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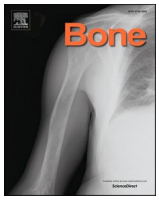

Full Length Article

Evidence that pyrophosphate acts as an extracellular signalling molecule to exert direct functional effects in primary cultures of osteoblasts and osteoclasts

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ABSTRACT

Extracellular pyrophosphate (PP_i) is well known for its fundamental role as a physiochemical mineralisation inhibitor. However, information about its direct actions on bone cells remains limited. This study shows that PP_i decreased osteoclast formation and resorptive activity by ≤50 %. These inhibitory actions were associated with reduced expression of genes involved in osteoclastogenesis (*Tnfrsf11a, Dcstamp*) and bone resorption (*Ctsk, Car2, Acp5*). In osteoblasts, PP_i present for the entire (0–21 days) or latter stages of culture (7–21/14–21 days) decreased bone mineralisation by ≤95 %. However, PP_i present for the differentiation phase only (0–7/0–14 days) increased bone formation (≤70 %). Prolonged treatment with PP_i resulted in earlier matrix deposition and increased soluble collagen levels (≤2.3-fold). Expression of osteoblast (*RUNX2*, *Bglap*) and early osteocyte (*E11*, *Dmp1*) genes along with mineralisation inhibitors (*Spp1, Mgp*) was increased by PP_i (≤3-fold). PP_i levels are regulated by tissue non-specific alkaline phosphatase (TNAP) and ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (NPP1). PP_i reduced NPP1 expression in both cell types whereas TNAP expression (≤2.5-fold) and activity (≤35 %) were increased in osteoblasts. Breakdown of extracellular ATP by NPP1 represents a key source of PP_i. ATP release from osteoclasts and osteoblasts was decreased ≤60 % by PP_i and by a selective TNAP inhibitor (CAS496014–12-2). Pertussis toxin, which prevents $G\alpha_i$ subunit activation, was used to investigate whether G-protein coupled receptor (GPCR) signalling mediates the effects of PP_i. The actions of PP_i on bone mineralisation, collagen production, ATP release, gene/protein expression and osteoclast formation were abolished or attenuated by pertussis toxin. Together these findings show that PP_i, modulates differentiation, function and gene expression in osteoblasts and osteoclasts. The ability of PP_i to alter ATP release and NPP1/TNAP expression and activity indicates that cells can detect PP_i levels and respond accordingly. Our data also raise the possibility that some actions of PP_i on bone cells could be mediated by a $G\alpha_i$-linked GPCR.

1. Introduction

Inorganic pyrophosphate (PP_i) was first identified as a potent endogenous inhibitor of biomineralisation over 50 years ago by the seminal work of Fleisch, Bisaz and colleagues [1,2]. PP_i acts in a physiochemical way to directly prevent hydroxyapatite crystal formation and propagation [3]. It is now widely accepted that PP_i acts as the body's own "water softener" regulating bone mineralisation and preventing harmful soft tissue calcification [4,5].

PP_i is a simple molecule comprised of two inorganic phosphates (P_i) joined by a hydrolysable ester bond. It is found both within cells and in the extracellular environment. Intracellular PP_i is a by-product of over 200 different enzyme reactions and is rapidly degraded by pyrophosphatases [6]. Extracellular PP_i is separately regulated and its levels are

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determined by the coordinated actions of several proteins. Specifically, it is generated by the ecto-nucleotide pyrophosphatase/phosphodiesterase (NPP) family of enzymes which hydrolyse nucleotide triphosphates (such as ATP) to the corresponding monophosphate and PP_i. Thus, breakdown of the ATP released from cells represents a key source of extracellular PP_i [5]. Many external factors have been shown to regulate ATP release from cells including mechanical stress, hypoxia and vitamin D [7–10] thereby indirectly influencing PP_i levels. Degradation of PP_i is catalysed by alkaline phosphatases, including tissue non-specific alkaline phosphatase (TNAP) to produce two P_i molecules. The membrane protein, Ank (product of the progressive ankyloses gene), was thought to contribute to PP_i levels by facilitating its transport from the intra-to-extracellular environment, but recent data has suggested that Ank exports ATP not PP_i [4,11]. In bone, TNAP and NPP1 work antagonistically to ensure that the PP_i-to-P_i ratio remains at a level conducive to regulated mineralisation [12–14].

The ability of extracellular PP_i to inhibit bone mineralisation has been well documented [5,15]. Consistent with this, deletion or inactivation of either TNAP or NPP1 has significant effects in vivo. For example, TNAP knockout mice display defective mineralisation and skeletal deformations, which are characteristic of the human disease, hypophosphatasia [16–18]. In contrast, NPP1 knockout mice (*Enpp1*$^{-/-}$) display ectopic calcification of the joints, spine, tendons and other collagen-rich soft tissues [12,19–21]. They also display reduced levels of trabecular and cortical bone in the femur and tibia [19,21,22]. Despite this extensive work, studies examining the direct functional effects of PP_i on bone cells are much more limited. Earlier work using osteoblast-like cells has shown that PP_i (100-500 μM) can promote differentiation and increase osteopontin (OPN) expression via activation of MAP kinase pathways [23,24]. Furthermore, mice lacking NPP1 or Ank display reduced osteoblast expression of OPN and lower serum levels of the protein [13,20]. PP_i can also regulate the expression and activity of NPP1, TNAP and Ank in osteoblasts [15,23,24].

Bisphosphonates (BPs), which are chemically stable analogues of PP_i, are well known for their ability to inhibit bone resorption and induce osteoclast apoptosis [25]. Despite the similarities in structure, the effects of PP_i on osteoclast formation and activity have not been reported to date. There is, however, some evidence that PP_i can regulate osteoclasts. For example, previous studies have shown that PP_i can promote osteoclast apoptosis [26]. In addition, NPP1 knockout mice display increased osteoclast activity in vivo [21].

The aim of this study was to use primary cells to explore the effects of PP_i on osteoclast and osteoblast formation and function in vitro. It will also determine whether the actions of PP_i in bone extend beyond its established role as a physiochemical mineralisation inhibitor.

2. Methods

2.1. Reagents

All tissue culture reagents were purchased from ThermoFisher UK; unless mentioned, all chemicals were obtained from Sigma Aldrich. Primary Antibodies were purchased from Abcam UK (TNAP, OPN, β-actin), ThermoFisher (NPP1), Cell Signalling (Runx2) and Proteintech (MGP, cathepsin K, carbonic anhydrase II). Secondary antibodies were from Jackson Immuno Research Europe.

2.2. Animals

The generation and characterisation of mice lacking NPP1 (*Enpp1*$^{-/-}$), which are on a 129Sv/TerJ genetic background, has previously been described [27]. Animals were bred from heterozygote (*Enpp1*$^{+/-}$) breeding pairs due to the inability of homozygotes to breed. These and all C57Bl/6 mice were housed under standard conditions with free access to food and water. All procedures complied with the UK (Scientific Procedures) Act 1986 and were reviewed and approved by the Royal Veterinary College Ethics Committee.

2.3. Osteoclast formation assay

Osteoclasts were isolated from the long bones of C57BL/6, *Enpp1*$^{+/+}$ or *Enpp1*$^{-/-}$ mice as described previously [28]. Cells (10^6) were plated onto 5 mm diameter dentine discs in 96-well plates in Minimum Essential Medium (MEM) supplemented with 10 % FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin, 100 nM PGE_2. 200 ng/ml M-CSF and 3 ng/ml RANKL (R&D Systems Europe Ltd., Abingdon, UK). After 24 h, discs containing adherent osteoclast precursors were transferred to 6-well trays (4 discs/well in 4 ml medium) for a further 6 days. Culture medium was acidified to ~pH 7.0 by the addition of 10 meq/l H^+ (as HCL) on day 7 to activate resorption [28]. PP_i (10-100 μM) was added from day 3 (precursors), day 5 (early osteoclasts) or day 7 (mature osteoclasts) of culture. To confirm that observed functional effects were mediated via PP_i rather than its breakdown product P_i, cells were also cultured with a selective TNAP inhibitor, CAS496014-12-2, (0.1-10 μM, Merck-Millipore, Watford, UK) or P_i (10-100 μM). To investigate potential involvement of G-protein coupled receptor (GPCR) signalling, PP_i was also co-administered with pertussis toxin (200-400 ng/ml).

After 9 days of culture, osteoclasts were fixed in 2.5 % glutaraldehyde and stained for tartrate-resistant acid phosphatase (TRAP). Mature osteoclasts were defined as TRAP-positive cells with 2 or more nuclei and/or clear evidence of resorption. Osteoclast number and area of resorption pits were assessed "blind" using transmitted light microscopy and reflected light microscopy and dot counting morphometry (ImageJ), respectively [28].

2.4. Osteoblast bone formation assay

Osteoblasts were isolated from the calvariae of 3–5 day old C57BL/6, *Enpp1*$^{+/+}$ or *Enpp1*$^{-/-}$ mice by trypsin/collagenase digestion as previously described [29,30]. Following isolation, cells were resuspended in αMEM and cultured for 2–4 days in 75 cm^2 flasks until confluent (in 5 % CO_2–95 % air at 37 °C). Cells were plated into 6-well trays in αMEM supplemented with 50 μg/ml ascorbic acid and 2 mM β-glycerophosphate, with half medium changes every 3 days. Unless stated, cells were cultured ±1-100 μM PP_i/P_i, 10 μM CAS496014-12-2 and/or pertussis toxin (200-400 ng/ml) for the duration of the culture.

2.5. Total RNA extraction and DNase treatment

Total RNA was extracted from PP_i-treated osteoclasts using TRIZOL® reagent (ThermoFisher, location) after 5 (early), 7 (mature) or 9 (mature, resorbing) days of culture, according to manufacturer's instructions. Osteoblast RNA was collected after 7 (differentiating) or 14 (mature) days of culture. Cells were treated with PP_i or P_i for the whole culture. Following extraction, RNA was treated with RNAse-free DNase I (35 U/ml) for 30 min at 37 °C. The reaction was terminated by heat inactivation for 10 min at 65 °C. Total RNA was quantified by measuring absorbance at 260 nM and stored at −80 °C until amplification by qRT-PCR.

2.6. Quantitative real time polymerase chain reaction (qRT-PCR)

Osteoclast and osteoblast RNA (50 ng) was transcribed and amplified using the qPCRBIO SyGreen one-step qRT-PCR kit (PCR Biosystems, London, UK), which allows cDNA synthesis and PCR amplification to be carried out sequentially. Reactions were performed according to the manufacturer's instructions with initial cDNA synthesis (45 °C for 10 min) and reverse transcriptase inactivation (95 °C for 2 min) followed by 40 cycles of denaturation (95 °C for 5 s) and detection (60 °C) for 30 s. All reactions were carried out in triplicate using RNAs derived from 5 different cultures. Data were normalised to β-actin and analysed using

the ΔΔCT method [31]. Primer sequences are shown in Table 1.

2.7. Western blotting

Total protein was extracted from mature osteoclasts (day 7), mature resorbing osteoclasts (day 9), differentiating osteoblasts (day 7) or mature, mineralising osteoblasts (day 14) treated with PP_i (10-100 μM). Cell layers were lysed in ice-cold radio immunoprecipitation (RIPA) lysis buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1 % SDS 1 mM phenyl methyl sulfonyl fluoride (PMSF), 1 mg/ml aprotinin, 1 mM Na_3VO_4 and 2.5 mg/ml deoxicolic acid). Cell homogenates were sonicated for 5 min and stored at −80 °C. Lysate protein concentrations were determined using the Bradford assay (Sigma-Aldrich, Poole, UK). Prior to loading total protein samples were denatured by incubating at 95 °C for 5 min in the presence of 5× reducing sample buffer (60 mM Tris-HCl pH 6.8, 25 % glycerol, 2 % SDS, 10 % β-mercaptoethanol and 0.1 % bromophenol blue). Protein samples (20 μg/lane) were loaded into SDS-PAGE (10 %) gels and resolved proteins transferred onto a polyvinylidenifluoride (PVDF) membrane (Amersham, Buckinghamshire, UK) by the use of a wet tank blotter (Bio-Rad, Watford, UK) at 150 V for 1 h. Membranes were then blocked with 5 % non-fat milk and incubated with β-actin (1:1000), Runx2 (1:500), OPN (1:200), NPP1 (1:100), TNAP (1:5000), MGP (1:200) cathepsin K (1:200) or carbonic anhydrase II (1:500) antibodies overnight at 4 °C with shaking. After washing, blots were incubated in horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. A peroxidase detection system (Immobilon™ Western, Merck-Millipore, Watford UK) and ChemiDoc™ XRS+ system (Bio-Rad, Watford, UK) were used for the visualisation of immunoreactivity. Quantification of blots was performed using ImageJ software with band densities normalised to the β-actin loading control to show the relative change in protein expression.

2.8. Immunofluorescence

Osteoclasts and osteoblasts were cultured on sterile 1 cm discs, cut from Melinex (Du Pont, Dumfries, UK), for 7 or 14 days, respectively. Cells were fixed with 4 % paraformaldehyde in 0.1 M phosphate buffer for 20 min at room temperature, washed 3 × 5 min in PBS and stored at 4 °C in PBS until staining. Each disc was incubated in a 2 % BSA in PBS blocking solution for 1 h. Discs were then incubated overnight at 4 °C in the primary antibody solution (1:200 in 2 % BSA in PBS). Cells were washed 3 × 5 min in PBS before incubation for 1 h with a Cy3-labelled secondary antibody solution (1:400 in PBS with 1 % BSA). After three further 5-min PBS washes, disc were mounted on to microscope slides using Prolong™ Diamond Antifade Mountant with DAPI (Thermofisher Scientific, UK) and viewed by fluorescence microscopy (Cy3 absorbance and emission at 550 nm and 570 nm, respectively).

Table 1
Primer sequences for qPCR.

	Sense (5′-3′)	Anti-sense (3′-5′)
β-actin (*Actb*)	gat gtg gca cca cac ctt ct	ggg gtg ttg aag gtc tca aa
RANK (*Tnfrsf11a*)	ggg tga ttt tct ttt ggt ggg tc	tgc tcg tga taa cta tgc ctg tg
c-fms (*Csf1r*)	gat gtg tga gca atg gca gtg tg	tca ggg tcc aag gtc cag tag g
DC-STAMP (*Dcstamp*)	cgc tgt gga cta tct gct gta	cct tgg gtt cct tgc ttc t
V-ATPase (*Atp6v0d2*)	aag cct ttg ttt gac gct gt	tga atg cca gca cat tca tc
Carbonic anhydrase 2 (*Car2*)	tga aga ttg gac ctg cct cac a	Agc aga ggc gga gtg gtc ag
TRAP (*Acp5*)	att tgt ggc tgt ggg cga ct	gca cgg ttc tgg cga tct ct
Cathepsin K (*Ctsk*)	gca ggg tcc cag act cca tcg	gct gaa agc cca cag gaa cca c
NPP1 (*Enpp1*)	gtc agt atg cgt gct aac	tgg cac act gaa ctg tag
Runx2 (*Runx2*)	acc ata aca gtc ttc aca aat cct	cag gcg atc aga gaa caa acta
TNAP (*Alpl*)	ggg acg aat ctc agg cta	tca ggg cat ttt tca agg tc
Osteopontin (*Spp1*)	gag agc cag gag agt gcc a	gct ttg gaa ctt gct tga cta tcg
Osteocalcin (*Bglap*)	ccg gga gca gtg tga gct ta	tag atg cgt ttg tag gcg gtc
MGP (*Mgp*)	tca cga aag cat gga gtc ct	gct gag ggg aca taa agg tg
Col1α1 (*Col1α1*)	cag cag att gag aac atc cgc ag	gag tgg cac atc ttg agg tcg c
ANK (*Ankh*)	cat cac caa cat agc cat cg	Aag gca gcg aga tac agg aa
E11 (*E11*)	aac aag tca ccc caa tag	cta aca aga cgc caa cta tga ttc
DMP1 (*Dmp1*)	tga aga gag gac ggg tgat t	ctt ctg atg act cac tgt tcg tg

2.9. Determination of TNAP and total NPP activity

Enzyme activity was measured in osteoclasts and osteoblasts cultured with PP_i or P_i (10-100 μM) at regular intervals throughout the culture. TNAP and total NPP activity were measured in cell lysates using colorimetric assays as previously described [29,32]. Enzyme activity was normalised to cell protein using the Bradford Assay (Sigma-Aldrich, Poole, UK).

2.10. Measurement of ATP release

ATP release from PP_i-treated osteoclasts and osteoblasts was measured at regular intervals throughout the culture. The effects of TNAP inhibition and pertussis toxin on PP_i-induced changes in ATP release were measured in mature cells. To avoid confounding effects of any treatments on the ATP measurements, culture medium was removed, cell layers washed, and cells incubated with serum-free DMEM (without additional PP_i or inhibitors). To quantify ATP release, samples were collected after 1 h and immediately measured using the luciferin-luciferase assay as described previously [9]. ATP release was normalised for cell number and viability monitored using a colorimetric lactate dehydrogenase (LDH) assay (Promega UK, Southampton).

2.11. Soluble collagen levels

Collagen production was determined in osteoblasts after 14 days of culture. Cells were transferred to medium containing 5 % FCS, 2 mM β-glycerophosphate, 50 μg/ml ascorbic acid and the lysyl oxidase inhibitor, β-aminoproponitrile (50 μg/ml), for the final 24 h of culture. The concentration of collagen accumulated in the tissue culture medium was assayed using a Sirius red dye-based kit (Sircol soluble collagen assay, Biocolor Ltd., Newtownabbey, UK) according to the manufacturer's instructions. Total protein concentrations in lysates were determined using the Bradford assay.

2.12. cAMP ELISA

Intracellular cAMP levels were measured in mature osteoblasts 1 h after treatment with a single dose of PP_i. Treatment with the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX), was used as a positive control to increase cAMP levels. The concentration of cAMP in cell lysates was measured using the mouse/rat cAMP parameter immunoassay kit (R&D Systems Europe Ltd) as per the manufacturer's instructions.

2.13. Statistics

Data were analysed using GraphPad Prism 9 software (San Diego, CA). All data are presented as bar graphs with points to show the values for individual experiments. Results show data from 4 to 6 individual experiments; each experiment was performed using cells isolated from different animals. Biological replicates comprised of cells derived from males and females. Within every experiment each group contained 3–7 technical replicates. To account for the inherent variation of using primary cells isolated from different animals, all data were analysed using a

randomised block ANOVA followed by a Fisher's LSD post hoc test as described by Festing [33].

3. Results

3.1. PP_i inhibits osteoclast formation and activity

Long-term treatment with PP_i (100 μM from day 3 of culture) decreased osteoclast number and resorptive activity by up to 50 % and 45 %, respectively (Fig. 1A & B). The representative transmitted and reflected light microscopy images in Fig. 1C show the inhibitory actions of long-term treatment with PP_i on osteoclast formation and activity. Treatment with PP_i from day 5 (early osteoclasts) decreased osteoclast number by up to 25 % (compared to ≤50 % from day 3) whilst a single dose to mature cells (from day 7) was without effect (Fig. 1D). The anti-resorptive effects of PP_i were unaffected by duration of exposure, with a single dose to mature cells inhibiting resorption to the same degree as long-term administration (Fig. 1E).

3.2. The effect of PP_i on bone mineralisation is dependent on osteoblast differentiation stage and the duration of exposure

When osteoblasts were cultured with PP_i for the duration of the culture (21 days) mineralised nodule formation was reduced by 45 % at 1 μM PP_i, increasing to a 95 % inhibition at 100 μM (Fig. 1F). However, if cells were exposed to 100 μM PP_i for only the differentiation phase of the culture (days 0–7 or days 0–14), the amount of bone formation was increased by up to 70 % (Fig. 1G). Addition of PP_i at only the latter stages of culture (days 7–21 or 14–21) inhibited bone mineralisation to the same extent as when PP_i was present throughout (Fig. 1G).

3.3. PP_i decreases the expression of genes modulating osteoclast formation and activity

qRT-PCR analysis was performed on key genes associated with osteoclast formation, fusion and resorptive activity in early, mature and mature resorbing osteoclasts (Fig. 2). Data are presented as fold-change relative to untreated cells. The mRNA expression of factors critical for osteoclastogenesis, *Tnfrsf11a* (RANK) and *Csf1r* (c-fms), was reduced in PP_i-treated osteoclasts (100 μM) (Fig. 2A & B). *Dcstamp* expression was decreased by PP_i in early osteoclasts but unaffected in mature and mature, resorbing cells (Fig. 2C). PP_i also decreased *Ankh* levels in mature, resorbing osteoclasts (Fig. DF). Genes associated with bone resorption (*Ctsk, Atp6v0d2, Car2, Acp5*) were all downregulated by PP_i, particularly in mature and mature, resorbing osteoclasts (Fig. 2E-H).

The effect of PP_i on the expression of selected proteins by osteoclasts was also examined (Fig. 2I). Consistent with the qRT-PCR data, PP_i (100 μM) caused small decreases in cathepsin K and carbonic anhydrase II protein expression in mature, resorbing osteoclasts (Fig. 2I & Supp. Fig. 1A-B).

3.4. PP_i promotes osteoblast differentiation and matrix deposition

Culture with PP_i (100 μM) increased the mRNA expression of early (*RUNX2*) and mature (*Bglap*) osteoblast genes by up to 2.5-fold (Fig. 3A-B). PP_i also promoted the expression of other mineralisation inhibitors (*Spp1* (Opn), *Mgp*) (Fig. 3C-D). The level of *Col1α1* expression was increased 2-fold but this did not reach statistical significance; *Ankh* expression was also unchanged (Fig. 3E-F). Levels of the early osteocyte markers (*E11, Dmp1*) were up to 3-fold higher (Fig. 3G & H). No effects of P_i on gene expression were observed at any stage (Supp. Fig. 2A-H).

Western blot analysis of selected proteins showed that the changes in mRNA expression were generally reflected at the protein level. PP_i caused a small trend towards increased OPN expression but this did not reach statistical significance (Fig. 3I & Supp. Fig. 1D). Expression of Runx2 and MGP was increased in differentiating and mature osteoblasts treated with PP_i (≥10 μM) (Fig. 3I & Supp. Fig. 1C & E).

As mentioned PP_i had no effect on *Col1α1* mRNA expression, however, soluble collagen levels were increased up to 2-fold in PP_i-treated osteoblasts (Fig. 3J). Furthermore, it was consistently observed that matrix deposition was evident at an earlier stage in cells cultured with 100 μM PP_i. Fig. 3K shows representative phase contrast images of the matrix deposition in differentiating and mineralising osteoblasts with and without PP_i.

3.5. Osteoclasts and their precursors express the enzymes needed for PP_i generation and metabolism

TNAP, the principal enzyme involved in PP_i hydrolysis, is typically thought of as a marker of the osteoblast phenotype. Here, we show that mature osteoclasts and their precursors express TNAP and that these cultures exhibit low levels of TNAP activity at all stages (Fig. 4A & C). It should be noted that (1) these are mixed cell cultures and so the TNAP activity could be attributed to stromal cells and (2) the level of TNAP activity detected is significantly lower (approximately 35-fold) than that seen in mature, mineralising osteoblasts (Fig. 4C). Osteoclasts also NPP1 express protein (Fig. 4A). Levels of total NPP activity are the same in early and mature osteoclasts but are 2.2-fold lower than in mature osteoblasts (Fig. 4D).

3.6. The effects of PP_i on TNAP and NPP1 expression and activity in osteoblasts and osteoclasts

TNAP activity was increased by up to 35 % in mature osteoblasts treated with 100 μM PP_i (Fig. 4E). This was accompanied by higher levels of mRNA (2.5-fold) and protein expression (Fig. 4B, H, Supp. Fig. 1G). Conversely, in osteoblasts isolated from *Enpp1*$^{-/-}$ mice, which have lower levels of extracellular PP_i, TNAP activity was reduced by up to 55 % (Fig. 4G). In contrast, P_i had no effect on osteoblast TNAP expression or activity (Supp. Fig. 2G and I). PP_i had no effect on TNAP activity or expression in osteoclast cultures at any stage (Fig. 4F, J).

Enpp1 mRNA and NPP1 protein expression were decreased in PP_i-treated osteoblasts at all stages of differentiation (Fig. 4B, I, Supp. Fig. 1F). In osteoclasts, PP_i also down-regulated *Enpp1* mRNA expression (Fig. 4K). Due to low protein levels western blot analysis could not reproducibly detect NPP1 in osteoclasts.

3.7. P_i regulates osteoclast resorptive activity but does not mediate the effects of PP_i

The low level of TNAP expression in osteoclast cultures means that there is the capacity to hydrolyse PP_i to generate P_i. Therefore, it was necessary to determine whether the observed functional effects of PP_i were mediated indirectly via P_i. Treatment with P_i (≤100 μM) had no effect on osteoclast number (Fig. 5A, B) but reduced osteoclast resorptive activity by up to 45 % (Fig. 5A, C). Representative transmitted and reflected light microscopy images show the decreased bone resorption in P_i-treated osteoclasts (Fig. 5C).

Culture with a selective TNAP inhibitor (0.1-10 μM CAS496014-12-2 (TNAPi)) reduced basal TNAP activity by 80 % (10 μM) (Fig. 5D). The TNAPi alone decreased osteoclast number by 25 % but had no effect on bone resorption (Fig. 5E & F). Furthermore, the effects of PP_i were potentiated by TNAP inhibition; for example, PP_i reduced osteoclast number by 54 % and 65 % in the absence or presence of the TNAPi, respectively. In terms of resorption, the PP_i mediated inhibition was 65 % when co-administered with the TNAPi compared to 40 % without (Fig. 5E & F).

3.8. PP_i decreases controlled ATP release from osteoblasts and osteoclasts

ATP released from cells represents a key source of extracellular PP_i [5]. Long-term culture of osteoclasts and osteoblasts with PP_i decreased

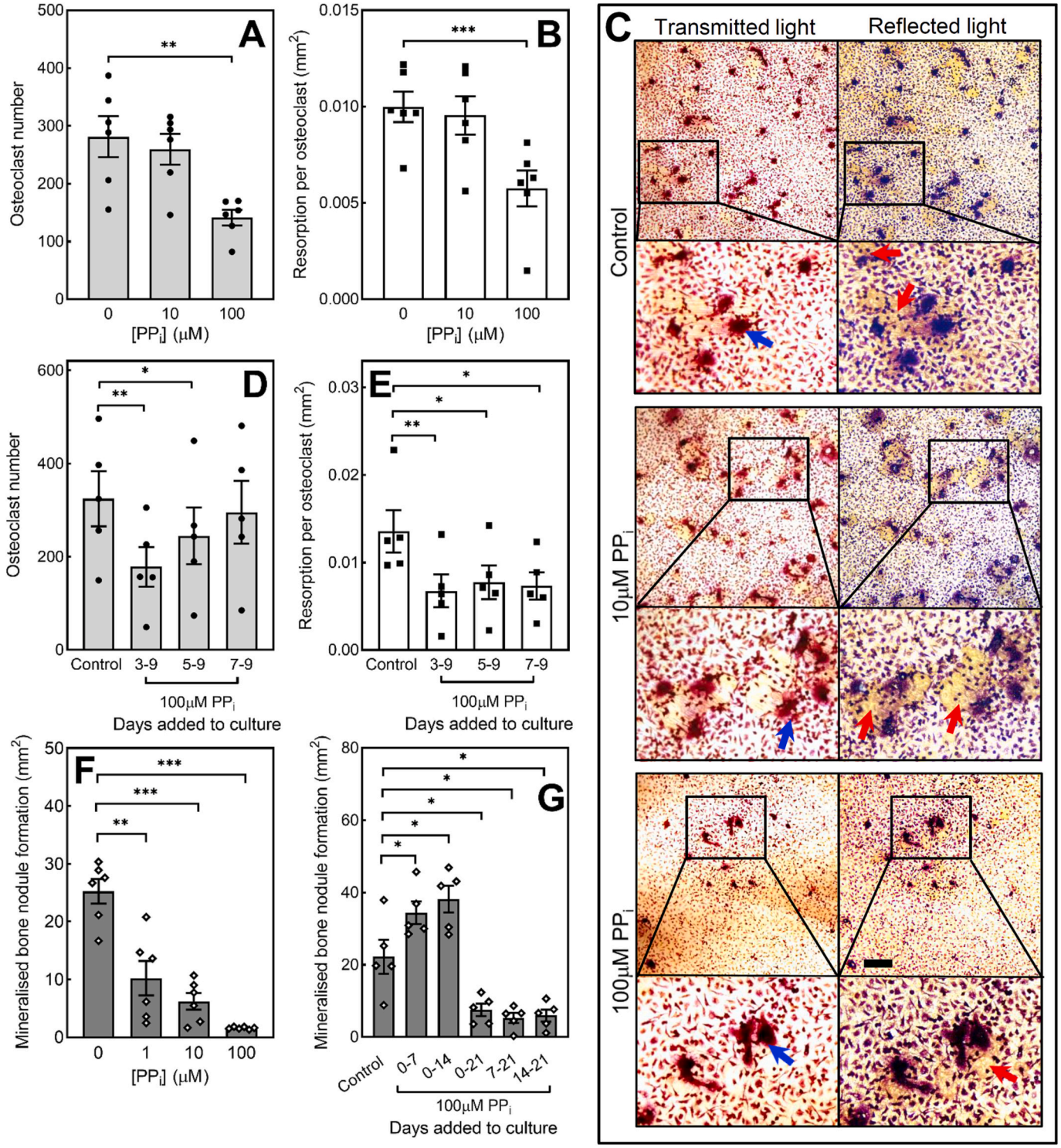

Fig. 1. The functional effects of PP_i on osteoclasts and osteoblasts.
Culture with PP_i (100 μM) decreases (A) osteoclast number and (B) bone resorption by ≤50 % and 45 %, respectively. (C) Representative transmitted and reflected light microscopy images showing the reduced osteoclast formation and resorptive activity in cells treated with PP_i. Osteoclasts are the large red cells as illustrated by the blue arrows in the transmitted light images. Resorption pits are the tan areas clearly visible in the reflected light images, illustrated by the red arrows. Scale bar = 50 μm. (D) The effects of PP_i on osteoclast number require prolonged exposure whilst (E) a single dose is sufficient to inhibit resorption. (F) PP_i (≥1 μM) dose dependently inhibits bone mineralisation by ≤95 %. (G) Exposure of osteoblasts to PP_i in the differentiation phase only increases bone formation by ≤70 %, whilst addition at the latter stages of culture inhibited mineralisation to the same level as constant PP_i treatment. Data shown as mean ± SEM of 4–5 independent experiments: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

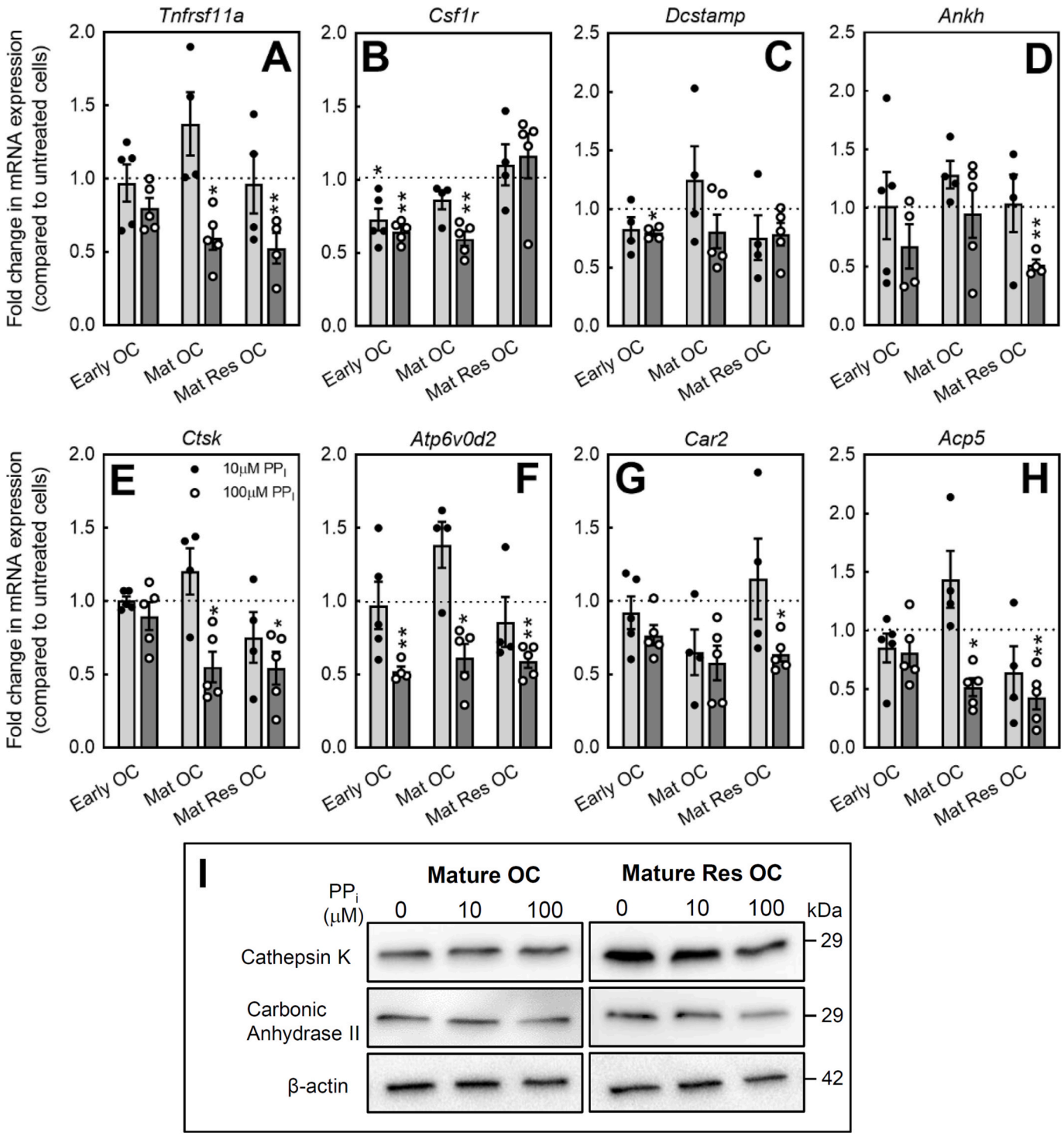

Fig. 2. The effects of PP_i on gene expression by osteoclasts.
The effect of PP_i (10-100 μM) on gene expression was examined in early, mature and mature, resorbing osteoclasts. (A, B) *Tnfrsf11a* and *Csf1r* mRNA levels were decreased by PP_i. (C) DC-stamp levels were slightly reduced in early osteoclasts whereas (D) *Ankh* expression was decreased in mature, resorbing osteoclasts. Genes associated with bone resorption including (E) *Ctsk,* (F) *Atp6v0d2,* (G) *Car2* and (H) *Acp5* were downregulated by PP_i. The dotted line represents control cells. Data shown as mean ± SEM of 4–5 different RNA sets: * = $p < 0.05$, ** = $p < 0.01$. (I) Western blots showing decreased expression of cathepsin K and carbonic anhydrase II in mature, resorbing osteoclasts cultured with PP_i. Blots are representative of images produced from 3 to 4 different protein sets, quantification is shown in supplementary data Fig. 1.

ATP release by up to 60 % (Fig. 6A & B). In osteoclasts, effects on ATP release were only observed in mature, multinucleate cells; PP_i had no effect on ATP release from early osteoclasts (Fig. 6A). A PP_i-induced decrease in ATP release was observed in both differentiating and mature, bone-forming osteoblasts (Fig. 6B).

Culture with the TNAPi (10 μM) also reduced ATP release (~75 %) in osteoblasts (Fig. 6C). At this level, the inhibitor reduced osteoblast TNAP activity by over 85 % (Fig. 6D). Co-administration of PP_i and the TNAPi did not prevent the actions on ATP release in either cell type (Fig. 6E & F). In the case of osteoblasts, the inhibitory effects of PP_i on

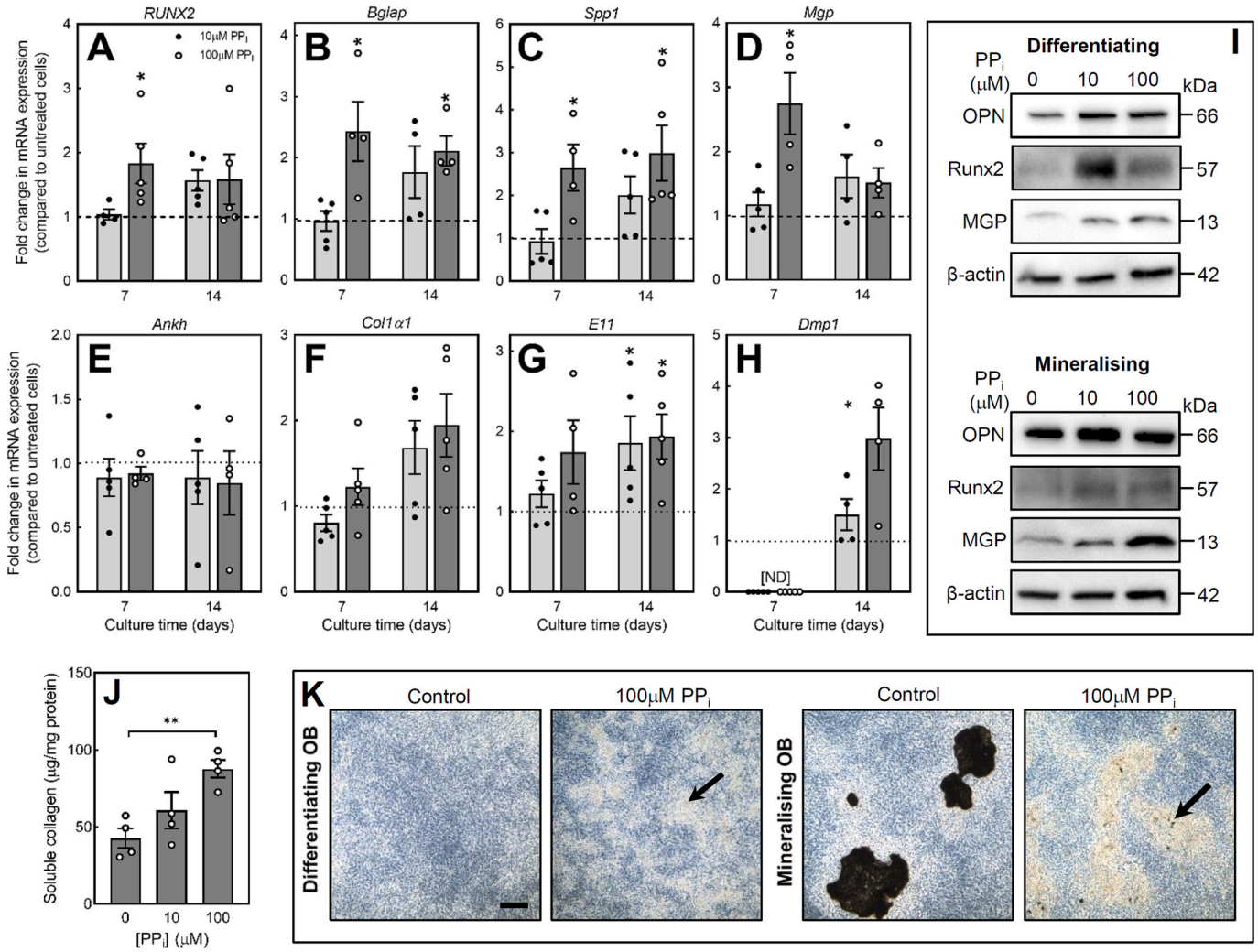

Fig. 3. The effects of PPi on gene and protein expression by osteoblasts.
The ability of PP_i (10-100 μM) to regulate gene expression was investigated in differentiating (day 7) and mature osteoblasts (day 14). mRNA levels of (A) *RUNX2,* (B) *Bglap* (C) *Spp1* and (D) *Mgp* were increased up to 3-fold in PP_i-treated osteoblasts. Expression of (E) *Ankh* and (F) *Col1α1* was unchanged by PP_i. Levels of the early osteocyte markers (G) *E11* and (H) *Dmp1* were up to 3-fold higher in PP_i-treated cells (ND = not detected). The dotted line represents control cells. (I) Western blot analysis showed PP_i increased protein levels of Runx2 and MGP in differentiating and mature osteoblasts. Blots are representative of images produced from 3 to 4 different protein sets; quantification is shown in supplementary data Fig. 1. (J) Soluble collagen levels were increased 2-fold in PP_i-treated osteoblasts. (K) Representative unstained phase contrast microscopy images showing the earlier appearance of matrix deposition (highlighted by the black arrow) in osteoblasts cultured with PP_i. Scale bar = 50 μm. Data shown as mean ± SEM of 4–5 different RNA sets/individual experiments: * = $p < 0.05$, ** = $p < 0.01$.

ATP release were potentiated in the presence of the TNAPi (Fig. 6E). P_i had no effect on ATP release in osteoclasts; however, ATP release from osteoblasts was increased up to 2.5-fold by P_i (100 μM) (Supp. Fig. 2J & K).

Osteoblasts derived from *Enpp1*$^{-/-}$ mice, which display reduced extracellular PP_i levels [20], showed ~45 % increase in ATP release from mineralising cells (Fig. 6G). ATP release from *Enpp1*$^{-/-}$ osteoclasts was unchanged (Fig. 6H).

3.9. The actions of PP_i on ATP release are inhibited by pertussis toxin

To investigate whether the effects of PP_i on ATP release could involve GPCR signalling, PP_i was co-administered with pertussis toxin (which prevents α_i subunit activation). In both osteoblasts and osteoclasts, the inhibitory effects of PP_i on ATP release were lost in the presence of pertussis toxin (Fig. 6I & J).

3.10. The functional effects of PP_i on osteoblasts may involve GPCR signalling

PP_i decreased intracellular cAMP levels by 30 % whereas the positive control, IBMX, increased cAMP by 33 % (Fig. 7A). Culture with PP_i (100 μM) alone decreased bone mineralisation by ~80 %. In the presence of pertussis toxin, the level of inhibition induced by PP_i was reduced to 45 % (compared to control, $p = 0.0839$) (Fig. 7B). The stimulatory effects of PP_i on soluble collagen levels were abolished in the presence of pertussis toxin (Fig. 7C). Representative phase contrast images illustrating these altered effects on collagen deposition and mineralisation are shown in Fig. 7D.

The stimulatory effects of PP_i on MGP and Runx2 protein expression were prevented by co-treatment with pertussis toxin (Fig. 7E & Supp. Fig. 1H-K). Analysis of gene expression showed that the PP_i-induced increase in mRNA levels of *Runx2, Bglap, Mgp* and *Spp1* were lost in the presence of pertussis toxin (Fig. 7F-I). Interestingly, the ability of PP_i to regulate the expression of *Alpl* and *Enpp1* was unaffected by pertussis toxin (Fig. 7J & K).

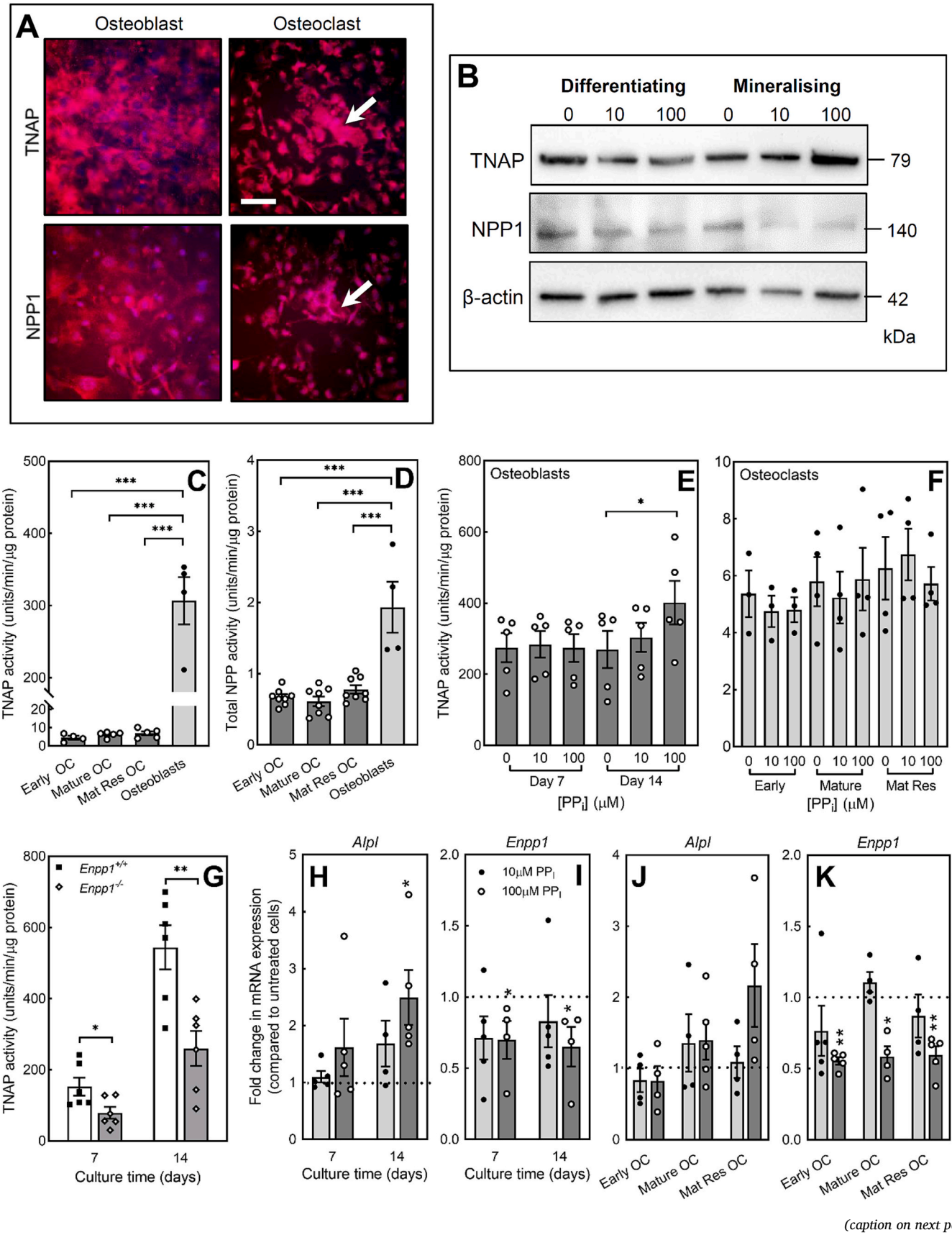

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Fig. 4. PP_i and the expression of TNAP and NPP1 by osteoblasts and osteoclasts.
(A) Immunofluorescence images showing expression of TNAP and NPP1 (red) by osteoblasts and osteoclasts. Multinucleate osteoclasts are highlighted by the white arrows and DAPI nuclear stain is shown in blue. (B) Western blots showing decreased NPP1 protein levels in osteoblasts treated with PP_i; a trend to increased TNAP expression was also observed. Blots are representative of data produced from 3 to 4 different protein sets, quantification is shown in supplementary data Fig. 1. (C) Osteoclasts display a low level of TNAP activity (≤35-fold lower than osteoblasts). (D) Total NPP activity in osteoclasts is ≤2.2-fold lower than in mature osteoblasts. (E) PP_i increases TNAP activity ≤35 % in mature osteoblasts but has no effect in differentiating cells or (F) osteoclasts. (G) Osteoblasts derived from *Enpp1*$^{-/-}$ mice display a 55 % reduction in TNAP activity. mRNA expression of (H) *Alpl* is increased ≤2–5-fold whilst (I) *Enpp1* expression is reduced in PP_i-treated osteoblasts. PP_i has no effect on (J) *Alpl* levels in osteoclasts but downregulates (K) *Enpp1* expression. The dotted line represents control cells. Data shown as mean ± SEM of 4–5 independent experiments/RNA sets: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.11. The role of GPCR signalling in mediating the effects of PP_i on osteoclasts

Treatment with PP_i (100 μM) reduced osteoclast formation by 60 %; this inhibitory action was abolished in the presence of pertussis toxin (Fig. 8A). Osteoclast resorptive activity was decreased by PP_i (60 %), pertussis toxin (40 %) and PP_i and pertussis toxin combined (40 %) (Fig. 8B). Representative reflected light images of these cultures are shown in Fig. 8C. Protein expression of cathepsin K and carbonic anhydrase II in mature, resorbing osteoclasts was decreased by PP_i: this inhibitory action was attenuated in the presence of pertussis toxin (Fig. 8D & Supp. Fig. 1L & M). In mature, resorbing cells expression of key osteoclast genes (*Tnfrsf11a, Ctsk, Atp6v0d2, Car2*) was reduced by PP_i; these inhibitory effects were lost in when PP_i was co-administered with pertussis toxin (Fig. 8E-J).

4. Discussion

The results presented in this study show that PP_i exerts several cell-mediated actions on bone cells. We show for the first time that PP_i can regulate osteoclast formation, resorptive activity and gene/protein expression. In osteoblasts, PP_i appears to exert contradictory actions in that it can promote differentiation whilst also reducing mineralisation both directly and indirectly (by increasing expression of other inhibitors such as MGP). In both cell types, PP_i can reduce cellular ATP release and influence the expression and activity of the enzymes involved in its generation and metabolism. Many of these processes can be blocked or attenuated via the α_I GPCR inhibitor, pertussis toxin. This suggests that the functional effects of PP_i could be mediated, at least in part, via GPCR signalling (summary Fig. 9).

In osteoclasts, extracellular PP_i reduced number and resorptive activity. These inhibitory effects on formation required PP_i levels to be elevated during the entire process of osteoclastogenesis. PP_i addition to mature osteoclasts had no effect on cell number suggesting that it could not cause apoptosis. In contrast, one dose of PP_i to mature cells induced an anti-resorptive effect. PP_i also had a significant effect on osteoclast gene expression, decreasing the mRNA levels of proteins involved in formation (e.g. RANK, c-fms) and bone resorption (e.g. cathepsin K, TRAP, carbonic anhydrase II). We also found that P_i (≤100 μM) reduced resorptive activity but had no effect on osteoclast number. Previous work has shown that P_i can regulate osteoclast formation and resorption, however, these studies used P_i at much higher concentrations (≥2 mM) suggesting that micromolar levels are not sufficient to regulate osteoclastogenesis [34,35]. Osteoclast cultures were found to display a low level of TNAP expression and activity in vitro; immunofluorescence showed TNAP expression in all the cells present including mature osteoclasts, precursors and stromal cells. This agrees with earlier studies that have reported osteoclast and bone marrow cell associated TNAP activity in vivo [36,37]. However, it is important to emphasise that TNAP was only weakly expressed in this culture system and was significantly lower than in osteoblasts. Furthermore, since these are mixed cell cultures, we could not determine which cell type was primarily responsible for the TNAP activity. Nonetheless, this observation made it important to establish if, in this culture system, PP_i was acting indirectly via hydrolysis to P_i. If the observed actions on osteoclasts were due to PP_i breakdown to P_i, then culture with a selective TNAP inhibitor would be expected to block any functional effects. This did not occur and co-administration with a TNAP inhibitor potentiated the actions of PP_i on osteoclast formation and activity. Together, these data indicate that the autocrine effects of PP_i are likely to be independent to those of P_i. Our results also suggest that PP_i can induce signalling pathways to regulate gene expression in osteoclasts that ultimately lead to a reduction in formation and bone resorption.

In osteoblasts, at concentrations that inhibit mineralisation, PP_i increases the expression of many marker genes (e.g. Runx2, OCN) as well as those associated with early osteocytes (e.g. E11, DMP1). We also observed in PP_i-treated cells that matrix deposition appeared earlier and collagen levels were higher. These findings are consistent with an earlier study, which suggested that PP_i can promote differentiation and matrix gene expression in osteoblast-like cells [23]. Interestingly, we also found that treatment with PP_i in the differentiation phase only was associated with an overall increase in the level of bone formed. In contrast, when PP_i was added to mature cells it exerted the same level of inhibition as when present throughout. Collectively, these data suggest that the overall functional effects of PP_i on osteoblasts are dependent on duration of exposure and the cell differentiation state. Extracellular P_i has been shown to regulate gene expression in osteoblasts (reviewed by [38]), however, these effects are only evident with concentrations ≥2 mM. Here, treatment with lower levels of P_i (≤100 μM) did not influence osteoblast gene expression. Therefore, at the concentrations used in this study, PP_i hydrolysis by TNAP will not produce P_i levels high enough to regulate gene expression. Thus, as with the effects on osteoclasts, these actions of PP_i on osteoblasts are likely to be independent of P_i.

Despite their structural similarities, the functional actions of PP_i show some differences to those of the BPs. Several nitrogen and non-nitrogen containing BPs (zoledronate, pamidronate, clodronate, alendronate) inhibit bone mineralisation in vitro with differing levels of potency [39–41]. However, unlike the beneficial actions seen with PP_i, this is associated with decreased proliferation, differentiation, collagen production and TNAP activity as well as increased apoptosis [39,40]. This suggests that the mechanisms underpinning the functional effects of PP_i and the BPs are likely to differ.

Previous work in vitro and in vivo work has suggested that PP_i can stimulate OPN protein expression [13,20,24]. Thus, it has been suggested that some of the inhibitory actions of PP_i on mineralisation are mediated indirectly via OPN [13,20]. In this study, PP_i (100 μM) exerted modest stimulatory effects on OPN mRNA expression and inconsistent actions on protein levels. The difference between this and previous in vitro work is most likely due to the concentration of PP_i used: Addison et al used PP_i at 500 μM, which is considerably higher than the 10-100 μM used here [24]. This suggests that the ability of PP_i to influence OPN expression is likely to be highly dependent on the local PP_i concentration. However, whilst the actions on OPN were small, we observed a striking increase in the protein levels of MGP. This suggests that a PP_i-induced increase in MGP protein may also contribute to the inhibitory effects of PP_i on mineralisation. This idea is supported by the observation that pertussis toxin abolishes the stimulatory effect of PP_i on MGP protein expression and attenuates the inhibitory effects of PP_i on bone mineralisation. Overall, our data support and build upon the earlier suggestion that, in addition to its physiochemical effects, PP_i also

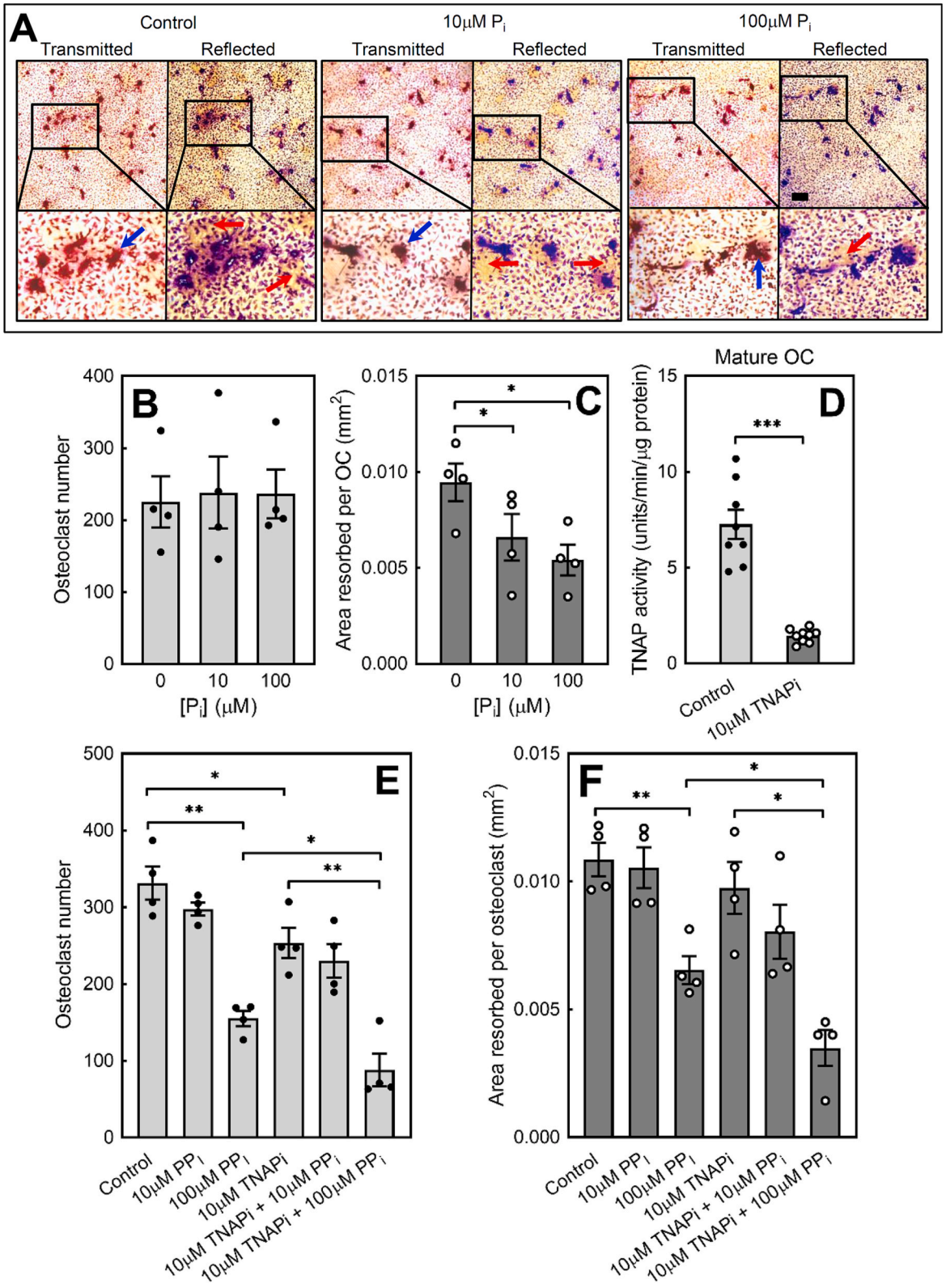

Fig. 5. The effects of PP_i on osteoclasts are unlikely to be mediated indirectly via P_i.
(A) Representative transmitted and reflected light microscopy images showing decreased resorption in osteoclasts cultured with P_i (≥10 μM). Osteoclasts are the large red cells as illustrated by the blue arrows in the transmitted light images. Resorption pits are the tan areas clearly visible in the reflected light images, illustrated by the red arrows. Scale bar = 50 μm. (B) P_i has no effect on osteoclast formation but (C) decreases resorption by up to 45 %. (D) Osteoclast TNAP activity decreases 80 % in the presence of a selective inhibitor. Culture with a TNAP inhibitor potentiated the effects of PP_i on (E) osteoclast number and (F) bone resorption. Data shown as mean ± SEM of 4–5 independent experiments: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

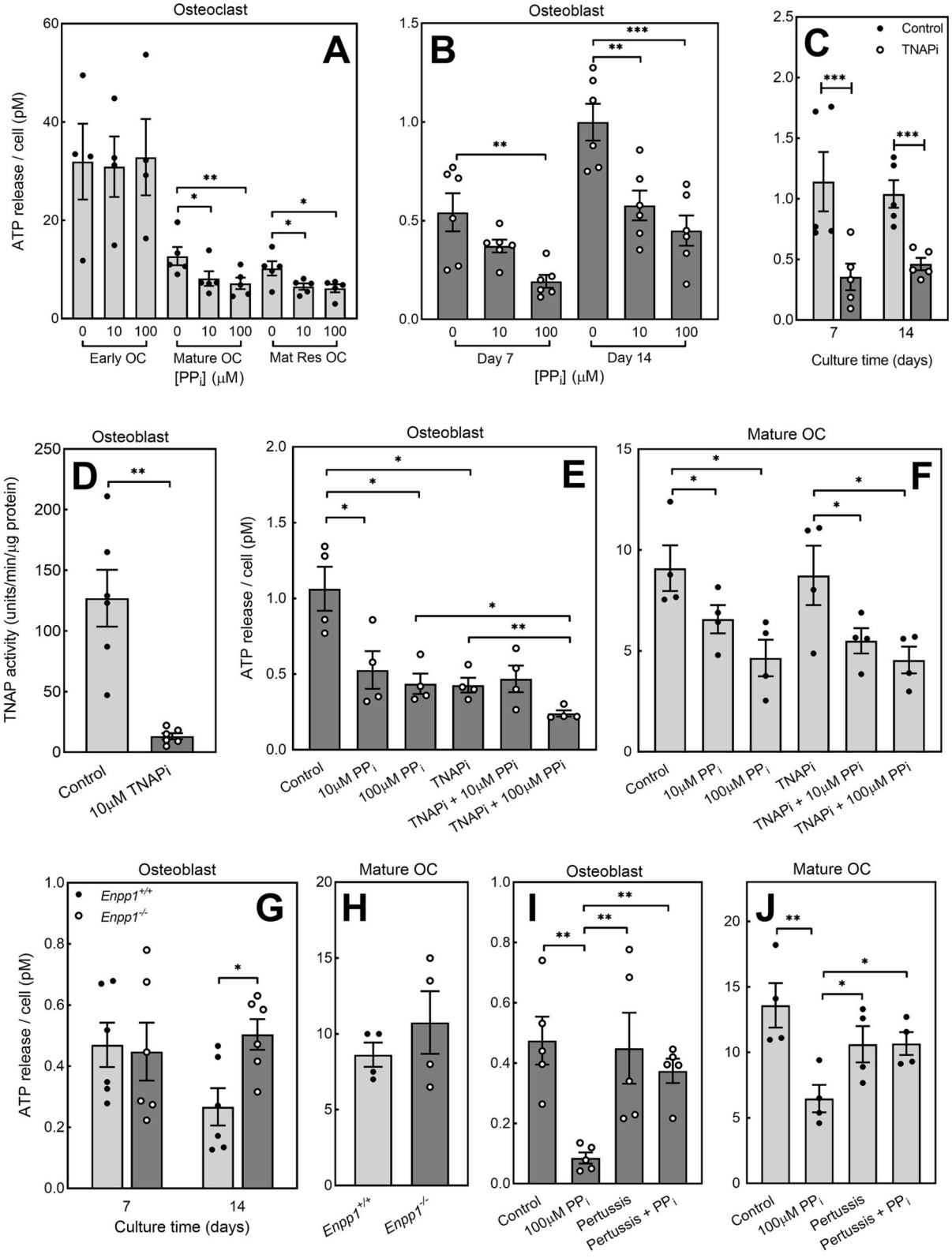

Fig. 6. PP_i decreases ATP release from osteoblasts and osteoclasts.
(A) PP_i decreased ATP release ≤60 % from mature and mature, resorbing osteoclasts. No effect was seen in early osteoclasts. (B) Osteoblasts (differentiating and mature) showed a ≤ 60 % reduction in ATP release in response to PP_i. (C) Culture of osteoblasts with a TNAPi (10 μM) decreased ATP release by ≤75 % and (D) reduced TNAP activity by over 85 %. (E) Co-administration of PP_i and a TNAPi potentiated the actions on ATP release from osteoblasts. (F) A TNAPi had no effect on the inhibitory actions of PP_i on ATP release from osteoclasts. (G) Mature osteoblasts derived from *Enpp1*$^{-/-}$ mice displayed increased levels (≤45 %) of ATP release. (H) Unchanged ATP release from *Enpp1*$^{-/-}$ osteoclasts. The inhibitory effects of PP_i on ATP release were lost in the presence of pertussis toxin in both (I) osteoblasts and (J) osteoclasts. Data shown as mean ± SEM of 4–6 independent experiments: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

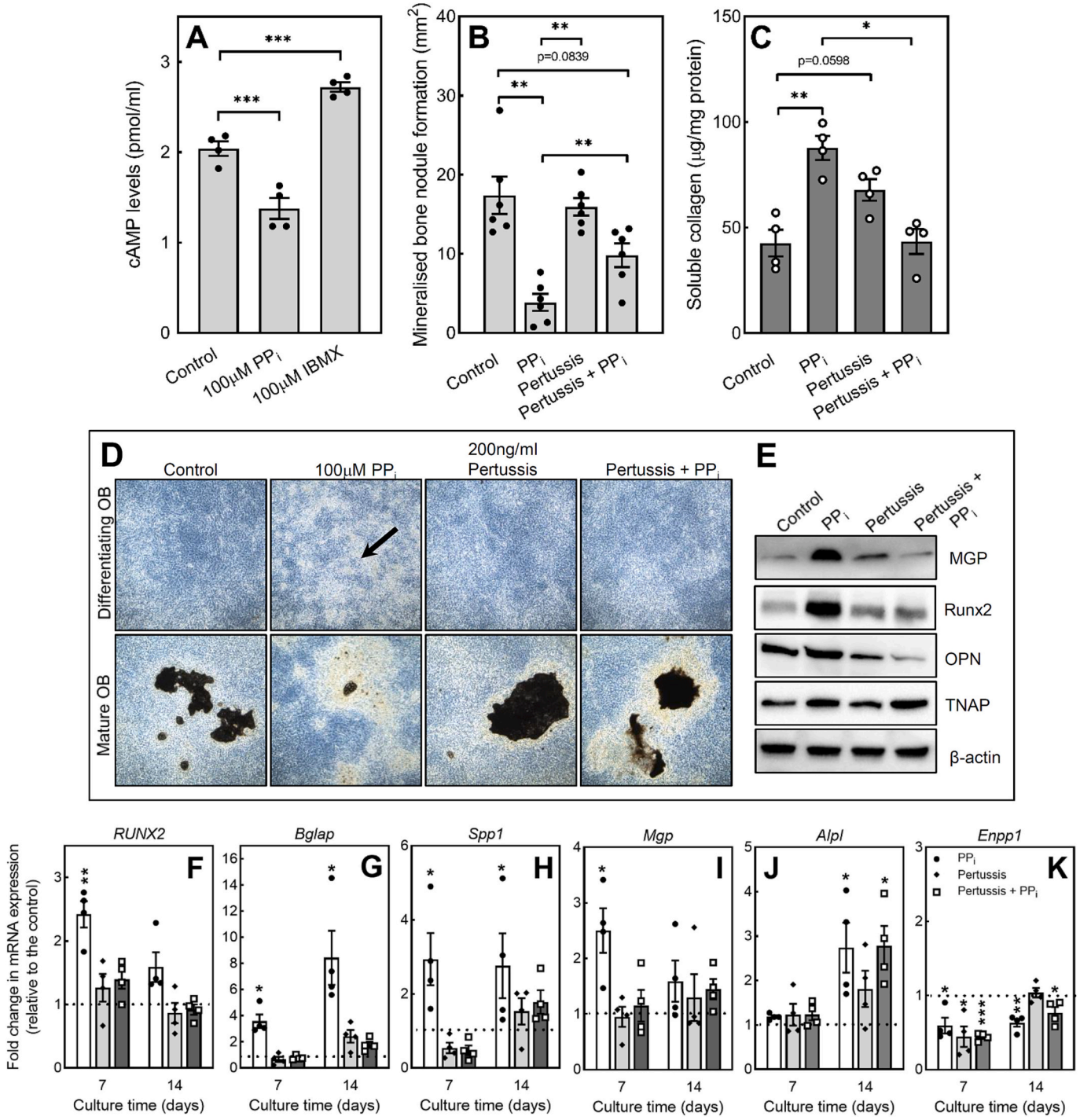

Fig. 7. The effects of pertussis toxin on the functional effects of PP_i in osteoblasts.
(A) PP_i decreased intracellular cAMP levels by 30 % in osteoblasts. Pertussis toxin (B) attenuated the inhibitory effects of PP_i on bone mineralisation and (C) blocked the stimulatory actions of PP_i on soluble collagen levels. (D) Phase contrast images showing the actions of PP_i on matrix deposition and mineralisation are reduced in the presence of pertussis toxin (matrix highlighted by the black arrow). (E) Western blots showing the PP_i-induced increase in MGP and Runx2 protein levels are prevented by pertussis toxin. Blots are representative of data produced from 3 to 4 different protein sets, quantification is shown in supplementary data Fig. 1. The stimulatory effects of PP_i on (F) *RUNX2*, (G) *Bglap,* (H) *Spp1* and (I) *Mgp* mRNA expression are prevented by pertussis. No effect of pertussis on (J) *Alpl* and (K) *Enpp1* expression. The dotted line represents control cells. Data shown as mean ± SEM of 4–6 independent experiments/RNA sets: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

functions indirectly via other proteins to regulate mineralisation.

As mentioned, TNAP and NPP1 play a key role in determining extracellular PP_i levels [12–14]. This study showed that PP_i (but not P_i) increases TNAP expression and activity in osteoblasts. Conversely, *Enpp1*$^{-/-}$ knockout osteoblasts, which have reduced PP_i levels [20], show decreased TNAP activity. In parallel, PP_i decreases NPP1 expression in osteoblasts. These findings are consistent with earlier studies showing that PP_i can regulate TNAP and NPP1 expression and activity [13,15,23]. Osteoclast expression of NPP1 has been reported previously [19] and, similar to osteoblasts, we observed PP_i decreased NPP1 mRNA

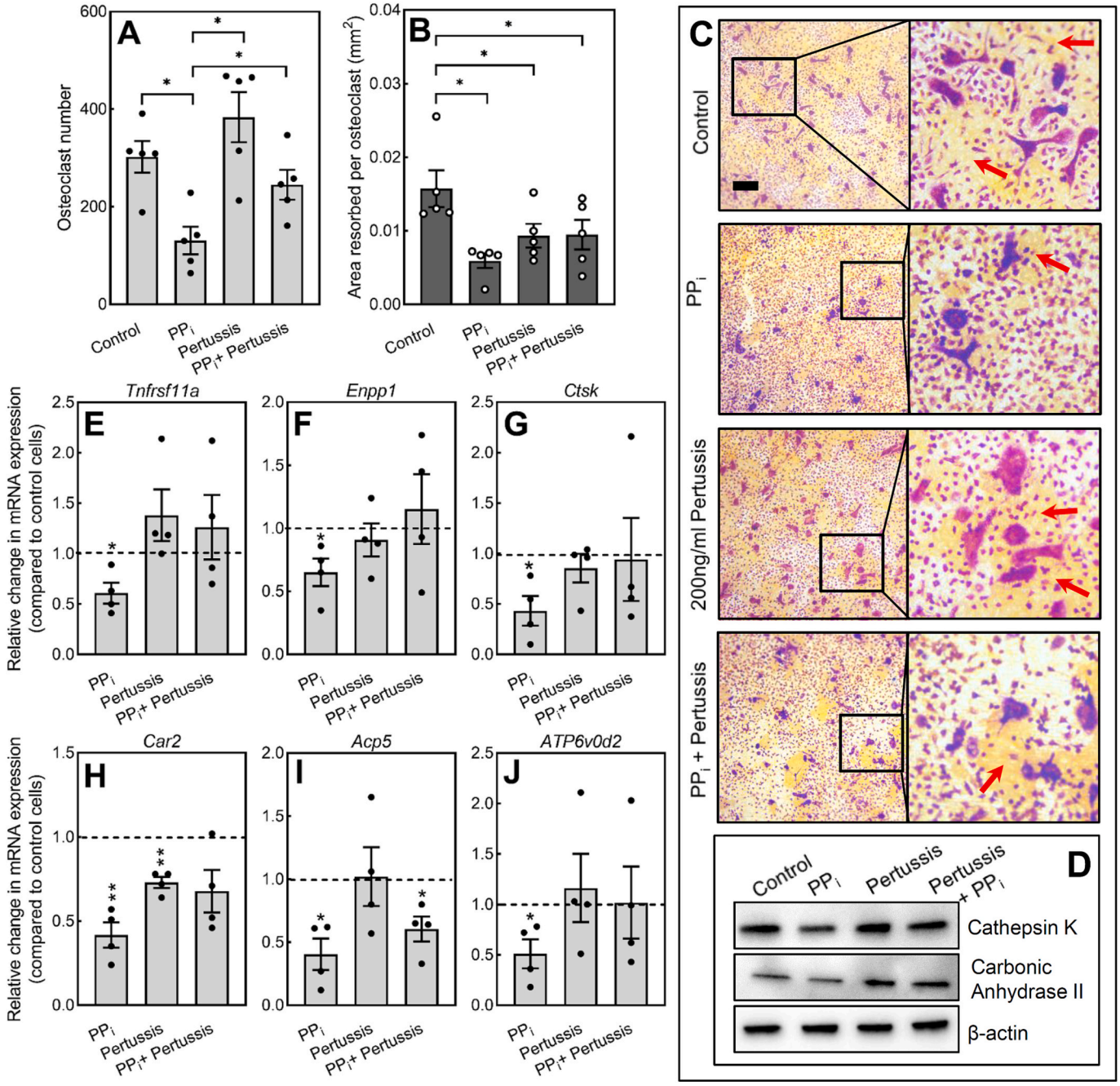

Fig. 8. The effects of pertussis toxin on the functional effects of PP_i in osteoclasts.
(A) The inhibitory effects of PP_i on osteoclast formation are prevented by pertussis toxin. (B) PP_i reduced osteoclast activity by 60 % whilst pertussis toxin and PP_i and pertussis toxin in combination decreased resorption by 40 %. (C) Representative reflected light images showing the effect of pertussis toxin on the functional effects of PP_i on osteoclasts. Resorption pits are the tan areas clearly visible in the images, illustrated by the red arrows. Scale bar = 50 μm. (D) Western blots showing the inhibitory effects of PP_i on carbonic anhydrase II (CAII) and cathepsin K expression are lost in the presence of pertussis toxin. Blots are representative of data produced from 3 to 4 different protein sets, quantification is shown in supplementary data Fig. 1. (E-J) qRT-PCR analysis showing the effects of PP_i on gene expression in osteoclasts are lost in the presence of pertussis toxin. Data shown as mean ± SEM of 4–5 independent experiments/RNA sets: * = $p < 0.05$, (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expression in these cells. Whilst a low level of TNAP expression and activity was detected in osteoclast cultures, PP_i did not regulate either of these parameters suggesting this enzyme is of limited importance in these cells. In agreement, organ cultures performed using calvariae derived from TNAP knockout mice suggest that the enzyme has no significant effect on osteoclast function [42]. Together these data indicate that increased PP_i levels can be detected by bone cells, particularly osteoblasts, resulting in the altered expression and activity of the enzymes involved in PP_i generation and metabolism.

This study shows that extracellular PP_i can also regulate controlled ATP release from osteoblasts and osteoclasts in a concentration dependent manner. Co-administration of a TNAP inhibitor did not prevent this action suggesting that PP_i, and not P_i, mediates this effect. An observation supported by the finding that P_i increases ATP release from osteoblasts and has no effect on osteoclasts. Furthermore, the TNAP inhibitor alone also reduced osteoblast ATP release, suggesting that the resulting change in endogenous PP_i levels was sufficient to be detected by the cells and induce a functional response. In agreement, a recent

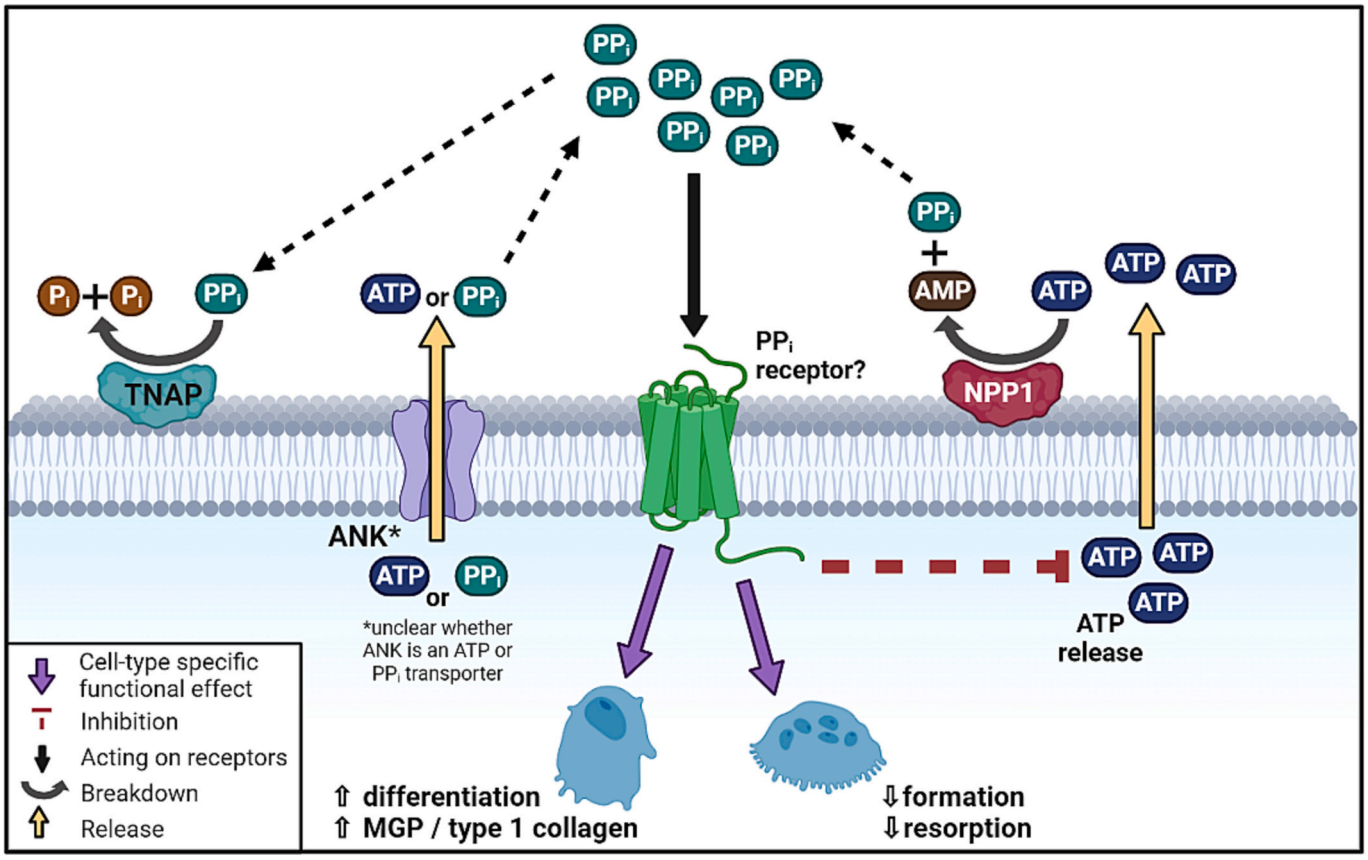

Fig. 9. Summary diagram showing the effects of PP_i.
ATP is released from cells in a controlled manner into the extracellular environment, where it can be hydrolysed by NPP1 to generate AMP and PP_i. ANK has historically been considered a PP_i transporter but there is now evidence to suggest it is an ATP transporter; therefore, either directly or indirectly this protein acts to increase extracellular PP_i levels. Evidence suggests that extracellular PP_i may act directly on cells via a GPCR to modulate function in a generic (e.g. inhibit ATP release) and cell-type specific manner (e.g. increased differentiation, MGP and collagen production in osteoblasts or inhibition of formation and activity in osteoclasts). TNAP hydrolyses PP_i to produce 2 x P_i for bone mineralisation. Image generated in Biorender.com.

study reported that haploinsufficient TNAP mice have reduced extracellular ATP levels [43]. Furthermore, we also observed that *Enpp1*$^{-/-}$ osteoblasts displayed increased levels of ATP release. Whilst changes in the activity of other ecto-nucleotidases could contribute to the reduction in ATP levels observed, these findings indicate that the cells themselves are releasing less ATP in response to increased extracellular PP_i levels. This suggests that, in addition to the actions on TNAP and NPP1, feedback mechanisms exist that regulate levels of extracellular PP_i by acting on cells to alter constitutive ATP release. Whilst previous studies had suggested this ability in osteoblasts [13,15,23], this work shows that the regulatory effects of PP_i are likely to be more extensive than originally thought.

The variety of direct PP_i-mediated effects on osteoblasts and osteoclasts raises the question of how bone cells might detect and respond to extracellular PP_i. Pertussis toxin is widely used in the study of GPCR pharmacology as it prevents the activation of the α_i subunit in GPCR signalling. Here, we found that many of the functional effects of PP_i were either prevented or attenuated when PP_i was co-administered with pertussis toxin. Furthermore, we also observed that acute PP_i treatment led to a decrease in intracellular cAMP levels in osteoblasts. These data provide evidence that $G\alpha_i$-linked GPCR signalling may be involved in transducing the extracellular signal into downstream functional effects. Addison et al reported that a PP_i-induced increase in OPN expression involved MAP kinase signalling [24]. $G\alpha_i$-linked GPCRs can activate the MAP kinase pathway, so this earlier work is consistent with the notion that PP_i is an extracellular signalling molecule that could engage with a cell surface GPCR. Indeed, the precedent for small, non-protein molecules binding to and activating GPCRs is well established as highlighted by known receptors for protons [44], ATP [45] and calcium [46]. It remains to be determined whether PP_i represents an additional agonist or allosteric modulator at a GPCR with known pharmacology or if it has its own as yet unidentified GPCR (from the approximate 120 orphan receptors). Small molecules can also act through different receptor subtypes as evidenced by extracellular nucleotides which can signal via both ligand-gated ion channels and GPCRs [45]. Thus, it is possible that PP_i interacts with multiple receptor types.

It should be acknowledged that the actions of PP_i on bone cells reported here require relatively high micromolar concentrations ($\geq$10 μM) albeit lower than used in previous studies [24]. Whilst the plasma concentration of PP_i is reported to be ~1-6 μM [47], the local levels of extracellular PP_i experienced by bone cells in vivo are unknown. However, the concentrations that induce functional effects in vitro are akin to those required to regulate mineralisation processes and to activate other cell surface receptors (e.g., activation of the P2X7 receptor requires ATP concentrations of $\geq$100 μM [48]). Since extracellular ATP is the primary source of PP_i, in situations where the ATP concentration is sufficient to activate the P2X7 receptor it is probable that PP_i levels would also be higher. Thus, it is plausible that, under certain conditions, cells could experience and respond to micromolar levels of PP_i in vivo.

In conclusion, this study has shown that PP_i exerts a range of functional effects on both osteoblasts and osteoclasts and provides evidence to support the notion that PP_i functions as an extracellular signalling molecule acting on bone cells, potentially via GPCRs. The intracellular mechanisms now require characterisation but the ubiquitous nature of

PP_i means that this signalling system could have widespread physiological and pathophysiological relevance beyond the skeletal system.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2023.116868.

CRediT authorship contribution statement

Lucie E. Bourne: Writing – review & editing, Validation, Investigation, Formal analysis. **Bethan K. Davies:** Writing – review & editing, Validation, Investigation, Formal analysis, Conceptualization. **Jose Luis Millan:** Writing – review & editing, Resources. **Timothy R. Arnett:** Writing – review & editing, Conceptualization. **Caroline P.D. Wheeler-Jones:** Writing – review & editing, Visualization. **Jacob A.C. Keen:** Writing – review & editing, Resources. **Scott J. Roberts:** Writing – review & editing, Resources. **Isabel R. Orriss:** Writing – review & editing, Writing – original draft, Validation, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors have no conflict of interest.

Data availability

Data will be made available on request.

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