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ACTIVATION OF THE $P2Y_2$ RECEPTOR REGULATES BONE CELL FUNCTION BY ENHANCING ATP RELEASE

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ABSTRACT

Bone cells constitutively release ATP into the extracellular environment where it acts locally via P2 receptors to regulate bone cell function. Whilst $P2Y_2$ receptor stimulation regulates bone mineralisation the functional effects of this receptor in osteoclasts remain unknown. This investigation used the $P2Y_2$ receptor knockout ($P2Y_2R^{-/-}$) mouse model to investigate the role of this receptor in bone. MicroCT analysis of $P2Y_2R^{-/-}$ mice demonstrated age-related increases in trabecular bone volume (≤48%), number (≤30%) and thickness (≤17%). *In vitro* $P2Y_2R^{-/-}$ osteoblasts displayed a 3-fold increase in bone formation and alkaline phosphatase activity whilst $P2Y_2R^{-/-}$ osteoclasts exhibited a 65% reduction in resorptive activity. Serum cross-linked c-telopeptide levels (CTX, resorption marker) were also decreased (≤35%). The resorption defect in $P2Y_2R^{-/-}$ osteoclasts was rescued by the addition of exogenous ATP, suggesting that an ATP deficit could be a key factor in the reduced function of these cells. In agreement, we found that basal ATP release was reduced up to 53% in $P2Y_2R^{-/-}$ osteoclasts. The $P2Y_2$ receptor agonists, UTP and 2-thioUTP, increased osteoclast activity and ATP release in wildtype but not $P2Y_2R^{-/-}$ cells. This indicates that the $P2Y_2$ receptor may regulate osteoclast function indirectly by promoting ATP release. UTP and 2-thioUTP also stimulate ATP release from osteoblasts suggesting that the $P2Y_2$ receptor exerts a similar function in these cells. Taken together, our findings are consistent with the notion that the primary action of $P2Y_2$ receptor signalling in bone is to regulate extracellular ATP levels.

INTRODUCTION

Adenosine triphosphate (ATP) has long been recognized for its role in intracellular energy metabolism; however, it is also exported to the extracellular environment where it acts as an important signalling molecule (Burnstock 2007a). Outside cells, ATP and related compounds act via purinergic receptors to modulate a range of biological processes. These receptors are classified into two groups; P1 and P2 receptors. There are four P1 receptors (A_1,A_{2a},A_{2b},A_3), which are activated by adenosine. The P2 receptors are further subdivided into the P2X ligand-gated ion channels and the P2Y G-protein-coupled receptors. P2X receptors are activated by ATP whilst P2Y receptors respond to nucleotides including ATP, adenosine diphosphate (ADP), uridine triphosphate (UTP) and uridine diphosphate (UDP) (Abbracchio and Burnstock 1994; Burnstock 2007b). Currently, seven P2X receptors (P2X1-7) and eight P2Y receptors ($P2Y_{1,2,4,6,11-14}$) have been identified (Burnstock 2007b).

The P2Y receptors display distinct pharmacology with some being activated by adenine-containing nucleotides ($P2Y_1$, $P2Y_{12}$, $P2Y_{13}$), whilst others are stimulated by uridine-containing nucleotides ($P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{14}$) (Burnstock 2007a, b). The primary agonist at the $P2Y_2$ receptor is UTP but it is also activated by ATP. Selective synthetic agonists (e.g. 2-thioUTP) are also available. Receptor stimulation activates phospholipase C and results in Ca^{2+} release from internal stores. Expression of the $P2Y_2$ receptor has been reported in many tissues including heart, blood vessels, lung, kidney and skeletal muscle (Burnstock 2007a).

Bone cells express multiple P2 receptor subtypes and knowledge of the functional effects of extracellular nucleotides in bone has increased significantly in recent years (Burnstock, et al. 2013; Gartland, et al. 2012; Noronha-Matos and Correia-de-Sa 2016; Orriss 2015). $P2Y_2$ receptor expression by osteoclasts has been widely reported (Bowler, et al. 1995; Buckley, et al. 2002; Hoebertz, et al. 2000; Orriss, et al. 2011b). Early work using cells from a human osteoclastoma suggested that ATP could act via the $P2Y_2$ receptor to promote bone resorption (Bowler et al. 1995). However, in a follow up study UTP failed to stimulate resorption, suggesting this was not the case (Bowler, et al. 1998). To date, there are no studies directly

describing the functional effects of $P2Y_2$ receptor activation on osteoclasts. In contrast, activation of several other P2Y receptor subtypes ($P2Y_1$, $P2Y_6$, $P2Y_{12}$, $P2Y_{14}$) has been associated with increased osteoclast formation and/or activity (Hoebertz, et al. 2001; Lee, et al. 2013; Orriss et al. 2011b; Su, et al. 2012; Syberg, et al. 2012b).

The role of the $P2Y_2$ receptor in osteoblasts has been more extensively investigated. $P2Y_2$ receptor expression by osteoblasts has been extensively reported (Bowler et al. 1995; Hoebertz et al. 2000; Maier, et al. 1997), with several studies describing that expression is differentiation-dependent with the highest levels seen in mature, bone forming cells (Noronha-Matos, et al. 2012; Orriss, et al. 2006). $P2Y_2$ receptor activation in osteoblast-like cells activates several intracellular signalling pathways including protein kinase C, p38 mitogen-activated protein kinase, c-Jun NH_2-terminal protein kinase and RhoA GTPase (Costessi, et al. 2005; Gardinier, et al. 2014; Katz, et al. 2006, 2008; Pines, et al. 2005). The $P2Y_2$ receptor has also been shown to mediate the Ca^{2+} mobilisation induced by oscillatory fluid flow (You, et al. 2002).

One of the first functional effects to be attributed to the $P2Y_2$ receptor was the inhibition of bone mineralisation by ATP and UTP (Hoebertz, et al. 2002; Orriss, et al. 2013; Orriss, et al. 2007). Consistent with this, initial skeletal analysis of 8-week old $P2Y_2$ receptor knockout mice ($P2Y_2R^{-/-}$) demonstrated large increases in trabecular and cortical bone parameters in the long bones (Orriss et al. 2007; Orriss, et al. 2011a). Furthermore, $P2Y_2$ overexpression leads to decreased bone formation (Syberg, et al. 2012a) and polymorphisms in the $P2Y_2$ receptor gene are associated with increased bone mineral density and a decreased risk of osteoporosis (Wesselius, et al. 2013). In contrast, a recent study using $P2Y_2R^{-/-}$ mice on a different genetic background, described small decreases in the trabecular bone in knockout animals (Xing, et al. 2014), this work additionally reported that the $P2Y_2$ receptor promotes bone mineralisation.

The $P2Y_2$ receptor may also have a functional role in mediating osteoblast mechanosensitivity. Studies suggest that the $P2Y_2$ receptor promotes mechanotransduction (Xing et al. 2014) and increases cell stiffness and cytoskeletal rearrangement in response to fluid shear stress (Gardinier et al. 2014).

Expression of the $P2Y_2$ receptor has also been reported in MLO-Y4 osteocyte-like cells (Kringelbach, et al. 2014). The same study also demonstrated controlled ATP release from these cells and reported that UTP, probably acting via the $P2Y_2$ or $P2Y_4$ receptors, increased this ATP release.

Available evidence thus indicates that the $P2Y_2$ receptor plays significant, although not yet fully defined roles in regulating bone remodelling. This study used the *P2Y$_2$R*$^{-/-}$ mouse, which was first generated almost 2 decades ago (Cressman, et al. 1999), to determine how $P2Y_2$ receptor-mediated signalling influences bone cell function *in vitro* and *in vivo,* with a particular focus on its effects in osteoclasts.

METHODS

Reagents

Tissue culture reagents were purchased from Life Technologies (Paisley, UK); unless mentioned, all chemicals were purchased from Sigma Aldrich (Poole, Dorset, UK). UTP and 2-thioUTP were purchased from Tocris Bioscience (Bristol, UK).

Animals

Mice lacking the $P2Y_2$ receptor gene ($P2Y_2R^{-/-}$) were obtained from Jackson Laboratories (Bar Harbor, Maine, USA). The generation and characterisation of $P2Y_2R^{-/-}$ mice (C57BL/6J background) has been previously described (Homolya, et al. 1999). All animals were housed under standard conditions with free access to food and water. Animals were bred from homozygote ($P2Y_2R^{-/-}$) and parental strain wildtype ($P2Y_2R^{+/+}$) breeding pairs. All procedures complied with the UK animals (Scientific Procedures) Act 1986 and were reviewed and approved by the Royal Veterinary College Research Ethics Committee.

Microcomputed x-ray tomographic (μCT) analysis of $P2Y_2R^{-/-}$ mice

The tibiae and femora were isolated from male 4, 8, 16 and 24-week old $P2Y_2R^{-/-}$ and $P2Y_2R^{+/+}$ mice (n=10), fixed in 10% neutral buffered formalin (NBF) for 24 hours and stored in 70% ethanol until scanning. μCT analysis of trabecular and cortical bone parameters was performed on the tibial and femoral metaphysis (SkyScan 1172, Bruker, Belgium). The appearance of the first cartilage bridge was used as a reference point, with an offset of 0.4mm and 2.5mm for trabecular and cortical bone, respectively. In all cases the length of bone analysed was 1mm. The μCT scanner was set at 50Kv and 200μA using a 0.5mm Al filter and a resolution of 4.3μm. Analysis of isolated bones was performed blind. The images were reconstructed, analysed and visualised using SkyScan NRecon, CTAn and CTVol software. Bone mineral density (BMD) was calibrated and calculated using hydroxyapatite phantoms with a known density.

Osteoblast formation assay

Osteoblasts were isolated from the calvariae of 3-5 day old *P2Y$_2$R*$^{+/+}$ or *P2Y$_2$R*$^{-/-}$ mice by trypsin/collagenase digestion as previously described (Orriss, et al. 2012b; Taylor, et al. 2014). Cells were cultured for up to 21 days in alpha Minimum Essential Medium, (*α*MEM) supplemented with 2mM β-glycerophosphate and 50μg/ml ascorbic acid, with half medium changes every 3 days. The total area of bone nodules formed was quantified by image analysis, as described previously (Orriss et al. 2012b).

Primary osteoblasts of bone marrow/stromal cell origin were obtained from the long bones of 6-week old male *P2Y$_2$R*$^{+/+}$ or *P2Y$_2$R*$^{-/-}$ animals. The collected cells were suspended in α-MEM and pre-cultured in a 75 cm^2 flask in 5% CO_2 at 37ºC. After 24 hours the α-MEM was replaced in order to eliminate non-adherent cells; adherent stromal cells were cultured for a further 7 days. When confluent, cells were plated into 6-well trays and cultured as above.

Alkaline phosphatase (TNAP) activity

Osteoblast TNAP activity was measured in cell lysates taken at defined stages of osteoblast differentiation as previously described (Orriss et al. 2012b; Taylor et al. 2014). TNAP activity was normalised to cell protein using Bradford reagent. Time points in osteoblast cultures were defined thus: proliferating (day 4, calvarial only); differentiating (day 7); mature (day 14) and mature, bone-forming (day 21)

Osteoclast formation assay

Osteoclasts were isolated from the long bones of 6-8 week-old male *P2Y$_2$R*$^{+/+}$ or *P2Y$_2$R*$^{-/-}$ mice as described previously (Orriss and Arnett 2012). Cells were plated onto 5mm diameter ivory discs (10^6 cells) in 96-multiwells in αMEM supplemented with 10% FCS, 5% gentamicin, 100nM PGE_2, 200ng/ml M-CSF and 3ng/ml receptor activator of nuclear factor $_{\kappa}$B ligand (RANKL, R&D Systems Europe Ltd, Abingdon, UK). After 24 hours, discs containing adherent osteoclast precursors were transferred to 6-well trays (4 discs/well in 4ml medium) for a further 6 days. Culture medium was acidified to pH~7.0 by the addition 10meq/l H^+ (as HCL) on day 7 to activate resorption (Orriss and Arnett 2012). $P2Y_2$ receptor agonists (10nM-10μM UTP or 2-

thioUTP) were added from day 3 of culture. Apyrase (a broad spectrum ecto-nucleotidase) was used to determine the effects of endogenous ATP.

Osteoclasts were fixed in 2.5% glutaraldehyde and stained to demonstrate tartrate-resistant acid phosphatase (TRAP). Osteoclasts were defined as TRAP-positive cells with 2 or more nuclei and/or clear evidence of resorption. The total number of osteoclasts and the plan surface area of resorption pits on each disc was assessed 'blind' by transmitted light microscopy and reflective light microscopy and dot-counting morphometry, respectively.

Measurement of serum bone markers

Blood was collected from 4, 8, 16 and 24-week old male $P2Y_2R^{-/-}$ and $P2Y_2R^{+/+}$ mice by cardiac puncture immediately after termination. Following clotting, samples were centrifuged at 500g and the serum frozen until analysis. Levels of the bone formation marker, N-terminal propeptide of type I collagen (P1NP) and the bone resorption marker, cross-linked C-telopeptide (CTX) were assayed using the P1NP and RatLaps™ ELISAs, respectively (Immunodiagnostics Systems Ltd, UK).

Histology

Histological analysis was performed on the femur of 8 and 24-week old male $P2Y_2R^{+/+}$ or $P2Y_2R^{-/-}$ mice. Tissues were fixed in 10% NBF, decalcified in 10% EDTA for three weeks and embedded in paraffin wax blocks. Serial sections were cut every 5µm and slides stained with TRAP counterstained with haematoxylin to visualise osteoclasts.

Total RNA extraction and DNase treatment

$P2Y_2R^{+/+}$ and $P2Y_2R^{-/-}$ osteoclasts were cultured on dentine discs for 9 days (mature, resorbing cells) before total RNA was extracted using TRIZOL® reagent (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Osteoblasts were cultured for 14 days (mature, bone-forming cells) before RNA collection. Extracted RNA was treated with RNase-free DNase I (35U/ml) for 30 min at 37°C. The reaction was terminated by heat inactivation at 65°C for 10

min. Total RNA was quantified spectrophotometrically by measuring absorbance at 260nM. RNA was stored at –80°C until amplification by qRT-PCR.

Quantitative real time polymerase chain reaction (qRT-PCR)

Osteoclast and osteoblast RNA (50ng) 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. qRT-PCR was performed according to 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 sec) and detection (60°C for 30 sec). All reactions were carried out in triplicate using RNAs derived from 4 different cultures. Data were analysed using the Pfaffl method of relative quantification (Pfaffl 2001). Primers were obtained from Qiagen Ltd (Manchester, UK).

Measurement of ATP release

Prior to measurement of ATP release, culture medium was removed, cell layers washed and cells incubated with serum-free DMEM (phenol red free). To measure the effects of $P2Y_2$ receptor deletion on basal ATP release, samples were collected after 1 hour and immediately measured luminometrically using the luciferin-luciferase assay, as described previously (Orriss, et al. 2009). All ATP measurements were normalised to cell number. Cell viability and cell number were determined using the CytoTox 96® colorimetric cytotoxicity assay (Promega UK, Southampton UK).

To examine the effects of acute exposure to UTP or 2-thioUTP (0.1-50μM) agonists were added to the serum-free DMEM and samples taken for quantification after 10, 30, 60 and 90 minutes. The luminescence of the DMEM (± UTP/2-thioUTP) was used as a background reading and subtracted from the relevant measurements. Standard curves used to calculate the ATP concentrations in the presence or absence of UTP/2-thioUTP are shown in **Fig. 5**. To investigate the effects of long-term treatment with $P2Y_2$ receptor agonists, osteoclasts and osteoblasts were cultured with UTP or 2-thioUTP (0.1-100μM) for 7 or 14 days, respectively. Fresh UTP/2-thioUTP was added at each medium exchange. On the day of assay culture

medium was removed and cells incubated with serum-free DMEM without agonists. Samples were collected after 1 hour and measured immediately.

To determine the effects of $P2Y_2$ deletion on ATP breakdown, cells were swapped to DMEM containing 1μM ATP and samples taken after 2, 5, 10, 30 and 60 minutes.

Statistical analysis

Data were analysed using GraphPad Prism 6 software (San Diego, CA). Results are expressed as means ± SEM for between 6-12 biological replicates. Statistical analyses of bone parameters were performed by two-tailed unpaired student's *t*-test. *In vitro* data were analysed using an unpaired student's *t*-test, one-way or two-way ANOVA, followed by a Bonferroni *post hoc* test. For all *in vitro* work, results are representative of experiments performed at least three times, using cells isolated from different animals.

RESULTS

P2Y$_2$R$^{-/-}$ mice show age-related increases in trabecular bone

High resolution μCT analysis revealed that *P2Y$_2$R$^{-/-}$* mice display increased levels of trabecular bone compared to age-matched *P2Y$_2$R$^{+/+}$* controls. These differences appear to be age-related with the biggest changes observed in the 24-week animals. Trabecular bone volume (BV/TV) was increased ≤46% in the femur and ≤48% in the tibia of *P2Y$_2$R$^{-/-}$* mice (**Fig. 1A-1B, 1O**). Trabecular number (Tb.N) was increased ≤27% in the femora **(Fig. 1C, 1O)** and ≤30% in the tibiae **(Fig. 1D, 1O).** Trabecular thickness (Tb.Th) was unchanged up to 8 weeks of age but increased ≤10% and ≤17% at 16 and 24 weeks, respectively **(Fig. 1E-1F, 1O).** Trabecular bone mineral density (Tb.BMD) was ≤12% higher in *P2Y$_2$R$^{-/-}$* mice **(Fig. 1G-1H).** No differences were observed in the cortical bone volume **(Fig. 1K-1L, 1O),** cortical thickness (**Fig. 1K-1L),** endosteal and periosteal diameter (**Fig. 1M-1N**) and bone length at any age.

Increased bone formation by osteoblasts from P2Y$_2$R$^{-/-}$ mice

The level of mineralised bone nodule formation was increased ~3-fold in *P2Y$_2$R$^{-/-}$* calvarial osteoblasts **(Fig. 2A, 2G)** and 5-fold in *P2Y$_2$R$^{-/-}$* long bone osteoblasts **(Fig. 2B).** P2Y$_2$ receptor deletion increased basal TNAP activity (≤3-fold) in calvarial and long bone osteoblasts at all stages of differentiation with the largest effects being observed in the mineralising cells **(Fig. 2C-2D).** Serum TNAP activity was up to 60% higher in *P2Y$_2$R$^{-/-}$* animals **(Fig. 2E)**; no differences were observed in the serum P1NP levels **(Fig. 2F).** No differences in total protein content were observed in any TNAP activity experiments.

Osteoclasts from P2Y$_2$R$^{-/-}$ mice exhibit defective resorption

Whilst no differences in osteoclast numbers were observed **(Fig. 3A, 3D)**, the level of resorption per osteoclast was decreased 75% in *P2Y$_2$R$^{-/-}$* cultures **(Fig. 3B, 3D).** Serum CTX levels were reduced up to 35% in *P2Y$_2$R$^{-/-}$* mice **(Fig. 3C).** Qualitative histology suggested that decreased numbers of osteoclasts were evident on the trabecular and endocortical bone surfaces of 24-week old *P2Y$_2$R$^{-/-}$*; however, no differences were observed in 8-week old animals **(Fig. 3E**).

Changes in gene expression in $P2Y_2R^{-/-}$ osteoclasts and osteoblasts

The effect of $P2Y_2$ receptor deletion on the expression of resorption associated genes and ecto-nucleotidases was investigated in mature, resorbing osteoclasts. mRNA expression of many genes (TRAP, ClCN7, RANK, *c-fms*) showed a downward trend but only cathepsin K expression was significantly reduced (4.8-fold). Osteoclasts express a range of ecto-nucleotidases that hydrolyse ATP (Hajjawi, et al. 2014) and NDPK (nucleoside disphosphokinase), which can regenerate ATP from ADP. $P2Y_2$ receptor deletion did not influence the expression of any of these genes (**Table 1**).

In osteoblasts, deletion of the $P2Y_2$ receptor increased osteocalcin (Ocn), osteopontin (Opn) and osteoprotegerin (OPG) expression 3.3, 6 and 4.5-fold, respectively. The mRNA expression of Col1α1, Runx2, TNAP, osteonectin, RANKL, MCSF and the ecto-nucelotidases was unchanged (**Table 1**).

Activation of the $P2Y_2$ receptor increases bone resorption

Treatment with UTP and 2-thioUTP had no effect on osteoclast formation in $P2Y_2R^{+/+}$ or $P2Y_2R^{-/-}$ cells (**Fig. 4A-4B**). However, the area resorbed per osteoclast was dose-dependently increased by up to 80% and 45% in $P2Y_2R^{+/+}$ cells treated with UTP and 2-thioUTP (≥100nM), respectively. No effects on resorption were seen in $P2Y_2R^{-/-}$ osteoclasts **(Fig. 4C-4D).**

Reversal of resorption defect in $P2Y_2R^{-/-}$ osteoclasts by extracellular ATP

$P2Y_2R^{-/-}$ osteoclasts displayed a 53% reduction in ATP release **(Fig. 4E)** but showed no difference in the rate of ATP breakdown **(Fig. 4F).** Apyrase (≥1U/ml), a broad spectrum ecto-nucleotidase that rapidly degrades ATP and ADP, inhibited bone resorption by up to 55% **(Fig. 4G).** To determine if reduced extracellular ATP was the cause of the decreased resorption seen in $P2Y_2R^{-/-}$ osteoclasts, cells were cultured with exogenous ATP (1-10μM). Treatment with ATP (≥1μM) fully rescued the resorption defect see in $P2Y_2R^{-/-}$ osteoclasts **(Fig. 4H).**

$P2Y_2$ receptor agonists increase ATP release from osteoclasts

In $P2Y_2R^{+/+}$ cells, 10 minutes after addition of UTP (≥1μM) extracellular ATP levels were doubled; the increase in ATP levels was sustained for up to 90 minutes post treatment (**Fig.**

5A). No effect of UTP on ATP release was seen in *P2Y$_2$R*$^{-/-}$ osteoclasts at any stage **(Fig. 5B-5D)**. Treatment with 2-thioUTP (≥0.1μM) also dose dependently increased extracellular ATP levels by ≤50% for up to 90 minutes in *P2Y$_2$R*$^{+/+}$ osteoclasts **(Fig. 5E)**; 2-thioUTP was without effect in *P2Y$_2$R*$^{-/-}$ cells (**Fig. 5F-5H).**

The effect of long-term treatment (7 days) with P2Y$_2$ receptor agonists on basal ATP release was also investigated in mature osteoclasts. In *P2Y$_2$R*$^{+/+}$ cells, UTP and 2-thioUTP (≥1μM) increased ATP release by up to 70% and 65% respectively **(Fig.5I-5J).** No increase in ATP release was seen in *P2Y$_2$R*$^{-/-}$ osteoclasts. Standard curves used to calculate ATP levels are shown in **Fig. 5K-5L.** In all experiments, cell viability was unchanged (not shown).

ATP release from osteoblasts is stimulated by UTP and 2-thioUTP

The rate of ATP breakdown was unchanged in *P2Y$_2$R*$^{-/-}$ osteoblasts **(Fig. 6A).** ATP release from *P2Y$_2$R*$^{-/-}$ cells was decreased (≤60%) at all stages of differentiation **(Fig. 6B).** Long-term treatment (14 days) with UTP and 2-thioUTP increased the levels of ATP release by up to 4-fold and 3-fold, respectively, in *P2Y$_2$R*$^{+/+}$osteoblasts **(Fig. 6C-6D)**. No effects were seen in *P2Y$_2$R*$^{-/-}$ osteoblasts.

Acute UTP treatment increased ATP release from *P2Y$_2$R*$^{+/+}$ osteoblasts up to 4-fold within 10 minutes; stimulatory effects were sustained for up to 60 minutes **(Fig. 6E)**. UTP was without effect in *P2Y$_2$R*$^{-/-}$ osteoblasts **(Fig. 6F-6H).** 2-thioUTP also enhanced ATP release (≤4-fold) from *P2Y$_2$R*$^{+/+}$, but not *P2Y$_2$R*$^{-/-}$ osteoblasts **(Fig. 6I-6L).**

DISCUSSION

This study examined the role of $P2Y_2$ receptor-mediated signalling in osteoclasts and osteoblasts. We found that global deletion of the $P2Y_2$ receptor resulted in greater amounts of trabecular bone and increased BMD. Culture of cells derived from *P2Y$_2$R*$^{-/-}$ mice revealed that osteoclast resorptive activity was decreased whilst bone mineralisation was increased. Mechanistic analysis revealed that $P2Y_2$ receptor activation (acute and prolonged) promotes ATP release from osteoclasts and osteoblasts.

Several P2Y receptors ($P2Y_1$, $P2Y_6$, $P2Y_{12}$, $P2Y_{14}$) and extracellular nucleotides (e.g. ATP, ADP, UDP) have been implicated in the regulation of osteoclast formation and activity (Hoebertz et al. 2001; Lee et al. 2013; Orriss et al. 2011b; Su et al. 2012; Syberg et al. 2012b). However, there are no reports directly describing the functional role of the $P2Y_2$ receptor in osteoclasts. This study found that the $P2Y_2$ agonists, UTP and 2-thioUTP, dose-dependently stimulated bone resorption. Consistent with a pro-resorptive role for UTP and the $P2Y_2$ receptor, we observed that *P2Y$_2$R*$^{-/-}$ animals had decreased serum CTX levels and that cultured *P2Y$_2$R*$^{-/-}$ osteoclasts displayed reduced resorptive activity and cathepsin K expression. UDP, the breakdown product of UTP, acts via the $P2Y_6$ receptor to promote osteoclast function (Orriss et al. 2011b). However, since the actions of UTP are lost in *P2Y$_2$R*$^{-/-}$ osteoclasts, it is unlikely that the effects observed here are due to $P2Y_6$ receptor-mediated signalling.

Earlier studies have reported that $P2Y_2$ receptor activation by ATP and UTP can both inhibit (Hoebertz et al. 2002; Orriss et al. 2007; Orriss, et al. 2012a) and promote (Xing et al. 2014) bone mineralisation. Consistent with its role as a negative regulator of bone mineralisation, we observed that *P2Y$_2$R*$^{-/-}$ osteoblasts exhibited increased levels of bone formation, Ocn expression and TNAP activity. Suprisingly, TNAP mRNA expression was unaffected in *P2Y$_2$R*$^{-/-}$ osteoblasts. This could indicate that $P2Y_2$ receptor signalling increases enzyme activity by influencing the post-translational modifications of TNAP rather than the overall expression level. We have previously shown that the effects of ATP and UTP are restricted to the mineralisation

process with collagen expression and activity being unaffected (Orriss *et al.*, 2007). The lack of effect of $P2Y_2$ receptor deletion on serum P1NP levels is consistent with these observations.

In agreement with the *in vitro* findings, our longitudinal μCT study revealed that $P2Y_2$ deletion led to age-related increases in trabecular bone and BMD. These data are also consistent with our earlier description of the bone phenotype of 8-week old $P2Y_2R^{-/-}$ animals (Orriss et al. 2011a), and the observation that $P2Y_2$ receptor overexpression leads to decreased bone formation (Syberg et al. 2012a). However, they are at variance to a recent report of reduced bone levels in $P2Y_2R^{-/-}$ mice (Xing et al. 2014). The reasons for these divergent results are unclear but given that parental strain has been shown to affect the phenotype of the P2X7 receptor knockout (Syberg, et al. 2012a), the differing genetic background of the animals studied (C57BL/6 compared to SV129 (Xing et al. 2014)) could be a factor. Variations in μCT methodology could also contribute; for example, this study analysed a 1mm region of the trabecular bone within the metaphyseal portion of the long bones at a resolution of 4.3μm. In contrast, Xing *et al* measured the trabecular bone within a narrow region of the diaphysis at a lower resolution (10.5μm) (Xing et al. 2014).

Unlike the observed effects in the trabecular bone, in both this study and that of Xing *et al* (Xing et al. 2014), cortical bone parameters were unaffected in $P2Y_2R^{-/-}$ mice. This suggests that $P2Y_2$ receptor deletion does not have significant effects on bone growth. Thus, $P2Y_2$ receptor-mediated signalling appears to be more important in bone undergoing rapid turnover. *In vivo,* osteoblast and ostoclast function are tightly coupled with osteoclast activation being dependent on osteoblasts. Gene expression analysis revealed a significant increase in osteoblast expression of OPG whilst RANKL expression was unchanged. If reflected *in vivo* this would reduce osteoclast formation and activity and could contribute to the decreased bone resorption seen in $P2Y_2R^{-/-}$ mice. In agreement, qualitative observations showed that osteoclast numbers on the trabecular and endocortical bone surfaces appeared reduced in these animals. Further bone histomorphometric analysis of *in vivo* parameters such as bone formation rate and osteoclast number would confirm this and build on the findings reported here.

Controlled ATP release has been demonstrated from numerous cell types including bone cells. Several studies have indicated that the primary method of ATP release from osteoblasts is vesicular exocytosis (Genetos, et al. 2005; Orriss et al. 2009; Romanello, et al. 2001), although the P2X7 receptor may also be involved (Brandao-Burch, et al. 2012). In osteoclasts, ATP release involves the P2X7 receptor (Brandao-Burch et al. 2012; Pellegatti, et al. 2011). Increasing evidence now suggests that ATP can act to enhance its own release; ATP or UTP-induced ATP release has been demonstrated from MLO-Y4 osteocyte-like cells (Kringelbach et al. 2014), leukocytes (De Ita, et al. 2016), urothelial cells (Mansfield and Hughes 2014) and cells from the carotid body (Zhang, et al. 2012). The $P2Y_2$ receptor is thought to mediate this increased ATP release in cells including osteocytes (Kringelbach et al. 2014) and leukocytes (De Ita et al. 2016). Therefore we investigated whether UTP could exert its functional effects on bone cells indirectly i.e. acting via the $P2Y_2$ receptor to induce ATP release. We found that *$P2Y_2R^{-/-}$* osteoblasts and osteoclasts showed reduced levels of basal ATP release. Furthermore, UTP and 2-thioUTP increased ATP release from these cells following both acute (≤90 minutes) and long-term (≤14 days) treatment. These stimulatory effects were lost in *$P2Y_2R^{-/-}$* cells suggesting that the increased extracellular ATP levels were mediated via $P2Y_2$ receptor signalling. For the long-term experiments, UTP and 2-thioUTP were present in the culture medium for the 7 or 14 days days prior to testing but not in the medium used for the subsequent ATP release assay. This suggests that repeated $P2Y_2$ receptor stimulation could induce changes to the cellular processes which regulate ATP efflux from bone cells. However, at present, the mechanisms by which this could occur are unknown. Interestingly, $P2Y_2$ receptor activation in osteoblast-like cells has been shown to induce to actin fibre formation in response to fluid shear stress (Gardinier et al. 2014). This ability to regulate cytoskeletal rearrangement could result in alterations in the vesicular release pathway.

Extracellularly, ATP is rapidly broken down by ecto-nucleotidases, restricting its actions to cells close to the release site (Zimmermann, et al. 2012). The rate of ATP breakdown and the mRNA expression of ecto-nucleotidases (NPPs, NTPdases) were unchanged in *$P2Y_2R^{-/-}$* cells. Thus, our findings suggest that the primary effect of $P2Y_2$ receptor activiation is to stimulate the

level of ATP release from bone cells rather than influence the rate of ATP degradation or regeneration.

Following release, ATP can act on other P2 receptors to influence the function of surrounding cells. In osteoclasts, ATP and its breakdown product ADP act via the $P2Y_1$ and/or $P2Y_{12}$ receptors to promote bone resorption (Hoebertz et al. 2001; Su et al. 2012). Thus, our finding that $P2Y_2$ receptor activation promotes ATP release suggest indrect actions of UTP on bone resorption (a potential mechanism of action is shown in **Fig. 7**). Consistent with this idea, we observed that addition of exogenous ATP rescued the resorption defect in *P2Y$_2$R*$^{-/-}$ osteoclasts; although not studied here ADP would be expected to have a similar effect. Furthermore, apyrase, which breaksdown all endogenous ATP, inhibited osteoclast activity. The use of apyrase is likely to cause a rapid accumulation of adenosine. We have shown that adenosine has no effect on osteoclast function (Hajjawi, et al. 2016) whilst others report it promotes resorption (Kara, et al. 2010). If the actions of apyrase were a consequence of higher adenosine levels, an increase (or no effect) in resorption would be expected. However, since we observed the opposite it is more likely that the functional effects of apyrase are due to reduced extracellular ATP levels.

The role of purinergic signalling in osteoblasts has been widely studied and for some P2 receptors multiple functional effects have been described (Burnstock et al. 2013; Gartland et al. 2012; Noronha-Matos and Correia-de-Sa 2016; Orriss 2015). The diverse range of experimental models and culture conditions employed *in vitro* has often resulted in conflicting or confounding results regarding these actions. This is particurly evident for the $P2Y_2$ and P2X7 receptors, stimulation of which has been shown to both inhibit and promote bone mineralisation (Noronha-Matos, et al. 2014; Orriss et al. 2012a; Orriss et al. 2007; Panupinthu, et al. 2007; Xing et al. 2014). The data presented here show that $P2Y_2$ deletion leads to increased levels of bone mineralisation. Based on our findings one potential mechanism of action is summarised in **Fig. 7.** We suggest that UTP acts at the $P2Y_2$ receptor to stimulate ATP release, once released ATP can then act via other P2 receptors to block bone mineralisation (Orriss et al.

2012a), as well as exerting a direct physiochemical blockade via its breakdown product, pyrophosphate (Orriss et al. 2007; Orriss, et al. 2016).

Fluid flow and mechanical stress are well known stimulators of osteoblast ATP release (Genetos et al. 2005; Romanello et al. 2001; Rumney, et al. 2012). This enhanced release of ATP has been implicated in mechanically-induced bone formation via increased prostaglandin E_2 (PGE_2) secretion (Genetos et al. 2005). However, the ATP levels required to induce PGE_2 production are 10-fold higher than those needed to inhibit mineralisation and may only occur following mechanical stress. These potentially confounding actions serve to illustrate the highly complex, local effects of purinergic signalling on bone cell function. Thus, how a bone cell responds to these signals is likely to be influenced by factors including local nucleotide concentration, receptor expression profile, ecto-nucleotidase expression and activity, and, for osteoblasts and osteocytes, degree of mechanical stress experienced.

In conclusion, this study describes, for the first time, a role for the $P2Y_2$ receptor in regulating osteoclast function. The *in vitro* findings also provide further support for the inhibitory actions of $P2Y_2$ receptor signalling on bone mineralisation under normal conditions. Taken together our findings indicate that the $P2Y_2$ receptor modulates bone homeostasis by regulating extracellular ATP levels and, consequently, local purinergic signalling.

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AUTHOR CONTRIBUTIONS

Experimental design, **IRO, TRA**; performed experimental work, **IRO, DG, KS, MORH, JJP**; wrote and revised manuscript, **IRO, TRA**.

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635

FIGURE LEGENDS

Figure 1. ***P2Y$_2$R$^{-/-}$ mice display age-related increases in trabecular bone.***

Trabecular bone volume (BV/TV) was increased by ≤46% and ≤48% in the **(A)** femur and **(B)** tibiae of *P2Y$_2$R$^{-/-}$* mice, respectively. Trabecular number (Tb.N) was increased **(C)** ≤27% in the femur and **(D)** ≤30% in the tibia. Trabecular thickness (Tb.Th) was ≤17% and ≤10% higher in the **(E)** femur and **(F)** tibia, respectively. **(G, H)** Trabecular BMD was increased ≤12%. **(I, J)** Cortical bone volume, **(K, L)** cortical thickness, **(M)** periosteal diameter and **(N)** endosteal diameter were unchanged. Values are means ± SEM (n=10), significantly different from controls: * = p<0.05, ** = p<0.01, *** = p<0.001. **(O)** Representative 3D volumetric images of the trabecular and cortical bone of 24-week old *P2Y$_2$R$^{-/-}$* and *P2Y$_2$R$^{+/+}$* mice

Figure 2. ***Increased bone formation by osteoblasts from P2Y$_2$R$^{-/-}$ mice***

In cultures of **(A)** calvarial and **(B)** long-bone osteoblasts from *P2Y$_2$R$^{-/-}$* mice the level of mineralised bone nodule formation was increased 3-fold and 5-fold, respectively. Basal TNAP activity was increased by ≤3-fold in *P2Y$_2$R$^{-/-}$* **(C)** calvarial and **(D)** long bone osteoblasts (*n* = 6). **(F)** Serum TNAP activity was increased up to 60% (*n* = 10). **(E)** Serum P1NP levels were unchanged in *P2Y$_2$R$^{-/-}$* mice (*n* = 10). Values are means ± SEM, significantly different from controls: * = p<0.05, ** = p<0.01, *** = p<0.001. **(G)** Representative whole well scans (unstained) and phase contrast microscopy images (alizarin red stained) showing the increased bone formation in cultures of *P2Y$_2$R$^{-/-}$* calvarial osteoblasts. Scale bars: whole well = 0.5cm, microscopy images = 50μm.

Figure 3. ***Osteoclasts from P2Y$_2$R$^{-/-}$ mice exhibit defective resorption***

P2Y$_2$ receptor deletion **(A)** had no effect on osteoclast number but **(B)** decreased resorption per osteoclast by 75% (*n* = 8). **(C)** Serum CTX levels were up to 35% lower in *P2Y$_2$R$^{-/-}$* mice (*n* = 10). Values are means ± SEM, significantly different from controls: * = p<0.05, *** = p<0.001. **(D)** Representative transmitted and reflective light microscopy images showing the decreased resorption seen in *P2Y$_2$R$^{-/-}$* osteoclast cultures. Scale bar = 50μm. **(E)**

Qualitative histology suggested that the number of TRAP-positive osteoclasts was reduced on the endocortical and trabecular bone surfaces in 24-week but not 8-week old $P2Y_2R^{-/-}$ mice. Scale bar = 100μm

Figure 4. ***The role of the $P2Y_2$ receptor and extracellular ATP in regulating bone resorption***

Treatment with **(A)** UTP **(B)** 2-thioUTP had no effect on osteoclast formation. The area resorbed per osteoclast was increased up to **(C)** 80% by UTP and **(D)** 45% by 2-thioUTP (≥10nM) in $P2Y_2R^{+/+}$ but not $P2Y_2R^{-/-}$ osteoclasts, **(E)** $P2Y_2R^{-/-}$ osteoclasts mice displayed a 53% reduction in basal ATP release. **(F)** ATP breakdown was unchanged in $P2Y_2R^{-/-}$ osteoclasts. **(G)** Culture with apyrase inhibited bone resorption in normal osteoclasts by up to 55%. **(H)** Addition of exogenous ATP (≥1μM) returned the level of resorption in $P2Y_2R^{-/-}$ osteoclast cultures to normal**.** Values are means ± SEM (n = 8), significantly different from controls: * = p<0.05, ** = p<0.01, *** = p<0.001.

Figure 5. ***The effect of UTP and 2-thioUTP on ATP release from osteoclasts***

(A) UTP (≥1μM) increased extracellular ATP release by ≤2-fold for up to 90 minutes post-treatment. **(B,C,D)** No effects of UTP on ATP released were seen $P2Y_2R^{-/-}$ cells. **(E)** 2-thioUTP (≥0.1μM) dose-dependently increased extracellular ATP levels by up to 50% **(F, G, H)** but had no effect in $P2Y_2R^{-/-}$ osteoclasts. Long-term treatment (7days) with **(I)** UTP and **(J)** 2-thioUTP treatment enhanced ATP release by up to 70% and 65%, respectively in $P2Y_2R^{+/+}$ but not $P2Y_2R^{-/-}$ osteoclasts. Values are means ± SEM (n = 10), significantly different from controls: * = p<0.05, ** = p<0.01, *** = p<0.001. Differences between $P2Y_2R^{+/+}$ and $P2Y_2R^{-/-}$: # = p<0.05, ## = p<0.01, ### = p<0.001. Standard curves used to calculate ATP concentrations in acute **(K)** UTP and **(L)** 2-thioUTP experiments.

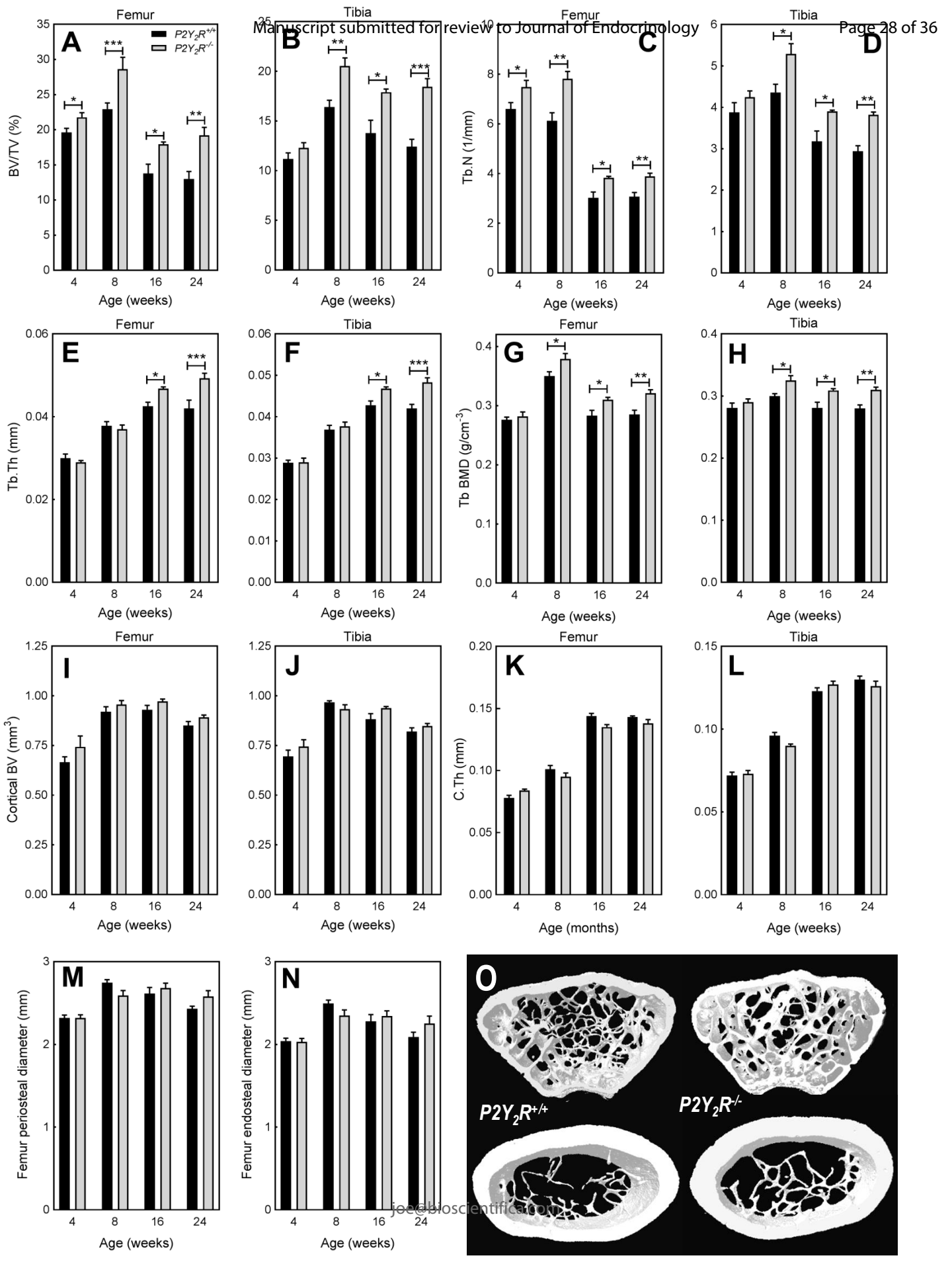
Figure 6. ***The role of the $P2Y_2$ receptor in ATP release from osteoblasts***

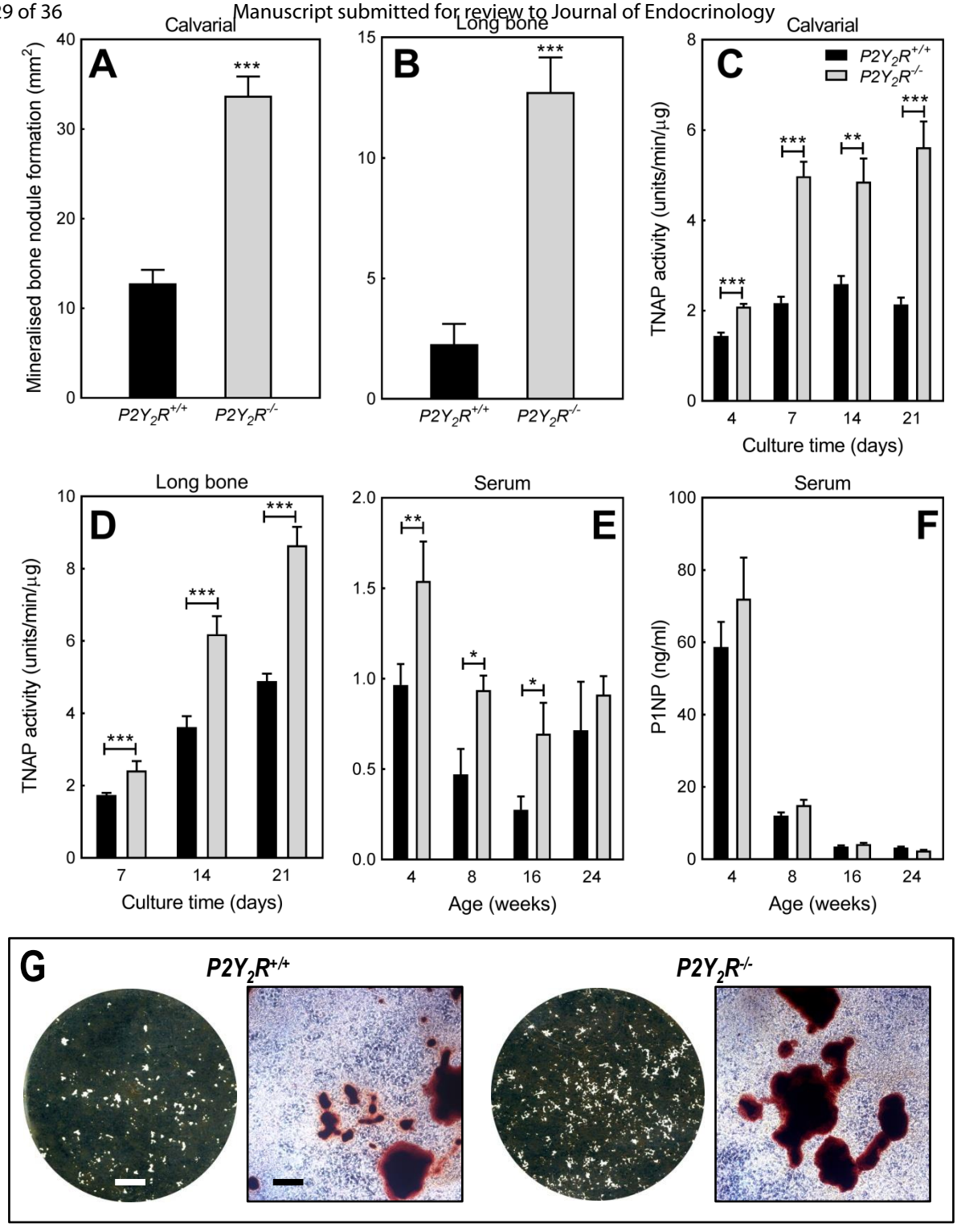
(A) No differences were observed in the rate of ATP breakdown between $P2Y_2R^{+/+}$ and $P2Y_2R^{-/-}$ osteoblasts. **(B)** Basal ATP release was up to 60% lower from $P2Y_2R^{-/-}$ osteoblast.

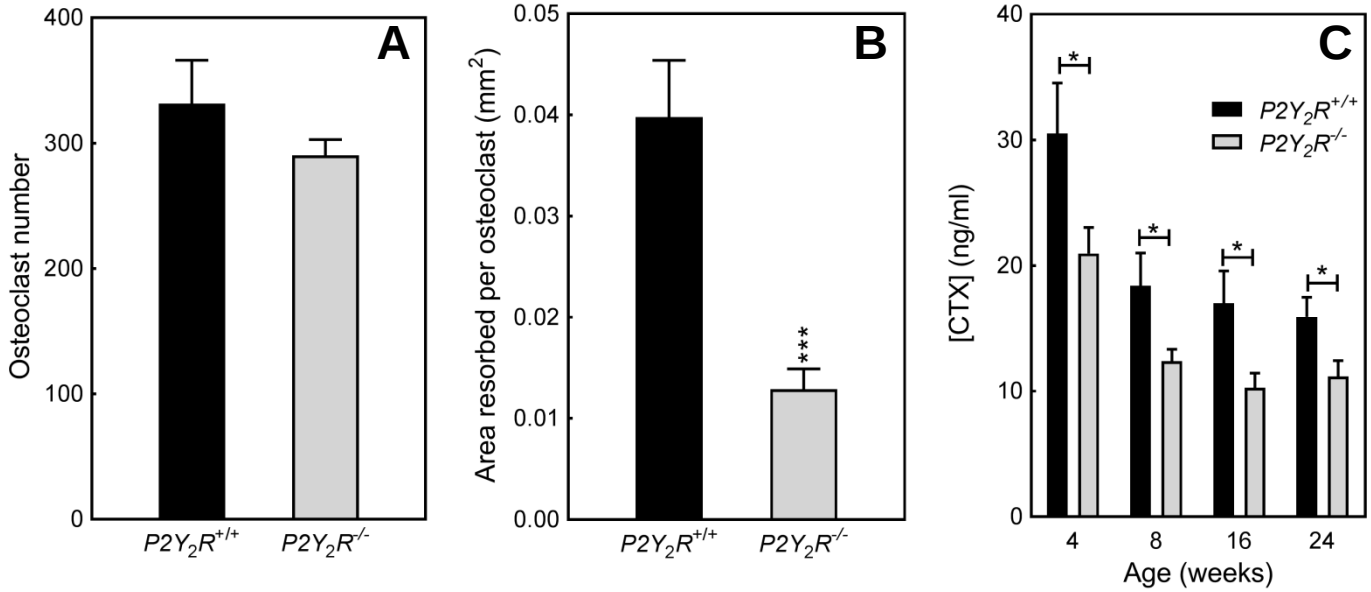
Increased ATP release from *P2Y$_2$R*$^{+/+}$ but not *P2Y$_2$R*$^{-/-}$ osteoblasts treated for 14 days with **(C)** UTP (≤4-fold) and **(D)** 2-thioUTP (≤3-fold). **(E)** Acute treatment with UTP (≥10μM) increased ATP release by ≤4-fold for up to 60 minutes. **(F,G,H)** No effect of UTP (10μM) on ATP release from *P2Y$_2$R*$^{-/-}$ osteoblasts. **(I)** ≥1μM 2-thioUTP also enhanced ATP release (≤4-fold) from *P2Y$_2$R*$^{+/+}$osteoblasts but was without effect in *P2Y$_2$R*$^{-/-}$ cells **(J,K,L).** Values are means ± SEM (*n* = 12), significantly different from controls: * = p<0.05, ** = p<0.01, *** = p<0.001. Differences between *P2Y$_2$R*$^{+/+}$ and *P2Y$_2$R*$^{-/-}$: # = p<0.05, ## = p<0.01, ### = p<0.001.

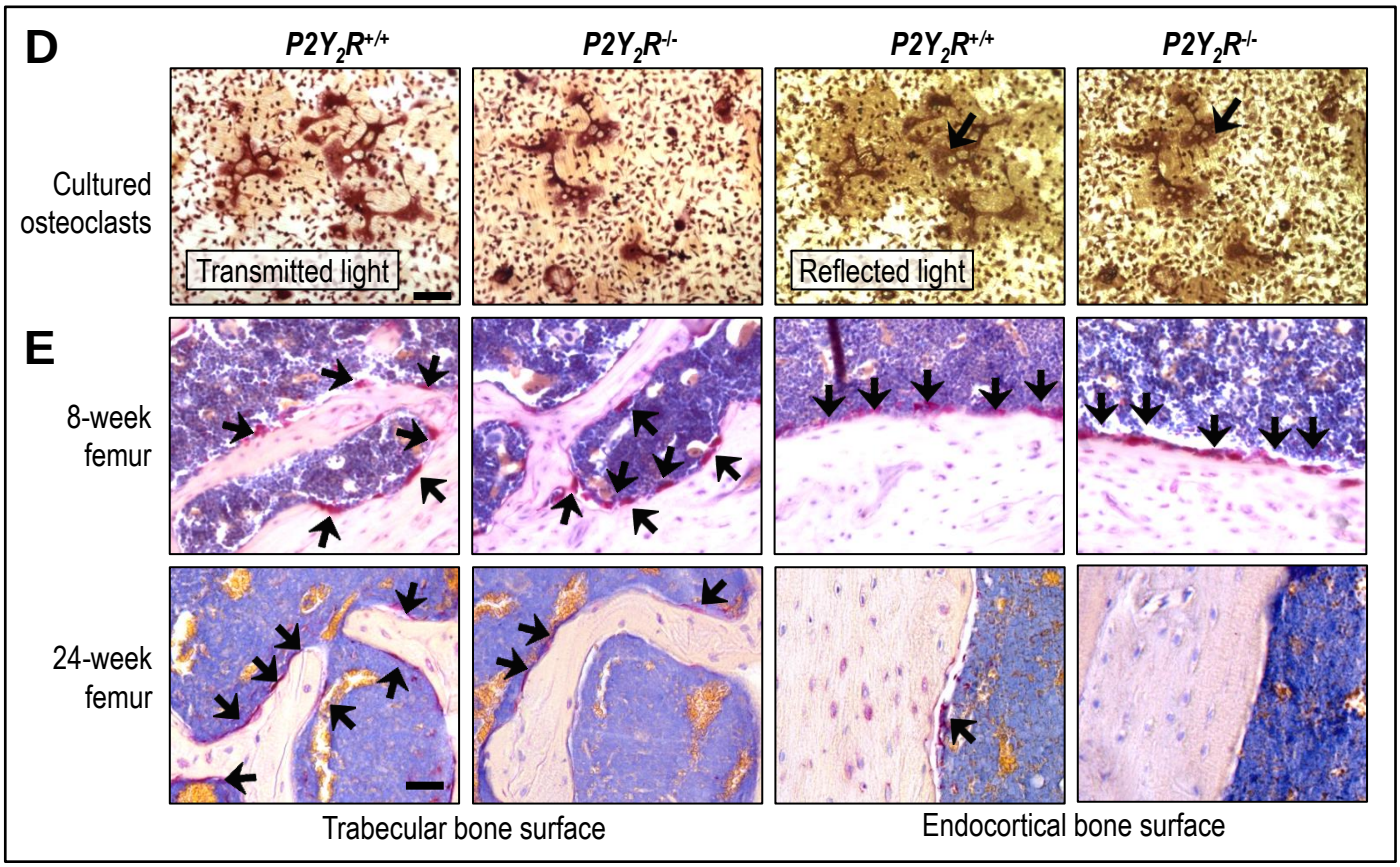
Figure 7. ***Proposed role of the P2Y$_2$ receptor in osteoclast and osteoblast function***

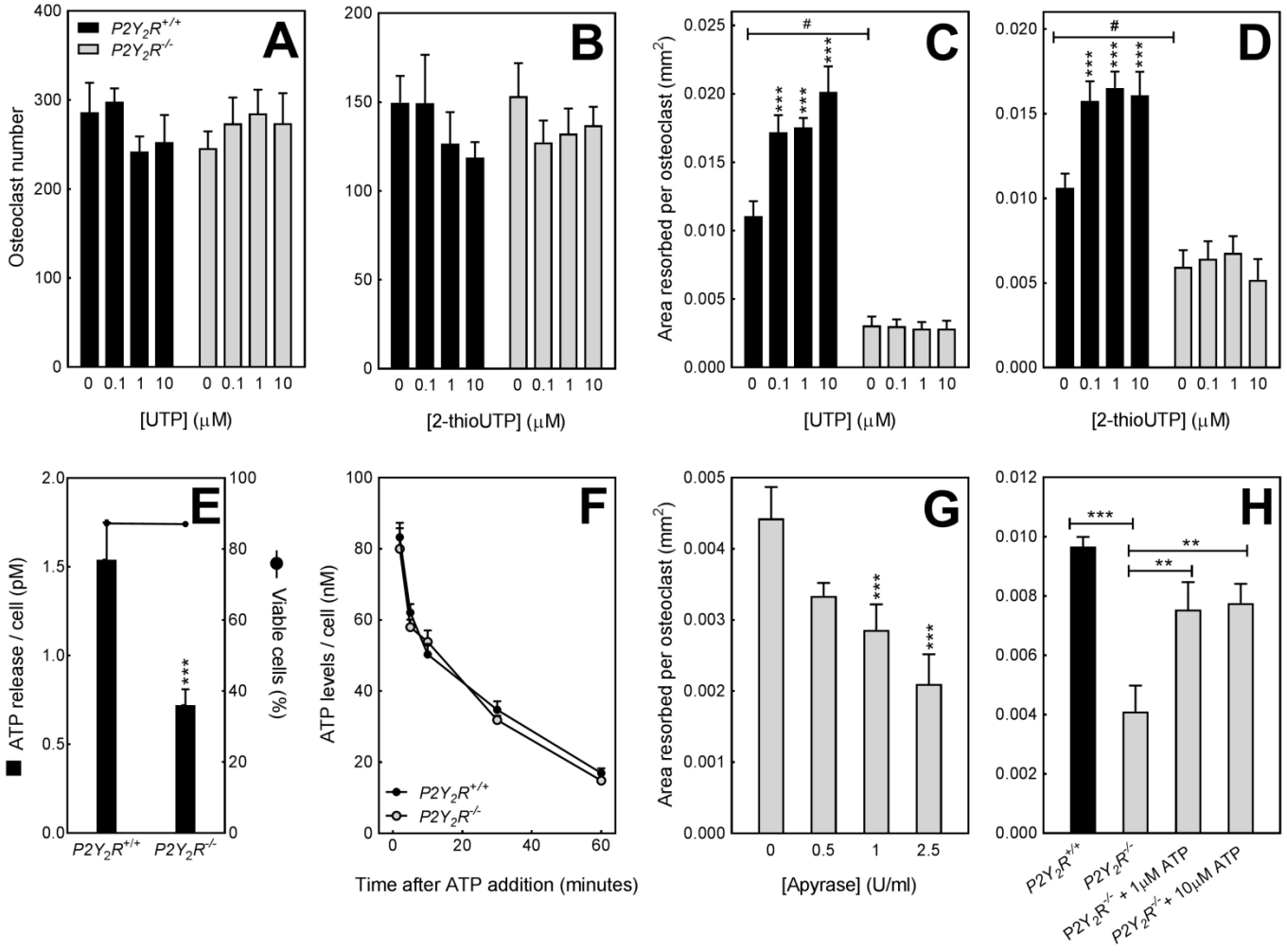
In osteoclasts, UTP acts via the $P2Y_2$ receptor to promote the release of ATP (via the P2X7 receptor). Once released ATP (and ADP) can act via the $P2Y_1$ and / or $P2Y_{12}$ receptors to stimulate bone resorption. UTP can also act via the $P2Y_2$ receptor to stimulate ATP release from osteoblasts (via vesicular exocytosis). ATP can then act via other P2 receptors (e.g. P2X1 or P2X7) to inhibit bone mineralisation. ATP can also be broken down by NPP1 to produce the mineralisation inhibitor, pyrophosphate (PP_i).

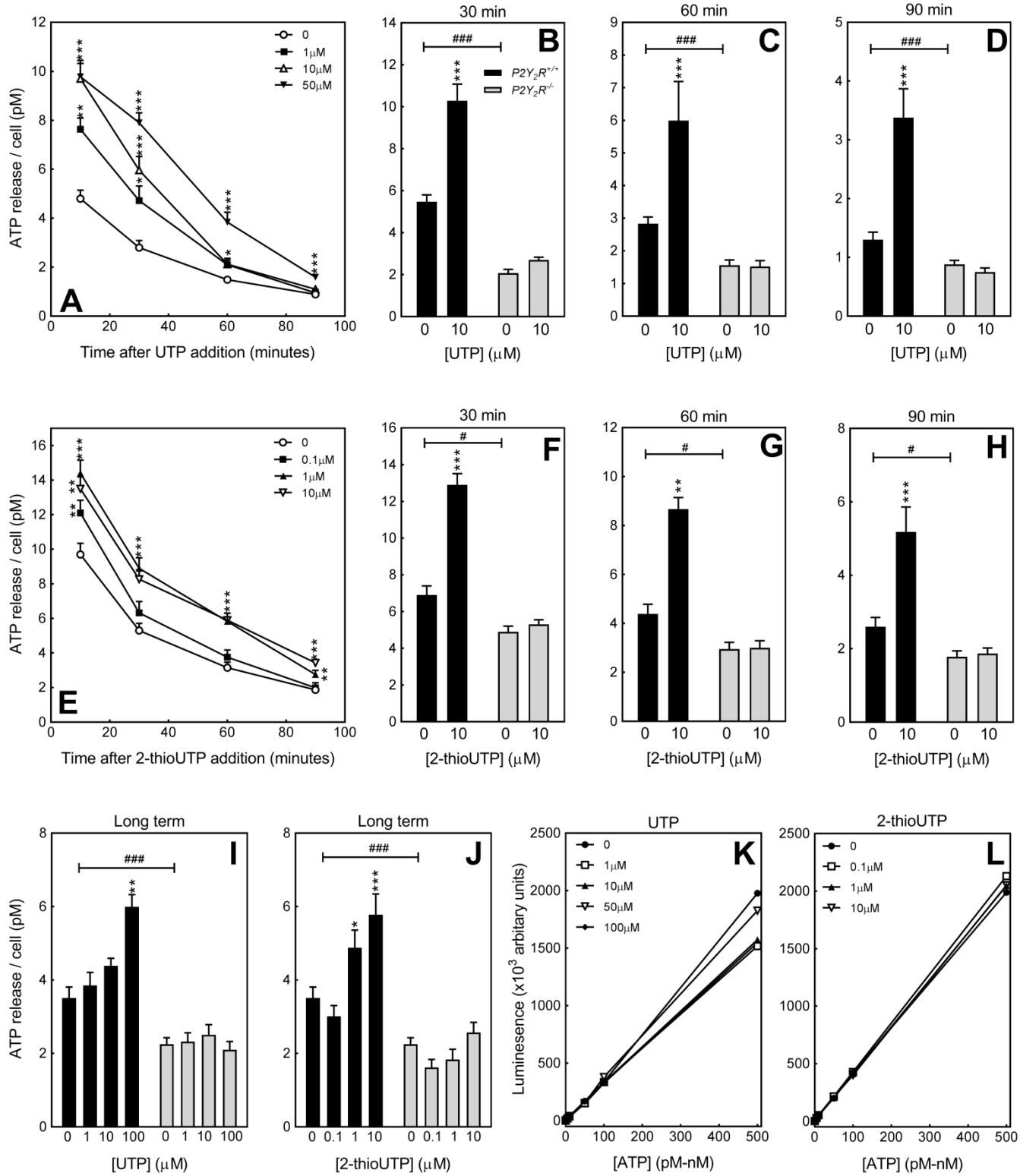


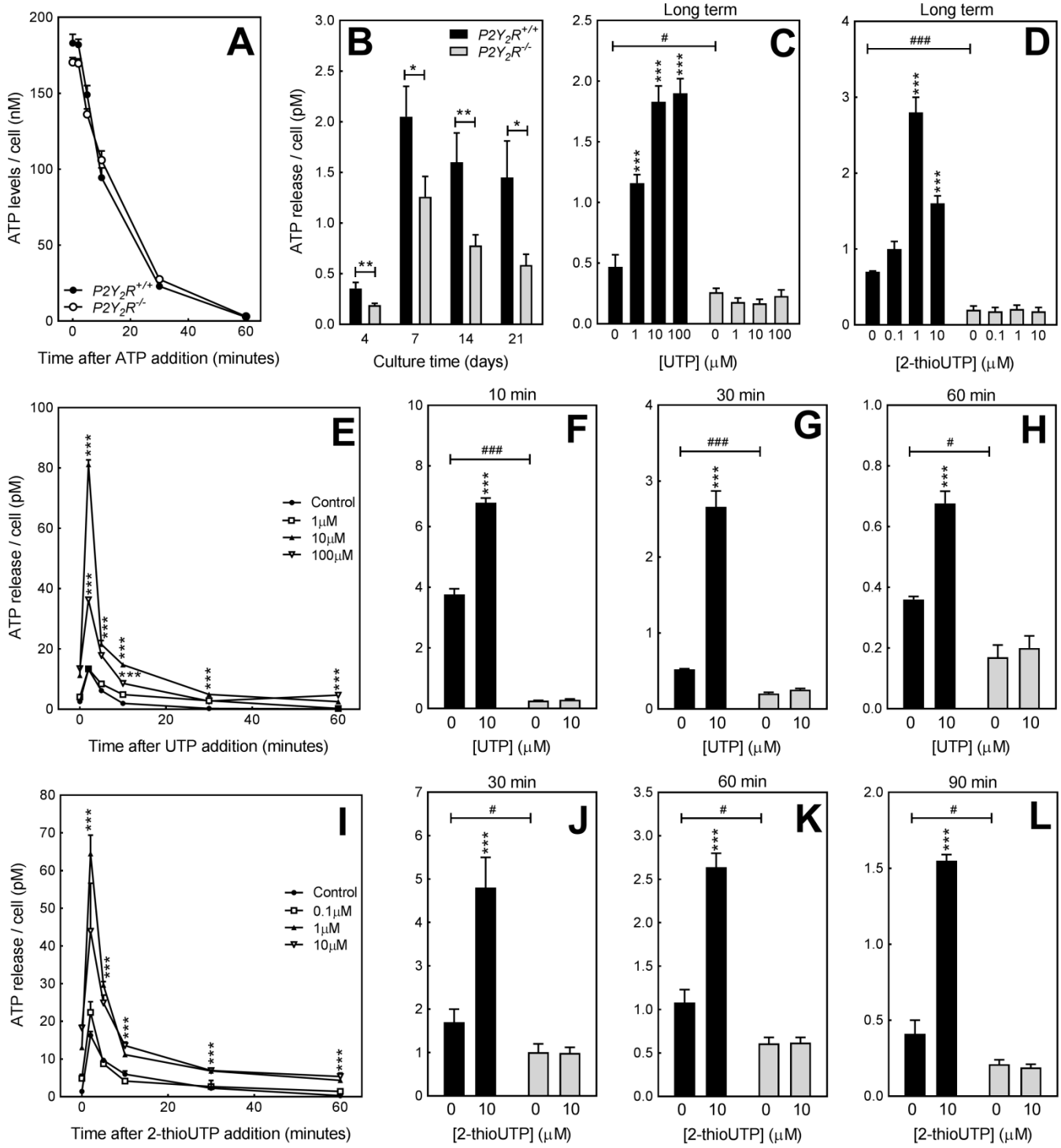

A
Calvarial
Mineralised bone nodule formation (mm²)
***
P2Y2R+/+
P2Y2R-/-
B
Long bone
***
C
Calvarial
P2Y2R+/+
P2Y2R-/-
TNAP activity (units/min/μg)
***
***
**
***
Culture time (days)
D
Long bone
TNAP activity (units/min/μg)
***
***
***
Culture time (days)
E
Serum
**
*
*
Age (weeks)
F
Serum
P1NP (ng/ml)
Age (weeks)
G
P2Y2R+/+
P2Y2R-/-


A
Osteoclast number
400
300
200
100
0
P2Y2R+/+
P2Y2R-/-
B
Area resorbed per osteoclast (mm2)
0.05
0.04
0.03
0.02
0.01
0.00
***
P2Y2R+/+
P2Y2R-/-
C
[CTX] (ng/ml)
40
30
20
10
0
*
*
*
*
P2Y2R+/+
P2Y2R-/-
4
8
16
24
Age (weeks)


D
P2Y2R+/+
P2Y2R-/-
P2Y2R+/+
P2Y2R-/-
Cultured osteoclasts
Transmitted light
Reflected light
E
8-week femur
24-week femur
Trabecular bone surface
Endocortical bone surface


A
B
C
D
E
F
G
H
P2Y2R+/+
P2Y2R-/-
Osteoclast number
[UTP] (μM)
[2-thioUTP] (μM)
Area resorbed per osteoclast (mm2)
ATP release / cell (pM)
Viable cells (%)
ATP levels / cell (nM)
Time after ATP addition (minutes)
[Apyrase] (U/ml)
P2Y2R-/- + 1μM ATP
P2Y2R-/- + 10μM ATP


A
ATP release / cell (pM)
Time after UTP addition (minutes)
0
1μM
10μM
50μM
B
30 min
C
60 min
D
90 min
P2Y2R+/+
P2Y2R-/-
###
[UTP] (μM)
E
Time after 2-thioUTP addition (minutes)
0.1μM
F
G
H
[2-thioUTP] (μM)
I
Long term
J
K
UTP
Luminesence (x10³ arbitary units)
100μM
[ATP] (pM-nM)
L
2-thioUTP


A
ATP levels / cell (nM)
Time after ATP addition (minutes)
P2Y2R+/+
P2Y2R-/-
B
ATP release / cell (pM)
Culture time (days)
C
Long term
[UTP] (μM)
D
Long term
[2-thioUTP] (μM)
E
ATP release / cell (pM)
Time after UTP addition (minutes)
Control
1μM
10μM
100μM
F
10 min
[UTP] (μM)
G
30 min
[UTP] (μM)
H
60 min
[UTP] (μM)
I
ATP release / cell (pM)
Time after 2-thioUTP addition (minutes)
Control
0.1μM
1μM
10μM
J
30 min
[2-thioUTP] (μM)
K
60 min
[2-thioUTP] (μM)
L
90 min
[2-thioUTP] (μM)

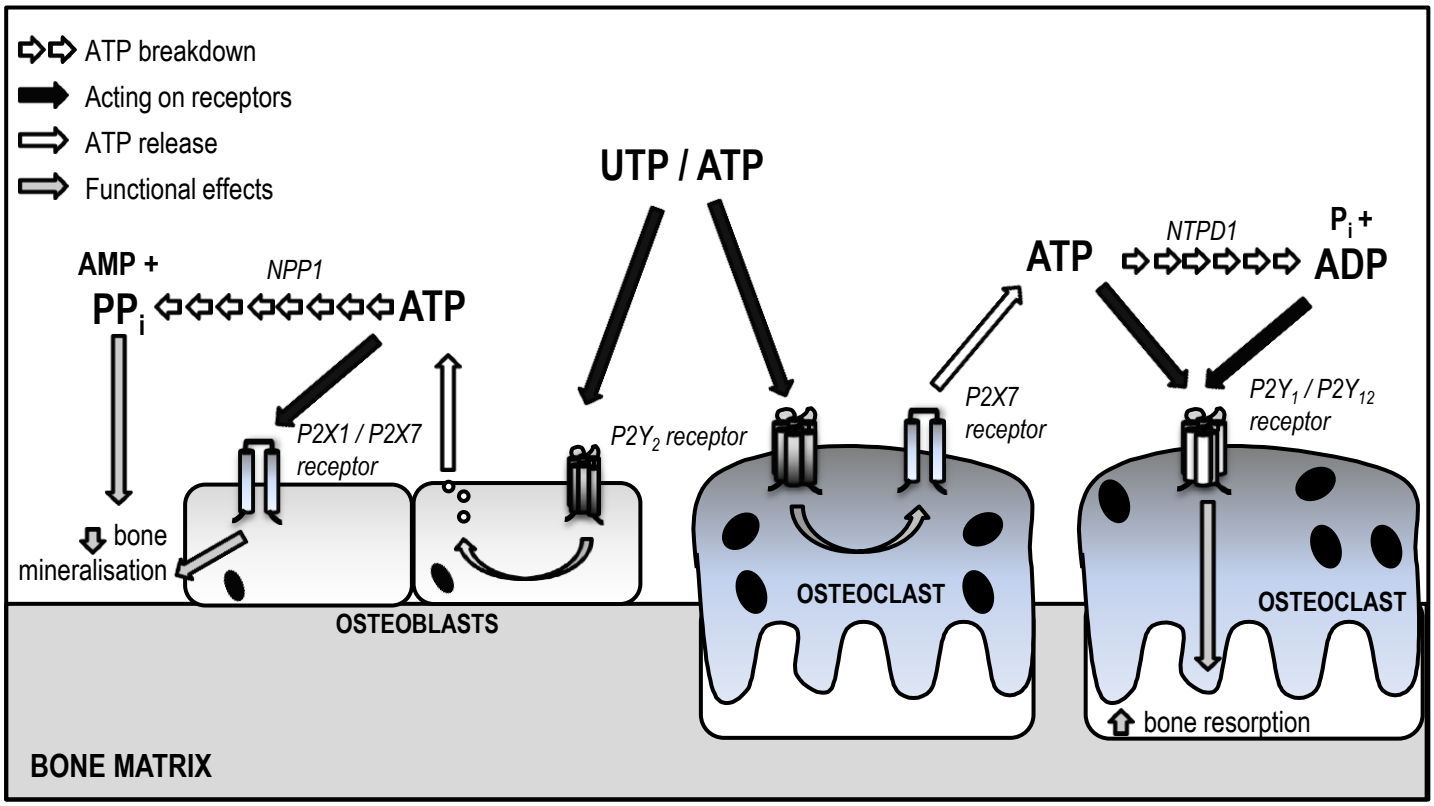

ATP breakdown
Acting on receptors
ATP release
Functional effects
UTP / ATP
AMP +
NPP1
PPi
ATP
NTPD1
Pi +
ATP
ADP
P2X1 / P2X7 receptor
P2Y2 receptor
P2X7 receptor
P2Y1 / P2Y12 receptor
bone mineralisation
OSTEOBLASTS
OSTEOCLAST
OSTEOCLAST
bone resorption
BONE MATRIX

Table 1: The effect of $P2Y_2$ receptor deletion on gene expression in osteoblasts and osteoclasts

Gene	Fold change in expression	Gene	Fold change in expression
Osteoclasts			
RANK	-2.6 ± 0.66	NPP1	-2.83 ± 0.47
Cathepsin K	-4.8 ± 0.13*	NPP3	1.14 ± 0.575
c-fms	-2.1 ± 0.52	NTPdase 1	-2.85 ± 0.50
TRAP	-2.3 ± 0.13	NTPdase 3	1.83 ± 0.57
CICN7	-1.2 ± 0.10	NDPK	-1.8 ± 0.27
V-ATPase	1.1 ± 0.29		
Osteoblasts			
Ocn	3.3 ± 0.78*	TNAP	1.94 ±.476
Opn	6.0 ± 0.14***	NPP1	-1.3 ± 0.48
On	1.49 ± 0.18	NPP3	-1.13 ± 0.20
Col1α1	1.67 ± 0.51	NTPdase 1	1.42 ± 0.41
Runx2	1.28 ± 0.07	NTPdase 3	1.22 ± 0.391
RANKL	1.28 ± 0.34	NDPK	1.44 ± 0.249
M-CSF	1.09 ± 0.33		
Opg	4.52 ± 0.13 **		

Data obtained from qPCR. Values are means ± SEM (*n* = 4). Significantly different from controls * = p<0.05, ** = p<0.01, ** = p<0.001.

RANK = receptor activator of nuclear factor $_K$B, *c-fms* = M-CSF receptor, TRAP = tartrate resistant acid phosphatase, CICN7 = chloride channel CICN7, NPP1/3 = ecto-nucleotide pyrophosphatase/phosphodiesterase 1/3, NTPdase = ecto-nucleoside triphosphate diphosphohydrolase, NDPK = nucleoside diphosphokinase, Ocn = osteocalcin, Opn = osteopontin, TNAP = alkaline phosphatase, On = osteonectin, Col1α1= collagen 1 alpha 1,

Runx2= runt related transcription factor 2, RANKL = receptor activator of nuclear factor $_{K}$B ligand, M-CSF = macrophage colony stimulating factor, Opg = osteoprotegerin