P2X7 receptors: role in bone cell formation and function

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Abstract

The role of the P2X7 receptor (P2X7R) is being explored with intensive interest in the context of normal bone physiology, bone-related diseases and, to an extent, bone cancer. In this review, we cover the current understanding of P2X7R regulation of bone cell formation, function and survival. We will discuss how the P2X7R drives lineage commitment of undifferentiated bone cell progenitors, the vital role of P2X7R activation in bone mineralisation and its relatively unexplored role in osteocyte function. We also review how P2X7R activation is imperative for osteoclast formation and its role in bone resorption via orchestrating osteoclast apoptosis. Variations in the gene for the P2X7R (P2RX7) have implications for P2X7R-mediated processes and we review the relevance of these genetic variations in bone physiology. Finally, we highlight how targeting P2X7R may have therapeutic potential in bone disease and cancer.

Key Words

- apoptosis
- bone
- cancer
- osteoblast
- osteoclast
- P2X7

Purinergic signalling

In the last four decades, extensive investigations have led to the recognition of ATP as a ‘molecular unit of energy’ to an extracellular messenger molecule. ATP-sensitive purinoceptors, omnipresent in invertebrate tissues, are involved in a wide variety of physiological roles (Burnstock 2013) and their function has also been demonstrated in invertebrates (Verkhratsky & Burnstock 2014). While they traditionally act as cell surface sensors (Khakh & North 2006), their participation in signalling within the intracellular compartment in mammals (Qureshi et al. 2007, Kuehnel et al. 2009, Stokes & Surprenant 2009, Toulme et al. 2010) and even protozoans (Fountain et al. 2007, Ludlow et al. 2009, Sivaramakrishnan & Fountain 2012) has also been recognised.

Purines (adenine, guanine and uridine) can act as signalling molecules in the form of their 5'-nucleotide triphosphates (such as ATP, GTP and UTP), diphosphates (ADP), monophosphate (AMP) or as a nucleoside (adenosine). They can activate one or more of the 19 receptors which are sorted into three classes: P1 (nucleoside) receptors; the metabotropic P2Y receptors and the ionotropic P2X, both of which are nucleotide triggered. Recently, a new family of purine receptors, AdeR or P0-receptors, responsive to adenine has been cloned from rodents (Bender et al. 2002, Gorzalka et al. 2005, von Kugelgen et al. 2008, Thimm et al. 2013), although the human homologue is yet to be identified.

Structural and stoichiometrical evidence suggests that all P2X receptor (P2XR) subunits trimerise to form functional receptors (Nicke et al. 1998, Barrera et al. 2005, Mio et al. 2005, Kaczmarek-Hajek et al. 2012) with the subunits forming either homomultimers or heteromultimers depending on the subtypes (Burnstock 2007). Each unit comprises two transmembrane domains (TM1 and TM2) with an intervening large extracellular loop and cytoplasmic N- and C-termini, and the primary agonist of all homomeric and heteromeric P2XR is ATP. The current view holds that ligand binding causes reduction in the
disulfide bridges between the cystine residues causing the movement of TM1 and TM2, allowing them to open the non-selective cation channel permeable to small monovalent and divalent cations (Browne et al. 2010). Subsequent elevation in the intracellular calcium concentration ([Ca\(^{2+}\)]_i) either by direct Ca\(^{2+}\) permeation or by activation of voltage-gated Ca\(^{2+}\) channels (Koshimizu et al. 2000) triggers a range of signalling cascades, resulting in both short- and long-term cellular events. P2XRs are classed as rapidly desensitising (P2X1 and P2X3) and slowly desensitising (P2X2, P2X4, P2X5 and P2X7) on the basis of the amplitude of the ATP-induced current (Koshimizu et al. 2000, North 2002), and continuous agonist application causes an increase in permeability, presumably caused by a progressive rotation and separation of TM1 and TM2 resulting in the formation of a membrane pore (Browne et al. 2010).

All the P2XRs share common and specific features as mentioned above, but the P2X7 receptor (P2X7R) differs from the other P2XRs in many ways. First, it has a very long cytoplasmic C-terminal tail, which is responsible for its unique properties and partly mediates P2X7R physiology by interacting with other proteins (Rassendren et al. 1997, Virginio et al. 1999). This permeability state allows permeation of larger cations with a molecular weight of up to 900 kDa, such as N-methyl-D-glucamine and fluorescein dyes such as the cationic propidium dye YO-PRO-1 and ethidium (Khakh et al. 1999), arguably due to an interaction with other proteins such as pannexin hemichannels (Pelegrin & Surprenant 2006). Thirdly, 2,3-(4-benzoyl)benzoyl ATP (BzATP) is more potent than ATP at P2X7 whereas ATP is the most potent agonist as pannexin hemichannels (Pelegrin & Surprenant 2006). Arguably due to an interaction with other proteins such as pannexin hemichannels (Pelegrin & Surprenant 2006).

P2X7R in cells of osteoblast lineage

Progenitor cells

Osteogenic precursors are derived from mesenchymal stem cells (MSCs), and the role of various purinergic receptor in dictating the commitment of MSCs as well as their fate in differentiation has been demonstrated (Zippel et al. 2012, Biver et al. 2013). Induction of osteogenic differentiation of human MSCs (hMSCs) has recently been shown to occur following shockwave treatment of hMSCs and is thought to be dependent upon P2X7R signalling (Sun et al. 2013). The authors demonstrated that cellular ATP was released following shockwave treatment and led to downstream p38 MAPK activation, and to c-Fos and c-Jun mRNA transcription. Treating hMSCs with apyrase (an enzyme that hydrolyses extracellular ATP), P2X7R-siRNA, PPADS (a non-selective P2 antagonist) and KN-62 (a P2X7R antagonist) completely abolished these downstream events, indicating a P2X7R-mediated effect. The shockwave-induced differentiation of MSCs, as measured by alkaline phosphatase activity, osteocalcin production and nodule formation, was significantly reduced by the targeted blockade of P2X7R adding further evidence of a P2X7R-mediated effect (Sun et al. 2013). In another study, bone marrow-derived MSC cultures from postmenopausal women showed a P2X7R-dependent enhancement in osteogenic differentiation and mineralisation (Noronha-Matos et al. 2014). The study described an initiating event underlying P2X7R-mediated plasma membrane blebbing in hMSC, which was induced by application of BzATP (100 \(\mu\)M) and involved cytoskeleton rearrangements due to P2X7R-dependent protein kinase C (PKC) and Rho-associated kinase activation. In addition, a delayed increase in the basal ALP activity of MSCs from postmenopausal women compared with those from the younger females was also demonstrated, suggestive of an impaired osteogenic commitment in ageing MSCs. However, assessment of ALP activity, expression of the transcription factors RUNX2 and osteolin, mineralised area and the number of bone nodules revealed that osteogenic differentiation and mineralisation in postmenopausal MSC cultures could be restored by P2X7R activation with BzATP (Noronha-Matos et al. 2014), highlighting the role of P2X7R in promoting mineralisation by MSCs. Taken together, these findings suggest that P2X7R promotes the differentiation of MSCs into mature bone cells and could potentially be used to positively drive bone formation.

Mature cells

As osteoblasts are responsible for bone formation (organic matrix, primarily composed of type I collagen with and eventual deposition of hydroxyapatite mineral), they are essential for the maintenance of bone mass. P2X7R expression has been consistently reported in human
and rodent cells of osteoblast lineage (osteoblast-like cell lines, calvarial and bone-derived primary osteoblasts) by RT-PCR, immunochemistry and cell permeabilisation experiments (Nakamura et al. 2000, Gartland et al. 2001, Orriss et al. 2006). It is noteworthy that only a subpopulation of bone-derived and calvarial osteoblasts demonstrate a positive nucleotide response (Gartland et al. 2001, Ke et al. 2003, Panupinthu et al. 2008) indicative of heterogeneity either in the cells in these in vitro assays or in P2X7R expression by the cells.

Biological effects of P2X7R activation include apoptosis in SaOS-2 osteoblast cell line (Gartland et al. 2001), induction of membrane blebbing in mouse calvarial osteoblasts and MC3T3-E1 osteoblastic cells (Panupinthu et al. 2007, Grol et al. 2012), production of lipid mediators in mouse calvarial osteoblasts (Panupinthu et al. 2008), substantial induction of transcription factor activating protein-1 (AP-1) in mouse MC3T3-E1 cells (Gavala et al. 2010) and reduced bone mineralisation and alkaline phosphatase activity in primary rat osteoblasts (Orriss et al. 2013). In addition, blockade or absence of P2X7R has been shown to inhibit propagation of intercellular calcium signalling between osteoblasts and osteoclasts in human bone marrow-derived cells (Jorgensen et al. 2002), significantly reduce ERK phosphorylation in response to fluid shear stress in mouse primary osteoblasts (Liu et al. 2008, Okumura et al. 2008) and prevented fluid shear-stress-induced IκBζ degradation and nuclear accumulation of nuclear factor kappa B (NFκB) in MC3T3-E1 osteoblasts (Genetos et al. 2011).

It is evident that functional P2X7R is required during osteogenesis (Fig. 1); however, contradicting evidence for the effects of P2X7R activation on osteoblast differentiation and matrix mineralisation in vitro makes the underlying mechanisms unclear. While rat calvaria-derived osteoblasts show inhibited mineralisation in the presence of P2X7R agonists (Orriss et al. 2012), a previous study showed an opposite effect of increased osteoblast differentiation and matrix mineralisation following P2X7R activation in cultures obtained from the same source (Panupinthu et al. 2008). The differences between culture methodologies in the two studies could in part explain the discrepancies in these studies. Orriss et al. attribute their findings to a P2 receptor-dependent and/or receptor-independent mechanism via hydrolysis of extracellular nucleotides to pyrophosphate (PPi). Indeed, the negative bone mineralisation effect of endogenously released extracellular ATP has subsequently been shown to be averted with apyrase treatment suggestive of an anti-osteogenic autocrine/paracrine mechanism involving other purinergic receptors and ATP derivatives (Orriss et al. 2013). In addition, PPi is a known inhibitor of mineralisation and given that biologically relevant PPi levels can be achieved with a single dose of 10 μM ATP or UTP in osteoblast cultures (Orriss et al. 2007), PPi generation could be a contributing mechanism to inhibit bone mineralisation. Panupinthu et al. (2008) describe that P2X7R-mediated production of lysophosphatidic acid (LPA) metabolites contributes to...
enhanced osteogenesis observed in their in vitro model. LPA signalling involves Rho-associated kinase, which has established roles in driving non-committed cells towards osteoblast lineage (McBeath et al. 2004). Therefore, it is likely that the observations of Panupinthu et al. involve osteoblast-autonomous mechanisms downstream of P2X7R activation. Further evidence for a positive role of P2X7R in osteoblasts include the observations of reduced ALP activity in osteoblasts from P2X7R knock out (KO) in vitro (Panupinthu et al. 2008), decreased periosteal bone formation in long bones of P2X7R KO mice (Ke et al. 2003) and their reduced osteogenesis in response to mechanical loading (Li et al. 2005). Furthermore, the significance of these observations is confirmed in humans as several polymorphisms in the P2RX7 imparting reduced function to the P2X7R are associated with increased osteoporosis risk in different human cohorts (Ohlendorff et al. 2007, Gartland et al. 2012b, Jorgensen et al. 2012). In addition, the truncated P2X7R isoform, P2X7RB, when co-expressed with the full variant P2X7RA demonstrates a significant enhancement of mineralisation in human osteosarcoma cell line (Giuliani et al. 2014), thus consolidating a positive role for fully functional P2X7R in maintenance of bone strength.

Other effects of P2X7R activation on osteoblast function include phospholipase D and A2 stimulation (Panupinthu et al. 2007, 2008) and mechanical stress-induced prostaglandin E2 release (Li et al. 2005), suggesting a coupling of P2X7R signalling with production of lipid mediators. In addition, Ca2+ influx following P2X7R activation causes sustained proton efflux dependent on glucose and phosphatidylinositol 3-kinase activity (Grol et al. 2012) and activation of PKC to mediate phosphorylation of ERK1/2 (Liu et al. 2008, Okumura et al. 2008), suggestive of more cross talk in osteoblast-like cells. A fluid shear stress-induced NF-κB nuclear localisation independent of both ERK1/2 LPA signalling has also been demonstrated through P2X7R in osteoblasts (Genetos et al. 2011). It seems likely that while the basal/transient activation of P2X7R is osteogenic, sustained stimulation could inhibit the function and activity of these bone-forming cells. In this context, it has been shown that while short-term application of BzATP induces reversible membrane blebbing without activating the key apoptotic mediator caspase-3 in murine osteoblastic cells (Li et al. 2005, Panupinthu et al. 2007), longer agonist stimulus in human osteoblast-like and primary bone-derived cells caused extensive plasma membrane blebbing and ultimately cell apoptosis (Gartland et al. 2001, Alqallaf et al. 2009). Table 1 summarises the effect of P2X7R expression on osteoblasts.

P2X7R in osteocytes

Osteocytes are terminally differentiated osteoblast cells which become incorporated within the mineralised matrix in the process of bone formation. Their slender cytoplasmic processes extend and interconnect to communicate with other bone cells on the bone surface, influencing bone remodelling. These cells are difficult to study because they are embedded within the bone, and how these osteocytes relay information to the osteoclasts and osteoblasts and whether purinergic signalling is involved in the process are still not clear.

Despite this, there is support for a role of the P2X7R in osteocyte signalling, with evidence of P2X7R protein expression and BzATP-induced pore formation in MLO-Y4 osteocytes provided nearly 10 years ago (Li et al. 2005). Furthermore, P2X7R-induced pore formation in MLO-Y4 osteocytes was shown to occur in response to fluid shear stress and led to the activation of downstream signals typically involved in mechanically induced bone formation, in particular the release of PGE2 (Yoshida et al. 2002, Li et al. 2007). However, contradictory evidence suggests that inhibiting P2X7R does not prevent fluid flow-induced release of PGE2 from MLO-Y4 osteocytes (Cherian et al. 2005). While a role of P2X7R activation in regulating mechanical load by osteocytes could be speculated, exactly how the activated ion channel creates an intracellular signal capable of an amplified mechanotransduction process remains unclear. Table 1 summarises the effect of P2X7R expression on osteoclasts.

P2X7R in cells of osteoclast lineage

Progenitor cells

Bone resorbing osteoclasts differentiate from the haematopoietic stem cells (HSCs) and cells of HSC lineage have also been shown to express an array of purinergic receptors (Lemoli et al. 2004, Wang et al. 2004a). A more specific role for the P2X7R has been suggested due to the observations that ATP at high concentrations (1 mM) reduced the number of murine HSCs, whereas the number of more committed, myeloid cells increased (Barbosa et al. 2011). The increased proliferation of HSCs caused a reduction in Notch expression, a marker of HSC quiescence, and compromised the ability of HSCs to repopulate the bone marrow. Concentrations lower than 1 mM failed to induce significant changes, indicating a P2X7R-mediated change in murine HSCs to a differentiated state from their primitive, undifferentiated state (Barbosa et al. 2011) and therefore suggest a role of P2X7R in promotion of haematopoiesis likely along the osteoclastic lineage.
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Mature cells

Osteoclasts, the bone-resorbing cells, are derived from monocytes and the P2X7R has a complex role in these cells of haematopoietic lineage (Fig. 2). P2X7R expression has been shown on authentic osteoclasts generated in vitro from small mammals such as rodents and rabbit (Hoebertz et al. 2000, Naemsch et al. 2001, Orriss et al. 2011) and has also confirmed in humans osteoclasts by immunocytochemistry both in vitro and in vivo (Jorgensen et al. 2002, Gartland et al. 2003a). Using RT-PCR, P2X7R expression was constitutively detected in human monocyctic precursors and throughout osteoclastogenesis in vitro (Buckley et al. 2002, Gartland et al. 2003a). However, in a recent study, P2X7R expression in primary mouse osteoclasts has been shown to be differentiation dependent, with higher mRNA and protein levels present in mature, resorbing cells compared with their precursors (Brandao-Burch et al. 2012). These confounding results in the expression of P2X7R mRNA transcripts could be attributed to the P2X7R specific differences in the investigated species (Roger et al. 2010, Bartlett et al. 2014), the different source of precursors used to derive mature osteoclasts and a potential contribution of human and murine P2X7R gene variants.

The functional consequence of P2X7R expression has also been extensively investigated. Blockade of P2X7R by a monoclonal antibody against the receptor’s external domain or specific P2X7R antagonists prevented osteoclast fusion, but not cell clumping, as previously described by our group (Gartland et al. 2003a, Agrawal et al. 2010). The role of P2X7R in cell fusion is consistent with previous findings in macrophage cell clones, where cells expressing the P2X7R fused spontaneously in vitro whereas the ones lacking P2X7R did not (Di Virgilio et al. 1999). However, P2X7R KO mice maintain their ability to form multinucleated osteoclasts in vivo and in vitro (Gartland et al. 2003b, Ke et al. 2003), suggesting that P2X7R might not play an exclusive role in driving cell fusion. Indeed, Pellegatti et al. recently demonstrated the existence of an ATP-mediated signalling loop controlling osteoclast fusion. In their study, addition of apyrase, and subsequent accumulation of adenosine, drove fusion whereas pharmacological blockade of P2X7R prevented fusion (Pellegatti et al. 2011). Therefore, the authors speculate that ATP release via the P2X7R pore is needed for osteoclast fusion, although the effect may be indirect involving other purinergic receptors. Since then, the role of P2X7R in driving LPA-stimulated osteoclast fusion has been highlighted (Hwang et al. 2013). The authors showed that siRNA-induced P2X7R knockdown in RAW 264.7 cells downregulated the expression of osteoclastic functional makers including nuclear factor of activated T cells 1 (NFATc1), cathepsin K, tartrate-resistant acid phosphatase (TRAP), AT6v0d2, c-Src, c-Jun, and Car2, in addition to suppression of the LPA-stimulated increase in osteoclast

Figure 2
Effect of P2X7R on osteoclastogenesis: extracellular ATP induces commitment of haematopoietic stem cells (HSCs) towards a more committed, myeloid progenitor/pre-osteoclast cell. Fusion of osteoclast precursors is blocked by antagonising P2X7R, possibly as P2X7R pore-mediated ATP release is needed for generation of adenosine which drives fusion. While it is clear that P2X7R activation promotes osteoclastogenesis via downstream events such as translocation of PKC, NF-κB nuclear localisation, NFATc1 activation, conflicting evidence exists regarding the significance of extracellular ATP in regulation of bone resorption. High extracellular ATP levels have been shown to both induce resorption and reduce resorption by initiating apoptosis. Intracellular ATP levels positively regulate survival and resorption.
diameter and bone resorptive capacity in differentiating cultures. The study suggests that P2X7R regulates the positive effect of LPA in osteoclast fusion and possibly also couples to other osteoclastogenic receptors such as osteoclast stimulatory transmembrane protein (OC-STAMP), providing important evidence in support of the role of P2X7R in osteoclastogenesis. It would be interesting to identify whether P2X7R is upstream of OC-STAMP in addition to confirming the findings using mature, resorbing osteoclasts to fully elucidate the effect of P2X7R activation on osteoclast formation.

P2X7R activation is also thought to be important in the differentiation and survival of osteoclasts in both a paracrine and autocrine manner. Stimulus by P2X7R specific agonist induces the membrane depolarisation in osteoclasts (Naemsch et al. 2001) and a subsequent increase in intracellular \([\text{Ca}^{2+}]\). This P2X7R-mediated \([\text{Ca}^{2+}]\) increase will result in the activation of key signalling molecules, such as PKC, NF-\(\kappa\)B, and NFATc1, which are dependent on elevations in cytosolic \([\text{Ca}^{2+}]\), and regulate an array of genes involved in osteoclast differentiation. Absence of P2X7R function causes loss of agonist-induced membrane translocation of PKC (Armstrong et al. 2009), a signal essential for osteoclast survival (Pereverzev et al. 2008); absence of NF-\(\kappa\)B nuclear localisation (Korcok et al. 2004), a transcription factor essential for osteoclastogenesis (Iotsova et al. 1997); and a loss of coupling leading to NFATc1 activation (Ferrari et al. 1999, Adinolfi et al. 2009), a master regulator of osteoclast differentiation (Takayanagi et al. 2002), further highlighting the direct involvement of P2X7R in intracellular signalling crucial to osteoclast differentiation and survival. We have data indicating that nuclear translocation of NFATc1 in RANKL and M-CSF primed monocytes and in mature, resorbing osteoclasts indeed depends on P2X7R activation (Agrawal & Gartland 2011), strengthening the role of P2X7R mediated events during differentiation of these bone resorbing cells. It seems likely that abolishing P2X7R activity would interfere with above signalling activities, thereby negatively influencing osteoclast function by affecting formation of ruffled border and subsequently bone resorption. In this regard, Hazama et al. (2009) reported that treatment of human osteoclasts with either BzATP or high concentrations of ATP increased bone resorption in vitro. Furthermore, the induction of resorption was accompanied by formation of sealing-zone like structures via the reorganisation of pre-existing cytoskeleton and the secretion of lytic granules at the site of osteoclast–matrix attachment. This seeming P2X7R-mediated augmentation of resorption appears to be absent in the presence of Brilliant Blue G, a selective P2X7R antagonist (Jiang et al. 2000), or in osteoclasts from KO mice (Armstrong et al. 2009, Hazama et al. 2009). Contrary to this, a recent study using murine bone marrow-derived osteoclasts has reported that extracellular ATP caused disruption of murine osteoclastic cytoskeleton and a subsequent reduction in survival and resorption (Miyazaki et al. 2012), probably by initiation of apoptosis as suggested previously (Ohlendorff et al. 2007). Miyazaki et al. show that osteoclastic bone resorption relies on the levels of intracellular ATP, which are dependent on mitochondrial function and steady extracellular ATP levels, as depletion of intracellular ATP led to increased resorption but shorter cell survival. Furthermore, either ATP hydrolysis or repletion of intracellular ATP by expression of anti-apoptotic protein Bcl-\(\_\)xl, completely reversed the inhibitory effect of extracellular ATP on osteoclast survival. Considering these two studies together suggests that extracellular ATP can act via two distinct mechanisms in controlling osteoclast cell survival – activation of purinergic signalling, particularly the P2X7R, and the control of mitochondrial energy regulation. However, the contradictory effects of ATP in these two studies remain unexplained, although some differences could be species specific (Donnelly-Roberts et al. 2009, Bartlett et al. 2014).

Activation of P2X7R in osteoclasts is imperative for cell fusion, can lead to initiation of apoptosis and is critical in determining the duration of cell survival and overall resorption (Table 1). It could be speculated that basal stimulus may cause a hypo or hyper stimulation of P2X7R due to its genetic variations, culminating in enhanced or reduced osteoclast formation and function respectively. Clearly a fine balance between the downstream consequences of P2X7R activation is needed and this may be achieved by modulating extracellular ATP concentrations.

### P2X7R-mediated ATP release by bone cells

There are several ways in which ATP can be released from the cell. A non-specific mechanism, following cell trauma, causes cytosolic ATP release along with the rest of the cytoplasmic contents and was probably the initial mechanism from which the entire purinergic network is thought to be evolved (Burnstock & Verkhratsky 2009). Controlled ATP release is thought to occur via vesicular exocytosis, in which ATP is part of a secretory vesicle; transmembrane ATP-binding cassette (ABC) proteins; gap junctions involving connexin and pannexin hemichannels and more recently the P2X7R.
ATP release in the context of bone has been shown to vary with the differentiation state of the cell (mature, bone-forming osteoblasts releasing up to several fold more ATP than undifferentiated, proliferating cells) (Orriss et al. 2009, Brandao-Burch et al. 2012), and the duration, direction and type of mechanical stimulus (Rumney et al. 2012a). The role of P2X7R regulated ATP release in response to fluid flow was initially described using human osteoblastic cell lines (Rumney et al. 2010). Recently, this has been confirmed using rat calvaria-derived osteoblasts (Brandao-Burch et al. 2012). However, the finding that P2X7R specific antagonists block ATP release from osteoblast by between 25 and 80% (Brandao-Burch et al. 2012) suggests that not all ATP release is via the P2X7R. Moreover, cultured calvarial osteoblasts from P2X7R null mice were previously shown to release similar amounts of ATP compared with WT cells following external stimulus such as fluid shear (Li et al. 2005). It is therefore likely that another pathway, such as gap junctions, could be involved. We have shown that ATP release from human osteoclasts derived from peripheral blood monocytes was reduced in the presence of P2X7R specific antagonists (Rumney et al. 2011), suggesting involvement of the P2X7R in ATP release from osteoclasts also. In support of this, ATP release through the P2X7R pore has been shown to be an important source of extracellular adenosine which acts to promote fusion of osteoclasts also. In support of this, ATP release through the P2X7R pore has been shown to be an important source of extracellular adenosine which acts to promote fusion of human osteoclast monocyte precursors (Pellegatti et al. 2011). In addition, constitutive release of ATP into extracellular microenvironment between 0.05 and 0.5 pmol/ml per cell was inhibited by at least 60% using commercially available P2X7R antagonists during murine osteoclastogenesis (Brandao-Burch et al. 2012).

While the exact mechanism of P2X7R-mediated ATP efflux from osteoblasts, osteocytes and osteoclasts remains unclear, it is likely that the participation of the P2X7R in ATP release may be indirect.

**P2rx7 KO mice: bone phenotypes**

The most extensively used strains of P2X7R KO mice are the Pfizer and GSK lines generated in 2001 and 2005 respectively (Solle et al. 2001, Chessell et al. 2005). Phenotype analysis of GSK KO females revealed no overall overt skeletal phenotype, whilst detailed bone analysis revealed a thickening of cortical bones but no differences in their trabecular bone volume compared with WT controls (Garland et al. 2003b). However, Pfizer KO mice of both genders showed reduced total and cortical bone mineral content (BMC) and decreased femoral periosteal circumference, abnormalities associated with the effects of disuse on the skeleton (Ke et al. 2003). Furthermore, the effect of P2rx7 deletion is more pronounced with age, and histomorphometric analyses showed reduced parameters of bone formation (mineralising surface, bone formation rate) with an increase in parameters of bone resorption (osteoclast number, percent osteoclast surface) in KO mice, supportive of a phenotype with an overall reduced bone mass (Ke et al. 2003). Compared with the WT controls, these KO mice are also show an apparent reduced sensitivity to mechanical loading (Li et al. 2005) and display impaired fracture healing (Li et al. 2009) again, abnormalities associated with the effects of disuse on the skeleton. However, recent evidence has revealed that none of these mice are true global KO as P2X7R splice variants have escaped deletion in both the Pfizer and GSK models due to C-terminal truncated and P2X7R(k) variants respectively (Adriouch et al. 2002, Nicke et al. 2009, Masin et al. 2012). As such, earlier studies describing the phenotype of P2rx7 KO (Table 2) need to be interpreted with caution.

Syberg et al. (2012b) performed an extensive analysis of the bone phenotype of ten most common inbred strains of mice. The authors showed that strains carrying the mutated 451L allele (C57BL/6 and DBA/2J), which confers a loss of ATP-induced pore formation (Adriouch et al. 2002), had weaker bones and lower levels of the bone resorption marker C-telopeptide collagen in comparison with the strains harbouring the functional P451 allele (BALB/cj and 129X1/SvJ) (Syberg et al. 2012b). As a follow-up study, the group showed that the bone phenotype of KO mice was influenced by their genetic background as alterations in bone parameters in the KO strain containing P451 allele (obtained by backcrossing the GSK P2rx7 KO mice onto BALB/cj background) in comparison with the strain containing the 451L allele (original C57BL/6) were more pronounced (Syberg et al. 2012a). Furthermore, the BALB/cj KO mice showed reduced serum C-terminal telopeptide (CTX), higher bone mineral density (BMD) and increased bone strength compared with their WT littermates (Syberg et al. 2012a). Whilst a fully functional, more sensitive P2X7R(k) variant has been demonstrated in certain tissues in the original GSK KO (Nicke et al. 2009), osteoclasts obtained from the long bones of BALB/cj KO mice do not express the P2X7R(k) variant (Hansen et al. 2011). We also have data confirming the complete absence of P2X7R function in these bone resorbing cells obtained using the BALB/cj strain. These studies demonstrate the role of the P2X7R in regulation of bone mass and highlight the importance of genetic background when looking at the functional effects of the P2X7R. For a review of use of these
mice models in other diseases, please refer to Volonte et al. (2012) and Bartlett et al. (2014).

**P2X7R single nucleotide polymorphisms**

The murine P2rx7 gene is polymorphic with 15 non-synonymous SNPs (http://www.ncbi.nlm.nih.gov/snp, access date 7 August 2014) but only the P451L SNP (change of proline at 451 to leucine) found in the cytoplasmic tail of P2X7R is validated with a reduced channel and pore function (Adriouch et al. 2002). In humans, however, there are more than 1700 SNPs (http://www.ncbi.nlm.nih.gov/snp, access date 7 August 2014) in the P2RX7 of which 146 are non-synonymous and only a small number of these have been functionally investigated. Functional P2RX7 SNPs have been associated with changes in bone turnover, thereby influencing bone quality. In different population-based cohorts, SNPs known to cause a functional change in P2X7R have been correlated with the change in bone strength in postmenopausal women (Gartland et al. 2012b, Jorgensen et al. 2012, Wesselius et al. 2013). The loss-of-function (LOF) SNP p.Arg307Gln is associated with higher bone loss in women in their postmenopausal years (Gartland et al. 2012b, Jorgensen et al. 2012), whilst both men and women with the gain-of-function (GOF) SNPs p.Gln460Arg and p.Ala348Thr are protected against bone loss in addition to a reduced fracture risk (Jorgensen et al. 2012, Wesselius et al. 2013). The findings reveal that loss of P2X7R function imparts weaker bone strength and enhanced bone loss compared with WT or GOF SNPs, data supported by the investigations from mouse models (Syberg et al. 2012a). The bone phenotype, influenced by loss of osteoclast apoptosis could potentially contribute to an increased fracture risk (Ohlendorff et al. 2007) in people with a LOF SNPs; however, the role of these SNPs on bone cell function remains unaddressed. The GOF p.Ala348Thr may increase susceptibility to inflammatory bone disorders such as rheumatoid arthritis (Al-Shukaili et al. 2011), further suggesting the role of P2RX7 SNPs in detection of ‘danger signals’ such as development of an inflammatory response. These studies suggest that detection of non-synonymous SNPs within the P2RX7 gene could prove helpful in identifying people at a greater risk of developing diseases and bone disorders.

**P2X7R and bone cancer**

Bone tissue provides a fertile setting for cancer cells and is a common metastatic site owing to this microenvironment. Signalling mechanisms involving purines and receptors...
have been implicated and studied in cancer (Di Virgilio 2012). While the effect of ATP in cancer has long been recognised (Rapaport 1983), it is only recently that the role of P2X7R has been explored. P2X7R expressing HEK cells showed increased proliferation, reduced apoptosis and more developed vascular network in vivo with strong P2X7R positivity in several human cancers (Adinolfi et al. 2012b). This is contradictory to previous reports indicating a pro-apoptotic effect of P2X7R in tumour cells (Greig et al. 2003, Schafer et al. 2003, Wang et al. 2004b, White et al. 2005, Shabbir & Burnstock 2009). Whether these differences are due to the potential preferential expression of P2X7R variants by different tumour cells is currently not known. In addition, tonic, as opposed to sustained P2X7R stimulus, might have a growth-promoting, rather than cytotoxic, effect on tumour growth (Adinolfi et al. 2005) by triggering growth promoting intracellular signalling events (Adinolfi et al. 2009, Di Virgilio et al. 2009). As per the current understanding, high levels of extracellular ATP found in the tumour microenvironment (Pellegatti et al. 2008) could involve P2X7R signalling in two different scenarios: i) as a death signal, tumour cells downregulate P2X7R expression and thus avoid apoptosis or ii) as a survival/growth-promoting signal, P2X7R expression causes enhanced invasiveness in primary and secondary sites.

The effect of the full-length P2X7RA and the truncated P2X7RB splice variants on bone tumour cell growth and function has recently been explored. Adinolfi et al. (2010) show that growth and matrix invasion were enhanced in cells transfected with P2X7RB and in a follow-up study, the variant showed positive expression in highly dense osteosarcomas, a primary tumour originating in long bones of limbs, in situ (Giuliani et al. 2014). The cells transfected with P2X7RB had the highest growth rate and increased NFATc1 activation. P2X7RB cells also had decreased mineralisation and decreased RANK-L:OPG ratio (Giuliani et al. 2014), highlighting the potential of P2X7R as a therapeutic target in osteosarcoma. In another cancer involving the bone, multiple myeloma, P2X7R activation has been associated with cell death in human RPMI-8226 cell line (Farrell et al. 2010); however, the contribution of P2RX7 variants and polymorphisms was not explored. A study by Paneesha et al. (2006) showed that that the 1513 A>C SNP of P2RX7 had no effect on the clinical prognostic markers and survival in patients with myeloma. Whilst a very recent, more comprehensive analysis identified individuals carrying the variant allele of the 151 + 1g>t polymorphism or a high number of LOF alleles in the P2RX7 gene to be at a greater risk of multiple myeloma than individuals not carrying these variant alleles (Vangsted et al. 2014). It is likely that the LOF alleles impart reduced P2X7R expression or activity and mitigate P2X7R-mediated apoptosis thus facilitating neoplastic cell growth and myeloma development. A higher activation of P2X7R might be needed in such patients for receptor-mediated cancer prevention, as suggested in other cancers (Gorodeski 2009). Cumulatively, the evidence points to involvement of P2X7R activity in cell proliferation and regulation of bone mass in tumourigenic bone neoplasms. A particular feature of all bone cancers is a debilitating pain, recently the blockade or absence of P2X7R function has been shown to exacerbate bone cancer pain, a separate pain state compared with neuropathic and inflammatory pain (Hansen et al. 2011). To understand how P2X7R-mediated events might culminate in bone-related cancer, please see Adinolfi et al. (2012a). While the above studies point to the role of P2X7R in primary bone cancer and related physiology, further investigations exploring the contribution of P2X7RB variant or its co-expression with P2X7RA and downstream signalling in bone cancer metastases are warranted.

**Conclusion and future perspectives**

Although both osteoblasts and osteoclasts express P2X7R, its function in their regulation remains complex. It is likely that while the basal/transient activation of P2X7R is osteogenic, sustained stimulation inhibits new bone formation and mineralisation. In a similar way, osteoclast formation requires P2X7R activation, but the bone resorptive ability could be inhibited in the presence of a sustained ATP stimulus. The duality of P2X7R signalling in these bone cells is further complicated by the existence of variations in the receptor, caused by splice isoforms and SNPs in the gene for P2X7R. Investigations have shed light on the influence of P2RX7 gene variations on bone cell function and bone remodelling, and need to be considered in future studies involving human subjects and rodent models. These variants could contribute to a diversity in P2X7R-mediated osteoblast, osteoclast and osteocyte function and overall bone health; therefore, a better understanding of P2X7R-mediated downstream signalling in bone cells would be helpful, particularly in bone-related conditions. Pharmacological blockade of P2X7R shows promising therapeutic option; however, the apparent differential activity between species and also between individuals (Bartlett et al. 2014) needs to be carefully considered, and studies with new generation antagonists will be important for supporting a role for P2X7R in bone diseases.
Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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References


Adinolfi E, Callegari MG, Cirillo M, Pinton P, Giorgi C, Cavagna D, Rizzuto R & Di Virgilio F 2009 Expression of the P2X7 receptor increases the Ca\(^{2+}\) content of the endoplasmic reticulum, activates NFATc1, and protects from apoptosis. *Journal of Biological Chemistry* **284** 10120–10128. (doi:10.1074/jbc.M805805200)


Kuehnel MP, Rybin V, Anand PK, Anes E & Griffiths G 2009 Lipoïds regulate P2X7-receptor-dependent actin assembly by phagosomes via ADP


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