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Bone phenotypes of P2 receptor knockout mice

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1. ABSTRACT

The action of extracellular nucleotides is mediated by ionotropic P2X receptors and G-protein coupled P2Y receptors. The human genome contains 7 P2X and 8 P2Y receptor genes. Knockout mice strains are available for most of them. As their phenotypic analysis is progressing, bone abnormalities have been observed in an impressive number of these mice: distinct abnormalities in $P2X_7^{-/-}$ mice, depending on the gene targeting construct and the genetic background, decreased bone mass in $P2Y_1^{-/-}$ mice, increased bone mass in $P2Y_2^{-/-}$ mice, decreased bone resorption in $P2Y_6^{-/-}$ mice, decreased bone formation and bone resorption in $P2Y_{13}^{-/-}$ mice. These findings demonstrate the unexpected importance of extracellular nucleotide signalling in the regulation of bone metabolism via multiple P2 receptors and distinct mechanisms involving both osteoblasts and osteoclasts.

2. INTRODUCTION

Nucleotides, such as ATP and UTP, are mainly intracellular, but they can be released into the extracellular fluids by various mechanisms. One of them is cell damage: both necrotic and apoptotic cells release ATP and other nucleotides that thus constitute "danger signals" (1,2). But they can also be released without cell lysis by specific mechanisms: exocytosis of secretory granules, vesicular transport and membrane channels, such as ABC transporters, pannexins and connexins (3,4). Exocytosis of nucleotides is typically observed during platelet aggregation and synaptic transmission. They are also released in response to various types of stress: hypoxia, pathogen invasion or mechanical stimulation (stretch, shear stress). Once in the extracellular fluid, nucleotides can activate two families of receptors: metabotropic P2Y

Table 1. P2X receptors

	Agonist	Potential coassembly
$P2X_1$	ATP	1, 2, 3, 5, 6
$P2X_2$	ATP	1, 2, 3, 5, 6
$P2X_3$	ATP	1, 2, 3, 5
$P2X_4$	ATP	4, 5, 6
$P2X_5$	ATP	1, 2, 3, 4, 5, 6
$P2X_6$	ATP	1, 2, 4, 5, 6
$P2X_7$	ATP	7

Column 3 gives the subscript of P2X subunits with which each P2X subunit can form oligomers.

Table 2. P2Y receptors

Group	Receptor	Agonist (human)	G protein
A	$P2Y_1$	ADP	G_q
	$P2Y_2$	ATP=UTP	G_q (+ G_i)
	$P2Y_4$	UTP	G_q (+ G_i)
	$P2Y_6$	UDP	G_q
	$P2Y_{11}$	ATP	G_q + G_s
B	$P2Y_{12}$	ADP	G_i
	$P2Y_{13}$	ADP	G_i
	$P2Y_{14}$	UDP-glucose, UDP	G_i

The pharmacology of some P2Y receptors exhibits species differences : whereas the human $P2Y_4$ is a UTP receptor, the rat and mouse $P2Y_4$ receptors are activated equipotently by ATP and UTP.

receptors coupled to G proteins (5) and fast P2X ion channels (6).

In the human genome there are 7 genes encoding P2X receptor subunits (Table 1) and 8 genes encoding P2Y receptors, that can subdivided in 2 subgroups based on structural features and coupling to specific G proteins (Table 2). Knockout mice have been generated for all P2Y receptors except $P2Y_{11}$, which is not present in murine genome. Phenotypes have also been described for the P2X receptor knockouts, except $P2X_5$ and $P2X_6$. The phenotypic analysis of these mice is revealing an increasing number of functions of these receptors. The most prominent results are listed below.

$P2X_1^{-/-}$ mice display male infertility due to reduced vas deferens contraction (7), reduced neurogenic vasoconstriction (8) and reduced autoregulation of renal blood flow (9). $P2X_2^{-/-}$ mice have a reduced ventilatory response to hypoxia (10). $P2X_3^{-/-}$ mice display urinary bladder hyporeflexia (11) and reduced pain response to formalin (12). A profound deficit of taste responses was found in the taste nerves of $P2X_2/P2X_3$ double knockouts, while loss of either $P2X_2$ or $P2X_3$ resulted only in a moderate change (13). $P2X_4^{-/-}$ mice are characterized by an impaired blood flow-dependent control of vascular tone (14) and a blunted tactile allodynia following spinal nerve injury (15). The study of $P2X_7^{-/-}$ mice revealed a crucial role of this receptor in the function of the inflammasome and the release of IL-1β (16,17).

Both $P2Y_1^{-/-}$ and $P2Y_{12}^{-/-}$ mice exhibit a defect in platelet aggregation by ADP and an increased resistance to thromboembolism (18,19). Furthermore atherosclerotic lesions were less in $P2Y_1^{-/-}$/apoE$^{-/-}$ mice, but this protection was independent from platelets and likely involve endothelial cells (20). The chemotactic attraction of both neutrophils (21) and monocytes (2) is decreased in $P2Y_2^{-/-}$ mice, while microglia migration and process extension to sites of brain damage is impaired in $P2Y_{12}^{-/-}$ mice (22). The phenotypic analysis of other P2Y knockouts is still at an early phase. The stimulatory effect of ATP on Cl^- and water secretion by intestinal epithelial cells was abolished in $P2Y_4$-deficient mice (23). The responses to UDP of vascular endothelial and smooth muscle cells as well as macrophages were impaired in $P2Y_6^{-/-}$ mice (24). A reduction in the hepatic clearance of HDL was observed in $P2Y_{13}^{-/-}$ mice (25). The contractile effect of UDP-glucose on the forestomach muscle was decreased in $P2Y_{14}^{-/-}$ mice (26).

This overview of P2 knockout mice phenotypes shows thus an involvement of these receptors in a broad range of functions and cell types : $P2Y_1$, $P2Y_6$ and $P2X_4$ in vascular endothelial cells, $P2X_1$ and $P2Y_6$ in vascular smooth muscle cells, $P2Y_1$ and $P2Y_{12}$ in platelets, $P2X_7$, $P2Y_2$ and $P2Y_6$ in immune cells, $P2X_4$ and $P2Y_{12}$ in microglia, $P2X_2$ and $P2X_3$ in neurons and $P2Y_{13}$ in hepatocytes. In an unexpected way, recent studies of knockout mice described below show that PX_7, $P2Y_1$, $P2Y_2$, $P2Y_6$ and $P2Y_{13}$ play all a role in bone via actions on osteoblasts or osteoclasts or both.

3. BONE PHENOTYPES OF $P2X_7^{-/-}$ MICE

Within the last ten years our understanding of the role of the $P2X_7$ receptor in the regulation of bone turnover and in mechanotransduction has progressed significantly. This has to a large extent resulted from the establishment of mouse models with a targeted disruption of the gene encoding the $P2X_7$ receptor. Overall $P2X_7^{-/-}$ mice are viable and fertile, and cannot be distinguished from wild type littermates by examination alone. Nevertheless, several defects have been described, including impaired IL-1β production in macrophages stimulated with ATP (27), an attenuated inflammatory response to induced arthritis (28) and enhanced susceptibility to multiple sclerosis (29).

Two $P2X_7^{-/-}$ mouse models have been created and both display a bone phenotype. Overall, the $P2X_7^{-/-}$ mice produced by the Pfizer group exhibit a reduced bone mass (30). In this study, young (2 months) and old (9 months) mice of both genders were examined. $P2X_7$ knockout animals displayed reduced total and cortical bone mineral content (BMC) and decreased periosteal circumference of the femur (30). These differences tended to be larger in older animals. Interestingly, the effect of $P2X_7$ receptor ablation was more pronounced in male mice than in females. Histomorphometric analyses of the bone tissue demonstrated reduced parameters of bone formation including mineralizing surface (MS/BS), mineral appositional rate (MAR) and bone formation rate (BFR/BS) as well as increased parameters of bone resorption such as osteoclast number per mm bone surface (OCN/BS) and percent osteoclast surface (OCS/BS), supporting the low

bone mass phenotype. Moreover calvarial sutures were wider in $P2X_7^{-/-}$ mice (31) This phenotype of decreased periosteal bone formation combined with increased trabecular bone resorption resembles the effects of disuse on the skeleton and mimics estrogen deficiency, suggesting that $P2X_7$ might regulate mechanotransduction. This has been confirmed in later studies (see below).

Another murine model, with ablation of the $P2X_7$ gene, has been produced by the group of Gary Buell at GSK (32). As with the Pfizer model, these $P2X_7^{-/-}$ mice also displayed a bone phenotype (33). In this study, young (6 weeks) and old (11 months) mice were examined, and no differences between knockout and wild type mice could be detected when looking at bone mineral density (BMD) at either age. Also, no differences were found in trabecular bone volume. However, in contrast to the bone phenotype described for the Pfizer model, Gartland *et al* showed that cortical thickness was increased in the $P2X_7^{-/-}$ animals compared to the wild types (33). Thus, the bone phenotypes described for the two models are conflicting. This contradiction could be related to the method of gene targeting or to the different genetic background of the inbred strains used to generate the knockout mice.

The $P2X_7^{-/-}$ construct of the GSK $P2X_7^{-/-}$ mice was created by insertion of a *LacZ* gene into the beginning of exon 1 of the $P2X_7$ gene (32), creating a frameshift mutation. Pfizer's $P2X_7^{-/-}$ strain was generated by deletion of the nucleotides 1527-1607 in exon 13 and insertion of a neomycin cassette in the 5' to 3' direction. In the generation of both strains, genomic DNA containing the $P2X_7$ gene was isolated from a genomic library drawn from 129/sv mice (32,34). The Pfizer $P2X_7^{-/-}$ mice were generated on 129/Ola x B6 x DBA/2 genetic background, and maintained on the B6 x DBA/2 background and later on the B6 background (27,30). The GSK $P2X_7^{-/-}$ mice were maintained on B6 background, but originate from a B6/129 hybrid (32,33). However, the most convincing explanation for the difference between the two models came in 2009, when Nicke *et al* demonstrated the expression of a functional $P2X_7$ splice variant in some tissues from the GSK $P2X_7^{-/-}$ mice (35). Staining by $P2X_7$ antibodies is detectable in brain (36-38) and in lymphocytes (34), at levels identical to wild type littermates. The staining pattern in neurons and lymphocytes is due to the expression of a $P2X_7$-like protein, absent from other tissues with less abundant alternative splicing. This unexpected staining pattern could be due to the expression of the splice variant $P2X_7$-k which escapes gene inactivation in the GSK $P2X_7^{-/-}$ mice (35). Although the Pfizer $P2X_7^{-/-}$ lymphocytes also express a $P2X_7$-like protein, it is non-functional (34). The $P2X_7$-k splice variant contains an alternative intracellular N terminus and first transmembrane domain, and appears to have higher sensitivity to the $P2X_7$ agonist BzATP when expressed in a HEK293 *in vitro* expression system. $P2X_7$-k is highly expressed in the spleen and has varying tissue specific expression. Preliminary results indicate that the splice variant is expressed at the mRNA but not protein level in osteoblasts (39), and its expression in bone and other bone cells remains unknown, so that results from studies in the GSK $P2X_7^{-/-}$ model should be interpreted with caution. The existence of the splice variant $P2X_7$-k indicates that the role of the $P2X_7$ receptor could have been misinterpreted, especially in tissues expressing the splice variant. For instance, the human $P2X_7$ receptor has been shown to participate in the fusion of multinucleated cells (8), but the murine $P2X_7$ receptor has been reported to be dispensable in formation of murine osteoclasts in the GSK $P2X_7^{-/-}$ model, since osteoclasts have been found in these mice (33). If the splice variant is expressed and functional in murine osteoclasts, they would have a high-activity $P2X_7$ receptor instead of a non-functional receptor.

A further complexity in the analysis of the bone phenotype of $P2X_7^{-/-}$ mice was recently revealed. It is known that several strains of mice including C57Bl/6 have a naturally occurring P451L mutation in the $P2X_7$ gene. This mutation reduces the sensitivity of the $P2X_7$ receptor to nucleotides, which may result in underestimation of the severity of the phenotype exhibited by knockout mice (30).Therefore $P2X_7^{-/-}$ mice were recently generated on a BALB/cJ background. Preliminary results indicate that these mice have an increase in BMD compared to wild types, which is related to decreased bone resorption (39).

Physical activity and thus mechanical stimulation is the most powerful anabolic stimulus to bone. As mentioned above, the structural changes in the Pfizer $P2X_7$-null animals resemble the changes observed in immobilized bone, suggesting that the $P2X_7$ receptor might be involved in the mechanotransductory cascade. In a highly interesting study by Li *et al* (40), the effect of *in vivo* mechanical loading on the ulnar bone in $P2X_7^{-/-}$ and wild type mice was examined. The right forearm of the mouse was loaded at 120 cycles per day for 3 consecutive days and the periosteal bone formation was determined. Interestingly, a marked reduction (73%) in the interlabeling distance (measure of bone formation) was observed in the $P2X_7^{-/-}$ animals compared to the wild types (40). Thus, the sensitivity to mechanical loading was significantly reduced indicating an important role of the $P2X_7$ receptor in bone mechanotransduction. *In vitro*, it was shown that both $P2X_7$ receptor-mediated pore formation and prostaglandin release in response to fluid shear stress were absent in osteoblasts from $P2X_7$-null animals, supporting the role of $P2X_7$ receptors in mechanotransduction in bone cells. A recent study has also investigated the role of the $P2X_7$ receptor in dental mechanotransduction (41). It was found that root resorption was increased and bone formation decreased in $P2X_7^{-/-}$ mice, confirming the role of the $P2X_7$ receptor in the mechanotransductory process in bone and related tissues. Fracture repair is another process where loading of the bone is important. Li *et al* recently examined the fracture healing process in adult $P2X_7$-null mice subjected to osteotomy (42). No differences could be detected in callus formation using standard imaging techniques. Furthermore, mechanical testing at the fracture site showed no statistically significant differences between $P2X_7$-null animals and wild type animals. However, a tendency towards a reduced strength at the fracture site was seen and the lack of statistical significance can be explained by the low power of the study. Histomorphometric analyses also revealed that mineralizing surface and bone formation were

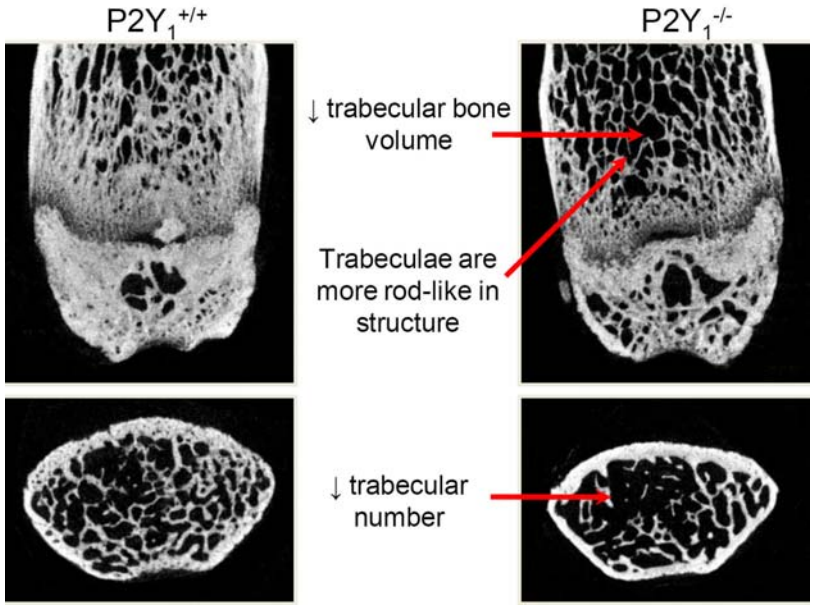

Figure 1. Decreased trabecular bone in $P2Y_1^{-/-}$ mice. µCT analysis of the femur and tibia from $P2Y_1^{-/-}$ mice demonstrated decreased trabecular bone volume and trabecular number; trabecular thickness was unchanged. An increased structural model index indicated the trabeculae were more rod-like in structure in the knockout animals.

significantly decreased in the $P2X_7^{-/-}$ animals. Thus, callus remodeling was significantly delayed, indicating a role for the $P2X_7$ receptor in callus formation and fracture repair, probably through effects in mechanotransduction.

4. BONE PHENOTYPE OF $P2Y_1^{-/-}$ MICE

Expression of the $P2Y_1$ receptor has been reported on both osteoblasts (43-45) and osteoclasts (43,46). In osteoblasts, activation of the $P2Y_1$ receptor has been reported to modulate cellular responses to systemic factors, such as parathyroid hormone, by increasing c-fos expression (44,47). Stimulation of the $P2Y_1$ receptor by ADP or ATP stimulates the formation and resorptive activity of rodent osteoclasts cultured *in vitro (*48).

The effect of $P2Y_1$-receptor deletion on skeletal structure was recently investigated using dual energy x-ray absorbtiometry (DEXA) scanning and micro computed tomography (µCT). DEXA analysis performed on whole animals demonstrated a 5% decrease in total BMD and a 7% decrease in total BMC in $P2Y_1^{-/-}$ mice (Figure 1). The long bones and spines of these animals exhibited 4-14% decreases in BMC and BMD; weight, lean tissue and fat content were unchanged. More detailed investigation by µCT showed that femoral and tibial trabecular bone volume (BV/TV) were decreased 35% and 23%, respectively, in the $P2Y_1^{-/-}$ mice. Trabecular number was also reduced (up to 32%) in the long bones of knockout animals, whereas trabecular thickness was unchanged. The structural model index (SMI) parameter gives an indication of trabecular structure; an increased SMI in the femur and tibia of $P2Y_1^{-/-}$ mice indicated that the trabeculae were more rod-like in shape. Cortical bone parameters were unaffected in the knockout animals (49). At present there are no studies detailing changes in the differentiation, survival and function of osteoblasts and osteoclasts derived from $P2Y_1^{-/-}$ mice.

5. BONE PHENOTYPE OF $P2Y_2^{-/-}$ MICE

The $P2Y_2$ receptor is expressed by both osteoblasts (42,44) and osteoclasts (42,45). Expression of the $P2Y_2$ receptor in osteoblasts increases strongly as differentiation proceeds (45) and this receptor has been implicated in the potent inhibition of bone mineralisation by ATP and UTP observed in osteoblast cultures (50,51). The $P2Y_2$ receptor may also have a role in the propagation of intercellular Ca^{2+} waves (51) and the stimulation of Erg1 and Runx2 expression in osteoblasts (53,54). Effects of $P2Y_2$ receptor activation on osteoclast formation and resorptive activity have not been reported.

Changes in the bone phenotype of $P2Y_2^{-/-}$ mice were first reported in 2007 when a study using DEXA analysis demonstrated increased BMC in the femora (9%) and tibiae/fibulae (17%) of knockout animals (51). Receptor deletion had no effect on weight, lean tissue and fat content (51). A subsequent µCT study of 2-month old $P2Y_2^{-/-}$ mice demonstrated large increases in the BV/TV of the long bones (43% and 21% in the femur and tibia, respectively) (Figure 2) (49). Trabecular thickness and trabecular number were also increased in the femora of $P2Y_2$-deficient animals, with decreased femoral SMI, indicating the presence of more plate-like trabeculae. Increased cortical bone volume (up to 25%) was additionally observed in the long bones of $P2Y_2^{-/-}$ animals (49). There are no published studies to date on the differentiation, survival and function of osteoblasts and osteoclasts derived from $P2Y_2^{-/-}$ mice.

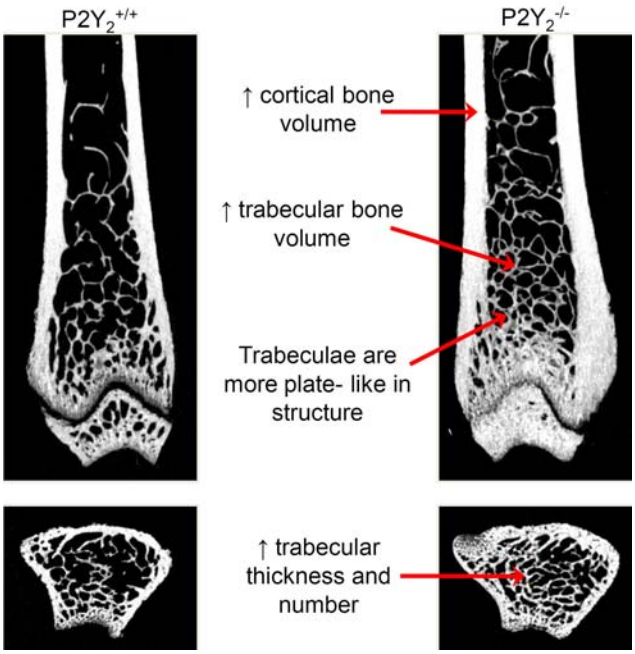

Figure 2. Increased trabecular and cortical bone in $P2Y_2^{-/-}$ mice. µCT analysis of the femur and tibia from $P2Y_2^{-/-}$ mice demonstrated increased trabecular bone volume, trabecular number, trabecular thickness and cortical bone volume. A decreased structural model index indicated the trabeculae were more plate-like in structure in the knockout animals.

6. PRELIMINARY RESULTS IN $P2Y_6^{-/-}$ MICE

The $P2Y_6$ receptor is widely expressed by both osteoblasts (45) and osteoclasts (55). The $P2Y_6$ receptor has been suggested to play a role in osteoclasts survival since receptor activation prevented their spontaneous apoptosis in culture (55). Recently, preliminary studies have reported an altered bone phenotype in the $P2Y_6$ receptor knockout (56). DEXA analysis showed increased bone content in the long bones and spine of the $P2Y_6R^{-/-}$ mice. µCT analysis demonstrated increased cortical thickness (up to 25%) and cortical bone volume (up to 18%) in the long bones of $P2Y_6^{-/-}$ mice. Additionally, the length of the long bones was increased up to 10% in the $P2Y_6^{-/-}$ mice. Cortical bone volume was also increased in the L3 lumbar vertebrae. In both the long bones and spine, no changes in the trabecular bone were observed. Modest reductions in osteoclast formation in M-CSF and RANKL-treated marrow cultures from $P2Y_6^{-/-}$ animals were observed, together with striking impairment of resorptive function in the mature cells. Calvarial osteoblasts derived from $P2Y_6R^{-/-}$ mice displayed reduced proliferation and mineralisation.

7. PRELIMINARY RESULTS IN $P2Y_{13}^{-/-}$ MICE

The recent availability of the $P2Y_{13}^{-/-}$ mice promises to provide researchers with an invaluable tool to further elucidate the role of this receptor in basic physiology. To that end, initial studies of the $P2Y_{13}^{-/-}$ mice have shown that when maintained under a normal diet these mice had normal weight gain, development, fertility, behaviour, haematological and plasma parameters - although hepatic cholesterol metabolism was impaired (25). The $P2Y_{13}^{-/-}$ mice exhibited normal immune response, and dendritic cells, which have previously been shown to express high levels of $P2Y_{13}$ receptor mRNA, were not affected by the $P2Y_{13}$ receptor gene deletion in terms of antigen presentation or lymphocyte activation (25).

In view of previous data suggesting that the cells responsible for maintaining skeletal health, especially osteoclasts (bone resorbing) and osteoblasts (bone building), would express the $P2Y_{13}$ receptor (57-59), we have made a detailed investigation of the skeletal phenotype of the $P2Y_{13}^{-/-}$ mice (60). Analysis of the bone micro-architecture of the $P2Y_{13}^{-/-}$ mice was performed using a Skyscan 1172 MicroCT machine at the scan resolution of 4.3µm. Results showed that $P2Y_{13}^{-/-}$ mice had significantly lower trabecular bone volume/tissue volume, significantly lower trabecular number and an increased cortical bone thickness. Histology of the tibia revealed a significant decrease in both osteoblast and osteoclast numbers on the endocortical surface of the $P2Y_{13}^{-/-}$ mice. We also performed *in vitro* cultures to assess the function of the bone cells. Primary osteoblasts derived from the calvaria of neonatal $P2Y_{13}^{-/-}$ mice showed reduced alkaline phosphatase activity, whilst there was also a reduction in the number and function of multinucleated osteoclasts derived from the mononuclear hematopoietic cell population of 10 weeks old female $P2Y_{13}^{-/-}$ mice. The molecular basis for the observed effect of $P2Y_{13}$ receptor deletion in osteoblasts and osteoclasts is currently under investigation. In summary, deletion of the $P2Y_{13}$ receptor in mice leads to an abnormal bone phenotype, including less trabecular bone but thicker cortical bone. This is a

consequence of reduced rates of bone turnover caused by decreased number and function of both osteoblasts and osteoclasts.

8. SUMMARY AND PERSPECTIVE

Unexpectedly all strains of P2-deficient mice studied so far have displayed a bone phenotype. No information is available on mice heterozygous for these deletions. Interestingly each of these phenotypes is unique. These studies emphasize thus the importance of nucleotide signalling in the control of bone metabolism and the unique role of each receptor.

Studies of the bone phenotype of $P2X_7^{-/-}$ mice have provided conflicting results between two distinct models generated at Pfizer and GSK respectively. Mice generated at Pfizer exhibited a decreased bone mass due to decreased bone formation and increased bone resorption (30). The GSK mice had a mild phenotype with an increased cortical thickness (33). This apparent contradiction can probably be explained by the expression of a functional splice variant of $P2X_7$ in the GSK mice (35). But these mice were generated in the B6 background which harbors a natural mutation of the $P2X_7$ gene. Preliminary results show an increased bone mass and decreased resorption in $P2X_7^{-/-}$ mice in the BALB/cJ background (39).

Studies have shown that ATP and ADP, acting via the $P2Y_1$ receptor, increase osteoclast formation and activity *in vitro (*48). Given this observation, the reduced trabecular bone observed in the long bones of $P2Y_1^{-/-}$ mice was somewhat unexpected. Thus, the effects of receptor deletion may predominantly affect osteoblasts or, given the defects in platelet aggregation and blood clotting seen in $P2Y_1^{-/-}$ mice (18), other systemic perturbations may indirectly influence bone cell function. Indeed there is growing evidence that hematopoietic cells such as megakaryocytes, which are known to express $P2Y_1$ (61), can influence osteoblasts and osteoclasts (62).

The $P2Y_2$ receptor is thought to mediate the inhibitory effects of ATP and UTP on bone mineralisation *in vitro (*50,51). Consistent with these observations, trabecular and cortical bone mass was increased in the $P2Y_2^{-/-}$ mice; thus, deletion of the $P2Y_2$ receptor could potentially limit the negative actions of extracellular nucleotides on bone.

Preliminary results indicate that $P2Y_6^{-/-}$ mice have an increased bone mass that can be explained by the decreased resorptive function of osteoclasts (56). Bone resorption was also decreased in $P2Y_{13}^{-/-}$ mice, but this was explained by a decrease in the osteoclast number rather than osteoclast function (60). In these mice, decreased bone resorption was associated with decreased bone formation : this decreased bone turnover resulted in opposite effects on trabecular (decreased volume) and cortical (increased thickness) bone. A $P2Y_{13}$ antagonist might thus be beneficial in osteoporosis which is characterized by an increased bone turnover.

These studies of knockout mice demonstrate thus that not less than five P2 receptors play a role in bone formation and/or resorption. This confirms the prediction that nucleotides acting via both P2X and P2Y receptors could play important roles in the regulation of bone metabolism (63,64). These important roles can be related to the fact that mechanical stimulation, which is an important factor in bone remodelling, stimulates the release of nucleotides.

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