The role of purinergic signalling in the musculoskeletal system

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Abstract

Accumulating evidence now suggests that purinergic signalling exerts significant regulatory effects in the musculoskeletal system. In particular, it has emerged that extracellular nucleotides are key regulators of bone cell differentiation, survival and function. This review discusses our current understanding of the effects of purinergic signalling in bone, cartilage and muscle.

1. Introduction

Adenosine triphosphate (ATP) has long been recognized for its role in intracellular energy metabolism; however, it is also an important extracellular signalling molecule. The potent actions of ATP were first described in 1929, yet it was 1972 before the concept of purinergic neurotransmission was proposed (Burnstock, 1972). Extracellular nucleotides, signalling via purinergic receptors, are now known to participate in numerous biological processes in both neuronal and non-neuronal tissues. The receptors for purines and pyrimidines are classified into two groups; P1 receptors and P2 receptors. There are four P1 receptor subtypes (A_1,A_{2a},A_{2b},A_3); these receptors are G-protein coupled and activated by adenosine. P2 receptors respond to a number of different nucleotides including ATP, adenosine diphosphate (ADP), uridine triphosphate (UTP) and uridine diphosphate (UDP). The P2 receptors are further subdivided into the P2X ligand-gated ion channels and P2Y G-protein-coupled receptors (Abbracchio et al., 1994; Burnstock et al., 1985; Ralevic et al., 1998). To date, seven P2X receptors (P2X1-7) and eight P2Y receptors ($P2Y_{1,2,4,6,11,12,13,14}$) have been identified; each receptor has been cloned, characterised and displays distinct tissue expression and pharmacology (Burnstock, 2007).

Over the last two decades, it has emerged that purinergic signalling plays a key regulatory role in the musculoskeletal system. In particular, the regulation of bone cell function by extracellular nucleotides has emerged as a particularly active and promising area of research. This review will summarise current understanding into the role of purinergic signalling in bone, cartilage and muscle.

2. Bone

Bone is a composite tissue containing inorganic mineral salts deposited within an organic collagenous matrix, and three cell types: osteoblasts, osteoclasts and osteocytes. Osteoblasts, which are derived from mesenchymal stem cells, are the bone-forming cells. When osteoblasts become incorporated within the bone matrix they may undergo a terminal differentiation to form an osteocyte. Osteocytes, the most abundant cells in bone (Jande et al., 1973), form a regular interconnected network of cells that is thought to regulate bone remodelling and mediate the responses to mechanical loading (Bonewald, 2011). Osteoclasts, the bone-resorbing cells, are usually multinuclear, and are formed by the fusion of mononuclear progenitors of the monocyte/macrophage lineage. The coordinated activity of these cells allows the skeleton to grow, adapt and repair itself; abnormalities in this process result in a variety of skeletal disorders.

The first indication that purinergic signalling could be important in modulating bone cell function came over 20 years ago when P1 (A_2-like) adenosine receptors were reported on osteoblasts (Lerner 1987). The expression of multiple P1 and P2 receptor subtypes in bone cells has now been widely reported (see review by (Burnstock et al., 2013)). More recently, analysis of knockout mouse models has provided evidence for the important role of purinergic signalling in bone remodelling *in vivo* (Gartland et al., 2003b; Ke et al., 2003; Orriss et al., 2011a; Su et al., 2012; Wang et al., 2012) **(Fig. 1)**

2.1. The role of purinergic signalling in osteoblast biology

P2 receptor expression by osteoblasts was first reported in the late 1980's when fluorescence studies demonstrated that extracellular nucleotides could transiently increase $[Ca^{2+}]_I$ and induce IP_3 formation (Kumagai et al., 1991; Kumagai et al., 1989). Subsequent pharmacological studies revealed that extracellular nucleotides interacted with at least two P2 receptors ($P2Y_1$ and $P2Y_2$) on rat osteoblast-like cells (Reimer et al., 1992; Yu et al., 1993b). The first molecular evidence for P2Y receptor expression was shown in 1995 by Bowler *et al*, who used RT-PCR and *in situ* hybridisation to demonstrate $P2Y_2$ receptor expression in human osteoblasts (Bowler et al., 1995). A detailed investigation of both single

cells and populations of human osteoblasts indicated that there was a heterogeneity in P2 receptor expression in any one culture (Dixon et al., 1997). This observation raised the possibility that the differentiation status of the osteoblast influences P2 receptor expression, an idea which was later confirmed in several separate studies (Nishii et al., 2009; Orriss et al., 2012; Orriss et al., 2006).

The expression of multiple P2 receptors by osteoblasts has now been widely reported **(Fig. 2)**. Studies using primary rat and mouse calvarial osteoblasts, human osteoblast-like cell lines (MG-63, OHS-4, SaM-1, SaOS-2, Te85) and rat osteosarcoma cell lines (UMR106-01, ROS17/2,8) have all described expression of most or all P2X and P2Y receptor subtypes in osteoblasts (see reviews (Burnstock et al., 2013; Gartland et al., 2012a)). P2 receptor mediated signalling has now been shown to exert many effects on osteoblast proliferation, differentiation, function, gene expression and cell signalling.

Extracellular nucleotides and the regulation of bone mineralisation

Initial functional studies showed that ATP and UTP (≥1μM), acting via the $P2Y_2$ receptor, strongly inhibited bone formation by cultured rat osteoblasts (Hoebertz et al., 2002). A follow up investigation demonstrated that ATP and UTP selectively inhibited the mineralisation of the organic matrix and alkaline phosphatase (TNAP) expression and activity (Orriss et al., 2007). It also provided further evidence for the involvement of the $P2Y_2$ receptor (Orriss et al., 2007). Subsequent skeletal analysis of $P2Y_2$ receptor knockout mice by dual energy x-ray absorbtiometry (DEXA) and micro computed tomography (μCT) demonstrated large increases in trabecular and cortical bone parameters in the long bones(Orriss et al., 2011a; Orriss et al., 2007) **(Fig. 1)**. Recently, it was also demonstrated that the P2X1, P2X3 and P2X7 receptor agonists, α,β-meATP, β,γ-methylene ATP and 2'(3')-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate (Bz-ATP) reduced bone mineralization *in vitro* (Orriss et al., 2012). This study used selective P2 receptor antagonists to suggest that the effects were mediated via the P2X1 and P2X7 receptor subtypes (Orriss et al., 2012). Furthermore, another investigation demonstrated increased bone mineralisation in osteoblast cultures treated with apyrase, suggesting that endogenous ATP released by osteoblasts acts as an important local brake on mineralisation (Orriss et al., 2013). Combined these data suggest that ATP acts as a key endogenous inhibitor of bone mineralisation.

The ATP concentration in cell cytosol is between 2-5mM. Following membrane damage or necrosis, all cells can release ATP into the extracellular environment, which can then act in an autocrine/paracrine manner to influence local purinergic signalling. Controlled ATP release has been demonstrated from numerous excitatory and non-excitatory cells. In the bone microenvironment, osteoblasts (Buckley et al., 2003; Genetos et al., 2005; Orriss et al., 2009; Romanello et al., 2001; Rumney et al., 2012), osteoclasts (Brandao-Burch et al., 2012) and MLO-Y4 osteocyte-like cells (Genetos et al., 2007; Kringelbach et al., 2014) have all been shown to constitutively release ATP.

Once released, nucleotides are rapidly broken down by an extracellular hydrolysis cascade. Molecular and functional characterisation has shown there are four families of ecto-nucleotidases: (1) the NTPdases (ecto-nucleoside triphosphate diphosphohydrolase); (2) the NPPs (ecto-nucleotide pyrophosphatase/phosphodiesterase); (3) alkaline phosphatases and, (4) ecto-5'-nucleotidase (Zimmermann et al., 2012). Many ecto-nucleotidases have overlapping specificities. For example, NTPdases catalyse the reactions: nucleotide triphosphate (NTP) → nucleotide diphosphate (NDP) + phosphate (Pi) and NDP → nucleotide monophosphate (NMP) + phosphate (P_i), whereas NPPs hydrolyse NTP → NMP + pyrophosphate (PP_i) or NDP → NMP + P_i. Thus, the combined activities of these ecto-enzymes limit the actions of extracellular nucleotides to cells within close proximity of the release site. Osteoblasts express three members of the NPP family (NPP1-3) (Hessle et al., 2002;

Johnson et al., 2000; Orriss et al., 2007) and at least six members of the NTPdase family (NTPdase 1-6)(Orriss et al., 2010). This hydrolysis of ATP and other NTPs by NPPs is particularly important in bone because the product, pyrophosphate (PP_i), is the key, local physicochemical inhibitor of mineralisation (Fleisch et al., 1962; Fleisch et al., 1961). Of the NPPs expressed in bone, NPP1 (previously called plasma cell membrane glycoprotein 1 or PC-1), is thought to be the most important in PP_i generation (Johnson et al., 2000). A recent, detailed study of the mouse model lacking NPP1 showed that this enzyme is essential for normal bone development and control of physiological bone mineralization (Mackenzie et al., 2012). There is now significant evidence suggesting that extracellular ATP is a key source of PP_i in bone (Orriss et al., 2012; Orriss et al., 2013; Orriss et al., 2007). Thus, it is likely that nucleotide triphosphates exert a dual inhibitory action on bone mineralisation via both P2 receptor mediated signalling and direct hydrolysis to PP_i **(Fig. 3)**

P2X receptors and osteoblasts

The mitogenic effects of ATP were first reported by Suzuki and colleagues in the early 1990's (Suzuki et al., 1993). Subsequent work showed that ATP acting via the P2X5 receptor promotes DNA synthesis in human osteoblast-like cells (Nakamura et al., 2000).

The role of the P2X7 receptor in osteoblast biology has been the focus of significant research albeit often with conflicting results. Expression of the P2X7 receptor was first demonstrated in a subpopulation of differentiated human osteoblasts (Gartland et al., 2001); later studies also described P2X7 receptor expression in rat (Orriss et al., 2006) and mouse osteoblasts (Ke et al., 2003). Early reports suggested that P2X7 receptor activation caused enhanced osteoblast apoptosis (Gartland et al., 2001). Subsequent studies suggested that P2X7 stimulation leads to increased membrane blebbing and bone formation; an effect thought to be mediated via increased production of lyophosphatidic acid (LPA) and prostaglandin E_2 (PGE_2)(Li et al., 2005; Panupinthu et al., 2008; Panupinthu et al., 2007). In contrast, a recent investigation found that Bz-ATP acting via the P2X7 receptor inhibited bone mineralisation (Orriss et al., 2012).

Work using selective P2X7 receptor antagonists indicated that this receptor also plays a role in mediating ATP release from osteoblasts (Brandao-Burch et al., 2012). Furthermore, the P2X7 receptor is thought to mediate the ERK1/2 activation, NF-κB translocation and PGE_2 synthesis caused by fluid shear stress in osteoblast-like cells (Genetos et al., 2005; Liu et al., 2008). Other suggested roles for the P2X7 receptor in osteoblasts include the regulation of metabolic acid production (Grol et al., 2012) and callus remodelling during fracture repair (Li et al., 2009).

To date, several studies using P2X7 receptor knockout mice from different genetic backgrounds have been performed. The initial investigation by Ke *et al.* reported a reduced bone mineral content and periosteal circumference accompanied by decreased bone formation parameters and increased bone resorption parameters (Ke et al., 2003). A separate study by Gartland *et al.* (Gartland et al., 2003b) reported no differences in bone mineral density or trabecular bone but an increase in cortical bone was observed. It was subsequently reported that the knockout model used by Gartland *et al.* expressed a P2X7 splice variant in some tissues (Nicke et al., 2009); therefore the results from this model should be interpreted with caution. Further complicating the analysis of this knockout mouse model, a recent study found that the genetic background strongly influenced the bone phenotype (Syberg et al., 2012b).

Several P2X receptors (P2X2, P2X3, P2X4, P2X6) expressed by osteoblasts still have no known functional effects and present interesting areas for future study. Of particular note is the P2X4 receptor, which displays the highest expression of all the P2 receptors present on osteoblasts (Orriss et al., 2012). Furthermore, since osteoblasts express all P2X receptor subtypes it is plausible that they may combine

to form functional heteromultimers. Heteromultimers often display the pharmacology of both participating subunits, thus increasing the diversity of ATP-mediated signalling and downstream functional effects. At present, it is not known whether functional heteromultimers are expressed by osteoblasts.

P2Y receptors and osteoblasts

Studies into the role of P2Y receptors in osteoblasts have identified a wide range of functional effects. The $P2Y_2$ receptor was initially thought to mediate intercellular signalling since an early report demonstrated that mechanically stimulated human osteoblasts propagate fast intercellular Ca^{2+} waves via the autocrine activation of $P2Y_2$ receptors (Jorgensen et al., 2000). A follow-up study showed that intercellular signalling between osteoblasts and osteoclasts was not mediated via P2Y receptors but instead appeared to require the P2X7 receptor (Jorgensen et al., 2002). However, in a separate investigation $P2Y_2$ receptors were shown to mediate the Ca^{2+} mobilisation induced by oscillatory fluid flow in mouse osteoblasts (You et al., 2002).

Activation of the $P2Y_2$ receptor in osteoblast-like cells has been shown to activate a number of intracellular signalling pathways including protein kinase C (PKC), p38 mitogen-activated protein kinase (p38 MAPK), and c-Jun NH_2-terminal protein kinase (JNK) (Costessi et al., 2005; Katz et al., 2006; Katz et al., 2008; Pines et al., 2005).

Purinergic signalling can also interact with other intracellular pathways to regulate osteoblast function. In early studies, P2 receptors were found to mediate the potentiation of parathyroid hormone (PTH) receptor-mediated increases in $[Ca^{2+}]_i$ and IP_3 in ostoebast-like cells (Kaplan et al., 1995; Sistare et al., 1995). Later work found that synergistic co-activation by PTH and ATP, acting via P2Y receptors, increased mRNA levels of *c-fos*, a transcription factor important in osteoblast activation and bone remodelling. The authors suggested that this may be a mechanism to generate strong localised responses to systemic growth and differentiation factors (Bowler et al., 1999). A follow-up study demonstrated that PTH potentiated nucleotide-induced $[Ca^{2+}]_i$ release in rat osteoblasts independently of G_q activation or cyclic adenosine monophosphate (cAMP) accumulation (Buckley et al., 2001). ATP can also act via P2Y receptors to induce the release of IL-6 (Ihara et al., 2005).

Clopidogrel (Plavix®), a selective $P2Y_{12}$ receptor antagonist, acts to inhibit platelet aggregation. It is an antithrombotic widely prescribed to reduce the risk of heart attack and stroke. A recent investigation by Syberg and colleagues found that low micromolar concentrations of clopidogrel inhibit osteoblast proliferation, differentiation and function and reduce cell viability *in vitro* (Syberg et al., 2012a). Reduced trabecular bone was also observed in ovariectomised mice treated with clopidogrel for 4 weeks (Syberg et al., 2012a). In contrast, a paper by Su *et al.* demonstrated increased bone in mice treated with clopidogrel (Su et al., 2012). The two investigations used very different dosing regimens (oral dosing in the former compared to clopidogrel treated water in the latter) and thus the divergent results may reflect variations in the final dose received by the animals. In agreement with this notion, a study looking at a cohort of Danish patients found that clopidogrel was associated with fracture risk; however, these effects were biphasic with high doses (the recommended range) being associated with increased fracture risk and low doses being associated with decreased fracture risk (Jorgensen et al., 2012).

Mice lacking the $P2Y_{13}$ receptor display reduced trabecular bone mass, decreased osteoblast numbers *in vivo* and a reduced rate of bone remodelling (Wang et al., 2012). Osteoblasts cultured from $P2Y_{13}$ knockout mice also displayed a down regulation of RhoA/ROCK I signalling and a reduced ratio of receptor activator of nuclear factor kappaB ligand (RANKL) / osteoprotegerin expression (Wang et al., 2012). A follow up study by the same group demonstrated that $P2Y_{13}$ knockout mice display enhanced

osteogenic responses to mechanical loading which the authors attribute to reduced levels of extracellular ATP metabolism (Wang et al., 2013). The $P2Y_{13}$ receptor has also been shown to play an important role in phosphate metabolism (Wang et al., 2014) and the terminal differentiation of osteoprogenitor cells into osteoblasts or adipocytes (Biver et al., 2013).

ATP release from osteoblasts

Controlled ATP release from osteoblast-like cells was first described by Romanello *et al* in 2001 (Romanello et al., 2001). Since then several studies have indicated that the primary method of ATP release from osteoblasts is by vesicular exocytosis (Genetos et al., 2005; Orriss et al., 2009; Romanello et al., 2005). However, it has been suggested that the P2X7 receptor may also be involved (Brandao-Burch et al., 2012). The amount of ATP released from osteoblasts depends on their differentiation state, with mature, bone-forming cells releasing up to seven-fold more than immature, proliferating cells (Orriss et al., 2009). Several studies have demonstrated enhanced ATP release in response to different external stimuli including hypoxia (Orriss et al., 2009), mechanical stress (Hecht et al., 2013), fluid flow (Genetos et al., 2005; Romanello et al., 2005), vitamin D (Biswas et al., 2009) and ultrasound (Alvarenga et al., 2010; Hayton et al., 2005). A recent investigation examined the effect of different forms of mechanical stimulation on ATP release from human osteoblast-like SaOS-2 cells, including turbulent fluid flow, laminar fluid flow, substrate strain and 3D compressive loading (Rumney et al., 2012). Rumney *et al* concluded that the concentration of ATP released in response to mechanical loading varied in a time-, direction- and strain-dependent manner representing a local mechanostat in bone that could influence bone mineralisation (Rumney et al., 2012). Osteoblasts cultured from $P2Y_{13}$ receptor knockout mice display increased levels of extracellular ATP suggesting a role for this receptor in the modulation of ATP metabolism (Wang et al., 2013).

P1 receptor signalling and osteoblasts

The role of adenosine and P1 receptor-mediated signalling in the regulation of bone cell function has become an area of increasing interest in recent years. Osteoblasts have been shown to express all four P1 receptor subtypes (Gharibi et al., 2011; Vincenzi et al., 2013); however, the actions of extracellular adenosine on osteoblasts appear to be less clear-cut than those of ATP. Initial work found that synthetic adenosine analogues elicited a receptor-mediated rise in cAMP levels in calvarial osteoblasts (Lerner et al., 1987). Subsequently, it was reported that adenosine acts as a mitogen for osteoblast-like cells by stimulating DNA synthesis (Shimegi, 1996). An important early study found that human osteoblast precursors produced extracellular adenosine, probably via the breakdown of released ATP, which modulated their secretion of Il-6 and osteoprotergerin (Evans et al., 2006).

Since the late 1990's, several studies have failed to find an effect of adenosine on mineralised bone nodule formation by rat calvarial osteoblasts (Jones et al., 1997; Hoebertz et al., 2002). However, another study indicated that adenosine, acting via the A_{2B} receptor, may increase the osteogenic differentiation of rat long bone mesenchymal stem cells (Gharibi et al., 2011). Furthermore, a synthetic A_{2B} receptor agonist has been shown to increase bone formation, and osteoblasts from A_{2B} receptor knockout mice display reduced activity *in vitro* (Carroll et al., 2012). Recently, it was reported that the adenosine generated by ecto-5'-nucleotidase (CD-73) was important in promoting osteoblast differentiation (Takedachi et al., 2012). In contrast, adenosine analogues, acting via the A_1 or A_{2A} receptors, have been shown to inhibit the differentiation of rodent osteoblast-like cells (Gharibi et al., 2012).

2.2. Purinergic signalling and mesenchymal stem cells

Osteoblasts are derived from mesenchymal stem cells (MSCs) and thus these cells play a key role within the bone. Although information is limited a role for extracellular nucleotides in the regulation of MSC proliferation and differentiation is emerging. Initial work showed that fluid flow induced vesicular release of ATP from human MSCs, which then stimulated proliferation via a P2 receptor-mediated increase in $[Ca^{2+}]_i$ (Riddle et al., 2007). More recent studies have shown that human MSCs express the P2X3-7 receptors and all P2Y receptors (Noronha-Matos et al., 2012; Zippel et al., 2012). P2 receptors and extracellular nucleotides are now thought to be important modulators of human MSC differentiation, with the P2X6, $P2Y_4$, $P2Y_6$ and $P2Y_{14}$ receptors identified as playing pivotal roles in this process (Ferrari et al., 2011; Zippel et al., 2012; Noronha-Matos et al., 2012). Furthermore, a detailed investigation using knockout mice identified that the $P2Y_{13}$ receptor regulates the terminal differentiation of MSCs into osteoblasts or adipocytes (Biver et al., 2013). It has also been reported that NTPDases influence which osteoblast progenitors are driven into proliferation or differentiation (Noronha-Matos et al., 2012).

To date, many of the studies investigating the role of adenosine in osteoblasts have utilised MSCs (as described above). In addition, A_1, A_{2A}, A_{2B} and A_3 receptor activation has been shown to increase the proliferation (mainly via A_1 and A_{2A} receptors) and differentiation (via A_{2B} receptors) of MSCs (Costa et al., 2011).

2.3. P2 receptors and osteocytes

Osteocytes are the most abundant cell type within bone (Jande et al., 1973) yet little is known about the role of purinergic signalling in their survival and function. Because they are embedded within the bone matrix, primary osteocytes are difficult to isolate in significant amounts; therefore most work to date has been performed on osteocyte-like cell lines. An early study found that the calcium signals induced by fluid flow were decreased by suramin and thapsigargin, suggesting involvement of ATP acting via P2Y receptors (Huo et al., 2008). Expression of functional P2X2, P2X7, $P2Y_2$, $P2Y_4$, $P2Y_{12}$ and $P2Y_{13}$ receptors on MLO-Y4 osteocyte-like cells has recently been described (Kringelbach et al., 2014)

Several studies have also reported that osteocytes release ATP in a controlled manner (Genetos et al., 2007; Kringelbach et al., 2014; Thompson et al., 2011). This release is enhanced by UTP and mechanical stimulation and is thought to be mediated primarily by vesicular exocytosis (Kringelbach et al., 2014). Mechanically induced ATP release can also be regulated by the $\alpha_2\delta_1$ auxillary subunit of T-type voltage sensitive calcium channels (Thompson et al., 2011).

Since ATP can inhibit bone mineralisation it is possible that release from osteocytes *in vivo* could act to reduce progressive, age-related mineral encroachment from the surrounding bone, thus preventing eventual cell ‘fossilation’ and death.

2.4. Purinergic signalling and osteoclasts

The first description of P2 receptor expression by osteoclasts was in the early 1990’s, when it was shown that ATP induced a rapid and transient increase in $[Ca^{2+}]_i$ in rabbit osteoclasts (Yu et al., 1993a). The human P2U ($P2Y_2$/$P2Y_4$) receptor was subsequently cloned and sequenced from osteoclastoma, indicating that this receptor was probably expressed by osteoclasts (Bowler et al., 1995). Early investigations also provided evidence for the presence of P2X receptors, giving rise to non-selective cation currents, and P2Y receptors, mediating Ca^{2+} release from intracellular stores (Weidema et al., 1997; Wiebe et al., 1999). Initially it was thought that P2X4 receptors mediated the ATP-activated non-selective cation currents in rabbit osteoclasts (Naemsch et al., 1999). However, it was subsequently found that the nucleotide-induced elevation of $[Ca^{2+}]_i$ arose primarily from activation of P2Y receptors (Weidema et al., 2001)(Weidema AF 2001).

Expression of multiple P2 receptor subtypes by osteoclasts has now been described (see review (Burnstock et al., 2013)). Initial studies demonstrated expression of mRNA and protein for the P2X2, P2X4, P2X7, $P2Y_1$ and $P2Y_2$ receptors by rat long bone-derived osteoclasts (Hoebertz et al., 2000). Osteoclasts generated from human peripheral blood were later shown to express the P2X1, P2X4, P2X7, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors (Buckley et al., 2002). Recently, osteoclasts derived from mouse bone marrow were found to express all P2 receptors, with the exception of the P2X6 and $P2Y_4$ receptors (Orriss et al., 2011b). This study also demonstrated large differences in the expression levels of the different P2 receptors, with the $P2Y_6$ receptor being the most abundant (Orriss et al., 2011b).

Functional effects of P2 receptor-mediated signalling in osteoclasts

The first indication that purinergic signalling could play a role in the regulation of osteoclast function came in 1995 when Bowler *et al.* reported that ATP stimulated resorption by cells from human osteoclastoma (Bowler et al., 1995). Originally, the $P2Y_2$ receptor was thought to mediate this effect; however, in a follow up study UTP failed to stimulate resorption suggesting this was not the case (Bowler et al., 1998). Subsequently ATP was found to increase the formation and activity of rodent osteoclasts, effects that were inhibited by suramin or apyrase (Morrison et al., 1998). These stimulatory actions on resorption were further enhanced when osteoclasts were first activated by culture in acidified medium (Morrison et al., 1998). Since the P2X2 receptor is the only P2 receptor that requires extracellular acidification to show its full sensitivity to ATP (Wildman et al., 1998) it was suggested that the P2X2 receptor could mediate these pro-resorptive effects. Further investigation revealed that ADP, 2-methThioADP and ATP all stimulated osteoclast formation and activity, suggesting involvement of P2Y receptors. Pharmalogical and cytochemical evidence indicated that these effects were mediated by the $P2Y_1$ receptor (Hoebertz et al., 2001).

DEXA and μCT analysis of $P2Y_1$ receptor knockout mice showed reduced trabecular bone in the long bones (Orriss et al., 2011a; Orriss et al., 2008) **(Fig. 1)**. Given the suggested role for the $P2Y_1$ receptor in promoting osteoclast activity this decrease in bone mass was unexpected. However, a recent study found that ADP still stimulated osteoclast formation and resorption in cells derived from $P2Y_1$ receptor knockout mice (Su et al., 2012), indicating the involvement of another P2Y receptor. Based on pharmacology, the $P2Y_{12}$ receptor was the logical alternative candidate for mediating these effects. The $P2Y_{12}$ receptor was only identified in 2001 (Hollopeter et al., 2001), the same year as the study by Hoebertz *et al.,* and thus it was not considered in the original investigation. Expression of the $P2Y_{12}$ receptor by osteoclasts has recently been reported (Orriss et al., 2011b) and $P2Y_{12}$ receptor knockout mice display impaired responses to ADP, increased trabecular bone and decreased arthritis associated bone loss (Su et al., 2012). Furthermore, the $P2Y_{12}$ receptor antagonist, clopidogrel, inhibits osteoclast formation, viability and resorptive activity (Syberg et al., 2012a). Taken together these data suggest a role for the $P2Y_{12}$ receptor in mediating the effects of ADP on osteoclast formation and activity.

The $P2Y_6$ receptor also appears to regulate osteoclast formation, activity and survival. UDP, acting via the $P2Y_6$ receptor, has been shown to prevent the apoptosis induced by tumour necrosis factor-α (TNF-α), induce the translocation and activation of NF-κB and stimulate osteoclast formation and activity (Korcok et al., 2005; Orriss et al., 2011b). Consistent with a role for this receptor in bone resorption, osteoclasts derived from $P2Y_6$ receptor knockout mice display defective activity (Orriss et al., 2011b). Analysis of $P2Y_6$ receptor knockout mice by μCT revealed increased cortical bone in the long bones and spine (Orriss et al., 2011b) **(Fig. 1)**.

The role of the $P2Y_{13}$ receptor in regulating osteoclast function is still unclear. However, $P2Y_{13}$ receptor knockout mice display reduced osteoclast numbers *in vivo* and protection from ovariectomy-induced bone loss in females (Wang et al., 2012).

The regulation of osteoclast formation, activity and survival by the P2X7 receptor has been the focus of many, often conflicting, studies. P2X7 receptor blockade with selective antagonists or a monoclonal antibody has been shown to inhibit osteoclast formation formation (Agrawal et al., 2010; Gartland et al., 2003a). Analogues of the P2X7 receptor antagonist, KN-62, have also been shown to induce osteoclast apoptosis (Penolazzi et al., 2005). Combined, these studies suggest a role for the P2X7 receptor in osteoclast formation. However, other investigations have demonstrated that P2X7 receptor knockout mice possess functional osteoclasts *in vivo* and that osteoclasts can be generated *in vitro*, using knockout precursor cells (Gartland et al., 2003b; Ke et al., 2003). Furthermore, prolonged exposure to ATP has also been shown to down-regulate P2X7 receptor function and inhibit osteoclast formation and activity (Hiken et al., 2004; Naemsch et al., 2001).

Other work has demonstrated that P2X7 receptor activation induces the translocation of NFκB (Korcok et al., 2004) and an isoform specific PKC in osteoclasts and their precursors (Armstrong et al., 2009). The P2X7 receptor may additionally play a role in the intercellular communication between osteoblasts and osteoclasts, cytoskeletal reorganisation at the sealing zone and the delivery and secretion of lytic granules into the resorption lacunae (Hazama et al., 2009; Jorgensen et al., 2002). Recently, it was reported that ATP release from osteoclasts is mediated via the P2X7 receptor (Brandao-Burch et al., 2012; Pellegatti et al., 2011). Thus, this receptor may promote osteoclast fusion by increasing the concentration of extracellular adenosine (via breakdown of released ATP) (Pellegatti et al., 2011). It has also been suggested that ATP stimulates human osteoclast activity indirectly through upregulation of osteoblast-expressed RANKL (Buckley et al., 2002).

At present, it is not known how activation of the other P2 receptor subtypes influences osteoclast formation and activity and this presents an interesting area for further study.

P1 receptors, adenosine and osteoclasts

A potential role for adenosine in regulating osteoclast function was first suggested in the 1980s when the P1 receptor agonist, 2-chloroadenosine, was shown to stimulate bone resorption in calvarial bones in organ culture (Lerner et al., 1983). However, a later investigation found that adenosine analogues had no effects on resorption in this culture system (Lerner et al., 1987). In agreement, several studies have reported that adenosine has no effect on the formation or resorptive activity of rodent osteoclasts *in vitro* (Hoebertz et al., 2001; Morrison et al., 1998). Adenosine was also reported to have no effect on intracellular calcium levels in rabbit osteoclasts (Korcok et al., 2004). However, more recent work has suggested that stimulation of the A_{2A} receptor by adenosine or selective agonists can both stimulate (Pellegatti et al., 2011) and inhibit (Mediero et al., 2012b) osteoclast formation. A_{2A} receptor activation has also been shown to prevent the osteolysis that occurs during prosthesis loosening, a common cause of joint implant failure (Mediero et al., 2012a).

Pellegatti *et al* (2011) reported that the A_1 receptor was only weakly expressed by osteoclasts and activation of the A_1 receptor was recently shown to have no effect on mouse osteoclasts (Pellegatti et al., 2011; He et al., 2012). In contrast, blockade or deletion of the A_1 receptor can reduce the formation of mouse osteoclasts in culture (Kara et al., 2010a) and A_1 receptor knockout mice display increased bone mineral density and resistance to ovariectomy-induced bone loss (Kara et al., 2010b)

Adenosine has also been reported to stimulate osteoclastogenesis indirectly. Evans *et al* (2006) suggested that the adenosine produced from the hydrolysis of released ATP acts on P1 receptors to cause IL-6 release and inhibition of osteoprotegerin secretion (Evans et al., 2006).

2.5. P2 receptor polymorphisms and bone

Single nucleotide polymorphisms (SNPs) have been described in several P2 receptor genes (P2X4, P2X7, $P2Y_1$, $P2Y_2$, $P2Y_6$, $P2Y_{13}$). To date, only SNPs in the genes for the $P2Y_2$, P2X4 and P2X7 receptors have been studied for an association with osteoporosis risk or bone turnover (see review (Wesselius et al., 2012)).

At present, two SNPs have been identified which alter $P2Y_2$ receptor activity. The first of these is located at position 1000 (cytosine to thymine), changing arginine to cysteine at residue 334 ($Arg^{334}Cys$) (Janssens et al., 1999). The second SNP is located at position 936 (guanine to cytosine) and changes arginine to serine at residue 312 ($Arg^{312}Ser$) (Buscher et al., 2006). A recent study examining the association between the $Arg^{312}Ser$ SNP and bone status found that subjects homozygous for the C (variant) allele of the $P2Y_2$ gene had a significantly increased bone mineral density (BMD) at menopause (Wesselius et al., 2013a). Furthermore, the rate of bone loss for up to ten years post-menopause was 20-30% lower than both the heterozygous and the homozygous for the common allele (Wesselius et al., 2013a).

Many SNPs have been identified in the P2X7 receptor gene (see the recent review by Wesselius *et al* (Wesselius et al., 2012) for detailed information). Loss of function polymorphisms in the P2X7 receptor have been associated with increased fracture risk, reduced BMD and osteoporosis (Gartland et al., 2012b; Husted et al., 2013; Ohlendorff et al., 2007). Recently, it was reported that a single SNP in the P2X4 receptor gene (tyrosine to cysteine at position 315) is associated with an increased risk of osteoporosis and lower lumbar spine BMD (Wesselius et al., 2013b).

3. Cartilage

Cartilage is a flexible connective tissue that is found throughout the body, including the surface of joints. It is composed of specialised cells called chondrocytes that synthesize large amounts of collagen, abundant ground substance rich in proteoglycans and elastin fibres. Unlike other connective tissues, cartilage does not have a blood supply and chondrocytes receive their nutrients by diffusion. This lack of vasculature means that, compared to other tissues, cartilage grows and repairs itself more slowly.

P2 receptor expression by chondrocytes

Purinergic receptor expression in cartilage was first suggested in 1990 when ATP was found to stimulate resorption of bovine nasal cartilage (Leong et al., 1990). The following year, ATP was found to increase the production of PGE_2 from articular chondrocytes (Caswell et al., 1991). Several early studies reported that chondrocytes were responsive to UTP as well as ATP indicating the presence of P2Y receptors (Caswell et al., 1992; Kaplan et al., 1996; Koolpe et al., 1997). Chondrocytes are now know to express multiple P2 receptor subtypes including the P2X1, P2X2, P2X3, P2X4, P2X7, $P2Y_1$ and $P2Y_2$ receptors (Koolpe et al., 1999; Knight et al., 2009; Varani et al., 2008b; Kudirka et al., 2007). Many of these receptors (P2X2, P2X4, P2X7 and $P2Y_1$) are expressed throughout all the zones of articular cartilage, whilst others (e.g. the $P2Y_2$ receptor) show more limited spatial expression (Knight et al., 2009). A number of investigations have shown increased $[Ca^{2+}]_i$ levels following treatment with ATP, ADP or UDP, indicating the presence of functional P2 receptors on chondrocytes (Bulman et al., 1995; Hung et al., 1997; Kono et al., 2006).

Functional effects of P2 receptor signalling in chondrocytes

To date, many of the studies describing the effects of purinergic signalling on chondrocytes have been conflicting with some reports suggesting that extracellular nucleotides negatively regulate cartilage metabolism whilst others describe beneficial effects of ATP. ATP has been reported to inhibit cartilage formation in micromass cultures (Meyer et al., 2001), promote proteoglycan breakdown and glycosaminoglycan release in bovine nasal cartilage (Brown et al., 1997) and increase the production of inflammatory mediators, nitric oxide (NO) and PGE_2 (Varani et al., 2008b). Furthermore, $P2Y_2$ receptor stimulation increased IL-1-mediated PGE_2 release from articular chondrocytes (Koolpe et al., 1999) and induced a rapid rise in PGE_2 synthesis via the ERK1/2 and p38 MAPK signalling pathways (Berenbaum et al., 2003). In contrast, early work suggested that ATP caused articular cartilage mineralisation by promoting matrix vesicle-mediated calcium deposition (Ryan et al., 1992; Hsu, 1992). Extracellular ATP and UTP have also been shown to stimulate cartilage proteoglycan and collagen accumulation and suppress inflammatory mediator (NO) production (Chowdhury et al., 2006; Croucher et al., 2000).

Other reported effects of purinergic signalling include increased chondrocyte differentiation (Fodor et al., 2009), elevated fibroblast growth factor-induced proliferation (Kaplan et al., 1996; Koolpe et al., 1997) and increased responsiveness to IL-1β and TNF-α (Caswell et al., 1992; Leong et al., 1993).

ATP release and breakdown in cartilage

The first direct evidence for constitutive ATP release from chondrocytes came in 2000 from a study by Graff *et al.* (Graff et al., 2000). Subsequent studies have shown that P2Y receptor stimulation increases $[Ca^{2+}]_i$ levels which trigger further release of ATP from adjacent cells (Kono et al., 2006; Millward-Sadler et al., 2004).

Ecto-nucleotidase activity in chondrocytes was first reported by Kanabe *et al* in 1983 (Kanabe et al., 1983). It was later shown that NTPDases, NPPs, alkaline phosphatase and ecto-5'-nucleotidase, as well as adenylate kinase and nucleoside diphosphokinase which mediate regeneration of ATP, are all present in cartilage (Graff et al., 2003). A recent study found that P1 receptor agonists decreased extracellular PP_i production in chondrocytes and cartilage explants, while ADP increased PP_i levels, suggesting a homeostatic role for P1 and P2 receptors in stabilizing concentrations of PP_i (Rosenthal et al., 2010).

P1 receptors and chondrocytes

Chondrocytes are known to express all the P1 receptors (Tesch et al., 2002; Varani et al., 2008a); however, information regarding the functional effects of receptor activation remains limited. Pulsed electro-magnetic fields have been shown to promote chondrocyte proliferation via the up-regulation of $A2_A$ and A_3 receptor expression (Varani et al., 2008a). Adenosine, acting via $A2_A$ receptors, can also reduce IL-1β-induced inflammation (Campo et al., 2012) and VEGF secretion (Vincenzi et al., 2013).

4. Muscle

There are three types of muscle, skeletal, cardiac and smooth within the body. Cardiac and smooth muscle are often termed involuntary as these muscles contract without conscious thought. Whilst cardiac muscle is only found within the heart, smooth muscle is more widely distributed being found within the walls of many internal organs. Skeletal (or voluntary) muscle is anchored to bones by tendons. It plays a key role in locomotion and posture and thus is a critical component of the musculoskeletal system. Within muscle, extracellular ATP comes from a number of sources including

motor nerves, stressed or exercising muscle and damaged or dying muscle and vascular endothelial cells (Buvinic et al., 2009; Mortensen et al., 2009; Tu et al., 2012).

Skeletal muscle and purinergic signalling

The first evidence for involvement of purinergic signalling in skeletal muscle came in the 1980's when ATP analogues were found to induce the lengthening of muscle fibres in rigor (Schoenberg, 1989). Activation of P2 receptors was later shown to induce IP_3 accumulation in cultured skeletal muscle cells (Keresztes et al., 1991). Many studies have demonstrated the expression of multiple P2 receptor subtypes by skeletal muscle (Banachewicz et al., 2005; Cheung et al., 2003; Deli et al., 2007; Janssens et al., 1996); however, the pattern of purinergic receptor expression is strongly influenced by developmental stage and species (see (Burnstock et al., 2013)).

There is now abundant evidence showing that purinergic signalling plays an important role in modulating the development and function of skeletal muscle (see review by (Burnstock et al., 2013) for detailed information). For example, several studies have shown that extracellular ATP can regulate myoblast proliferation and the differentiation of mammalian skeletal muscle (Martinello et al., 2011; Ryten et al., 2002; Sciancalepore et al., 2012). Extracellular nucleotides, acting via the $P2Y_1$ receptor, can also enhance muscle contraction and influence muscle excitability (Choi et al., 2003; Voss, 2009).

5. Summary and future directions

Our knowledge about the role purinergic signalling in the musculoskeletal system has increased significantly in the past twenty years. In particular, it is now evident that P2 receptor-mediated signalling exerts complex, local effects on bone cell function. The effects of this signalling system are influenced by a number of factors including the receptor subtype, the extracellular nucleotides present locally and the expression of ecto-nucleotidases. At present, there is abundant evidence for the negative actions of extracellular nucleotides, with particularly notable effects on osteoblast function and matrix mineralisation. However, given that purinergic signalling also exerts some positive effects on bone cells, it is likely that a balance exists *in vivo* between the positive and negative actions of this complex signalling system. Selective receptor agonists and antagonists for the P2 receptor subtypes involved in bone remodelling are being developed, which ultimately could lead to new ways to treat bone disease.

Bone, cartilage and muscle are often studied in isolation and the ability of one tissue to influence another is often overlooked. However, given the significant ATP release and widespread expression of purinergic receptors in the musculoskeletal system crosstalk between these tissues is likely and presents an important area for future work.

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Figure Legends

Figure 1. Bone phenotypes of $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptor knockout mice

Representative µCT images of the femurs of 8-week old $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptor knockout mice show that receptor deletion causes significant changes in the bone structure. **(A)** $P2Y_1$ null mice have decreased trabecular bone volume and trabecular number. **(B)** $P2Y_2$ receptor knockout animals display increased trabecular bone volume, thickness and number. **(C)** $P2Y_6$ null mice have a higher cortical bone volume and thickness.

Figure 2. Expression of P2 receptors by osteoblasts

The expression of P2 receptors by primary rat osteoblasts was studied by immunofluorescence using specific primary polyclonal antibodies, Cy3-labelled anti-rabbit secondary antibody (red) and DAPI nuclear stain (blue). Expression of protein for all the P2 receptors was detected in in rat osteoblasts. Scale bar = 25µm.

Figure 3. Extracellular ATP acts via P2-receptor dependent and independent mechanisms to inhibit bone mineralisation.

Osteoblasts constitutively release ATP into the bone microenvironment, where it can act via two different mechanisms to inhibit mineralisation. (1) *P2-receptor dependent:* activation of the $P2Y_2$, P2X1 or P2X7 receptor decreases TNAP activity and PP_i breakdown. (2) *Receptor independent:* hydrolysis of ATP by NPP1 produces PP_i. The overall effect of either mechanism is increased levels of extracellular PP_i, the key physicochemical inhibitor of mineralisation.

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Figure 1

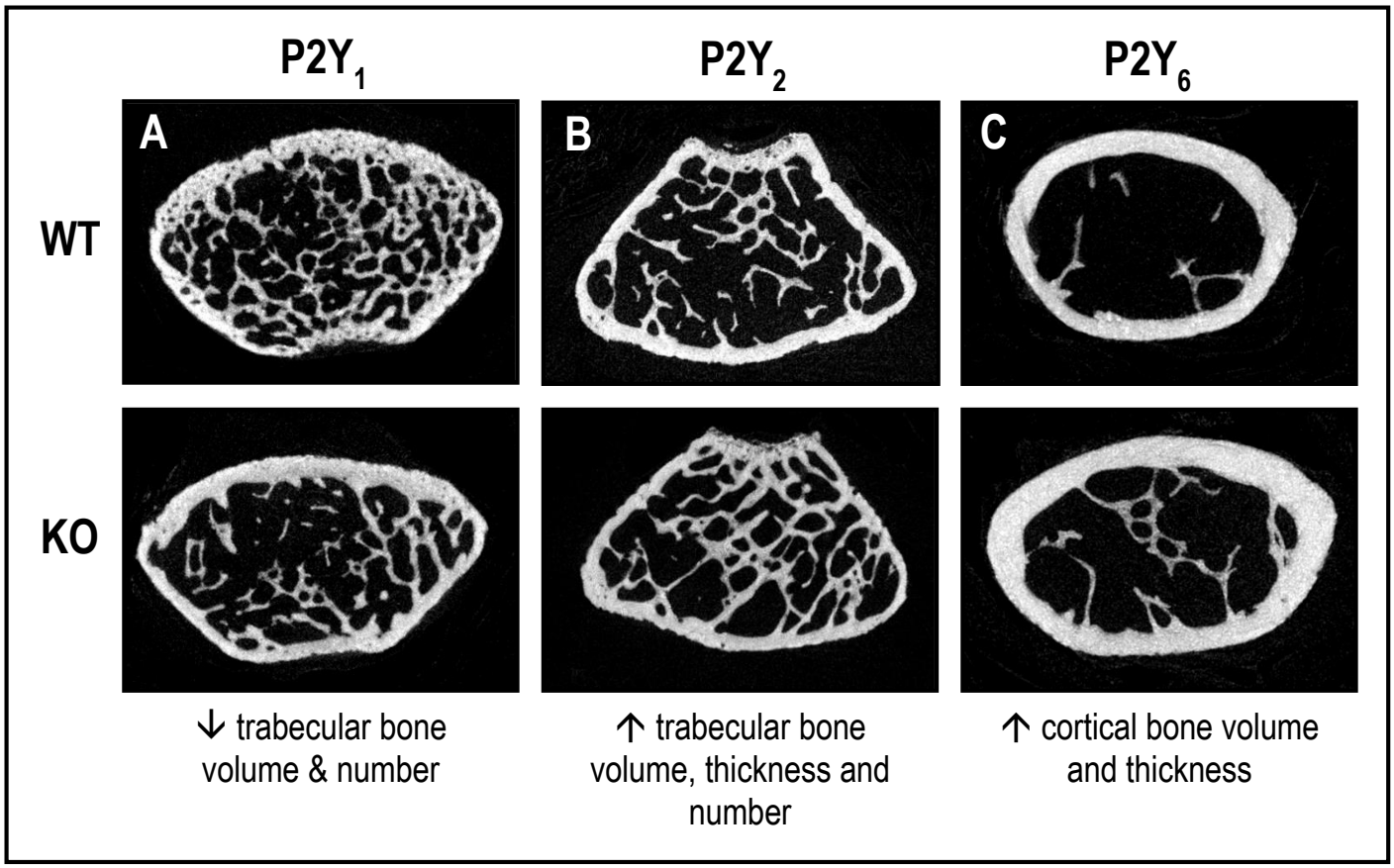
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Figure 2

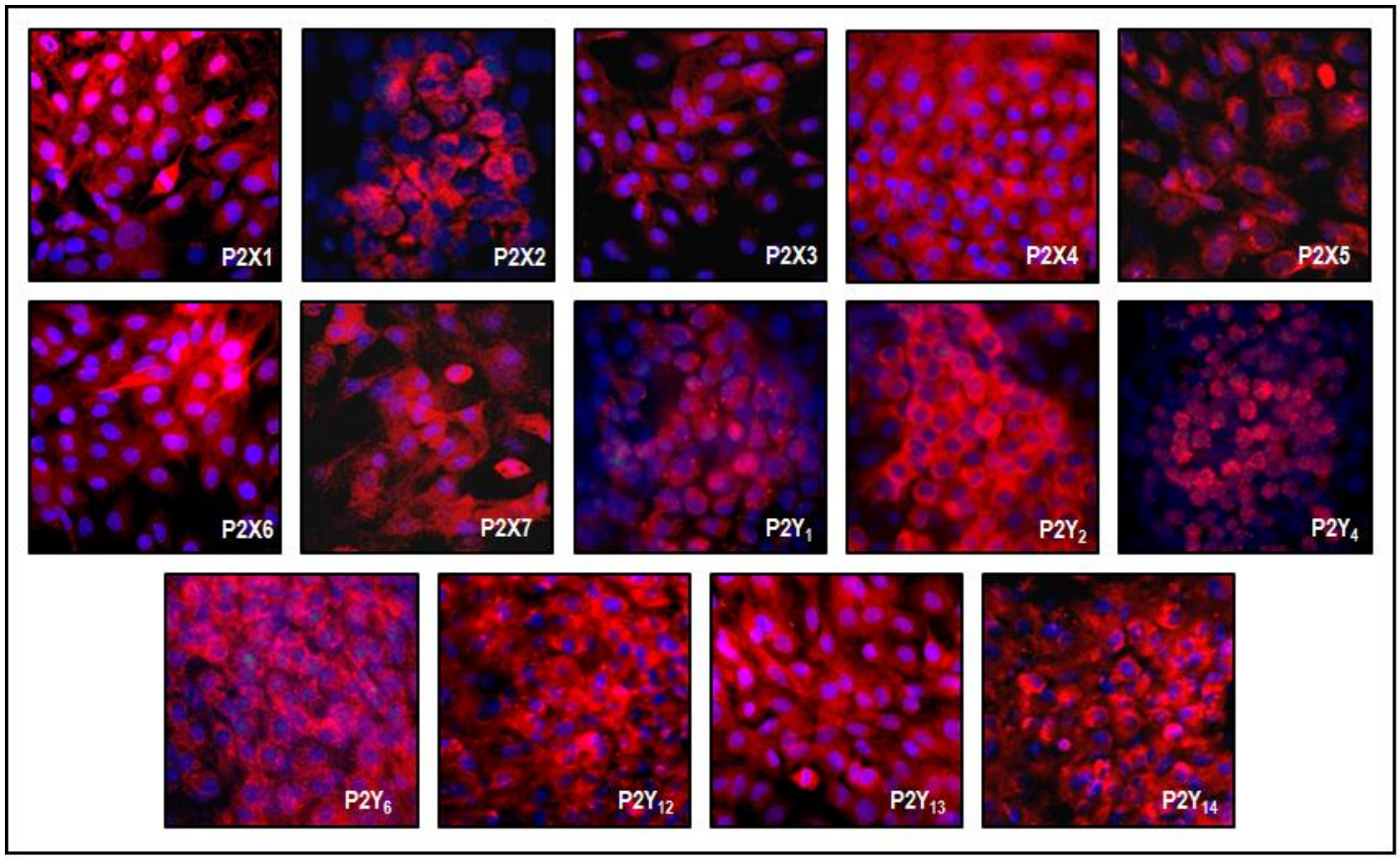
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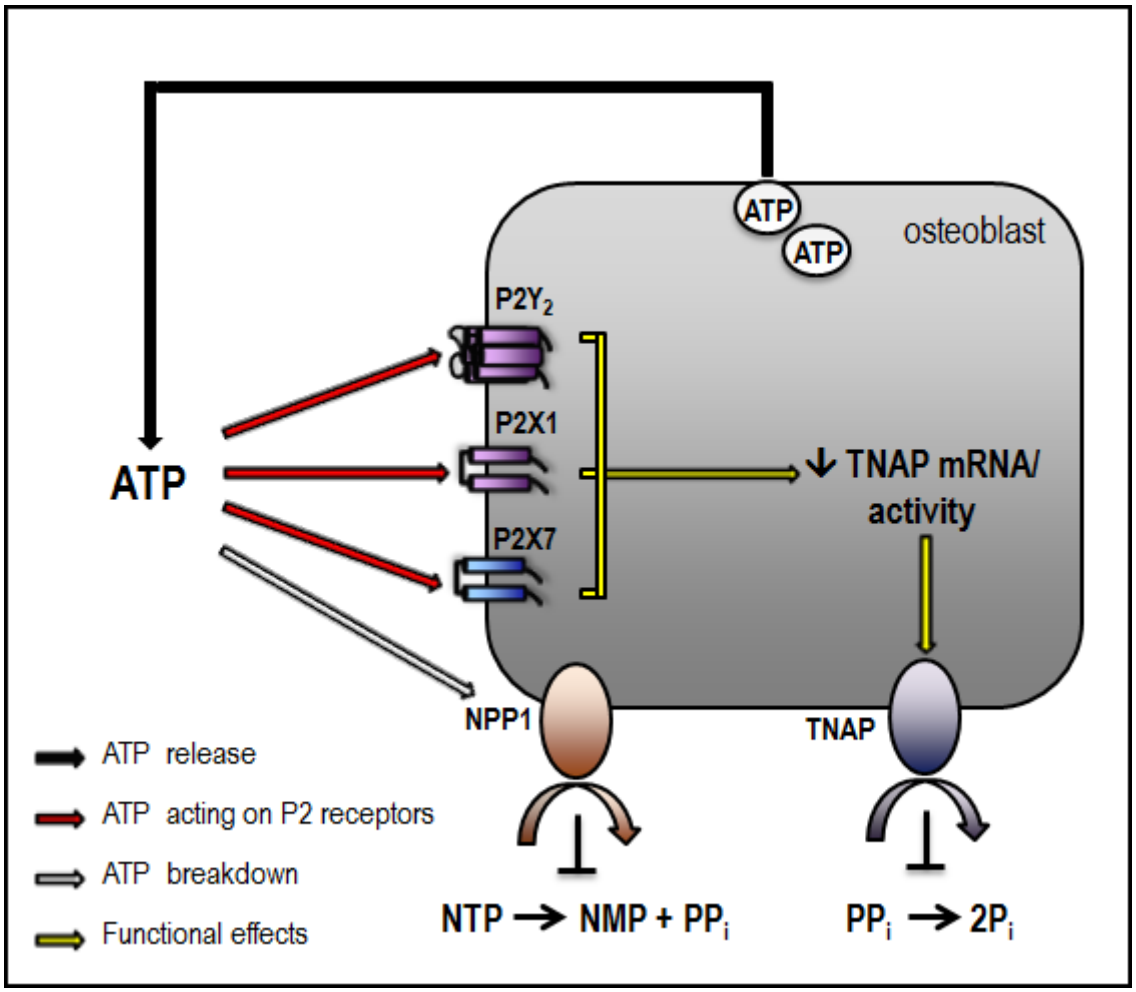
Figure 3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ATP breakdown
Functional effects
NTP → NMP + PPi
PPi → 2Pi