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Role of purinergic receptor polymorphisms in human bone

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1. ABSTRACT

Osteoporosis is a multifactorial disease with a strong genetic component. Variations in a number of genes have been shown to associate with bone turnover and risk of osteoporosis. P2 purinergic receptors are proteins that have ATP or other nucleotides as their natural ligands. Various P2Y and P2X receptor subtypes have been identified on bone cells. Several cellular functions in bone tissue are coupled to P2-receptor activation, including bone resorption, cytokine release, apoptosis, bone formation, and mineral deposition. Furthermore, ATP release and P2 purinergic signalling is a key pathway in the mechanotransductory process, where mechanical stimulation on bone leads to anabolic responses in the skeleton. A number of single nucleotide polymorphisms have been identified in the P2 receptor genes, where especially the P2X7 subtype has been the focus of extensive investigation where several polymorphisms have been shown to have functional implications on receptor function; moreover, some polymorphisms are associated with alterations in bone turnover and bone mass. This review focuses on variations in P2 receptor genes and the association to bone turnover and –quality.

2. INTRODUCTION

ATP has long been recognized as an intracellular energy source. Since the concept of purinergic neurotransmission by extracellular ATP was first introduced in the 1970s (reviewed in (1, 2)), it has become well-accepted that ATP is an important messenger molecule in the extracellular space for cell-cell communication. Nowadays it is clear that ATP plays an important regulatory role in a wide variety of biological processes in different cell types and organs (reviewed in (3, 4)).

ATP can be released into the extracellular space via different mechanisms, i.e. lytic release as a result of cell lysis (necrosis, pyroptosis), and non-lytic release either via exocytosis (vesicular release) or via plasma membrane channels or pores. ATP release can be triggered under several conditions, such as traumatic shock and inflammation, and by various mechanical and other stimuli, such as shear stress, hypotonic swelling, hypoxia, stretching and hydrostatic pressure (reviewed in (5)). Whereas the intracellular concentration of ATP is in the low millimolar range, the extracellular ATP concentration

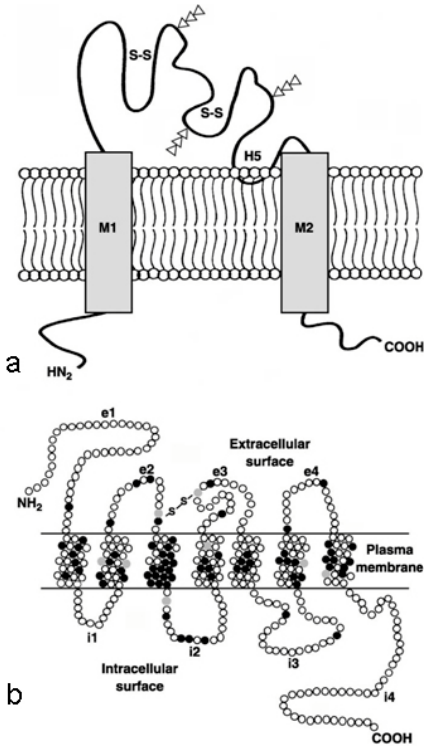

Figure 1. (a) Diagram depicting the transmembrane topology for P2X receptor protein showing both N-terminus and C-terminus in the cytoplasm. Two putative membrane-spanning segments (M1 and M2) traverse the lipid bilayer of the plasma membrane and are connected by a hydrophilic segment of 270 amino acids. This putative extracellular domain is shown containing two disulfide-bonded loops (S–S) and three N-linked glycosyl chains (triangles). Reproduce with permission from (111). (b) Schematic diagram of the sequence of the P2Y1 receptor showing its differences from P2Y2 and P2Y3 receptors. Filled circles represent amino acid residues that are conserved among the three receptors. Reproduce with permission from (112).

is in the nanomolar range under normal physiological circumstances (6, 7). The extracellular level of ATP is controlled by a number of ecto-enzymes which are located in the extracellular compartment, either located on the cell surface or in the interstitial medium. These ecto-enzymes rapidly dephosphorylate ATP into ADP, AMP and adenosine, which are also important in the purinergic signalling cascade (reviewed in (5)).

Extracellular ATP acts through binding to specific receptors on the cell surface. These receptors are called purinergic P2 receptors and classified in P2X (Figure 1A) and P2Y (Figure 1B) receptor subclasses (8). So far seven P2X (P2X1-7) receptor subtypes and eight P2Y (P2Y1,2,4,6,11-14) receptor subtypes have been identified. The P2X family of receptors are ligand-gated ion channels. P2X subtypes share the same general structure, having intracellular N- and C-termini, two membrane-spanning domains and a large extracellular loop containing 10 conserved cysteine residues (reviewed in (9)). Binding of ATP to a P2X receptor causes a conformational change in the shape of the receptor, which results in pore formation and membrane depolarisation. This leads to an increase of intracellular [Ca^{2+}] both by direct Ca^{2+} ion influx and by an inward Na^{+} current that depolarises the cell and opens voltage-operated Ca^{2+} channels (reviewed in (10-12)). In comparison with other P2X receptor subtypes, the structure of the $P2X_7$ subtype differs somewhat in that it has a significantly longer cytoplasmic C-terminus. Short exposure to ATP stimulates the $P2X_7$ receptor, like the other P2X subtypes, to open a reversible membrane channel permeable to small cations (Na^{+}, Ca^{2+}, K^{+}), whereas sustained stimulation with ATP or repeated stimulation with sequential ATP pulses induces formation of a large pore that permeabilizes the plasma membrane to molecules up to 900 Da (13).

P2Y (Figure 1B) receptors are G protein-coupled receptors. They belong to the superfamily of seven transmembrane-spanning receptors, and are amongst the smallest G protein-coupled receptors (reviewed in (14)). In addition to ATP, ADP and pyrimidine nucleotides (UTP and UDP) are also natural ligands for different P2Y receptor subtypes. However, one exception to this is the P2Y14 receptor which is only activated by naturally occurring UDP sugars (UDP-glucose), but not by ATP/UTP. Binding of a ligand to a P2Y receptor induces, for the majority of P2Y receptors, an increase in intracellular inositol (1,4,5)-triphosphate (IP_3), triggering release of Ca^{2+} ions from thapsigargin-sensitive stores into the cytosolic compartment (reviewed in (15)). Some P2Y receptors signal through the cyclic adenosine monophosphate (cAMP) pathway by either stimulating (P2Y11) or inhibiting (P2Y12,13) adenylate cyclase activity.

Table 1. Expression of P2 receptors on osteoblasts, osteoclasts and osteocytes

Celltype	Source	Cell-line	P2 receptor	Evidenced by	Ref.
Osteoblast	Human	MG-63	P2X4,5,6,7 P2Y1,2,4,6	RT-PCR RT-PCR	(92) (48, 93)
		SaM-1	P2X4,5,6 P2Y2,6	RT-PCR RT-PCR	(28) (28)
		SaOS-2	P2X7 P2Y1,12 P2Y2	RT-PCR, Southern blot, immunostaining, pharmacology RT-PCR RT-PCR, Southern blot	(25, 94) (6, 56) (56, 95)
		Te85	P2X7 P2Y2	RT-PCR, Southern blot RT-PCR, Southern blot	(25) (95)
		HOBIT	P2Y1,2	RT-PCR	(46, 48)
		OHS-4	P2Y1,2,4,6	RT-PCR	(96)
		Primary cells	P2X7 P2Y1 P2Y2	RT-PCR, Southern blot, immunostaining, pharmacology RT-PCR RT-PCR, Southern blot, Western blot	(25, 94) (56, 97) (56, 94, 95)
	Mouse	MC3T3-E1	P2X2,3,4,5,7 P2X7 P2Y1,2,4,6,13,14 P2Y2	RT-PCR RT-PCR, Western blot RT-PCR pharmacology	(98, 99) (98-100) (101)
		TBR31-2	P2Y1,2,4,6	RT-PCR	(102)
		Primary cells	P2X7	RT-PCR, Western blot, pharmacology	(21, 30, 33)
	Rat	UMR-106	P2Y1 P2Y2	pharmacology Northern blot, pharmacology	(56, 103) (20, 41, 104)
		Primary cells	P2X2,5,7 P2Y1,2,4,6	RT-PCR, in situ hybridisation, Western blot, immunostaining, pharmacology RT-PCR, in situ hybridization, Western blot, immunostaining, pharmacology	(33, 38, 105) (24, 38, 105)
Osteoclast	Human	Primary cells	P2X1,4,5,6,7 P2X7 P2Y1,2,4,6,11 P2Y1 P2Y1,2	RT-PCR RT-PCR, Southern blot, immunostaining, pharmacology RT-PCR RT-PCR, pharmacology RT-PCR, in situ hybridization, Southern blot	(65) (26, 35, 45, 106) (45) (22, 56, 95)
	Mouse	Primary cells	P2X7	Pharmacology	(30)
	Rat	Primary cells	P2X2,4,7 P2Y1,2	in situ hybridization, immunostaining in situ hybridization, immunostaining	(105, 107) (24, 105)
	Rabbit	Primary cells	P2X4 P2Y1,2,6	RT-PCR, Southern blot RT-PCR	(108) (109)
Osteocyte	Mouse	MLO-Y4	P2X7	Western blot	(21)

Since it has been shown that purinergic signalling via these P2 receptors plays a central role in bone physiology (reviewed in (16, 17)), it is nowadays suggested that aberrations in P2 receptor genes also could play an important role in the pathogenesis of osteoporosis. The present review provides a short update of the current knowledge on the role of P2 receptors in bone and will focus on genetic variations within the P2 receptor genes in relation to osteoporosis.

3. ATP AND PURINERGIC SIGNALLING IN BONE

Kumagai and co-workers (18) were the first to show that ATP plays a role in bone physiology. In rat osteosarcoma UMR-106 cells, these authors demonstrated that extracellular ATP increased intracellular Ca^{2+} and IP_3 concentration in these osteoblast-like cells, which was suggested to be mediated through P2 receptor activation. Subsequent experiments by Schlof *et al.* and Yu and Ferrier (19, 20) showing that ATP induced intracellular Ca^{2+} elevation in non-transformed human bone cells as well as in rabbit osteoclasts, suggested that elevation of intracellular Ca^{2+} may be a common response of both osteoblasts and osteoclasts to extracellular ATP.

These early experiments fuelled the notion that extracellular ATP might influence osteoblast and osteoclast function, and thus may play a regulatory role in the process of bone remodelling. Since then, a number of P2X and P2Y receptors have been identified on both osteoblasts and osteoclasts (Table 1). Thus far, expression has been demonstrated of P2X2,3,4,5,6,7 and P2Y1,2,4,6,12,13,14 receptor subtypes on osteoblasts and of P2X1,2,4,5,6,7 and P2Y1,2,4,6,11 subtypes on osteoclasts (Table 1). In osteocytes, only expression of the P2X7 receptor subtype has been demonstrated so far (21). Even though most P2 receptor subtypes appear to be expressed on bone cells, P2X7, P2Y1 and P2Y2 are currently considered as the most important receptor subtypes involved in the process of bone remodelling.

3.1. Effects of ATP and purinergic P2 receptors on osteoclast function

An effect of ATP on osteoclast function was initially shown in giant cell tumour osteoclasts. ATP exerted a small stimulatory effect on resorption pit formation by these cells (22). Next, potent stimulatory effects of ATP on the resorptive function of normal mammalian osteoclasts were observed by the group of Arnett (23). These authors showed that incubation of an osteoclast-containing bone cell culture from neonatal rats with a low concentration of ATP (0.2-2 μM) resulted in an up to 5.6-fold stimulation of resorption pit formation (23). It was unclear whether this stimulation occurred via a direct effect of ATP on osteoclasts, an indirect effect on osteoblasts, or both. In addition to ATP, ADP and 2-MeSADP, a selective P2Y1 receptor agonist, has also been shown to enhance resorption pit formation by osteoclasts,

suggesting that the stimulatory effect on bone resorption was being mediated via the P2Y1 receptor (24).

Gartland and co-workers (25) provided further evidence that ATP could enhance bone resorption. In a co-culture of human osteoclasts and UMR-106 rat osteoblast-like cells, it was shown that ATP enhanced osteoclastic bone resorption. In contrast, ATP had no effect on resorption in a culture of only osteoclasts, suggesting that the stimulatory effect on resorption that was observed in the co-culture was probably mediated via an effect of ATP on UMR-106 rat osteoblast-like cells (25). This was confirmed by Buckley *et al.* (26), who showed that ATP did not influence resorption in cultures containing only osteoclasts, whereas resorption was enhanced by ATP when osteoclasts were co-cultured with UMR-106 osteoblasts, possibly due to upregulated expression of RANKL by these latter cells. Upregulated RANKL expression as well as downregulated OPG expression in response to ATP has also been observed in human SaOS-2 osteoblast-like cells (26, 27). Additionally, in SaM osteoblast-like cells it was shown that extracellular ATP, probably acting on P2Y receptors, stimulated the production of IL-6 (28) which is known to stimulate bone resorption in concert with RANKL.

In contrast to the above findings, which suggest that ATP at low extracellular concentrations (0.2-2 μM) could enhance osteoclastic resorption, there is also evidence suggesting that ATP at a higher concentration (200-2000 μM) may exert an opposite effect on osteoclast resorption. Morrison *et al.* (23) showed that bone resorption was inhibited by ATP, which was likely due to a cytotoxic effect on osteoclasts following $P2X_7$ receptor activation. More evidence for an apoptotic effect of the $P2X_7$ receptor was demonstratd by Gartland *et al.* (29). Using human osteoclastoma-derived osteoclast-like cells these authors found that activation of the $P2X_7$ receptor by Benzoyl-benzoyl-ATP (BzATP) inhibited bone resorption *in vitro* through initiation of apoptosis. Furthermore Ke *et al.* showed a significant lower trabecular bone mass in P2X7 receptor KO mice accompanied by an increased osteoclast surface and number (30). Lastly also Ohlendorff *et al.* (31) showed an involvement of the P2X7 receptor in ATP-mediated cytotoxic effects on human osteoclasts. They found a single nucleotide loss-of-function polymorphism within the P2X7 receptor (Glu496Ala) to be associated with diminished ATP-induced osteoclast apoptosis *in vitro* (31).

3.2. Effects of ATP and purinergic P2 receptors on osteoblast function

Besides a role of P2 receptors in osteoclast resorption, numerous studies also describe a role for ATP in the regulation of bone formation by osteoblasts. Similar to osteoclast function, ATP seems to exert opposite effects on osteoblast function depending on extracellular ATP concentrations and P2 receptor subtype. In MC3T3-E1 osteoblast-like cells, it was shown that ATP induced mineral deposition (32). More recently, it was shown that ATP through binding to P2X7 receptors stimulated osteogenesis via activation of alkaline phosphatase, which promotes mineralization by hydrolyzing inorganic pyrophosphate (PPi) (33). In the same study it was also shown that P2X7-mediated stimulation of phospholipase D (PLD) resulted in production of phosphatidic acid and lysophosphatidic acid (LPA) (33, 34). As LPA up-regulated, via binding to its receptor, the expression of osteoblast marker genes and stimulated mineralization, the authors suggested the existence a P2X7-LPA signalling axis involved in regulating osteoblast differentiation and osteogenesis during skeletal remodelling and mechanotransduction (33). In contrast, activation of P2Y2 receptors by ATP has been shown to strongly inhibit mineralized bone nodule formation (35-37), in combination which reduced expression and activity of alkaline phosphatase (38).

Taken together, the above results would suggest that bone formation may be enhanced by extracellular ATP via P2X7 receptor activation, whereas bone formation may be reduced via activation of P2Y2 receptors. Additional evidence for these opposite effects of ATP on osteogenesis derives from studies using knockout models. In mice lacking the P2X7 receptor, decreased periosteal bone formation in long bones and reduced osteogenesis relative to wild-type mice was observed (21, 30, 33). In contrast, mice lacking the P2Y2 receptor, a small increase (9-17%) in bone mineral content of hindlimbs was observed compared to wild-type mice (38). These data from knock-out mice provide strong evidence for a role of P2X7/P2Y2 receptors in bone development.

3.3. Role of ATP and purinergic P2 receptors in mechanotransduction

Mechanical stimulation is well-known to be essential for the generation of new bone, i.e. osteogenesis, but the exact mechanisms through which mechanical stimulation of bone is transduced into biochemical signals at the cellular level is still not clearly understood. It is thought that interaction between osteocytes and osteoblasts on the bone surface is the main mechanism for mechanotransduction, via production of signalling molecules by osteocytes that modulate bone cell activity. Since it was recently shown that osteocytes released ATP in response to mechanical stimulation (21, 39), ATP may be one of the signalling molecules contributing to transduction of mechanical stimuli in the bone microenvironment.

In addition to contributing to osteocyte-to-osteoblast signalling, earlier experiments also indicated that ATP may be involved in the communication among osteoblasts and osteoclasts after mechanical stimulation. In 1992, it was found that mechanical stimulation of a single cell in monolayer of primary cultures of rat calvarial cells and in rat osteosarcoma cells induced intercellular calcium signalling (40). Subsequently it was demonstrated in UMR-106 cells that these so-called calcium waves among osteoblasts were partly propagated by the paracrine action of ATP through binding to P2Y2 receptors on neighbouring cells, inducing elevation of intracellular calcium (41, 42). Further studies using human osteoblast-like initial transfectant (HOBIT) cells and MC3T3-E1 mouse

osteoblastic cells showed that ATP was released by osteoblasts in response to a number of different mechanical stimuli, including hypotonic stress, medium displacement and shear stress, and that it was this ATP release which could initiate intercellular calcium signalling between osteoblasts (43, 44). Moreover, the intercellular calcium signalling mediated by ATP release was shown to occur not only among osteoblasts but also between osteoblasts and osteoclasts. Mechanical stimulation of osteoblasts that were co-cultured with osteoclasts induced intercellular calcium signalling to osteoblasts as well as to nearby osteoclasts, via binding of ATP to P2X7 receptors (45). Thus, ATP release after mechanical loading may provide a mechanism for crosstalk between osteocytes, osteoblasts and osteoclasts.

In HOBIT cells it has been shown that ATP release after mechanical stimulation resulted in stimulation of protein kinase C (PKC)-dependent pathways, ultimately leading to activation of the transcription factor early growth response protein-1 (EGR-1), which is responsible for the activation of several genes involved in the control of cell proliferation, apoptosis and biology of osteoblasts (46, 47). Via the same pathway also the transcription factor runt-related transcription factor 2 (RUNX2), which is essential in controlling osteoblast differentiation, was shown to be stimulated by ATP released following mechanical stress (48). More recent studies showed that the $P2X_7$ receptor is involved in the fluid flow-induced extracellular signal-regulated kinase (ERK) activation, which is essential for the activation of various osteogenic genes including, *c-fos,* COX2, RUNX2 and RANKL (21, 49, 50). Okumura showed complete inhibition of fluid flow-induced ERK activation by a P2X7 receptor antagonist, indicating that $P2X_7$ receptor functions as an initial activator in fluid flow-induced ERK activation (49). Liu *et al.* found partial involvement of the P2X7 receptor in fluid flow-induced ERK activation using osteoblasts derived from P2X7 knockout mice. In addition, they suggested that P2Y receptor were involved in fluid flow-induced ERK activation because U73122 (an inhibitor of the P2YR pathway) partially inhibited this activation (50). Furthermore, it has been shown that prostagladin-E_2 (PGE_2), an osteogenic factor that contributes to the regulation of bone remodelling (51), was released by both osteoblasts and osteocytes as a result of activation of a P2 receptor (39, 52). The authors suggested that a metabotropic P2Y receptor was involved in this response, since Reich *et al.* (53) showed that flow-induced PGE_2 release was mediated through a G-protein. P2X7 receptor KO models showed that the PGE_2 release may also mediated through P2X7 receptors (21, 33).

3.4. Interaction of purinergic signalling with systemic factors

Besides direct effects of ATP on bone cells, ATP has also been shown to potentiate the response of bone cells to systemic factors. In UMR-106 rat osteoblastic cells, it was shown that the effect of PTH on the intracellular Ca^{2+} concentration was augmented by binding of ATP to $P2Y_2$ receptors. PTH was not able to activate phospholipase C (PLC) without prior activation of P2 receptors (54, 55). Furthermore, it was demonstrated that activation of the P2Y1 receptor subtype potentiated PTH-induced *c-fos* gene expression (56). This gene plays an important role in the skeletal system. Mice lacking the *c-fos* gene fail to develop osteoclasts, while upregulated *c-fos* expression leads to osteosarcomas (57, 58).

Taken together, the above data deriving mostly from *in vitro* studies provide evidence for a versatile role of ATP in the process of bone remodelling. Although the exact role of ATP *in vivo* is not completely understood yet, it is clear that the effects of ATP ultimately depend on: a) the P2 receptor expression profile at specific skeletal sites (i.e. degree of expression of P2Y1,2 vs. P2X7), b) the presence of systemic factors at these specific sites, and c) the nature of mechanical stress (i.e. inducing low-level ATP release vs. massive ATP release).

4. SINGLE NUCLEOTIDE POLYMORPHISMS IN OSTEOPOROSIS

Osteoporosis is a multifactorial disease which is influenced by both environmental and genetic factors. A number of twin and family studies have shown that genetic factors play a significant role in the development of osteoporosis(59, 60). Several candidate genes have been associated with low bone mass and osteoporosis. The first to be investigated was the vitamin D receptor gene. Though the initial studies were promising, subsequent studies showed that polymorphisms in this gene had only minor influence on bone mass. Other genes with products important for the regulation of bone mass have also been investigated. These include genes coding for collagen type I (*COLIA1*), the estrogen receptor (*ESR1*), and parathyroid hormone (*PTH*). As collagen type 1 is the primary collagen in bone, it is among the best examined. In an extensive meta analysis by Stuart Ralston *et al* including more than 26,000 persons, a single nucleotide polymorphism in intron 1, which encodes the alpha-1 chain of collagen type 1 was shown to be associated to bone mass of the lumbar spine so that individuals homozygote for the variant allele had a two to three percent decreased bone mineral density compared to the rest of the population (61). This association was confirmed in a study by Uitterlinden, where associations to both lumbar spine and hip bone mineral density was demonstrated (62). Also polymorphisms in ESR1 have been shown to be associated to bone mass and fracture risk. Two meta analyses by Ioannidis have demonstrated that individuals with the XX genotype at the XbaI restriction site have increased BMD (63)and decreased fracture risk (64)compared to individuals with the xx and Xx genotypes. Finally, studies have demonstrated that polymorphisms in the PTH gene are also associated to bone mass. In a Japanese population, carriers of the variant allele b for the *BST* BI restriction site in the PTH gene had a 7.7% lower BMD than individuals homozygous for the normal allele. Thus, a number of genes important for the regulation of bone mass and structure have been identified and mutations and polymorphisms affecting the expression of the gene products have implications on bone mass and strength. However, the effect sizes are relatively small, as osteoporosis is a polygenetic disease.

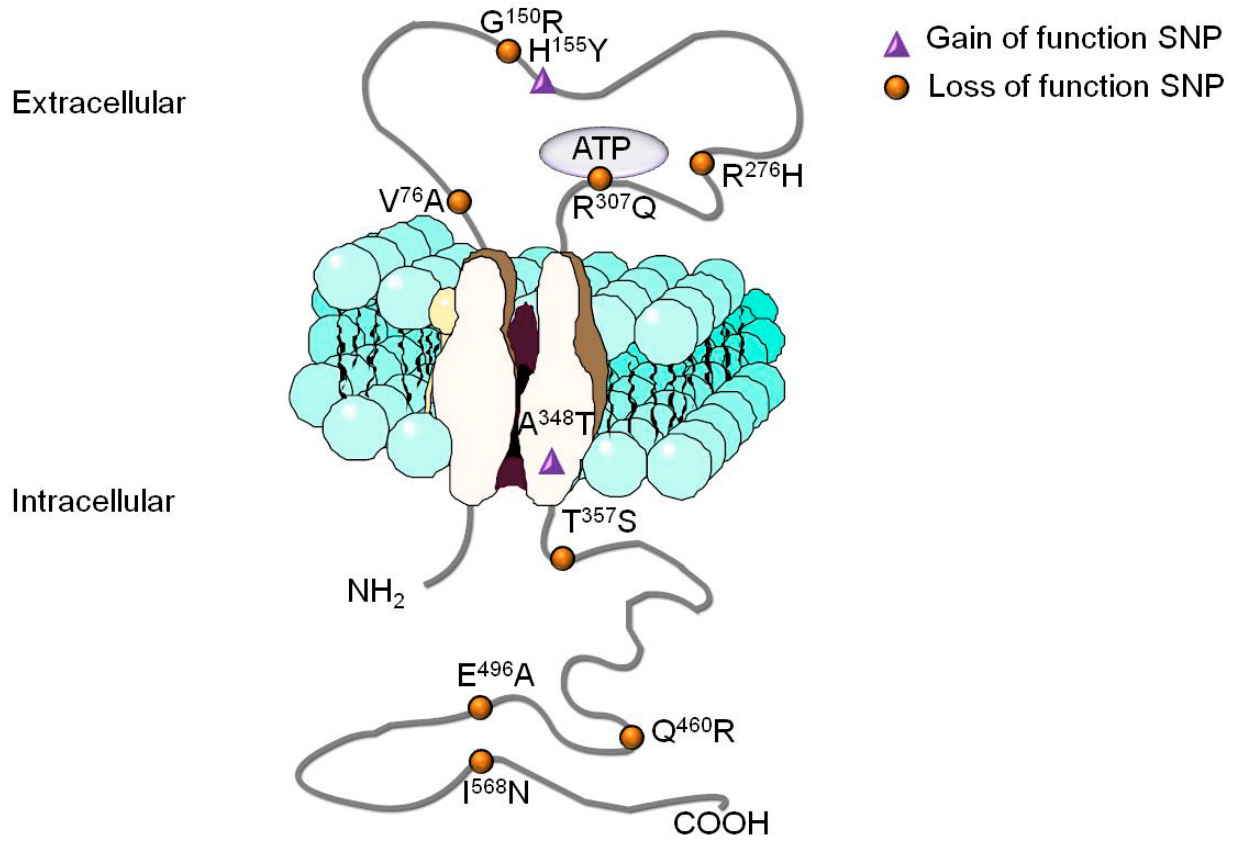

Figure 2. Schematic presentation of the structure of the P2X7 receptor showing the sites of the functionally characterized single nucleotide polymorphisms in the receptor and the corresponding amino acid changes. SNP: single nucleotide polymorphism.

As P2 receptors have been shown to be involved in most processes in the regulation of bone turnover (17), it is conceivable that alterations caused by genetic variation in the function of these receptors may affect the finely tuned balance between bone formation and bone resorption, subsequently affecting the bone status of the organism. However, only few studies have so far investigated the association between single nucleotide polymorphisms in the P2 receptor genes and osteoporosis risk, and these have mainly focused on the P2X7 and P2Y2 receptors: the following section is therefore focusing on SNPs in these two receptors. Although SNPs have also been described in genes for the other P2 receptors, no studies have addressed effects of these P2 receptor SNPs on bone turnover yet.

4.1. Functional P2X7 receptor SNPs

The P2X7 receptor gene is highly polymorphic with more than 260 single nucleotide polymorphisms (SNPs) found in the International HapMap project (www.hapmap.org). The P2X7 gene is located on the long arm of chromosome 12 (12q24), spans over 53kb and consist of 13 exons. To date at least 12 of the SNPs described by the International HapMap project have been demonstrated to be non-synonymous, meaning that they lead to an amino acid base change, and they have also been shown to affect P2X7 receptor function (Table 2) (Figure 2). Most of the impressive amount of work characterizing the effects of the twelve functional SNPs investigated so far has been carried out by Wiley and colleagues over the last decade using biological and genetic studies.

In 2001 Gu *et al.* were the first to describe a SNP in the P2X7 receptor at position 1513 (adenine to cytosine) in exon 13, coding for a glutamic acid to alanine acid at residue 496 ($Glu^{496}Ala$). This SNP was found to be present in low frequency in the Caucasian population (65). The $Glu^{496}Ala$ polymorphism is located in the carboxyl termini of the receptor, which is necessary for its permeability properties. It was shown in human leukocytes that the glutamate to alanine exchange at amino acid position 496 was associated with a loss of receptor function in terms of ATP-induced pore formation and apoptosis induction. Other studies confirmed the loss of function of the $P2X_7$ receptor due to the $Glu^{496}Ala$polymorphism (66-69). Boldt *et al.* demonstrated that the $Glu^{496}Ala$ polymorphism did not change the cation–selective channel function of the P2X7 receptor in transfected oocytes and human embryonic kidney cells, but particularly induced a lack in formation of non-selective pores (70).

A second SNP associated with loss of function of the P2X7 receptor is a polymorphism at position 1729 (thymine to adenine) in exon 13, localized within a

Table 2. Polymorphisms in the P2X7 receptor gene

SNP*	Base change	Amino Acid change	Minor allele freq.	Effect	References
rs35933842	151+1g→t	Null allele	0.01	No expression of receptor mRNA	(68, 74)
rs17525809	253T→C	$Val^{76}Ala$	0.06	Loss-of-function	(76)
rs28360447	474G→A	$Gly^{150}Arg$	0.02	Loss-of-function SNP	(76)
rs208294	489C→T	$His^{155}Tyr$	0.5	Gain-of-function SNP	(67, 76)
rs7958311	835G→A	$His^{270}Arg$	0.26	Minor loss-of-function	[75]
rs7958316	853G→A	$Arg^{276}His$	0.01	Loss-of-function SNP	(77)
rs28360457	946G→A	$Arg^{307}Gln$**	0.01	Loss-of-function	(68, 73)
rs1718119	1068G→A	$Ala^{348}Thr$**	0.29	Gain-of-function SNP	(67, 76, 78)
rs2230911	1096C→G	$Thr^{357}Ser$**	0.10	Loss-of-function	(67, 75, 76)
rs2230912	1405A→G	$Gln^{460}Arg$**	0.16	Minor loss-of-function Marker of gain-of-function	(67, 76, 77)
rs3751143	1513A→C	$Glu^{496}Ala$**	0.19	Loss-of-function SNP (pore formation)	(31, 65, 66, 70, 85, 110)
rs1653624	1729T→A	$Ile^{568}Asn$**	0.02	Loss-of-function SNP (trafficking defect)	(72, 76)

*non-synonymous SNPs according to the International HapMap project, **polymorphisms within the same haplotype block

trafficking motif in the carboxyl tail of the receptor. It changes isoleucine to asparagine at residue 568 ($Ile^{568}Asn$), which causes loss of receptor function (68, 69, 71). Wiley *et al.* showed that this loss of function was due to a trafficking defect (72). In transfected human embryonic kidney cells it was shown that the mutated P2X7 receptor was non-functional, due to failure of trafficking of the mutant receptor to the cell surface as shown by the absence of surface $P2X_7$ immunoreactivity (72).

A third loss-of-function SNP is located in the extracellular domain of the P2X7 receptor gene within exon 9 at position 946 (guanine to adenine). It changes arginine to glutamine at residue 307 ($Arg^{307}Gln$). Lymphocytes from subjects heterozygous for the A allele showed reduced pore and channel formation, while the expression of the mutant P2X7 receptor was normal (73). Double heterozygous subjects (i.e. subjects heterozygous for both $Arg^{307}Gln$ and $Ile^{568}Asn$ polymorphisms) failed to show any functional $P2X_7$ receptor (73) .

A fourth P2X7 SNP was found by Skarrat *et al.* during a search for new mutations leading to functional changes in the P2X7 receptor, who described a splice site mutation (guanine to thymine) at position +1 of the first intron (74). This mutation causes a null allele in 1-2% of the Caucasian population, due to absent levels of mRNA from the mutant allele, presumably involving the nonsense-mediated mRNA decay pathway (68, 74).

In 2005 a fifth non-synonymous SNP was found in the $P2X_7$ gene, located in exon 11 at position 1096 (cytosine to guanine), which changes threonine to serine ($Thr^{357}Ser$) in a region of the carboxyl terminus that has been proposed as a β-arrestin-2 binding motif on the receptor (75). Subjects heterozygous and homozygous for the $Thr^{357}Ser$ polymorphism had reduced ATP-induced $ethidium^+$ influx compared with wild-type subjects, indicating partial loss of the $P2X_7$ receptor-mediated pore formation and apoptosis of macrophages (75). However, an almost neutral effect of the $Thr^{357}Ser$ polymorphism was shown more recently (67, 69).

Cabrini *et al.* described the first SNP with a gain-of-function effect on the P2X7 receptor (67). The SNP is located in the extracellular portion of the receptor at position 489 (cytosine to thymine), changing amino acid 155 from histidine to tyrosin ($His^{155}Tyr$). This SNP was shown to induce an increased Ca^{2+} influx in both primary lymphocytes and human embryonic kidney cells (67). However, Denlinger *et al.* showed no functional effect of the $His^{155}Tyr$ polymorphism in native cells (69).

The seventh SNP yet described that influences the function of the P2X7 receptor is the 474 (guanine to adenine) polymorphism, changing glycine to arginine ($Gly^{150}Arg$). This polymorphism was shown to reduce receptor function in native cells (69).

A recent report by Roger *et al* showed that a SNP located at position 253 inducing an amino acid shift from valine to alanine ($Val^{76}Ala$) in the ectodomain is associated to a moderately reduced function of the receptor (76). This study was performed in HEK293 cells transfected with the contructs for the different genotypes. No studies have so far tested the effect in primary cells.

Very recently, Stokes *et al* showed that also the SNP located at position 853 giving rise to an $Arg^{276}His$ change results in an almost complete abolishment of ethidium bromide uptake in HEK-293 cells transfected with the contructs with the mutant sequence for this SNP as compared to the normal variant (77). Thus, the $Arg^{276}His$ SNP is also a major loss-of-function polymorphism.

In 2005 another gain-of-function SNP was identified (67). This SNP is located at position 1068 and changes alanine to threonine ($Ala^{348}Thr$). Interestingly, the initial study describing the SNP, demonstrated that it was associated with a slight reduction in agonist-induced calcium influx in primary lymphocytes (67). In contrast, later reports showed that HEK-293 cells transfected with the contruct containing the mutated gene at this position, actually had an increased inward current as determined by patch clamp (76)as well as an increased dye-uptake and thus pore formation (78). Interestingly, the SNP is not only a gain-of-function but is also located in a haplotype block together with $Thr^{357}Ser$, $Glu^{496}Ala$ and the $Gln^{460}Arg$ that is associated with an even more pronounced gain-of-function of the receptor. The $Gln^{460}Arg$ (1405A>G) mutant allele is by itself associated with minor loss-of-function of the receptor (67, 76) when constructs carrying the mutant allele is transfected into HEK-293 cells. However, in combination with mutants of the $Ala^{348}Thr$ SNP in a

haplotype block very strong potentiation of the receptor function is seen (see below) (77).

4.2. Effect of combined genotypes and haplotypes in the P2X7 receptor gene

According to the International HapMap project the P2X7 gene includes six 'haplotype' blocks. Six non-synonymous P2X7 receptor SNPs described by the International HapMap project lie within the same haplotype block, which spans from exon 9 and beyond the 3' terminal of the gene. Recent data have also shown pairwise LD between these SNPs (79). Furthermore two of the SNPs within this haplotype block ($Gln^{460}Arg$ and $Glu^{496}Ala$) are shown to be in strong LD with the gain of function SNP at position 489 ($His^{155}Tyr$) (79). Therefore it was tested whether this latter polymorphism could rescue the loss of function of the $Gln^{460}Arg$ and the $Glu^{496}Ala$ polymorphisms. It was shown that the Tyr^{155} mutation rescued the functional effect of Arg^{460} mutation, whereas the Tyr^{155} mutation could not rescue to functional decrease of the Ala^{496} mutation (79). The strong LD between the $His^{155}Tyr$ and the $Glu^{496}Ala$ polymorphisms could also explain the result shown by Cabrini *et al.*, who showed that the gain of function of the Tyr^{155} mutation was completely lost in subjects with the Ala^{496} mutation (67).

As mentioned above, one of the haplotype blocks published in the HapMap project contains the following four SNPs: $Ala^{348}Thr$, $Thr^{357}Ser$, $Gln^{460}Arg$, and $Glu^{496}Ala$. It is positioned in exon 11 to exon 13, and two of the SNPs are known to confer loss of function; i.e. $Thr^{357}Ser$ and $Glu^{496}Ala$. In a study in a large Australian sample of 3,430 subjects genotyped for the 12 non-synonymous *P2RX7* SNPs, the predicted haplotype block contained only five variants covering >99% of the genotyped subjects (77). The P2X7-1 variant was the most frequent of the haplotype blocks with no variant alleles for the four SNPs (coinherited alleles: GCAA). The P2X7-4 variant contained variant alleles for both $Ala^{348}Thr$ and $Gln^{460}Arg$ (coinherited alleles: ACGA), while the remaining three variants (P2X7-2, P2X7-3, P2X7-5) each contained one variant allele (coinherited alleles respectively: ACAA, GCAC, and GGAA). However, interestingly when comparing the functional characteristics of the P2X7 receptor in HEK-293 cells transfected with constructs of either the P2X7-1 or P2X7-4 haplotype block, a significantly higher receptor function was found in the cells carrying the P2X7-4 variant, and the function was higher than what could be expected from the contribution of the Thr^{348} gain-of-function. Furthermore, the increase in receptor function was not only in ethidium bromide uptake, but also in ATP-induced IL-1beta production (77), a feature unique to the P2X7 receptor among the P2 family. Thus, as the variant allele Arg^{460} is only found in the P2X7-4 variant this implies, that the Arg^{460} is a marker of gain-of-function.

4.3. Role of P2X7 receptor SNPs in osteoporosis

Data on associations between bone diseases and P2X7 receptor SNPs have been reported by the group of Jørgensen. These authors investigated the association of three non-synonymous SNPs ($Glu^{496}Ala$, $Ile^{568}Asn$ and $Gln^{460}Arg$) with bone mass, bone loss after menopause, and vertebral fracture incidence after menopause in a cohort of approximately 2,000 Danish postmenopausal women (The Danish Osteoporosis Prevention Study). It was shown that presence of the Ala^{496} mutation was associated to increased fracture incidence at 10 years after menopause (31). This increased fracture incidence could be due to an increased number of osteoclasts, since it was shown earlier in lymphocytes that cells homozygous for the rare allele showed a decreased apoptosis compared to wild-type cells (65). *In vitro* studies confirmed that osteoclasts from women homozygous for Ala^{496} were significantly less susceptible to undergo apoptosis than osteoclasts from individuals not carrying the variant allele. Furthermore, the authors found that also the $Ile^{568}Asn$ polymorphism was significantly associated with an increased 10-year fracture incidence. In addition, the $Ile^{568}Asn$ polymorphism showed an association with effect of hormone replacement therapy. Postmenopausal women carrying the variant allele of the $Ile^{568}Asn$ SNP had greatly increased benefit of HRT compared to the homozygotes for the reference allele (31). In the same cohort it was recently investigated whether the $Glu^{496}Ala$ and the $Ile^{568}Asn$ polymorphisms influenced hip structural geometry. However, ,no significant association was found (80). In addition, a preliminary report presents evidence that perimenopausal women with the Gln^{307} mutation had a significantly lower lumbar spine BMD than women without this mutation, whilst a combined group of subjects who had any of the LOF SNPs had nearly nine-fold greater annualized percent change in LS-BMD than subjects who were wild type at the SNP positions (Gartland *et al.*, abstract).

In a recent study the relation of four total loss-of-function SNPs in the P2X7 receptor gene with the susceptibility to aseptic osteolysis and with the risk of revision of total hip arthroplasty (THA) in a smaller cohort (81) was evaluated. Interestingly, the authors found an overrepresentation of carriers of the variant alleles of the $Arg^{307}Gln$, $Glu^{496}Ala$, $Ile^{568}Asn$, and the intronic splice site mutation in the group receiving THA revision compared to those with unrevised functional prosthesis. . Furthermore, the carriage of the $^{Gln}307$ allele was associated with greater cumulative hazard of THA revision. However, the authors conclude that it is only a preliminary study, and the investigation in larger THA cohorts is warranted. However, these initial trends could imply that P2X7 receptor SNPs resulting in loss-of-function of the receptor might involve impaired prosthesis fixation and subsequently also reduced bone healing which has wide ranging implications.

4.4. Role of P2X7 receptor SNPs in other human diseases

Several association studies have determined P2X7 receptor SNPs as a susceptibility gene in relation to infections with intracellular pathogens such as tuberculosis as well as in mood disorders. Firstly, the P2X7 receptor has been shown to be important in the ATP-mediated killing of phagocytosed Mycobacterium tuberculosis. It has been shown that extracellular ATP via P2X7 can activate a bactericidal pathway that leads to death of the bacteria and initiates apoptosis of the infected macrophage carrying the

live mycobacteria (82-85). Subsequently, epidemiological studies have shown an association between the $Glu^{496}Ala$ loss-of-function polymorphism and reactivation of latent tuberculosis, with a 3.5 times increased life-time risk in carriers of the variant allele (85, 86).

4.5. Functional P2Y2 receptor SNPs

The P2Y2 receptor gene is located on the long arm of chromosome 11 (11q13.4), spans over 18kb coding for 377 amino acids and consists of 1 exon. Three splice variants are known from this receptor. To date, 71 SNP are found within the P2Y2 receptor gene according to the international Hapmap project, with at least 4 leading to an amino acid change. Two of these 4 non-synonymous SNP are known to affect the function of the P2Y2 receptor.

Janssens *et al.* (87) described the first non-synonymous polymorphism leading to a functional change of the P2Y2 receptor in 1999. This SNP is located at position 1000 (cytosine to thymine), changing arginine to cysteine at residue 334 ($Arg^{334}Cys$). Within this study it was shown that the Cys^{334} mutation produced slower accumulation of intracellular inositol triphosphates (IPs) induced by either ATP, UTP or ATPγS compared to wild type. However, no impact on the intracellular Ca^{2+}, the downstream molecule regulated by IPs, could be assessed. A few years later Buscher *et al.* showed an effect of the $Arg^{334}Cys$ on intracellular Ca^{2+}. They showed, by measuring the 100umol/l ATP-stimulated Ca^{2+} increase, that cells expressing the homozygous Cys^{334} variant showed significantly increased transient Ca^{2+} influx (88). Thus, presence of the mutant allele seems to be associated with increased response of the receptor to ATP.

The second SNP that affects the function of the $P2Y_2$ receptor was described in 2006 by the group of Insel (88). This SNP is located at position 936 (guanine to cytosine) and changes arginine to serine at residue 312 ($Arg^{312}Ser$). Cells expressing the homozygous Ser^{312} variant showed an increased transient Ca^{2+}-influx compared to wild-type cells (88).

4.6. Role of P2Y2 receptor SNPs in osteoporosis

As P2Y2 has been shown to have effects on both bone formation and bone resorption *in vitro* and *in vivo* in studies in P2Y2 knock-out mice,, showing changes in bone mass between knock-out and wildtype animals, it is conceivable that changes in P2Y2 receptor function as a result of genetic mutations or polymorphisms might affect bone mass and quality in humans. However, only recently a single study showed a positive association between SNPs in the P2Y2 receptor and bone status in humans. Again, in the Danish Osteoporosis Prevention Study cohort, the association between the $Arg^{312}Ser$ SNP and bone status at and after menopause was examined. It was found that subjects homozygous for the C (variant) allele of the P2Y2 gene had a significantly increased BMD at menopause, and that the rate of bone loss from 0 to five and ten years after menopause was 20-30% lower than both the heterozygous and the homozygous for the common allele (89). This was accompanied by a decrease in urinary bone resorption markers, but with no change in bone formation markers. This would suggest that the difference might be due to reduced osteoclastic activity. However, so far no *in vitro* studies on the association of the SNP to bone cell function have been published.

4.7. Role of P2Y2 receptor SNPS in other human diseases

While several studies have demonstrated the association between P2X7 receptor SNPs and human disease, only very few studies have examined how the genetic variation in the P2Y2 receptor gene affects the risk of disease. Two recent studies in Japanese men have investigated the association of P2RY2 SNPs to essential hypertension (90) and myocardial infarction (91), respectively. They were able to demonstrate an association to the non-synonymous SNPs as well as to certain haplotypes. In another study, the distribution of genotypes for the $Arg^{312}Ser$ SNP in patients with cystic fibrosis and controls was examined. It was found that subjects with cystic fibrosis more frequently were carriers of the Arg^{312} allele than the controls and that this was accompanied by a decreased receptor function in terms of calcium response to ATP stimulation as described above (88).

5. PERSPECTIVES

Although tremendous progress has been made in uncovering genetic variations within the P2 receptor genes that lead to a functional change of the receptor, only a few studies have focused on the association between these genetic variations and human bone phenotype.

Despite the hypothesis that functional changes in the P2 receptor genes may affect human bone mass, because it has been shown that P2 receptors play a central role in the bone physiology, future research is required in order to determine the exact role of genetic alterations leading to functional changes of the P2 receptor in human bone phenotype and human bone disease.

It is not only single nucleotide polymorphisms in the P2X7 or P2Y2 receptor genes that may be associated with the etiology and pathophysiology of human bone disease; single nucleotide polymorphisms in other P2 receptor genes (e.g. P2Y1, P2X4, P2Y6 and P2Y13) also might show an important association.

Research into genetic variations in the P2 receptor genes can be used to improve the diagnosis as well as risk assessment of bone diseases such as osteoporosis, which may lead to more effectively designed therapeutic or preventive treatments. Given the high disease load of osteoporosis and its projected dramatic rise over the next decades, such progress would be of great potential.

6. CONCLUSION

Thus, only a few studies have focused on the association of P2 receptor SNPs with bone mass and fracture incidence. However, as a number of these receptors are involved in the regulation of bone metabolism, the continued exploration of the role of genetic alterations in

P2 receptor genes will possibly be of great importance in the understanding of the mechanisms leading to osteoporosis, though further studies within this field are highly warranted.

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8. REFERENCES

1. G Burnstock, G Campbel, D Satchell, A Smythe: Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. *Br J Pharmacol* 40, 668-88 (1970)

2. Burnstock G: Purinergic nerves. *Pharmacol Rev* 24, 509-81 (1972)

3. Burnstock G, GE Knight: Cellular distribution and functions of P2 receptor subtypes in different systems. *Int Rev Cytol* 240, 231-304.

4. Dubyak GR, C el-Moatassim: Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. *Am J Physiol* 265, C577-606 (1993)

5. Yegutkin GG: Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. *Biochim Biophys Acta* 1783, 673-94 (2008)

6. Buckley KA, SL Golding, JM Rice, JP Dillon, JA Gallagher: Release and interconversion of P2 receptor agonists by human osteoblast-like cells. *Faseb J* 17, 1401-10 (2003)

7. Dixon CJ, JA Tattersall, JA Gallagher, PH Cobbold, WB Bowler: Real-time measurement of ATP release from human osteoblasts and keratinocytes. *Drug Dev Res* 43 (1998)

8. Burnstock G, C Kennedy: Is there a basis for distinguishing two types of P2-purinoceptor? *Gen Pharmacol* 16, 433-40 (1985)

9. Buell G, G Collo, F Rassendren: P2X receptors: an emerging channel family. *Eur J Neurosci* 8, 2221-8 (1996)

10. Soto F, M Garcia-Guzman, W Stuhmer: Cloned ligand-gated channels activated by extracellular ATP (P2X receptors) *J Membr Biol* 160, 91-100 (1997)

11. Khakh BS, RA North: P2X receptors as cell-surface ATP sensors in health and disease. *Nature* 442, 527-32 (2006)

12. Surprenant A, RA North: Signaling at purinergic P2X receptors. Ann Rev Physiol 71, 333-59 (2009)

13. Adinolfi E, C Pizzirani, M Idzko, E Panther, J Norgauer, F Di Virgilio, D Ferrari: P2X(7) receptor: Death or life? Purinergic signalling2005 Sep;1(3):219-27.

14. Boarder MR, GA Weisman, JT Turner, GF Wilkinson: G protein-coupled P2 purinoceptors: from molecular biology to functional responses. *Trends Pharmacol Sci* 16, 133-9 (1995)

15. James G, AM Butt: P2Y and P2X purinoceptor mediated Ca(2+) signalling in glial cell pathology in the central nervous system. *Eur J Pharmacol* 447, 247-60 (2002)

16. Hoebertz A, TR Arnett, G Burnstock: Regulation of bone resorption and formation by purines and pyrimidines. Trends Pharmacol Sci 24, 290-7 (2003)

17. Orriss IR, G Burnstock, TR Arnett: Purinergic signalling and bone remodelling. Curr Opin Pharmacol 10, 322-30 (2010)

18. Kumagai H, B Sacktor, CR Filburn: Purinergic regulation of cytosolic calcium and phosphoinositide metabolism in rat osteoblast-like osteosarcoma cells. *J Bone Min Res* 6, 697-707 (1991)

19. Schofl C, KSR Cuthbertson, CA Walsh, C Mayne, P Cobbold, A von zur Muhlen, RD Hesch, JA Gallagher: Evidence for P_2-purinocepters on human osteoblast-like cells. *J Bone Min Res* 7, 485-91 (1992)

20. Yu H, J Ferrier J: Osteoblast-like cells have a variable mixed population of purino/nucleotide receptors. *FEBS letters* 328, 209-14 (1993)

21. Li J, D Liu, HZ Ke, RL Duncan, CH Turner: The $P2X_7$ nucleotide receptor mediates skeletal mechanotransduction. The J Biol Chem 280, 42952-9 (2005)

22. Bowler WB, A Littlewood-Evans, G Bilbe, JA Gallagher, CJ Dixon: P2Y2 receptors are expressed by human osteoclasts of giant cell tumor but do not mediate ATP-induced bone resorption. *Bone* 22, 195-200 (1998)

23. Morrison MS, L Turin, BF King, G Burnstock, TR Arnett: ATP is a potent stimulator of the activation and formation of rodent osteoclasts. *J Physiol* 511, 495-500 (1998)

24. Hoebertz A, S Meghji, G Burnstock, TR Arnett: Extracellular ADP is a powerful osteolytic agent: evidence for signaling through the P2Y(1) receptor on bone cells. *Faseb J* 15, 1139-48 (2001)

25. Gartland A, RA Hipskind, JA Gallagher, WB Bowler: Expression of a $P2X_7$ receptor by a subpopulation of human osteoblasts. *J Bone Min Res* 16, 846-56 (2001)

26. Buckley KA, RA Hipskind, A Gartland, WB Bowler, JA Gallagher: Adenosine triphosphate stimulates human osteoclast activity via upregulation of osteoblast-expressed receptor activator of nuclear factor-kappa B ligand. *Bone* 31, 582-90 (2002)

27. Hayton MJ, JP Dillon, D Glynn, JM Curran, JA Gallagher, KA Buckley: Involvement of adenosine 5'-triphosphate in ultrasound-induced fracture repair. *Ultrasound Med Biol* 31, 1131-8 (2005)

28. Ihara H, K Hirukawa, S Goto, A Togari: ATP-stimulated interleukin-6 synthesis through P2Y receptors on human osteoblasts. *Biochem Biophys Res Comm* 326, 329-34 (2005)

29. Gartland A, JA Gallagher, WB Bowler: Activation of $P2X_7$ receptors expressed by human osteoclastoma modulates bone resorption. Calc Tiss Int 64, S56 (1999)

30. Ke HZ, H Qi, AF Weidema, Q Zhang, N Panupinthu, DT Crawford, WA Grasser, VM Paralkar, M Li, LP Audoly, CA Gabel, WS Jee, SJ Dixon, SM Sims, DD Thompson: Deletion of the P2X7 nucleotide receptor reveals its regulatory roles in bone formation and resorption. *Mol Endocrinol* 17, 1356-67 (2003)

31. Ohlendorff SD, CL Tofteng, JE Jensen, S Petersen, R Civitelli, M Fenger, B Abrahamsen, AP Hermann, P Eiken, NR Jorgensen: Single nucleotide polymorphisms in the $P2X_7$ gene are associated to fracture risk and to effect estrogen treatment. *Pharmacogenet Genomics* 17, 555-67 (2007)

32. Nakano Y, WN Addison, MT Kaartinen: ATP-mediated mineralization of MC3T3-E1 osteoblast cultures. *Bone* 41 (2007)

33. Panupinthu N, JT Rogers, L Zhao, L Pastor Solano-Flores, F Possmayer, SM Sims, SJ Dixon: P2X7 receptors on osteoblasts couple to production of lysophosphatidic acid: a signaling axis promoting osteogenesis. *J Cell Biol* 181, 859-71 (2008)

34. Panupinthu N, L Zhao, F Possmayer, HZ Ke, SM Sims, SJ Dixon: P2X7 nucleotide receptors mediate blebbing in osteoblasts through a pathway involving lysophosphatidic acid. J Biol Chem 282, 3403-12 (2007)

35. Orriss IR, GE Knight, S Ranasinghe, G Burnstock, TR Arnett: Osteoblast responses to nucleotides increase during differentiation. *Bone* 39, 300-9 (2006)

36. Hoebertz A, S Mahendran, G Burnstock, TR Arnett: ATP and UTP at low concentrations strongly inhibit bone formation by osteoblasts: a novel role for the P2Y2 receptor in bone remodeling. *J Cell Biochem* 86, 413-9 (2002)

37. Jones SJ, C Gray, A Boyde, G Burnstock: Purinergic transmitters inhibit bone formation by cultured osteoblasts. *Bone* 21, 393-9 (1997)

38. Orriss IR, JC Utting, A Brandao-Burch, K Colston, BR Grubb, G Burnstock, TR Arnett: Extracellular nucleotides block bone mineralization in vitro: evidence for dual inhibitory mechanisms involving both $P2Y_2$ receptors and pyrophosphate. *Endocrinology* 48, 4208-16 (2007)

39. Genetos DC, CJ Kephart, Y Zhang, CE Yellowley, HJ Donahue: Oscillating fluid flow activation of gap junction hemichannels induces ATP release from MLO-Y4 osteocytes. *J Cell Physiol* 212, 207-14 (2007)

40. Xia SL, J Ferrier: Propagation of a calcium pulse between osteoblastic cells. *Biochem Biophys Res Comm* 186, 1212-9 (1992)

41. Jorgensen NR, ST Geist, R Civitelli, TH Steinberg: ATP- and gap junction-dependent intercellular calcium signalling in osteoblastic cells. *J Cell Biol* 139, 497-506 (1997)

42. Jorgensen NR, Z Henriksen, C Brot, EF Eriksen, OH Sorensen, R Civitelli, TH Steinberg: Human osteoblastic cells propagate intercellular calcium signals by two different mechanisms. *J Bone Miner Res* 15, 1024-32 (2000)

43. Romanello M, B Pani, M Bicego, P D'Andrea P: Mechanically induced ATP release from human osteoblastic cells. *Biochem Biophys Res Comm* 289, 1275-81 (2001)

44. Romanello M, P D'Andrea P: Dual mechanism of intercellular communication in HOBIT osteoblastic cells: a role for gap-junctional hemichannels. *J Bone Min Res* 16, 1465-76 (2001)

45. Jorgensen NR, Z Henriksen, OH Sorensen, EF Eriksen, R Civitelli, Steinberg TH: Intercellular calcium signaling occurs between human osteoblasts and osteoclasts and requires activation of osteoclast $P2X_7$ receptors. *J Biol Chem* 277, 7574-80 (2002)

46. Pines A, M Romanello, L Cesaratto, G Damante, L Moro, P D'Andrea, G Tell: Extracellular ATP stimulates the early growth response protein 1 (Egr-1) via a protein kinase C-dependent pathway in the human osteoblastic HOBIT cell line. *Biochem J* 373, 815-24 (2003)

47. Romanello M, A Codognotto, M Bicego, A Pines, G Tell, P D'Andrea: Autocrine/paracvrine stimulation of purinergic receptors in osteoblasts: contribution of vesicular ATP release. *Biochem Biphys Res Comm* 331, 1429-38 (2005)

48. Costessi A, A Pines, P D'Andrea, M Romanello, G Damante, L Cesaratto L, F Quadrifoglio, L Moro, G Tell: Extracellular nucleotides activate Runx2 in the osteoblast-like HOBIT cell line: a possible molecular link between mechanical stress and osteoblasts' response. *Bone* 36, 418-32 (2005)

49. Okumura H, D Shiba, T Kubo, T Yokoyama: P2X7 receptor as sensitive flow sensor for ERK activation in osteoblasts. *Biochem Biphys Res Comm* 372, 486-90 (2008)

50. Liu D, DC Genetos, Y Shao, DJ Geist, J Li, HZ Ke, CH Turner, RL Duncan: Activation of extracellular-signal regulated kinase (ERK1/2) by fluid shear is Ca(2+)- and ATP-dependent in MC3T3-E1 osteoblasts. *Bone* 42, 644-52 (2008)

51. Suzawa T, C Miyaura, M Inada, T Maruyama, Y Sugimoto, F Ushikubi, A Ichikawa, S Narumiya, T Suda: The role of prostaglandin E receptor subtypes (EP1, EP2, EP3, and EP4) in bone resorption: an analysis using specific agonists for the respective EPs. *Endocrinology* 141, 1554-9 (2000)

52. Genetos DC, DJ Geist, D Liu, HJ Donahue, RL Duncan: Fluid shear-induced ATP secretion mediates prostaglandin release in MC3T3-E1 osteoblasts. *J Bone Min Res* 20, 41-9 (2005)

53. Reich KM, JA Frangos: Protein kinase C mediates flow-induced prostaglandin E2 production in osteoblasts. *Calc Tiss Int* 52, 62-6 (1993)

54. Kaplan AD, WJ Reimer, RD Feldman, SJ Dixon: Extracellular nucleotides potentiate the cytosolic Ca^{2+}, but not cyclic adenosine 3',5'-monophosphate response to parathyroid hormone in rat osteoblastic cells. *Endocrinology* 136, 1674-85 (1995)

55. Sistare FD, BA Rosenzweig, JG Contrera: P2 purinergic receptors potentiate parathyroid hormone receptor-mediated increases in intracellular calcium and inositol triphosphate in UMR-106 rat osteoblasts. *Endocrinology* 136, 4489-97 (1995)

56. Bowler WB, CJ Dixon, C Halleux, R Maier, G Bilbe, WD Fraser, JA Gallagher, RA Hipskind: Signaling in human osteoblasts by extracellular nucleotides. *J Biol Chem* 274, 14315-24 (1999)

57. Johnson RS, BM Spiegelman, V Papaioannou: Pleiotropic effects of a null mutation in the c-fos proto-oncogene. *Cell* 71, 577-86 (1992)

58. Grigoriadis AE, ZQ Wang, MG Cecchini, W Hofstetter, R Felix, HA Fleisch, EF Wagner: c-Fos: a key regulator of osteoclast-macrophage lineage determination and bone remodeling. *Science* 266, 443-8 (1994)

59. Stewart TL, SH Ralston. Role of genetic factors in the pathogenesis of osteoporosis. *J Endocrinol* 166, 235-45 (2000)

60. Ralston SH, B de Crombrugghe: Genetic regulation of bone mass and susceptibility to osteoporosis. Genes *Develop* 20, 2492-506 (2006)

61. Ralston SH, AG Uitterlinden, ML Brandi, S Balcells, BL Langdahl, P Lips, R Lorenc, B Obermayer-Pietsch, S Scollen, M Bustamante, LB Husted, AH Carey, A Diez-Perez, AM Dunning, A Falchetti, E Karczmarewicz, M Kruk, JP van Leeuwen, JB van Meurs, J Mangion, FE McGuigan, L Mellibovsky, F del Monte, HA Pols, J Reeve, DM Reid, W Renner, F Rivadeneira, NM van Schoor, RE Sherlock, JP Ioannidis: Large-scale evidence for the effect of the COLIA1 Sp1 polymorphism on osteoporosis outcomes: the GENOMOS study. *PLoS Med* 3, e90 (2006)

62. Uitterlinden AG, H Burger, Q Huang, F Yue, FE McGuigan, SF Grant, A Hofman, JP van Leeuwen, HA Pols, SH Ralston: Relation of alleles of the collagen type Ialpha1 gene to bone density and the risk of osteoporotic fractures in postmenopausal women. *NEJM* 338, 1016-21 (1998)

63. Ioannidis JP, I Stavrou, TA Trikalinos, C Zois, ML Brandi, L Gennari L, O Albagha, SH Ralston, A Tsatsoulis: Association of polymorphisms of the estrogen receptor alpha gene with bone mineral density and fracture risk in women: a meta-analysis. *J Bone Miner Res* 17, 2048-60 (2002)

64. Ioannidis JP, SH Ralston, ST Bennett, ML Brandi, D Grinberg, FB Karassa, BL Langdahl, JB van Meurs, L Mosekilde, S Scollen, OM Albagha, M Bustamante, AH Carey, AM Dunning, A Enjuanes, JP van Leeuwen, C Mavilia, L Masi, FE McGuigan, X Nogues, HA Pols, DM Reid, SC Schuit, RE Sherlock, AG Uitterlinden AG. Differential genetic effects of ESR1 gene polymorphisms on osteoporosis outcomees. *JAMA* 292, 2105-14 (2004)

65. Gu BJ, W Zhang, RA Worthington, R Sluyter, P Dao-Ung, S Petrou, JA Barden, JS Wiley: A Glu-496 to Ala polymorphism leads to loss of function of the human $P2X_7$ receptor. *J Biol Chem* 276, 11135-42 (2001)

66. Wiley JS, LP Dao-Ung, BJ Gu, R Sluyter, AN Shemon, C Li, J Taper, J Gallo, A Manoharan: A loss-of-function polymorphic mutation in the cytolytic P2X7 receptor gene and chronic lymphocytic leukaemia: a molecular study. *Lancet* 359, 1114-9 (2002)

67. Cabrini G, S Falzoni, SL Forchap, P Pellegatti, A Balboni, P Agostini, A Cuneo, G Castoldi, OR Baricordi, F Di Virgilio: A His-155 to Tyr polymorphism confers to gain-of-function to the human $P2X_7$ receptor of human leukemic lymphocytes. *J Immunol* 175, 82-9 (2005)

68. Fernando SL, BM Saunders, R Sluyter, KK Skarratt, JS Wiley, WJ Britton: Gene dosage determines the negative effects of polymorphic alleles of the P2X7 receptor on adenosine triphosphate-mediated killing of mycobacteria by human macrophages. *J Infect Dis* 192, 149-55 (2005)

69. Denlinger LC, DB Coursin, K Schell, G Angelini, DN Green, AG Guadarrama, J Halsey, U Prabhu, KJ Hogan, PJ Bertics: Human P2X7 pore function predicts allele linkage disequilibrium. Clin Chem 52, 995-1004 (2006)

70. Boldt W, M Klapperstuck, C Buttner. S Sadtler, G Schmalzing, F Markwardt: Glu496Ala polymorphism of human P2X7 receptor does not affect its

electrophysiological phenotype. *Am J Physiol* 284, C749-56 (2003)

71. Wiley JS, L-P Dao-Ung, C Li, AN Shemon, BJ Gu, ML Smart ML, SJ Fuller, JA Barden, S Petrou, R Sluyter: An Ile-568 to Asn polymorphism prevents normal trafficking and function of the human $P2X_7$ receptor. *J Biol Chem* 278, 17108-13 (2003)

72. Wiley JS, L-P Dao-Ung, C Li, AN Shemon, BJ Gu, ML Smart,: An Ile-568 to Asn polymorphism prevents normal trafficking and function of the human $P2X_7$ receptor. *J Biol Chem* 278, 17108-13 (2003)

73. Gu BJ, R Sluyter, KK Skarratt, AN Shemon, L-P Dao-Ung, SJ Fuller, JA Barden, AL Clarke, S Petrou, JS Wiley: An Arg^{307} to Gln polymorphism within the ATP-binding site causes loss of function of the human $P2X_7$ receptor. *J Biol Chem* 279, 31287-95 (2004)

74. Skarratt KK, SJ Fuller, R Sluyter, LP Dao-Ung, BJ Gu, JS Wiley: A 5' intronic splice site polymorphism leads to a null allele of the P2X7 gene in 1-2% of the Caucasian population. *FEBS letters* 579, 2675-8 (2005)

75. Shemon AN, R Sluyter, SL Fernando, AL Clarke, L-P Dao-Ung, KK Skarratt, BM Saunders, KS Tan, BJ Gu, SJ Fuller, WJ Britton, S Petrou, JS Wiley: A Thr^{357} to Ser polymorphism in homozygous and compound heterozygous subjects causes absent or reduced $P2X_7$ function and impairs ATP-induced mycobacterial killing by macrophages. *J Biol Chem* 281, 2079-86 (2006)

76. Roger S, ZZ Mei, JM Baldwin, L Dong, H Bradley, SA Baldwin, AM Surprenant LH Jiang: Single nucleotide polymorphisms that were identified in affective mood disorders affect ATP-activated P2X7 receptor functions. *J Psychi Res* 44, 347-55 (2009)

77. Stokes L, SJ Fuller, R Sluyter, KK Skarratt, BJ Gu, JS Wiley: Two haplotypes of the P2X(7) receptor containing the Ala-348 to Thr polymorphism exhibit a gain-of-function effect and enhanced interleukin-1beta secretion. *Faseb J* 24, 2916-27 (2010)

78. Sun C, J Chu, S Singh, RD Salter: Identification and characterization of a novel variant of the human P2X(7) receptor resulting in gain of function. *Purinergic Signal* 6, 31-45 (2009)

79. Fuller SJ, L Stokes, KK Skarratt, BJ Gu, JS Wiley: Genetics of the P2X7 receptor and human disease. *Purinergic Signal* 5, 257-62 (2009)

80. Nissen N, JS Madsen, EM Bladbjerg, JE Beck Jensen, NR Jorgensen, B Langdahl, B Abrahamsen, K Brixen: No association between hip geometry and four common polymorphisms associated with fracture: the Danish osteoporosis prevention study. *Calc Tiss Int* 84, 276-85 (2009)

81. Mrazek F, J Gallo, A Stahelova, M Petrek: Functional variants of the P2RX7 gene, aseptic osteolysis, and revision of the total hip arthroplasty: a preliminary study. *Hum Immunol* 71, 201-5 (2009)

82. Saunders BM, SL Fernando SL, R Sluyter, WJ Britton, JS Wiley: A Loss-of-Function Polymorphism in the Human P2X(7) Receptor Abolishes ATP-Mediated Killing of Mycobacteria. *J Immunol* 171, 5442-6 (2003)

83. Fairbairn IP, CB Stober, DS Kumararatne, DA Lammas: ATP-mediated killing of intracellular mycobacteria by macrophages is a P2X(7)-dependent process inducing bacterial death by phagosome-lysosome fusion. *J Immunol* 167, 3300-7 (2001)

84. Kusner DJ, J Adams: ATP-induced killing of virulent Mycobacterium tuberculosis within human macrophages requires phospholipase D. *J Immunol* 164, 379-88 (2000)

85. Fernando SL, BM Saunder, R Sluyter, KK Skarratt, H Goldberg, GB Marks, JS Wiley, WJ Britton: A polymorphism in the P2X7 gene increases susceptibility to extrapulmonary tuberculosis. *Am J Resp Crit Care Med* 175, 360-6 (2007)

86. Nino-Moreno P, D Portales-Perez, B Hernandez-Castro, L Portales-Cervantes, V Flores-Meraz, L Baranda, A Gomez-Gomez, V Acuna-Alonzo, J Granados, R Gonzalez-Amaro: P2X7 and NRAMP1/SLC11 A1 gene polymorphisms in Mexican mestizo patients with pulmonary tuberculosis. Clin Exp Immunol 148, 469-77 (2007)

87. Janssens R, P Paindavoine, M Parmentier, JM Boeynaems : Human P2Y2 receptor polymorphism: identification and pharmacological characterization of two allelic variants. Br J Pharmacol 127, 709-16 (1999)

88. Buscher R, A Hoerning, HH Patel, S Zhang, DB Arthur, H Grasemann, F Ratjen, PA Insel: P2Y2 receptor polymorphisms and haplotypes in cystic fibrosis and their impact on Ca2+ influx. Pharmacogenet Genomics 16, 199-205 (2006)

89. Henriksen Z. The Arg312Ser Single Nucleotide Polymorphism in the P2Y2 Receptor Gene is Associated with Postmenopausal Rate of Bone Loss. The American Society for Bone and Mineral Research, abstract 2009.

90. Wang Z, T Nakayama, N Sato, Y Izumi, Y Kasamaki, M Ohta, M Soma, N Aoi, Y Ozawa, Y Ma: The purinergic receptor P2Y, G-protein coupled, 2 (P2RY2) gene associated with essential hypertension in Japanese men. *J Hum Hypertens* 24, 327-35 (2009)

91. Wang J, SF Nagueh: Current perspectives on cardiac function in patients with diastolic heart failure. *Circulation* 119, 1146-57 (2009)

92. Nakamura E, Y Uezono, K Narusawa, I Shibuya, Y Oishi, M Tanaka, N Yanagihara, T Nakamura, F Izumi: ATP activates DNA synthesis by acting on P2X receptors in human osteoblast-like MG-63 cells. *Am J Physiol* 279, C510-9 (2000)

93. Maier R, A Glatz, J Mosbacher, G Bilbe: Cloning of P2Y6 cDNAs and identification of a pseudogene: comparison of P2Y receptor subtype expression in bone and brain tissues. *Biochem Biophys Res Comm* 240, 298-302 (1997)

94. Henriksen Z, N Nissen, NR Jorgensen: Functional P2X7 purinergic receptors are expressed in differentiated human osteoblasts. *J Bone Min Res* abstract SU208 (2006)

95. Bowler WB, MA Birch, JA Gallagher, G Bilbe: Identification and cloning of human P2U purinocepter present in osteoclastoma, bone and osteoblasts. *J Bone Miner Res* 10, 1137-45 (1995)

96. Maier R, A Glatz, J Mosbacher, G Bilbe: Cloning of P2Y6 cDNAs and identification of a pseudogene: comparison of P2Y receptor subtype expression in bone and brain tissue. *Biochem Biophys Res Comm* 237, 297-302 (1997)

97. Dixon CJ, WB Bowler, CA Walsh, Gallagher JA: Effects of extracellular nucleotides on single cells and populations of human osteoblasts: contribution of cell heterogeneity to relative potencies. *Br J Pharmacol* 120, 777-80 (1997)

98. Okumura H, D Shiba, T Kubo, T Yokoyama: P2X7 receptor as sensitive flow sensor for ERK activation in osteoblasts. *Biochem Biophys Res Comm* 372, 486-90 (2008)

99. Qi J, L Chi, J Faber, B Koller, AJ Banes: ATP reduces gel compaction in osteoblast-populated collagen gels. *J Appl Physiol* 102, 1152-60 (2007)

100. Li J, D Liu, HZ Ke, RL Duncan, CH Turner: The P2X7 nucleotide receptor mediates skeletal mechanotransduction. *J Biol Chem* 280, 42952-9 (2005)

101. You J, CR Jacobs, TH Steinberg, HJ Donahue: P2Y purinocepters are responsible for oscillatory fluid flow-induced intracellular calcium mobilisation in osteoblastic cells. *J Biol Chem* 277, 48724-9 (2002)

102. Nishii N, N Nejime, C Yamauchi, N Yanai, K Shinozuka, T Nakabayashi T. Effects of ATP on the intracellular calcium level in the osteoblastic TBR31-2 cell line. *Biol Pharm Bull* 32, 18-23 (2009)

103. Buckley KA, SC Wagstaff, G McKay, A Gaw, RA Hipskind, G Bilbe, JA Gallagher, WB Bowler: Parathyroid hormone potentiates nucleotide-induced $[Ca^{2+}]_i$ release in rat osteoblasts independently of G_q activation or cyclic monophosphate accumulation. *J Biol Chem* 276, 9565-71 (2001)

104. Sistare FD, BA Rosenzweig, B Jordan. Separate P2T and P2U puurinergic receptors with similar second messenger signaling pathways in UMR-106 osteoblasts. *J Pharmacol Exp Ther* 269, 1049-61 (1994)

105. Hoebertz A, A Townsend-Nicholson, R Glass, G Burnstock, TR Arnett. Expression of P2 receptors in bone and cultured bone cells. *Bone* 27, 503-10 (2000)

106. Gartland A, KA Buckley, RA Hipskind, WB Bowler, JA Gallagher: P2 receptors in bone: modulation of osteoclast formation and activity via $P2X_7$ activation. *Crit Rev Eukaryot Gene Expr* 13, 237-42 (2003)

107. Naemsch LN, X Du, SM Sims, SJ Dixon: P2 nucleotide receptors in osteoclasts. *Drug Dev Res* 53, 130-9 (2001)

108. Naemsch LN, AF Weidema, SM Sims, TM Underhill, SJ Dixon: P2X(4) purinoceptors mediate an ATP-activated, non-selective cation current in rabbit osteoclasts. *J Cell Sci* 112, 4425-35 (1999)

109. Korco, LN Raimundo, HZ Ke, SM Sims, SJ Dixon: Extracellular nucleotides act through P2X7 receptors to activate NF-kappaB in osteoclasts. *J Bone Miner Res* 19, 642-51 (2004)

110. Sluyter R, AN Shemon, JS Wiley: Glu^{496} to Ala polymorphism in the P2X7 receptor impairs ATP-induced IL-1beta release from human monocytes. *J Immunol* 172, 3399-405 (2004)

111. Brake AJ, MJ Wagenbach, D Julius: New structural motif for ligang-gated ion channels defined by an ionotropic ATP receptor. *Nature* 371, 519-23 (1994)

112. Barnard EA, G Burnstock, TE Webb: G protein-coupled receptors for ATP and other nucleotides a new receptor family. *Trends Pharmacol Sci* 15, 67-70 (1994)

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