoclasts was evaluated using Seahorse XF-96.

Results: GIPR is robustly expressed in mature human osteoclasts. GIP inhibits osteoclastogenesis, delays bone resorption, and increases osteoclast apoptosis by acting upon multiple signaling pathways (Src, cAMP, Akt, p38, Akt, NFκB) to impair nuclear translocation of nuclear factor of activated T cells-1 (NFATc1) and nuclear factor-κB (NFκB). Osteoblasts also expressed GIPR, and GIP improved osteoblast survival. Decreased bone resorption and improved osteoblast survival were also observed after GIP treatment of osteoclast–osteoblast co-cultures. Antagonizing GIPR with GIP(3–30)NH_2 abolished the effects of GIP on osteoclasts and osteoblasts.

Conclusions: GIP inhibits bone resorption and improves survival of human osteoblasts, indicating that drugs targeting GIPR may impair bone resorption, whilst preserving bone formation.

Keywords: GIPR, bone remodeling, osteoporosis, resorption, Akt1/2, c-Src, NFATc1, NFκB

Significance

Short-term treatment with glucose-dependent insulinotropic polypeptide (GIP) acutely decreases markers of bone resorption and transiently increases bone formation markers in humans. We aimed to investigate the mechanisms by which GIP mediates these effects. We demonstrated that GIP acts directly on primary human osteoclasts and osteoblasts to reduce osteoclast activity and differentiation and improve osteoblast survival through multiple GIP receptor (GIPR)-mediated signaling pathways. Targeting the GIPR is an emerging therapy for obesity and type-2 diabetes. Our data indicate that GIPR agonism may improve bone mass by decreasing bone resorption, but not bone formation. Thus, GIPR agonists could potentially be repurposed as treatment for patients with osteoporosis, or individuals with type-2 diabetes with increased fracture risk.

† C.M.G. and M.F. contributed equally to this manuscript and should be considered joint senior authors.

Received: August 16, 2022. **Revised:** October 26, 2022. **Editorial Decision:** November 18, 2022. **Accepted:** November 19, 2022

Introduction

Bone is a dynamic tissue, remodeled throughout life by bone resorbing osteoclasts and bone forming osteoblasts to adapt to physiological or mechanical demands. Osteoporosis is caused by an imbalance in bone remodeling, characterized by decreased bone mass and increased fracture risk and affects >10 million individuals in the United States and >27 million in Europe.[1,2] Deciphering how bone remodeling is regulated is central for the development of anti-osteoporotic treatments, which reduce fracture risk by decreasing bone resorption, stimulating bone formation, or through dual effects on bone resorption and formation.[2]

Rodent and human studies indicate that the gut-secreted hormone glucose-dependent insulinotropic polypeptide (GIP) regulates bone remodeling.[3] GIP acts on the GIP receptor (GIPR), a G protein-coupled receptor, to mediate its primary function at pancreatic β-cells, stimulating glucose-dependent insulin secretion.[4] Both GIPR agonists and antagonists prevent weight gain in preclinical models and have been pursued as anti-obesity therapies, and pharmacological agents targeting GIPR and the glucagon-like peptide-1 (GLP-1) receptor show promising results for the treatment of type-2 diabetes (T2D).[5]

In humans, GIP infusion suppressed the bone resorption marker C-terminal telopeptide of type-I collagen (CTX),[6-9] and transiently increased the bone formation marker procollagen type 1 N propeptide (P1NP) in healthy men and type-1 diabetics[8,10] but not in overweight or hypoparathyroid individuals.[7,9] Importantly, pre-treatment with GIP(3–30)NH_2, a high-affinity GIPR antagonist,[11] abolished GIP-induced CTX and P1NP responses in healthy men.[10] Consistent with this, homozygous transgenic mice with a *Gip* truncation had reduced bone volume and increased osteoclast surface[12] and *Gip* overexpression increased bone mineral density, and an osteoblast activity marker, while reducing osteoclast numbers and bone resorption markers.[13] By contrast, reports on the bone phenotypes of global *Gipr* knockout mice ($Gipr^{-/-}$) are inconsistent. $Gipr^{-/-}$ mice with deletion of *GIPR* exons 1–6 have reduced BMD, decreased circulating markers of bone formation markers, and elevated osteoclast numbers and resorption markers;[14-16] however, another murine model with deletion of *GIPR* exons 4–5 showed fewer osteoclasts, increased trabecular bone volume and more active osteoblasts, despite reductions in bone strength.[17]

Although clinical studies support that GIP acutely regulates bone resorption and formation, and murine models indicate GIP and GIPR influence bone mass and strength, the direct effects of GIP and GIPR antagonism on signaling and activity of primary mature human bone cells are unknown. We recently demonstrated GIPR mRNA expression on primary human osteoclasts and osteoblast-like cell-lines,[9] and previous studies show GIP may activate cAMP and/or intracellular calcium (Ca_i^{2+}) signaling in human and/or rodent cell-lines.[18,19] Several GIPR targeting therapies are in development, and the recent FDA approval of tirzepatide, a dual GIP and GLP-1 receptor agonist, for use in T2D, highlights the importance of understanding how GIP regulates whole-body metabolism, including bone remodeling. We therefore conducted a comprehensive analysis of GIP effects on primary human osteoclasts and osteoblasts.

Methods

Detailed methods are in Supplementary Appendix. The number of replicates and statistical analyses are described in figure legends.

Cell culture

Primary human osteoclasts were differentiated from human $CD14^+$ monocytes isolated from anonymous blood donations.[20] Primary human osteoblast-lineage cells were obtained from bone specimens from patients receiving hip replacement surgery, as described.[21] Informed consent was obtained with approval from local ethics committees in Denmark (S-2011-0114 and S-20120193) and the UK (ERN_14-0446).

Expression studies

Bone specimens were obtained from proximal femurs from adolescents during corrective surgery for coxa valga.[22] Paraffin sections were subjected to in situ hybridization using an RNAScope 2.5 high-definition procedure using hybridized 20-ZZ-pair probes (477541, ACD Bioscience), directed against the human GIPR 384-1553 mRNA region.[22,23]

RNA was extracted using Trizol. For PCR, 500 ng of total RNA was used for reverse transcription using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystem) and primers in Table S1. RNA-sequencing was performed according to manufacturer's instructions (TruSeq2, Illumina). Differential gene expression was analyzed using DESeq2.[24]

Osteoclast activity assays

Osteoclasts were seeded on bone slices in monocultures or co-cultures with osteoblasts and exposed to vehicle or GIP for 72 hours. Media was collected for TRAcP activity and bone resorption assays performed.[25] For number of nuclei studies, differentiating osteoclasts were exposed to vehicle or GIP for 7 days, then fixed and stained with Giemsa and May–Grünwald.[26]

Functional assays

Cells were plated on bone slices in 96-well plates and exposed to either vehicle or GIP for 0–120 minutes for cAMP and 30 minutes for AlphaLISA assays. LANCE cAMP and AlphaLISA assays were performed following manufacturer's instructions using a Pherastar (BMG Labtech) plate reader. Western blots were performed as described.[27]

Apoptosis and cell viability (ATP) assays were performed using Caspase-Glo 3/7 and CellTiterGlo (Promega) on cells incubated with vehicle or GIP for 3 days. For bioenergetic profiling, mature osteoclasts were seeded in a Seahorse 96-well plate (Agilent Technologies), incubated with GIP or vehicle and measurements performed as described.[28]

Microscopy

Time-lapse recordings of osteoclastic actin ring formation on bone slices were performed as described.[29] For intracellular calcium imaging, osteoclasts were loaded with Fura-2 (ThermoFisher) and imaged on a Crest X-light spinning disk system.[30] For NFATc1 and NFκB imaging, cells were pre-incubated with inhibitors, then exposed to vehicle or GIP, followed by fixation and immunostaining with total NFATc1 or p-p65 primary antibodies and secondary Alexa Fluor 488. TUNEL staining was performed using a TUNEL-FITC kit (Abcam). Images were captured using a Zeiss LSM780 confocal microscope.

Results

GIP acts directly on osteoclast GIPR to reduce bone resorption

We confirmed GIPR expression by in situ hybridization (Figure 1A) on osteoclasts differentiated from human CD14$^+$ monocytes and showed *GIPR* mRNA expression increases during osteoclastogenesis (Figure 1B). GIP decreased osteoclastic resorptive activity in human osteoclasts and osteoclast–osteoblast co-cultures (Figure 1C; Figure S1A, B). Pre-treatment of mature osteoclasts with the GIPR antagonist, GIP(3–30)NH_2, prevented GIP-mediated reductions in bone resorption (Figure 1D). GIPR expression also correlated with the resorptive activity of mature osteoclasts (Figure 1E). Osteoclasts resorb bone by forming pits (round cavitas made by osteoclasts that are immobile during bone resorption) or trenches (elongated excavations as a result of osteoclasts moving across the bone surface while resorbing).[20] The ratio between pits and trenches was not affected by GIP, indicating that GIP is unlikely to affect osteoclast mobility or the bone resorption pattern (Figure S1). GIP reduced the number of nuclei per osteoclast during osteoclastogenesis (Figure 1F, G), and reduced TRAcP activity in mature osteoclasts, but not in pre-osteoclasts (Figure 1H), consistent with induction of GIPR mRNA expression during late osteoclastogenesis.

GIPR activation enhances cAMP signaling in human osteoclasts

GIPR couples to Gs signaling pathways, which activate adenylate cyclase and increase cAMP. To determine whether GIP activates these pathways, we measured cAMP accumulation using LANCE assays in osteoclasts exposed to vehicle or GIP. GIP, but not vehicle, increased cAMP levels after 30 minutes (Figure S1C) which was reversed by pre-treatment with GIP(3–30)NH_2 (Figure 1I). GIP also increased cAMP in osteoclast–osteoblast co-cultures after 30 minutes, but not in those pre-treated with GIP(3–30)NH_2 (Figure S1D).

Stimulation of cAMP can enhance the cAMP response element binding protein (CREB) pathway to increase bone resorption.[31] No difference in the accumulation of phosphorylated CREB was identified in osteoclast monocultures or osteoclast–osteoblast co-cultures exposed to vehicle or GIP (Figure S1E, F), indicating GIPR and cAMP likely modify other signaling pathways in human osteoclasts.

GIPR activation reduces human osteoclast Src signaling

We next assessed signaling pathways known to affect osteoclast activity. Phosphorylation of the tyrosine kinase c-Src is important for bone resorption via actin ring formation.[32] AlphaLISA assays revealed that GIP suppresses the phosphorylation of Src-Tyr419 in osteoclast and osteoclast–osteoblast co-cultures, which was abolished by pre-treatment with GIP(3–30)NH_2 (Figure 2A; Figure S2A).

Elevations in cAMP can reduce Src activity in a PKA-dependent pathway.[33] We therefore hypothesized that activation of GIPR-cAMP pathways could enhance PKA and reduce p-Src generation (Figure 2B). However, pre-treatment of osteoclasts with the PKA inhibitor H-89, had no effect on GIP-induced reductions in p-Src (Figure 2C). Treatment with forskolin, which activates adenylate cyclase and should phenocopy treatments that activate cAMP, also did not affect p-Src concentrations in osteoclasts (Figure 2D), indicating it is unlikely that the canonical GIPR-Gs-cAMP-PKA pathway modulates p-Src levels. Moreover, pre-treatment of osteoclasts with a PKC inhibitor (GF109203X), a calcium chelator (BAPTA), and an Akt1/2 inhibitor had no effect on p-Src concentrations, suggesting GIPR does not activate p-Src by these pathways, although a Src inhibitor (3,4-methylenedioxy-β-nitrostyrene) significantly reduced p-Src concentrations (Figure S2B–D). Thus, GIPR-mediated Src inhibition may occur by direct interaction with the receptor, components of its signaling pathway, or by crosstalk with other membrane receptors that recruit Src kinases.[34]

As GIP reduced p-Src and bone resorption, we predicted that actin ring formation may be impaired. In osteoclasts exposed to GIP, the median time for initiation of actin ring formation was 26.2 hours, which was significantly longer than for vehicle-treated cells (10.9 hours) (Figure 2E, F; Movie S1). Thus, GIP impairs bone resorption by reducing p-Src signaling and delaying actin ring formation.

GIP reduces PI3K-Akt signaling in human osteoclasts

PI3K can promote bone resorption by c-Src recruitment[35] and activation of Akt.[36] To investigate if GIP affects PI3K signaling, we measured Akt1/2/3 phosphorylation using an Akt1/2/3 (p-Akt) AlphaLISA assay, following 30 minutes exposure of osteoclasts to GIP or vehicle. P-Akt was reduced by GIP in osteoclasts and osteoclast–osteoblast co-cultures, which was reversed by pre-treatment with GIP(3–30)NH_2 (Figure 3A; Figure S3A). Pre-treatment of cells with the PI3K inhibitor wortmannin, phenocopied GIP actions on p-Akt, while co-treatment with GIP and wortmannin had an additive effect on p-Akt, which was reversed by pre-treatment with GIP(3–30)NH_2 (Figure 3B). Thus, GIP acts on PI3K to reduce p-Akt generation in osteoclasts. However, as co-treatment with wortmannin and GIP could still further reduce p-Akt, GIP may activate additional pathways (Figure 3C).

To determine whether p-Akt is also reduced by GIP-mediated increases in cAMP, we pre-treated osteoclasts with H-89 (Figure 3D). This prevented GIP-induced reductions in p-Akt, indicating GIP also reduces p-Akt via a cAMP-PKA pathway in osteoclasts. Moreover, a Src inhibitor phenocopied these effects (Figure 3E), indicating that the GIP-c-Src pathway could also contribute to reductions in p-Akt.

GIP reduces calcium oscillations to impair p-Akt generation

Exposure to a stable GIP analog for 48 hours reduced Ca_i^{2+} signaling in mouse osteoclast-like cell-lines.[37] We tested acute effects of GIP by single-cell microfluorimetry with the calcium-indicating dye Fura-2. Quantification of the 340/380 ratio showed that GIP induced a greater Ca_i^{2+} maximal stimulatory response (E_{max}), than observed in vehicle-treated osteoclasts (Figure 3F–H; Figure S3). However, vehicle-treated osteoclasts had more frequent Ca_i^{2+} oscillations of similar amplitude than GIP-treated osteoclasts (Figure 3I–K; Figure S3). The amplitude of these oscillations was significantly higher with GIP, consistent with elevated E_{max} (Figure 3L, M; Figure S3). Thus, GIP reduces Ca_i^{2+} oscillation frequency, which may reduce calcium-mediated gene expression, consistent with previous studies showing the frequency, rather than E_{max} of oscillations, is important for NFATc1 activation.[38,39]

As p-Akt can be stimulated by elevations in Ca_i^{2+},[40] we hypothesized that reductions in Ca_i^{2+} oscillations could contribute to the observed GIP-induced p-Akt reductions. Indeed,

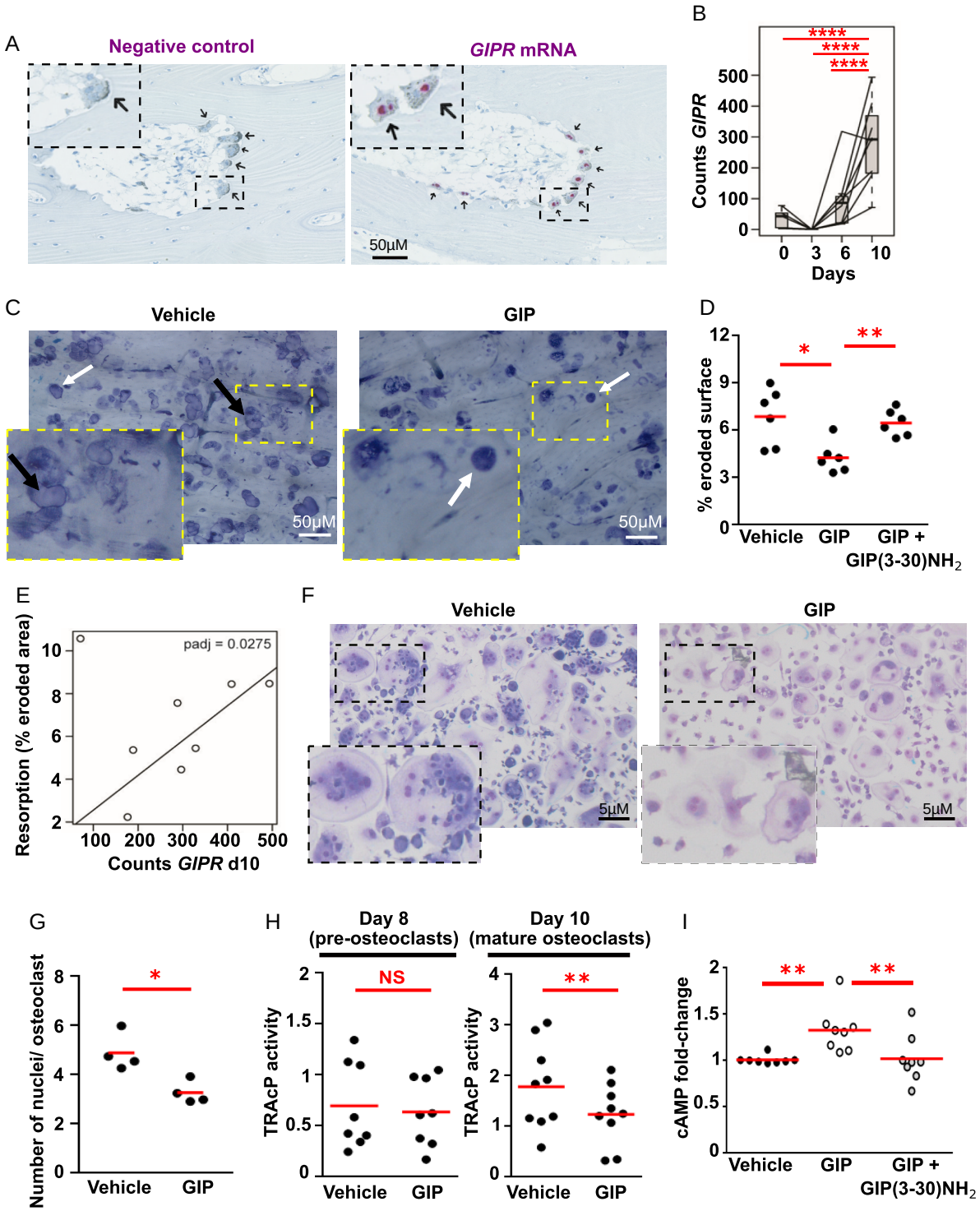

Figure 1. GIP reduces bone resorption and activates cAMP signaling in primary human osteoclasts. (A) In situ hybridization showing *GIPR* mRNA expression (magenta) in human osteoclasts (arrows). (B) Quantification of *GIPR* mRNA expression during osteoclast differentiation by RNA-sequencing ($n = 8$ donors). DEseq2-adjusted *P*-values modeled for donor and timepoint. (C) Representative images and (D) quantification of resorption pits and trenches formed on bone slices by osteoclasts exposed to vehicle or 10-nM GIP, visualized using toluidine blue ($n = 6$ donors). White arrows show pits and black arrows show trenches. (E) Baseline resorptive activity of osteoclasts over *GIPR* mRNA expression at day 10 of osteoclast differentiation ($n = 8$ donors). DEseq2-adjusted *P*-values modeled for resorptive activity. (F) Representative images and (G) quantification of number of nuclei per osteoclast visualized using May–Grünwald–Giemsa stain ($n = 4$ donors). (H) Quantification of osteoclast activity measured by TRAcP staining. Osteoclasts were exposed to vehicle, 10-nM GIP or GIP with the GIPR antagonist, GIP(3–30)NH_2, for 3 days in panels C–H. (I) Quantification of cAMP generated in osteoclasts exposed to vehicle, GIP, or GIP + GIP(3–30)NH_2 for 30 minutes measured by the LANCE assay. Each dot represents one donor with mean or median shown in red in panels D, G, H, I. $^{****}P < .0001$, $^{**}P < .01$, $^{*}P < .05$. Statistical analyses were performed by one-way ANOVA with Dunnett's correction for multiple comparison testing for panel D; paired *t*-test for panels G and H; and Kruskal–Wallis one-way ANOVA with Dunn's multiple comparisons test for panel I.

p-Akt was lower in osteoclasts pre-treated with the calcium chelator BAPTA, such that p-Akt responses were not different to GIP-treated cells. However, BAPTA did not reduce p-Akt levels when compared to vehicle-treated cells (Figure 3N),

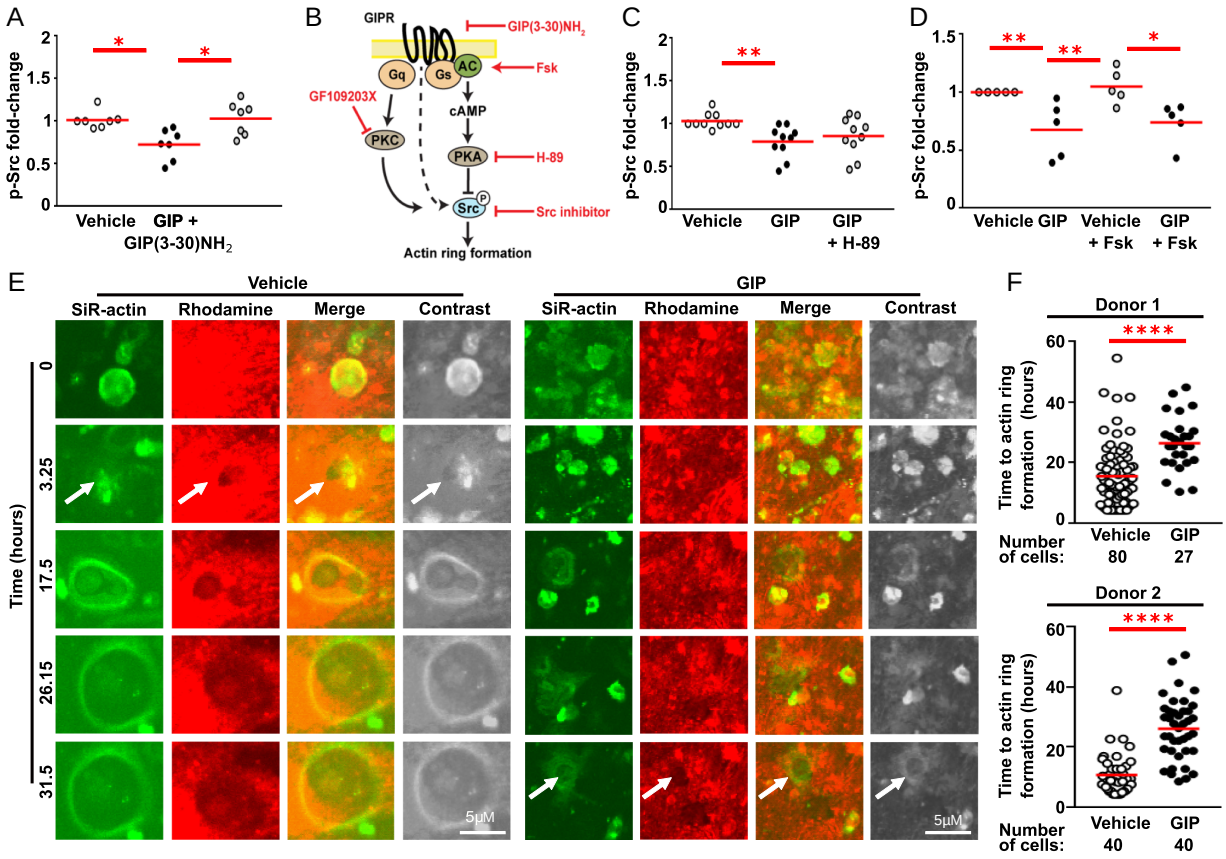

Figure 2. GIP reduces c-Src signaling and actin ring formation in human osteoclasts. (A) Quantification of phosphorylated c-Src (p-Src) in osteoclasts exposed to vehicle, GIP, or GIP with GIP(3–30)NH_2 for 30 minutes measured by AlphaLISA. (B) Cartoon showing signaling pathways that may affect p-Src signaling. Inhibitors/activators are in red. (C–D) Effect of pre-treatment with (C) the PKA inhibitor H-89 or (D) the adenylate cyclase activator forskolin (Fsk) on GIP-mediated p-Src responses in osteoclasts. p-Src was normalized to GAPDH in panels A, C, D ($n = 7$ in A, $n = 10$ in panel C, $n = 5$ donors in panel D). Each point in panels A, C, D represents one donor, with mean or median shown in red. (E) Representative time-lapse images and (F) quantification of initiation of actin ring formation in two donors. Arrows indicate the actin ring. Each dot represents one cell measured with median shown in red. $^{****}P < .0001$, $^{**}P < .01$, $^{*}P < .05$. Statistical analyses were performed by Kruskal–Wallis one-way ANOVA with Dunn's multiple comparisons test for A; one-way ANOVA with Sidak's and Dunnett's multiple comparisons tests, respectively, for panels B and C; nested *t*-test for panel F.

suggesting that multiple signaling pathways may act to reduce p-Akt downstream of GIPR in human osteoclasts.

GIP impairs NFATc1 nuclear translocation

Ca_i^{2+} oscillations and Akt can activate NFATc1, which upon nuclear translocation regulates expression of genes that regulate osteoclast differentiation and function.[41] We hypothesized that GIP may reduce NFATc1 translocation by the Ca_i^{2+}-Akt and cAMP-PKA-Akt pathways. Osteoclasts exposed to vehicle or pre-treated with GIP(3–30)NH_2 had significantly higher concentrations of NFATc1 in the nuclear fraction compared to GIP treatment alone (Figure 4A–C; Figure S4). Pre-treatment of cells with H-89 prevented the GIP-induced impairment in NFATc1 nuclear translocation (Figure 4A–C; Figure S4) indicating GIPR-cAMP-PKA contributes to NFATc1 translocation. Co-treatment of cells with GIP and an Akt1/2 inhibitor further inhibited NFATc1 translocation, indicating GIP-mediated reductions in Akt1/2 may also contribute to this pathway (Figure 4A–D; Figure S4).

GIP reduces p38 signaling in human osteoclasts

Src can activate p38, which promotes NFATc1 phosphorylation and interacts with Akt pathways.[42] GIP reduced phosphorylated p38 (p-p38) in osteoclasts and osteoclast–osteoblast co-cultures compared to vehicle or pre-treatment with GIP(3–30)NH_2 (Figure 5A, B; Figure S5A). Pre-treatment with H-89, forskolin or a Src inhibitor had no effect on p-p38 responses (Figure S5B–D), indicating that the cAMP-PKA and Src pathways are unlikely to regulate p-p38. In contrast, pre-treatment of osteoclasts with the calcium chelator BAPTA reduced p-p38 in vehicle-treated cells, and combined GIP and BAPTA treatment had similar effects to GIP (Figure 5C). The Akt1/2 inhibitor had no effect on p-p38 (Figure S5E), however, inhibition of p38 impaired the generation of p-Akt (Figure 5D). Thus, GIP-induced reductions in p-p38 contribute to the inhibition of p-Akt in osteoclasts.

GIP reduces NFκB signaling in human osteoclasts

NFκB regulates osteoclast differentiation and function[43] and can be stimulated by PI3K-Akt and p38,[44] which are reduced by GIP (Figures 3–5). We therefore assessed phosphorylation of the p65 NFκB subunit (p-p65) by AlphaLISA, and showed reduced p-p65 in GIP-treated osteoclasts and osteoclast–osteoblast co-cultures, which was reversed by pre-treatment with GIP(3–30)NH_2 (Figure 5E; Figure S5F).

Following activation, p65 translocates to the nucleus, where it regulates gene expression. Confocal imaging showed that GIP reduced the amount of p-p65 in nuclear fractions to similar

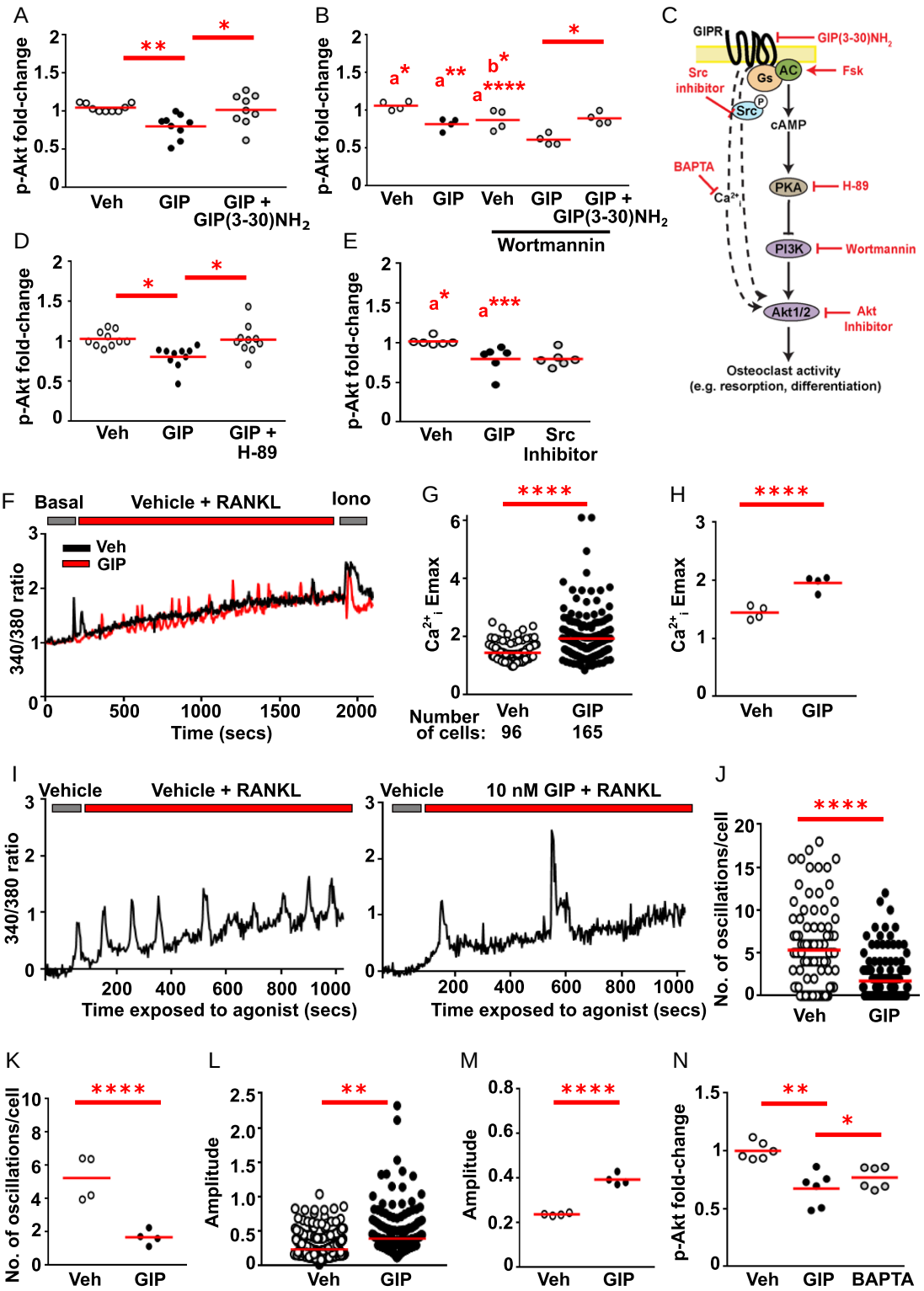

Figure 3. GIP reduces p-Akt signaling in human osteoclasts. (A) Quantification of phosphorylated Akt1/2/3 (p-Akt) generated in osteoclasts by vehicle (veh), GIP, or GIP with GIP(3–30)NH_2 measured by AlphaLISA. (B) Effect of pre-treatment with the PI3K inhibitor wortmannin. (C) Cartoon showing GIPR signaling pathways that may act on Akt. Inhibitors/activators are in red. (D–E) Effect of pre-treatment with (D) the PKA inhibitor H-89 and (E) a Src inhibitor on p-Akt responses ($n = 9$ in panels A and B, $n = 10$ in panel D and $n = 6$ donors in panel E). Each dot represents one donor measured with median shown in red in panels A, B, D, E. (F) Normalized mean fluorescence intensity ratio (340/380 nm) of Fura-2-AM calcium imaging in an individual cell exposed to vehicle or GIP. Data are normalized to the 340/380 ratio at 0 seconds for each cell. (G–H) Maximal Ca_i^{2+} responses in all osteoclasts. Panel (G) shows E_{max} of all cells measured. Panel (H) shows average for each of the 4 donors. Panel (I) Close-up of oscillations in a cell exposed to vehicle (left) and GIP (right). (J–K) Total number of Ca_i^{2+} oscillations and (L–M) amplitude of oscillations from all cells measured. Panels (K) and (M) show averages for each of the four donors. Data for individual donors are shown in Figure S3. (N) Effect of pre-treatment with the calcium chelator BAPTA on p-Akt ($n = 6$ donors). p-Akt was normalized to GAPDH in AlphaLISA assays. Each dot represents one cell measured with median shown in red. $^{****}P < .0001$, $^{***}P < .001$, $^{**}P < .01$, $^{*}P < .05$. Comparisons to vehicle-treated cells are labeled as (a) and to GIP-treated cells as (b). Statistical analyses were performed using Kruskal–Wallis one-way ANOVA with Dunn's multiple comparisons test for panels A, B, D, E, N; Mann–Whitney test for panels G, J, L; unpaired *t*-test for panels H, K, and M.

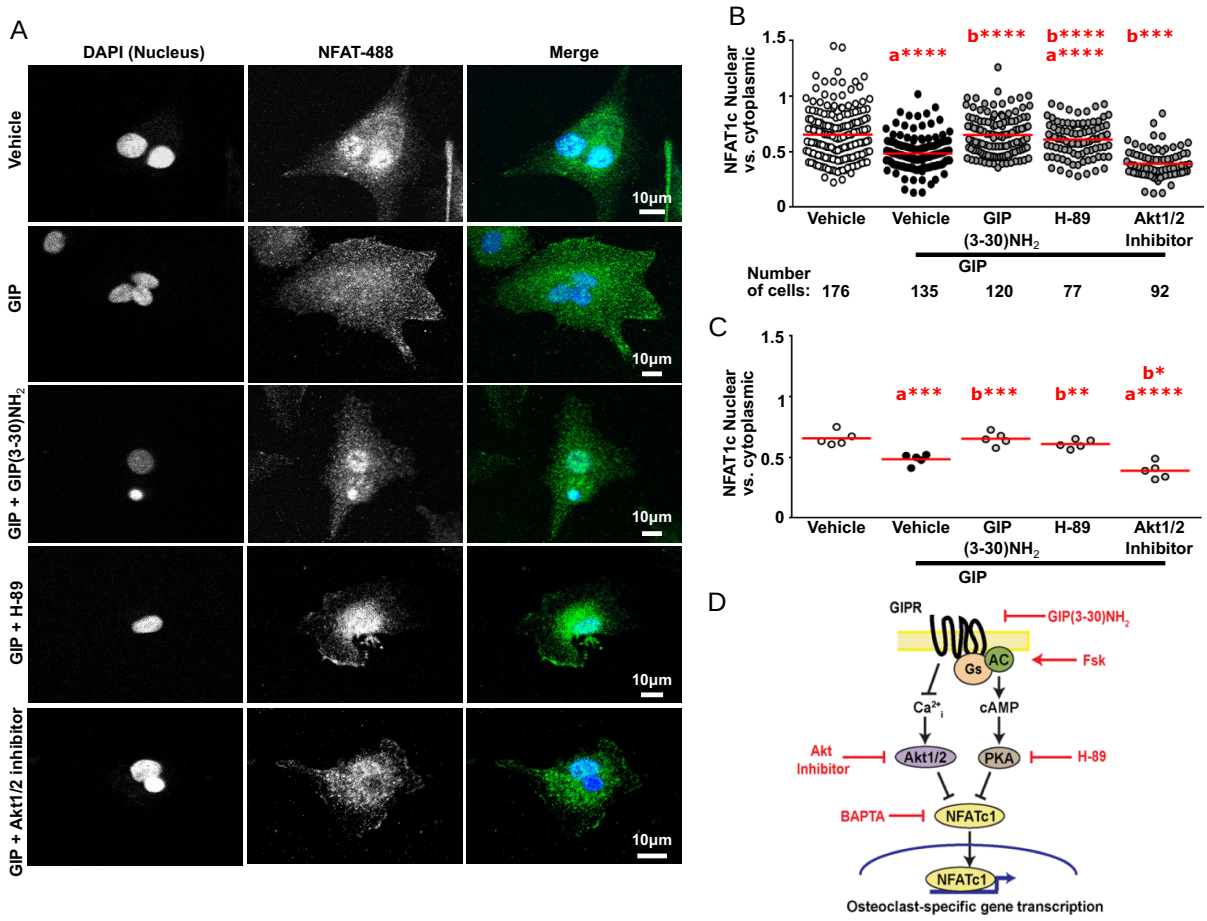

Figure 4. GIP reduces NFATc1 nuclear translocation in human osteoclasts. (A) Representative images of NFATc1 in osteoclasts exposed to vehicle or GIP +/– inhibitors of signaling. DAPI was used to label nuclei, and AlexaFluor488 to fluorescently label NFATc1. (B–C) NFATc1 nuclear and cytoplasmic ratios in osteoclasts from $n=5$ donors. Panel (B) shows ratios for all cells measured. Each dot represents one donor measured with median shown in red in panel C. Data for individual donors are shown in Figure S4. Comparisons to vehicle-treated cells are labeled as (a), and to GIP-treated cells as (b). $^{****}P<.0001$, $^{***}P<.001$, $^{**}P<.01$, $^{*}P<.05$. (D) Cartoon showing GIPR signaling pathways that converge on NFATc1 activation. Inhibitors/activators are in red. Statistical analyses were performed by one-way ANOVA with Dunnett's multiple comparisons test for panel B; and Kruskal–Wallis one-way ANOVA with Dunn's multiple comparisons test for panel C.

concentrations to that observed in cells exposed to an inhibitor of NFκB nuclear translocation (Figure 5F–H; Figures S5, 6). Therefore, GIP impairs the phosphorylation of NFκB subunits and reduces their nuclear translocation. Pre-treatment of osteoclasts with H-89 or forskolin had no effect on vehicle or GIP-induced NFκB nuclear translocation, while inhibitors of Src, Ca_i^{2+}, and p38 reduced NFκB nuclear translocation compared to vehicle-treated cells, indicating these three pathways activate NFκB signaling in human osteoclasts (Figure 5G, H; Figures S5-6). Combined treatments with GIP and inhibitors of Ca_i^{2+} and p38 phenocopied the GIP effects, indicating that Ca_i^{2+} and p38 may act downstream of GIP to inhibit NFκB signaling pathways (Figure 5G, H; Figures S5-6).

GIP reduces expression of genes involved in osteoclast activity and apoptosis

As GIP reduced NFATc1 and NFκB nuclear translocation, we hypothesized that GIP may affect osteoclast gene expression. RNA-seq on mature osteoclasts showed 911 differentially expressed genes between osteoclasts exposed to vehicle or GIP, with enrichment of genes involved in bone resorption, ATP production and apoptosis, correlating with osteoclast activity data (Figure 6A–C). More than 40 genes were involved in lysosome and osteoclast function (Figure 6D). Among the downregulated genes we found those encoding TRAP, cathepsin K and the calcitonin receptor, which are known to be regulated by NFATc1. Using the ISMARA algorithm[45] we quantified the impact of NFATc1 activity on global gene expression, which increases during osteoclastogenesis, and decreases upon GIP treatment in mature osteoclasts (Figure 6E). Genes predicted at higher confidence to be regulated by NFATc1 showed increasing specificity of osteoclast activity (Figure 6F) and higher susceptibility to be repressed by GIP treatment (Figure 6G, H).

Genes involved in apoptosis or mitochondrial function, which is important for osteoclast differentiation and resorptive activity,[46] were differentially expressed in osteoclasts exposed to GIP (Figure 6B). Consistent with this, we showed that osteoclasts exposed to GIP had significantly more terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)-positive cells and higher caspase-3/7 activity, which was not present in cells pre-treated with GIP(3–30)NH_2 (Figure 6K). Additionally, GIP reduced intracellular ATP concentrations in mature osteoclasts, but not immature cells, and reduced the total number of osteoclasts (Figure 6M). To determine whether alterations in osteoclast energy metabolism

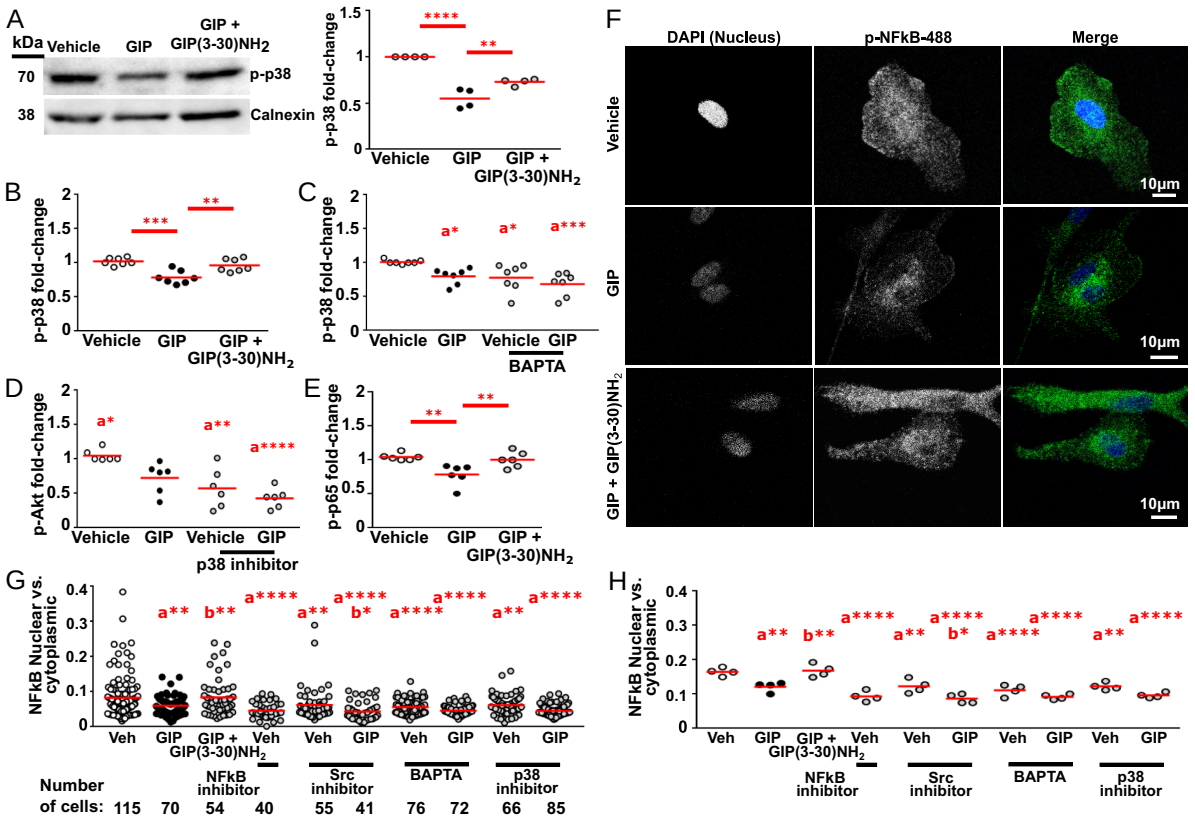

Figure 5. GIP reduces p38 and NFκB signaling in human osteoclasts. (A) Western blot analysis of phosphorylated p38 (p-p38) in osteoclasts exposed to vehicle or GIP for 30 minutes. (Right) Densitometry analysis of p-p38 relative to the calnexin loading control from 4 blots, each representing an independent donor. (B) Quantification of p-p38 measured by AlphaLISA. (C) Effect of pre-treatment with the calcium chelator BAPTA on p-p38 responses. (D) Effect of p38 inhibition on p-Akt in vehicle and GIP-treated osteoclasts. (E) Quantification of phosphorylated p65 (p-p65), an NFκB subunit, in osteoclasts exposed to vehicle or GIP for 30 minutes measured by AlphaLISA ($n=7$ in B, $n=6$ donors in C–E). Data were normalized to GAPDH in AlphaLISA assays. (F) Representative images of p-NFκB in osteoclasts exposed to vehicle or GIP ± GIP(3–30)NH_2 for 60 minutes. DAPI was used to label nuclei and AlexaFluor488 to fluorescently label p-NFκB. (G–H) p-NFκB nuclear and cytoplasmic ratios in osteoclasts from $n=4$ donors. Panel (G) shows ratios for all cells measured. Panel (H) shows average for each of the four donors. Data for individual donors are shown in Figure S5. Comparisons to vehicle-treated cells are labeled as (a) and to GIP-treated cells as (b). ****$P<.0001$, ***$P<.001$, **$P<.01$, *$P<.05$. Each dot represents one donor measured with mean or median shown in red in panels A, B, C, D, E, H. Statistical analyses were performed by one-way ANOVA with Tukey's multiple comparisons tests for panels A and B, with Dunnett's test for panels D and G, and with Holm-Sidak's test for panel H. Kruskal–Wallis one-way ANOVA with Dunn's test was used for panels D and G.

affect ATP production, we performed bioenergetic profiling using extracellular flux assays. Acute treatment with GIP for 30 minutes did not affect basal metabolism (Table S2). However, exposure of osteoclasts to GIP for 3 days produced a small but significant increase in basal respiration (Table S3; Figure 6N). No other differences were observed. Thus, GIP does not have a major effect on osteoclast bioenergetics despite lowering intracellular ATP concentrations.

GIP increases p-Akt via a cAMP-PKA pathway and decreases cell apoptosis in human osteoblasts

We confirmed *GIPR* expression in proliferating human osteoblasts and showed GIP significantly elevated cAMP following 15 and 30 minutes stimulation, which was prevented by pre-treatment with GIP(3–30)NH_2, indicating direct activation of osteoblastic GIPR (Figure 7A; Figure S7A, B). Additionally, GIP increased p-Akt concentrations, which have previously been shown to enhance osteoblast differentiation.[47] Pre-treatment with GIP(3–30)NH_2, H-89 or wortmannin inhibited GIP-induced increases in p-Akt, demonstrating GIPR activates PKA and PI3K to stimulate p-Akt in osteoblasts (Figure 7B, C). In contrast to osteoclasts, Ca_i^{2+}, NFATc1 nuclear translocation, and p38 signaling were unaffected by GIP in osteoblasts (Figure 7D–H; Figure S7C–E).

As Akt signaling promotes cell survival pathways we assessed osteoblast apoptosis. GIP-treated osteoblasts had fewer TUNEL-positive cells when compared to vehicle (Figure 7I, J), and reduced caspase3/7 activity (Figure 7K). Pre-treatment with GIP(3–30)NH_2, H-89, wortmannin or an Akt1/2 inhibitor prevented the GIP-induced reduction in caspase3/7 activation (Figure 7K), indicating that GIPR activation of cAMP-PKA and PI3K-Akt signaling pathways are involved in GIP-mediated reductions in osteoblast apoptosis. Consistent with this, GIP increased ATP generation in osteoblasts, which was reduced by GIP(3–30)NH_2, H-89, wortmannin and an Akt1/2 inhibitor (Figure 7L). In line with our findings that GIP has opposing effects on cell viability in osteoclasts and osteoblasts, there was no significant difference in the viability of osteoclast–osteoblast co-cultures exposed to vehicle or GIP (Figure S7F). As cell viability was increased in

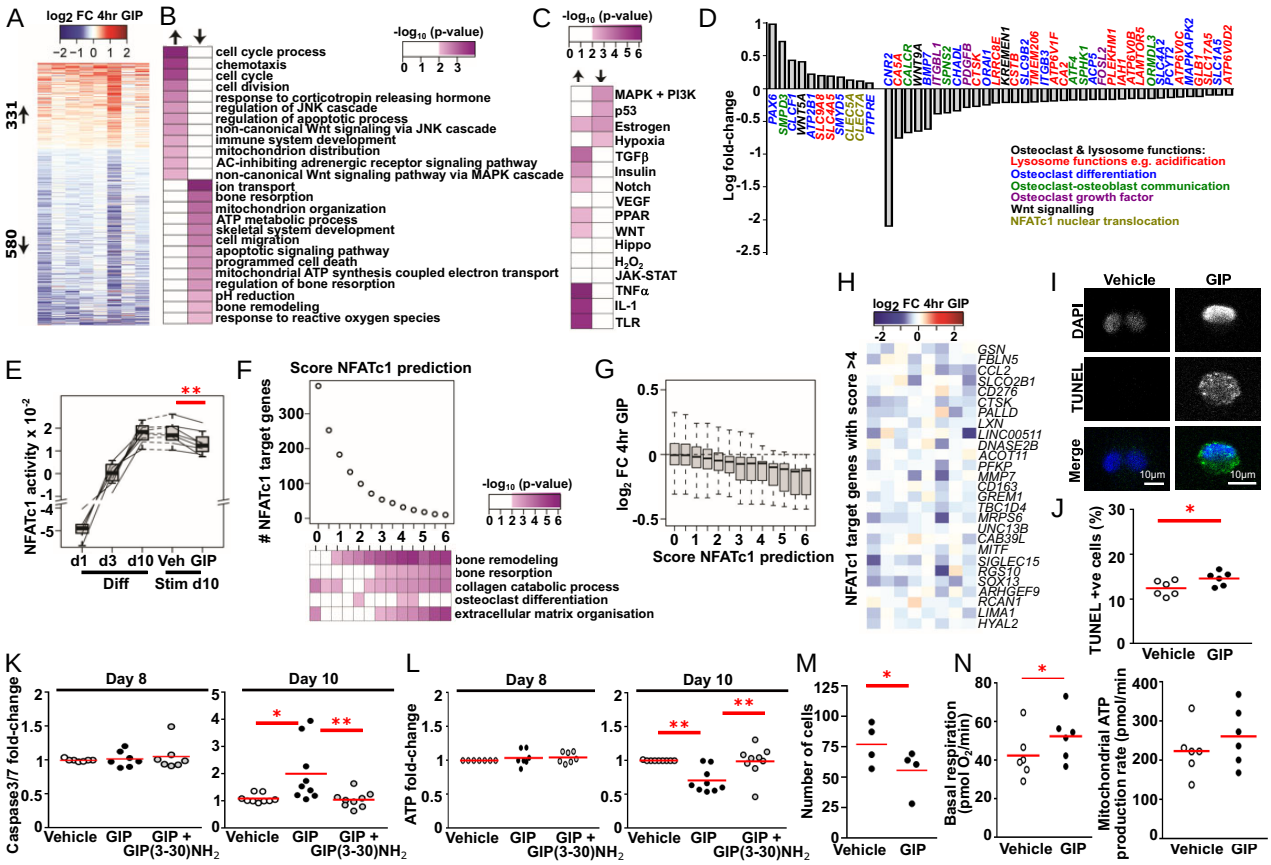

Figure 6. GIP reduces expression of genes involved in osteoclast function and induces apoptosis. (A) Heat map showing log fold changes (FC) for genes with significant changes (*P*-value <.05) in expression upon 4 hours of GIP treatment in mature osteoclasts ($n=8$ donors, DEseq2 *P*-values modeled for donor and treatment). (B) Heat map showing enrichment for gene ontology terms among the up- and downregulated genes. (C) Heat map showing enrichment for targets of the indicated signaling pathways (SPEED2) among the up- and downregulated genes. (D) Log fold-change of significantly differentially expressed genes with known roles in osteoclast activities, comparing mature osteoclasts exposed to GIP for 4 hours to vehicle-treated cells. (E) ISMARA-based motif activity of NFATc1 during osteoclast differentiation and in response to 4 hours GIP treatment in mature osteoclasts. (F) Heat map showing the enrichment of selected osteoclast related terms among NFATc1 targets with increasing confidence. (G) Box plot quantifying log fold changes in gene expression of ISMARA-based NFATc1 targets that were grouped according to prediction score upon 4 hours of GIP treatment. (H) Heat map showing the log fold changes for predicted NFATc1 target genes (ISMARA score above 4) in expression upon 4 hours of GIP treatment. (I) Representative images and (J) quantification of the percentage of TUNEL-FITC-positive (+ve) osteoclasts following 3 days of GIP exposure. DAPI was used to stain nuclei ($n=6$ donors). (K) Caspase3/7 activity and (L) intracellular ATP levels, measured by CaspaseGlo and CellTiterGlo, respectively, in immature (day 8) and mature (day 10) osteoclasts exposed to vehicle, GIP, or GIP with GIP(3–30)NH_2 for 3 days ($n=7$ for day 8, $n=9$ donors for day 10). (M) Total number of osteoclasts per bone slice following vehicle or GIP exposure ($n=4$ donors). (N) Basal respiration and mitochondrial ATP production rate in osteoclasts exposed to vehicle and GIP ($n=6$ donors). $^{**}P<.01$, $^{*}P<.05$. Each dot represents one donor measured, with mean or median shown in red in panels K–N. Statistical analyses were performed by unpaired *t*-test for panel J; Kruskal–Wallis one-way ANOVA with Dunn's test for panels K and L; and paired *t*-test for panels M and N.

osteoblast monocultures we assessed osteoblast activity by quantification of alkaline phosphatase activity and measurement of the RANKL/osteoprotegerin (OPG) ratio. No changes were detected in alkaline phosphatase enzymatic activity in osteoblast monocultures and osteoclast–osteoblast co-cultures or in the RANKL/OPG ratio in co-cultures (Figure S7G–I) after 3 days of GIP treatment.

Discussion

Our studies demonstrated that GIPR is robustly expressed on primary human osteoclasts, increasing during differentiation, and utilizes multiple signaling pathways to reduce osteoclast differentiation and bone resorption (Figure 8). GIPR is also expressed on proliferating mature human osteoblasts, and GIP improves osteoblast survival via cAMP-Akt signaling.

Endogenous GIP contributes to the postprandial suppression of bone resorption by up to 25% in humans,[48] and GIP abruptly decreases biomarkers of bone resorption.[6-9] However, these studies did not determine whether changes in bone turnover were due to direct effects on GIPR on bone cells, or indirect effects via extra-skeletal GIPR. A major advantage of our studies is in the use of the GIPR antagonist, GIP(3–30)NH_2, to demonstrate direct effects of GIP on GIPR in primary human osteoclasts and osteoblasts. Other gut-derived hormones, including GLP-1 and GLP-2, have been described to affect bone,[7,49] and adoption of a similar strategy to investigate bone cell signaling and activity with specific antagonists would help determine whether these hormones induce direct effects on human bone cells.

We showed that GIP stimulates the canonical GIPR-cAMP pathway in human osteoclasts, and that this pathway contributes to reduced osteoclast activity. This is consistent with previous studies that showed forskolin, which elevates cAMP, impairs bone resorption.[50] Previous studies in an osteoclast mouse cell-line showed a stable GIP analog did not activate

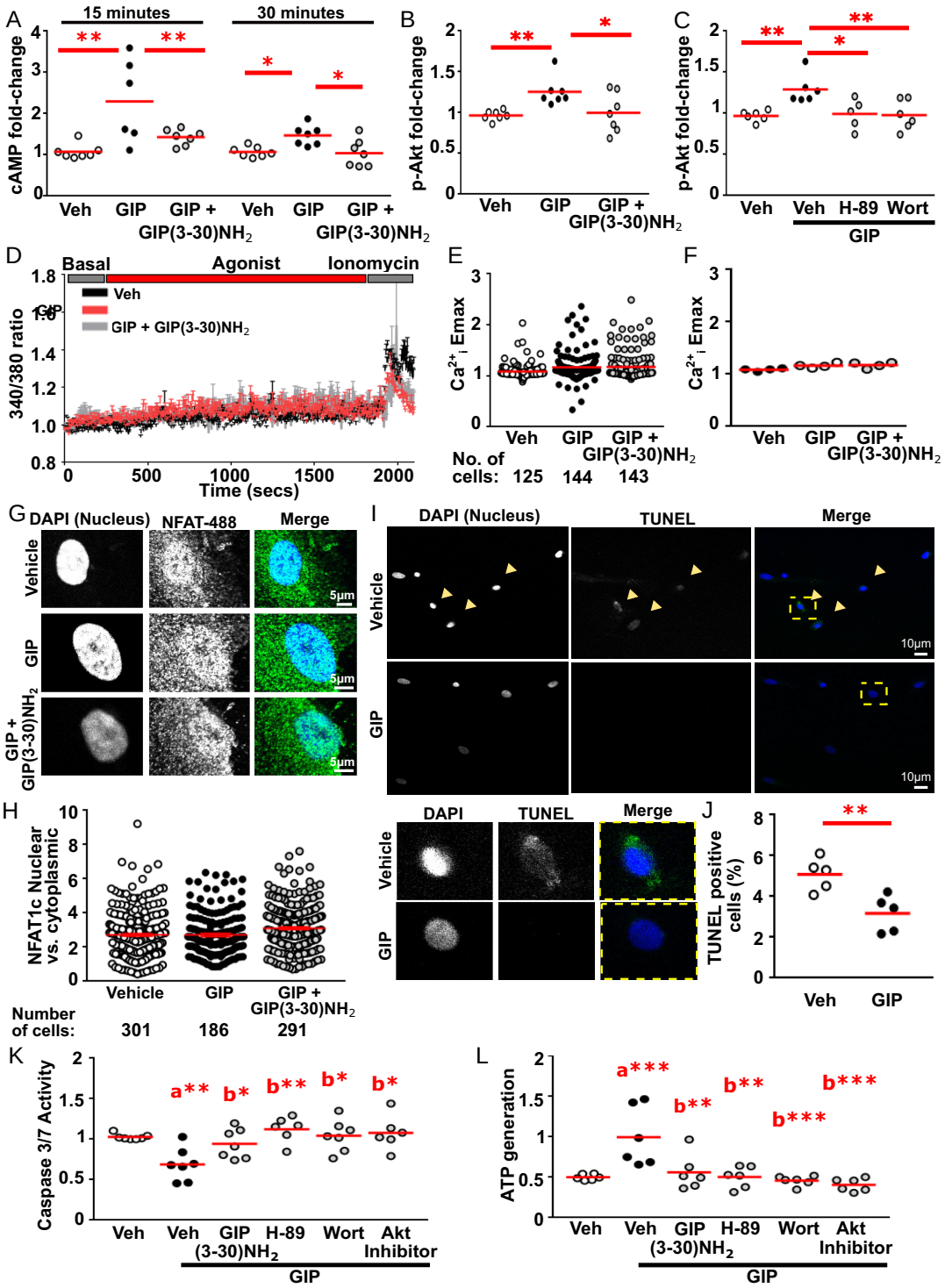

Figure 7. GIP increases cAMP and p-Akt signaling, and reduces apoptosis in human osteoblasts. (A) Quantification of cAMP in osteoblasts exposed to vehicle (veh), GIP or GIP + GIP(3–30)NH_2 measured by LANCE assays ($n = 7$ donors). (B–C) Quantification of phosphorylated Akt1/2/3 (p-Akt) in osteoblasts exposed to vehicle or GIP for 30 minutes measured by AlphaLISA. Cells were pre-treated with vehicle and (B) GIP(3–30)NH_2, (C) the PKA inhibitor H-89 and PI3K inhibitor wortmannin ($n = 7$ for B, $n = 6$ donors for C). p-Akt concentrations were normalized to GAPDH in AlphaLISA assays. (D) Normalized mean fluorescence intensity ratio of Fura-2-AM. Data are normalized to the 340/380 ratio at 0 seconds for each cell. Data show mean + SEM ($n = 4$ donors). (E–F) Quantification of the maximal Ca_i^{2+} responses from data in panel D. Panel (E) shows E_{max} of all cells measured. Panel (F) shows average for each of the four donors. (G) Representative images of NFATc1 and (H) quantification of nuclear and cytoplasmic ratios in osteoblasts exposed to vehicle or GIP for 60 minutes. DAPI was used to label nuclei and AlexaFluor488 to fluorescently label NFATc1 ($n = 5$ donors). (I) Representative images of osteoblasts stained with TUNEL to detect apoptotic cells and DAPI to detect nuclei, with zoomed images of cells indicated by a yellow box shown below. (J) Quantification of the percentage of TUNEL-FITC-positive cells ($n = 5$ donors). (K) Caspase-3/7 activity and (L) ATP generation, measured by CaspaseGlo and CellTiterGlo, respectively, in osteoblasts exposed to vehicle, GIP or GIP +/– inhibitors of signaling ($n = 6$ donors). Comparisons to vehicle-treated cells are labeled as (a), and to GIP-treated cells labeled as (b), in panels J and K. ***$P < .001$, **$P < .01$, *$P < .05$. Each dot represents one donor measured with mean or median shown in red in panels A–E and G–H. Statistical analyses were performed by Kruskal–Wallis one-way ANOVA with Dunn's test for panels A, B, E, F, H, K; one-way ANOVA with Dunnett's multiple comparisons test for C and L; and unpaired *t*-test for panels J and M.

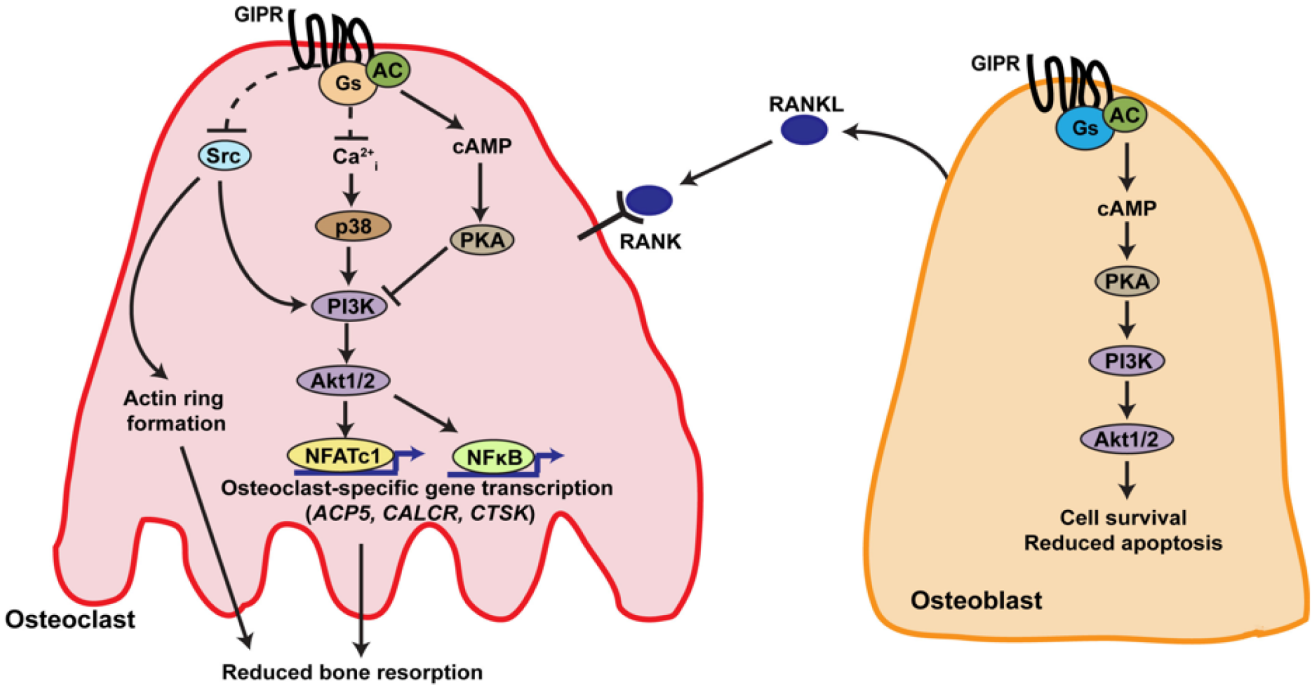

Figure 8. Summary diagram showing signaling pathways activated by GIP in human osteoclasts and osteoblasts. GIPR activates canonical Gs-mediated cAMP-PKA signaling in osteoclasts to reduce NFATc1 nuclear translocation and osteoclast-specific gene transcription. GIPR also inhibits Src phosphorylation to reduce actin ring formation, and impairs intracellular calcium signaling (Ca_i^{2+}) to reduce p38-PI3K-Akt signaling and reduce NFATc1 and NFκB nuclear translocation and gene transcription. Combined actions of GIP on these signaling pathways reduces bone resorption. GIP activation of GIPR on osteoblasts activates cAMP-PKA and PI3K-Akt signaling to reduce apoptosis and improve cell survival. GIP actions on osteoclasts rely on intact RANKL–RANK signaling between osteoblasts and osteoclasts. AC, adenylate cyclase; ACP5, acid phosphatase type 5 (TRAP) gene; CALCR, calcitonin receptor gene; CTSK, cathepsin K gene; Gs, stimulatory G protein; NFATc1, nuclear translocation of nuclear factor of activated T cells 1; NFκB, nuclear factor-κB; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A; RANKL, receptor activator of NFκB ligand.

canonical cAMP signaling[37] and there are several possible reasons for these differences. Different signaling pathways could exist between species, or between primary and immortalized cells. However, we were able to verify other GIP-induced signaling effects (i.e., inhibition of Ca_i^{2+} and impaired NFATc1 nuclear translocation) observed in these mouse cell-lines. Alternatively, experimental differences could explain why cAMP induction was not previously observed. We used a highly sensitive LANCE cAMP assay, which unlike the FRET probe used in the mouse cell-lines, does not require transfection. Therefore, it will be important to investigate the effects of GIPR analogs in primary human bone cells, rather than mouse cell-lines, to assess possible effects on osteoclast activity.

Our examination of multiple signal readouts demonstrated that GIP regulates several signaling pathways in human osteoclasts and that these have distinct effects on gene expression. Thus, NFATc1 nuclear translocation is regulated by cAMP-PKA and Ca_i^{2+} signaling pathways, whereas NFκB is regulated by Src and Ca_i^{2+}-p38-Akt, but not by cAMP-PKA. This could explain why GIP affects multiple aspects of osteoclast function (actin ring formation, gene expression, differentiation, cell survival, resorption). We showed that GIP-induced signaling events in human bone cells occur rapidly (phosphorylation events by 30 minutes, nuclear translocation by 60 minutes and gene expression by 4 hours), consistent with known acute signaling by GIPR.[51] Previous studies showed that NFATc1 nuclear translocation was impaired by chronic exposure (48 hours) to GIP;[37] however, chronic GIPR activation may desensitize GIPR activity.[52] We demonstrate that GIP-mediated effects on human osteoclasts are due to acute GIPR activation, rather than indirect effects of receptor desensitization. Our studies showed that osteoclasts differentiated in the presence of GIP have fewer nuclei per cell and a reduced total number of osteoclasts. It is possible that GIP could impair osteoclast fusion or adhesion during differentiation and this remains to be explored in future studies. However, it is unlikely that GIP has a significant effect on mobility of resorbing osteoclasts, as there was no evidence that GIP affected the extent of resorption pits (indicative of non-mobile cells) versus trenches (indicating more mobile cells), when compared to vehicle-treated cells.

Despite an enrichment in differentially expressed genes associated with metabolic pathways and oxidative phosphorylation, and lower ATP concentrations in osteoclasts exposed to GIP, we did not identify changes in ATP production. The most likely explanation for the observed lower ATP concentrations is that GIP reduces the total number of osteoclasts and the number of nuclei per osteoclast, possibly due to increased apoptosis. However, the differences between total ATP concentrations and ATP production in GIP-stimulated osteoclasts could reflect more complex dynamics between intracellular and extracellular ATP, previously shown to regulate osteoclast survival and bone resorption.[53]

GIP had a more pronounced effect on osteoclasts than osteoblasts, presumably due to higher osteoclastic GIPR expression. Importantly, osteoblast GIPR remains functional, resulting in reduced apoptosis and improved survival of osteoblasts, which

may explain the transient and small increase in bone formation markers observed in some human studies.[8,10,49] We could not demonstrate any changes in osteoblast activity using two methodologies in osteoblast monocultures or osteoclast–osteoblast co-culture. However, it is possible that GIP affects osteoblast differentiation or other osteoclast–osteoblast co-factors (e.g., hormones, cytokines and growth factors that mediate crosstalk) and this remains to be explored in future studies. Current anti-resorptive treatments for osteoporosis inhibit osteoclast activity, and through coupling mechanisms impair osteoblast activity, leading to reduced bone remodeling, which may compromise skeletal integrity with long-term exposure.[54] Bone anabolic treatments induce increases in bone formation and resorption, as observed with PTH-based therapies, or transiently increase bone formation, with a concomitant reduction in bone resorption, as seen with the sclerostin-antibody romosozumab.[55] Our co-culture studies demonstrate that GIP-mediated reductions in bone resorption can occur in a more physiologically relevant system, and indicate that GIP decreases bone resorption, while bone formation is maintained. This could lead to larger gains in bone mass and possibly lower fracture risk. Although it remains to be studied in clinical studies if these observed changes are maintained with long-term GIPR agonism, drugs targeting GIPR, including tirzepatide,[56-58] could represent a new class of anti-osteoporotic therapeutics with long-term advantages over currently approved anti-resorptive drugs.

In conclusion, our studies show that GIP acts via multiple signaling pathways to reduce differentiation and bone resorption in primary human osteoclasts and activates GIPR to improve cell survival in human osteoblasts. Stable GIPR agonists may reduce bone resorption without impairing bone formation, thus supporting studies of long-term effects of GIPR agonism on bone and exploration of such agonists as treatments for patients with increased fracture risk associated with osteoporosis and T2D.

Acknowledgements

M.S.H., K.S., T.L.A., M.K., C.M.G., and M.F. designed experiments; C.M.G., J.B.O., M.M.R., B.H., and R.S.H. provided materials; M.S.H., L.L.C., N.W.H., P.F.-G., R.W., C.M., and C.M.G. performed experiments and analyzed data. A.R. performed RNA-seq analysis. M.S.H., C.M.G., and M.F. wrote the manuscript. All authors reviewed and edited the manuscript.

Supplementary Data

Supplementary material is available at *European Journal of Endocrinology* online.

Funding

This work was funded by grants from the Novo Nordisk Foundation (NNF18OC0052699 (M.S.H.) and NNF18OC0055047 (M.F.)), Region of Southern Denmark (ref: 18/17553 (M.S.H)) and Odense University Hospital (ref: A3147 (M.F.)), and an Academy of Medical Sciences Springboard Award supported by the British Heart Foundation, Diabetes UK, the Global Challenges Research Fund, the Government Department of Business, Energy and Industrial Strategy and the Wellcome Trust (ref: SBF004\1034, C.M.G).

Conflicts of interest: None declared.

Data Availability

Raw RNA-seq data and DESeq2 processed data that have been generated in this study are available under the GEO accession number GSE201100. Requests for other data and materials will be made available upon reasonable request to C.M.G. (c.gorvin@bham.ac.uk) or M.F. (mmfnielsen@health.sdu.dk).

References

1. Holroyd C, Cooper C, Dennison E. Epidemiology of osteoporosis. *Best Pract Res Clin Endocrinol Metab*. 2008;22(5):671-685. https://doi.org/10.1016/j.beem.2008.06.001
2. Kenkre JS, Bassett J. The bone remodelling cycle. *Ann Clin Biochem*. 2018;55(3):308-327. https://doi.org/10.1177/0004563218759371
3. Hansen MS, Frost M. Alliances of the gut and bone axis. *Semin Cell Dev Biol*. 2021;123:74-81. https://doi.org/10.1016/j.semcdb.2021.06.024
4. Kieffer TJ, McIntosh CH, Pederson RA. Degradation of glucose-dependent insulinotropic polypeptide and truncated glucagon-like peptide 1 in vitro and in vivo by dipeptidyl peptidase IV. *Endocrinology*. 1995;136(8):3585-3596. https://doi.org/10.1210/endo.136.8.7628397
5. Killion EA, Lu SC, Fort M, Yamada Y, Veniant MM, Lloyd DJ. Glucose-dependent insulinotropic polypeptide receptor therapies for the treatment of obesity, do agonists = antagonists? *Endocr Rev*. 2020;41(1):bnz002. https://doi.org/10.1210/endrev/bnz002
6. Nissen A, Christensen M, Knop FK, Vilsboll T, Holst JJ, Hartmann B. Glucose-dependent insulinotropic polypeptide inhibits bone resorption in humans. *J Clin Endocrinol Metab*. 2014;99(11):E2325-E2329. https://doi.org/10.1210/jc.2014-2547
7. Bergmann NC, Lund A, Gasbjerg LS, *et al*. Separate and combined effects of GIP and GLP-1 infusions on bone metabolism in overweight men without diabetes. *J Clin Endocrinol Metab*. 2019;104(7):2953-2960. https://doi.org/10.1210/jc.2019-00008
8. Christensen MB, Lund A, Calanna S, *et al*. Glucose-dependent insulinotropic polypeptide (GIP) inhibits bone resorption independently of insulin and glycemia. *J Clin Endocrinol Metab*. 2018;103(1):288-294. https://doi.org/10.1210/jc.2017-01949
9. Skov-Jeppesen K, Hepp N, Oeke J, *et al*. The antiresorptive effect of GIP, but not GLP-2, is preserved in patients with hypoparathyroidism- a randomized crossover study. *J Bone Miner Res*. 2021;36(8):1448-1458. https://doi.org/10.1002/jbmr.4308
10. Gasbjerg LS, Hartmann B, Christensen MB, *et al*. GIP's effect on bone metabolism is reduced by the selective GIP receptor antagonist GIP(3-30)NH2. *Bone*. 2020;130:115079. https://doi.org/10.1016/j.bone.2019.115079
11. Hansen LS, Sparre-Ulrich AH, Christensen M, *et al*. N-terminally and C-terminally truncated forms of glucose-dependent insulinotropic polypeptide are high-affinity competitive antagonists of the human GIP receptor. *Br J Pharmacol*. 2016;173(5):826-838. https://doi.org/10.1111/bph.13384
12. Nasteska D, Harada N, Suzuki K, *et al*. Chronic reduction of GIP secretion alleviates obesity and insulin resistance under high-fat diet conditions. *Diabetes*. 2014;63(7):2332-2343. https://doi.org/10.2337/db13-1563
13. Xie D, Zhong Q, Ding KH, *et al*. Glucose-dependent insulinotropic peptide-overexpressing transgenic mice have increased bone mass. *Bone*. 2007;40(5):1352-1360. https://doi.org/10.1016/j.bone.2007.01.007
14. Xie D, Cheng H, Hamrick M, *et al*. Glucose-dependent insulinotropic polypeptide receptor knockout mice have altered bone turnover. *Bone*. 2005;37(6):759-769. https://doi.org/10.1016/j.bone.2005.06.021
15. Tsukiyama K, Yamada Y, Yamada C, *et al*. Gastric inhibitory polypeptide as an endogenous factor promoting new bone formation after food ingestion. *Mol Endocrinol*. 2006;20(7):1644-1651. https://doi.org/10.1210/me.2005-0187

16. Mieczkowska A, Irwin N, Flatt PR, Chappard D, Mabilleau G. Glucose-dependent insulinotropic polypeptide (GIP) receptor deletion leads to reduced bone strength and quality. *Bone*. 2013;56(2):337-342. https://doi.org/10.1016/j.bone.2013.07.003
17. Gaudin-Audrain C, Irwin N, Mansur S, *et al.* Glucose-dependent insulinotropic polypeptide receptor deficiency leads to modifications of trabecular bone volume and quality in mice. *Bone*. 2013;53(1):221-230. https://doi.org/10.1016/j.bone.2012.11.039
18. Bollag RJ, Zhong Q, Phillips P, *et al.* Osteoblast-derived cells express functional glucose-dependent insulinotropic peptide receptors1. *Endocrinology*. 2000;141(3):1228-1235. https://doi.org/10.1210/endo.141.3.7366
19. Mieczkowska A, Bouvard B, Chappard D, Mabilleau G. Glucose-dependent insulinotropic polypeptide (GIP) directly affects collagen fibril diameter and collagen cross-linking in osteoblast cultures. *Bone*. 2015;74:29-36. https://doi.org/10.1016/j.bone.2015.01.003
20. Merrild DM, Pirapaharan DC, Andreasen CM, *et al.* Pit- and trench-forming osteoclasts: a distinction that matters. *Bone Res*. 2015;3:15032. https://doi.org/10.1038/boneres.2015.32
21. Pirapaharan DC, Olesen JB, Andersen TL, *et al.* Catabolic activity of osteoblast lineage cells contributes to osteoclastic bone resorption in vitro. *J Cell Sci*. 2019;132(10):jcs229351. https://doi.org/10.1242/jcs.229351
22. Lassen NE, Andersen TL, Pløen GG, *et al.* Coupling of Bone resorption and formation in real time: new knowledge gained from human haversian BMUs. *J Bone Miner Res*. 2017;32(7):1395-1405. https://doi.org/10.1002/jbmr.3091
23. Abdelgawad ME, Delaisse JM, Hinge M, *et al.* Early reversal cells in adult human bone remodeling: osteoblastic nature, catabolic functions and interactions with osteoclasts. *Histochem Cell Biol*. 2016;145(6):603-615. https://doi.org/10.1007/s00418-016-1414-y
24. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15(12):550. https://doi.org/10.1186/s13059-014-0550-8
25. Soe K, Delaisse JM. Glucocorticoids maintain human osteoclasts in the active mode of their resorption cycle. *J Bone Miner Res*. 2010;25(10):2184-2192. https://doi.org/10.1002/jbmr.113
26. Hobolt-Pedersen AS, Delaisse JM, Soe K. Osteoclast fusion is based on heterogeneity between fusion partners. *Calcif Tissue Int*. 2014;95(1):73-82. https://doi.org/10.1007/s00223-014-9864-5
27. Gorvin CM, Rogers A, Hastoy B, *et al.* AP2sigma mutations impair calcium-sensing receptor trafficking and signaling, and show an endosomal pathway to spatially direct G-protein selectivity. *Cell Rep*. 2018;22(4):1054-1066. https://doi.org/10.1016/j.celrep.2017.12.089
28. Fernandez-Guerra P, Gonzalez-Ebsen AC, Boonen SE, *et al.* Bioenergetic and Proteomic Profiling of Immune Cells in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome Patients: An Exploratory Study. *Biomolecules*. 2021;11(7):961. https://doi.org/10.3390/biom11070961
29. Søe K, Delaissé JM. Time-lapse reveals that osteoclasts can move across the bone surface while resorbing. *J Cell Sci*. 2017;130(12):2026-2035. https://doi.org/10.1242/jcs.202036
30. Viloria K, Nasteska D, Briant LJB, *et al.* Vitamin-D-binding protein contributes to the maintenance of alpha cell function and glucagon secretion. *Cell Rep*. 2020;31(11):107761. https://doi.org/10.1016/j.celrep.2020.107761
31. Sato K, Suematsu A, Nakashima T, *et al.* Regulation of osteoclast differentiation and function by the CaMK-CREB pathway. *Nat Med*. 2006;12(12):1410-1416. https://doi.org/10.1038/nm1515
32. Destaing O, Sanjay A, Itzstein C, *et al.* The tyrosine kinase activity of c-Src regulates actin dynamics and organization of podosomes in osteoclasts. *Mol Biol Cell*. 2008;19(1):394-404. https://doi.org/10.1091/mbc.e07-03-0227
33. Abrahamsen H, Vang T, Tasken K. Protein kinase A intersects SRC signaling in membrane microdomains. *J Biol Chem*. 2003;278(19):17170-17177. https://doi.org/10.1074/jbc.M211426200
34. Luttrell DK, Luttrell LM. Not so strange bedfellows: G-protein-coupled receptors and Src family kinases. *Oncogene*. 2004;23(48):7969-7978. https://doi.org/10.1038/sj.onc.1208162
35. Miyazaki T, Sanjay A, Neff L, Tanaka S, Horne WC, Baron R. Src kinase activity is essential for osteoclast function. *J Biol Chem*. 2004;279-(17):17660-17666. https://doi.org/10.1074/jbc.M311032200
36. Kim S, Jee K, Kim D, Koh H, Chung J. Cyclic AMP inhibits Akt activity by blocking the membrane localization of PDK1. *J Biol Chem*. 2001;276(16):12864-12870. https://doi.org/10.1074/jbc.M001492200
37. Mabilleau G, Perrot R, Mieczkowska A, *et al.* Glucose-dependent insulinotropic polypeptide (GIP) dose-dependently reduces osteoclast differentiation and resorption. *Bone*. 2016;91:102-112. https://doi.org/10.1016/j.bone.2016.07.014
38. Yang YM, Kim MS, Son A, *et al.* Alteration of RANKL-induced osteoclastogenesis in primary cultured osteoclasts from SERCA2+/- mice. *J Bone Miner Res*. 2009;24(10):1763-1769. https://doi.org/10.1359/jbmr.090420
39. Dolmetsch RE, Xu K, Lewis RS. Calcium oscillations increase the efficiency and specificity of gene expression. *Nature*. 1998;392-(6679):933-936. https://doi.org/10.1038/31960
40. Yano S, Tokumitsu H, Soderling TR. Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. *Nature*. 1998;396(6711):584-587. https://doi.org/10.1038/25147
41. Moon JB, Kim JH, Kim K, *et al.* Akt induces osteoclast differentiation through regulating the GSK3beta/NFATc1 signaling cascade. *J Immunol*. 2012;188(1):163-169. https://doi.org/10.4049/jimmunol.1101254
42. Lin J, Lee D, Choi Y, Lee SY. The scaffold protein RACK1 mediates the RANKL-dependent activation of p38 MAPK in osteoclast precursors. *Sci Signal*. 2015;8(379):ra54. https://doi.org/10.1126/scisignal.2005867
43. Iotsova V, Caamano J, Loy J, Yang Y, Lewin A, Bravo R. Osteopetrosis in mice lacking NF-kappaB1 and NF-kappaB2. *Nat Med*. 1997;3(11):1285-1289. https://doi.org/10.1038/nm1197-1285
44. Gingery A, Bradley E, Shaw A, Oursler MJ. Phosphatidylinositol 3-kinase coordinately activates the MEK/ERK and AKT/NFkappaB pathways to maintain osteoclast survival. *J Cell Biochem*. 2003;89(1):165-179. https://doi.org/10.1002/jcb.10503
45. Balwierz PJ, Pachkov M, Arnold P, Gruber AJ, Zavolan M, van Nimwegen E. ISMARA: automated modeling of genomic signals as a democracy of regulatory motifs. *Genome Res*. 2014;24(5):869-884. https://doi.org/10.1101/gr.169508.113
46. Lemma S, Sboarina M, Porporato PE, *et al.* Energy metabolism in osteoclast formation and activity. *Int J Biochem Cell Biol*. 2016;79:168-180. https://doi.org/10.1016/j.biocel.2016.08.034
47. Mukherjee A, Rotwein P. Selective signaling by Akt1 controls osteoblast differentiation and osteoblast-mediated osteoclast development. *Mol Cell Biol*. 2012;32(2):490-500. https://doi.org/10.1128/MCB.06361-11
48. Helsted MM, Gasbjerg LS, Lanng AR, *et al.* The role of endogenous GIP and GLP-1 in postprandial bone homeostasis. *Bone*. 2020;140:115553. https://doi.org/10.1016/j.bone.2020.115553
49. Gabe MBN, Skov-Jeppesen K, Gasbjerg LS, *et al.* GIP and GLP-2 together improve bone turnover in humans supporting GIPR-GLP-2R co-agonists as future osteoporosis treatment. *Pharmacol Res*. 2022;176:106058. https://doi.org/10.1016/j.phrs.2022.106058
50. Lerner UH, Ransjo M, Klaushofer K, *et al.* Comparison between the effects of forskolin and calcitonin on bone resorption and osteoclast morphology in vitro. *Bone*. 1989;10(5):377-387. https://doi.org/10.1016/8756-3282(89)90134-8
51. Gabe MBN, van der Velden WJC, Smit FX, Gasbjerg LS, Rosenkilde MM. Molecular interactions of full-length and truncated GIP peptides with the GIP receptor - A comprehensive review. *Peptides*. 2020;125:170224. https://doi.org/10.1016/j.peptides.2019.170224

52. Killion EA, Chen M, Falsey JR, *et al*. Chronic glucose-dependent insulinotropic polypeptide receptor (GIPR) agonism desensitizes adipocyte GIPR activity mimicking functional GIPR antagonism. *Nat Commun*. 2020;11(1):4981. https://doi.org/10.1038/s41467-020-18751-8
53. Miyazaki T, Iwasawa M, Nakashima T, *et al*. Intracellular and extracellular ATP coordinately regulate the inverse correlation between osteoclast survival and bone resorption. *J Biol Chem*. 2012;287(45):37808-37823. https://doi.org/10.1074/jbc.M112.385369
54. Russell RG. Bisphosphonates: the first 40 years. *Bone*. 2011;49(1):2-19. https://doi.org/10.1016/j.bone.2011.04.022
55. Chavassieux P, Chapurlat R, Portero-Muzy N, *et al*. Bone-forming and antiresorptive effects of romosozumab in postmenopausal women with osteoporosis: bone histomorphometry and microcomputed tomography analysis after 2 and 12 months of treatment. *J Bone Miner Res*. 2019;34(9):1597-1608. https://doi.org/10.1002/jbmr.3735
56. Frías JP, Davies MJ, Rosenstock J, *et al*. Tirzepatide versus Semaglutide Once Weekly in Patients with Type 2 Diabetes. *N Engl J Med*. 2021;385(6):503-515. https://doi.org/10.1056/NEJMoa2107519
57. Nørregaard PK, Deryabina MA, Tofteng Shelton P, *et al*. A novel GIP analogue, ZP4165, enhances glucagon-like peptide-1-induced body weight loss and improves glycaemic control in rodents. *Diabetes Obes Metab*. 2018;20(1):60-68. https://doi.org/10.1111/dom.13034
58. Finan B, Yang B, Ottaway N, *et al*. A rationally designed monomeric peptide triagonist corrects obesity and diabetes in rodents. *Nat Med*. 2015;21(1):27-36. https://doi.org/10.1038/nm.3761