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Nucleotides as regulators of bone cell function and mineralisation

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A thesis submitted for the fulfilment of the degree of
Doctor of Philosophy

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University College London

2014

Declaration of authorship

I, Mark Omar Raimes Hajjawi, declare that this thesis titled '**Nucleotides as regulators of bone cell function and mineralisation**' and the work presented in it are my own. I confirm that:

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Abstract

Most cells, including bone cells, release ATP into the extracellular environment. A considerable body of previous work has shown that ATP, acting through the P2 receptors, inhibits bone formation by osteoblasts and increases bone resorption by osteoclasts. This work focuses on the action of two key breakdown products of ATP, pyrophosphate and adenosine on bone cell function. Pyrophosphate, a ubiquitous physicochemical inhibitor of mineralisation, is formed from extracellular ATP by the action of ecto-nucleotide pyrophosphatase phosphodiesterases (NPPs); in bone these enzymes act in opposition to alkaline phosphatase. Adenosine, which can be generated in a number of ways from ATP, has been previously reported to stimulate both osteoblast and osteoclast function. However, using *in vitro* cultures, I found that it had little or no effect on the differentiation and bone forming capacity of rat osteoblasts, nor on the formation and resorptive function of mouse osteoclasts. I investigated the possibility that osteocytes, which form an interconnected cellular network within bone, might regulate mineralisation via NPPs. I found that cultured, primary osteocyte-like cells derived from mouse bone expressed *Enpp1* mRNA. Osteocyte lacunae in the femora of *Enpp1*^{-/-} mice imaged by scanning electron microscopy were found to be reduced in area by about 35%; indirect estimates of lacunar size using microCT imaging were in agreement. These results are consistent with the notion that ATP-derived pyrophosphate is important for maintenance of osteocyte lacunae size. *Enpp1*^{-/-} mouse bones (humerus) were found to have reduced cortical bone diameter, reduced cortical porosity and an increased endosteal diameter compared to wild types, suggesting that the knockout phenotype also involves increased bone resorption and decreased bone formation. Histology and microCT of *Enpp1*^{-/-} mice confirmed inappropriate joint mineralisation and showed that cartilage in the trachea and ear pinna was also mineralised, as were whisker sheaths. Osteoblasts, osteoclasts and osteocytes cultured *in vitro* from *Enpp1*^{-/-} mice were found to release less ATP compared to cells from *Enpp1*^{+/+} mice in static conditions and after fluid flow stimulation. *Enpp1*^{-/-} osteoblasts and osteoclasts also contained higher levels of intracellular ATP. *Enpp1*^{-/-} osteoblasts showed increased bone production *in vitro* compared to *Enpp1*^{+/+}; no effects of *Enpp1* knockout on the formation or resorptive activity of osteoclasts were noted. Sclerostin, an osteocyte-derived inhibitor of WNT signalling and bone formation, was found to increase *Enpp1* mRNA expression and NPP activity of osteoblasts, without affecting ALP *in vitro*. These results emphasise the importance of ATP and its breakdown product pyrophosphate in regulating mineralisation.

Acknowledgments

I would like to thank Prof Tim Arnett and Dr Isabel Orriss for giving me the opportunity, and allowing me the privilege, to undertake my PhD under their supervision. Both have given me more than I could ever repay and share a part in any successes I have from this day forwards.

I would also like to thank Prof Alan Boyde from QMUL for performing some of the electron microscopy in this thesis and offering sage words of advice, and Dr Chris Scotton from Exeter University for assisting me in the practical aspects of histology.

Finally, I would like to thank my wife Rachel.

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Chapter 1

Introduction

The structure and composition of mammalian bone reflects its dynamic and varied functions. Bone requires mechanical strength because it has a major role in movement and locomotion, yet is also a key endocrine organ and regulator of calcium and phosphate homeostasis. Bone is also the primary site of haematopoiesis and has a key role within the immune system (Schwartz & Heath 1947; Meyer, Jr. *et al.* 1989; Le & Mougiakakos 2012).

Development of the skeleton

Each bone within the mammalian body is different. However, all bones of the mammalian skeleton are formed in one of two different ways, either by intramembranous ossification or endochondral ossification. The bones of the axial skeleton (vertebrae and ribs) and the bones of the appendicular skeleton (limbs) are formed by endochondral ossification. The flat bones of the skull are formed by the process of intramembranous ossification.

Endochondral ossification

Endochondral bone formation involves a cartilaginous template of the bone being created first, which then develops into mineralised bone. During development mesenchymal stem cells (MSCs) condense, these cells then differentiate into chondrocytes. Chondrocytes proliferate and secrete type II collagen and aggrecan (chondroitin sulphate proteoglycan 1). Certain chondrocytes in the centre of this embryonic clustering stop proliferating, become hypertrophic, and begin to secrete a matrix rich in collagen type X. These hypertrophic chondrocytes signal to perichondral cells to influence their differentiation into osteoblasts. The osteoblasts begin to form a collar of bone. The hypertrophic chondrocytes also attract blood vessels and cause them to invade the tissue by releasing vascular endothelial growth factor (VEGF). The hypertrophic chondrocytes then undergo

apoptosis, leaving behind a scaffold composed of type 2 collagen. There is evidence to suggest that these chondrocytes do not undergo apoptosis in a classical way, but undergo “chondroapoptosis”, which is preceded by an increase in the endoplasmic reticulum and Golgi apparatus of the cell (Roach & Clarke 2000). The chondrocytes further away from the hypertrophic chondrocytes continue to proliferate; this causes the bone to lengthen. As the bone enlarges further secondary ossification sites appear at the ends of the bone in a very similar process. In the long bones, the cartilage that remains between the primary and secondary ossification centres is called the growth plate (epiphyseal plate). In the growth plate below the secondary ossification centre, proliferating chondrocytes form orderly columns, these cells act to continue lengthening the bone postnatally (reviewed in Ortega *et al.* 2004).

Intramembranous ossification

Like endochondral ossification, intramembranous ossification starts with mesenchymal condensations. During intramembranous ossification MSCs differentiate directly into osteoblasts, without the intermediate collagen scaffold formation by chondrocytes. In the mammalian skull, neural crest derived MSCs proliferate and form the flat bones (Helms *et al.* 2005). The calvarial sutures form where two opposing bone formation fronts meet.

Bone cells

Osteoblasts

Osteoblasts are the bone forming cells; they are formed from MSCs by a multistep series of events. MSCs can differentiate into a number of different cell types, such as adipocytes (Friedenstein *et al.* 1976), chondrocytes (Mardon *et al.* 1987), myocytes (Wakitani *et al.* 1995) and fibroblasts (Friedenstein *et al.* 1987). MSCs are found in a wide variety of tissues: the Wharton’s jelly of an umbilical cord (Lee *et al.* 2004), adipose tissue (Zuk *et al.* 2002), amniotic fluid (Sessarego *et al.* 2008) and muscle (Jankowski *et al.* 2002). It has been suggested that MSCs can be found circulating in the blood (Eghbali-Fatourehchi *et al.* 2005; Kassis *et al.* 2006; He *et al.* 2007). Pools of MSCs reside in the bone marrow stroma; it is believed that it is

from these MSCs that osteoblasts develop (Haynesworth *et al.* 1992; Pittenger *et al.* 1999).

A number of studies have identified key genes which mediate the differentiation of MSCs. The formation of adipocytes is promoted by *PPAR γ* and *C/EBP α* (Mueller *et al.* 2002; Tang *et al.* 2003), the formation of chondrocytes from MSCs requires *Sox9* (de *et al.* 2000) and the formation of myocytes is under the control of *MyoD* (Emerson 1990). The formation of osteoblasts from MSCs requires a number of factors, including: *Runx2* (previously known as *cbfa1*) (Banerjee *et al.* 1997; Ducy *et al.* 1997; Komori *et al.* 1997), *Osterix* (Nakashima *et al.* 2002; Skillington *et al.* 2002), WNTs (Monroe *et al.* 2012) and bone morphogenic proteins (BMPs) (Urist 1965).

Runx2^{-/-} mice have been shown to have a normal cartilaginous skeleton at day 15 of gestation, but it was under mineralised compared to wild type skeletons (Komori *et al.* 1997; Otto *et al.* 1997). By day 18 of gestation, wild type mice tibias had developed a bone marrow cavity and were showing signs of vasculature invasion into the bone, whereas *Runx2*^{-/-} mice had neither of these developments, indicating the importance of *Runx2* in early bone formation and mineralisation. These *Runx2*^{-/-} mice did not form osteoblasts, had reduced alkaline phosphatase (ALP) activity and died immediately after birth. They died of asphyxiation due to the hypomineralisation of the rib cage; ribs lacking deposited mineral are not strong enough to provide the negative pressure needed for lung expansion (Komori *et al.* 1997; Otto *et al.* 1997). In addition to osteoblast formation, *Runx2* is required for osteoblast function. Osteocalcin, is an osteoblast specific calcium binding protein, the expression of which is under the control of *Runx2* (Ducy *et al.* 1997). Osteocalcin has been reported to be important in bone mineral deposition (Boskey *et al.* 2002) and induces chemotaxis in osteoclasts (Chenu *et al.* 1994). Further evidence is developing that shows decarboxylated-osteocalcin may stimulate the secretion of insulin from the pancreas (Lee *et al.* 2007; Ferron *et al.* 2008).

The expression of the zinc finger containing transcription factor *Osterix* (*Osx*) is under the control of *Runx2*; the expression of *Osterix* is reduced in *Runx2*^{-/-} mice (Nakashima *et al.* 2002). However, the expression of *Runx2* is not affected in *Osx*^{-/-}

mice (Nishio *et al.* 2006), indicating that Osterix acts downstream of *Runx2*. Similar to *Runx2*^{-/-} mice, *Osx*^{-/-} mice lack osteoblasts and have an unmineralised skeleton, but there are phenotypic differences between *Runx2*^{-/-} mice and *Osx*^{-/-} mice. Knockout of *Runx2* leads to an underdeveloped perichondrium (outer layer of connective tissue) in the mid shaft of long bones, whereas knockout of *Osx* causes ectopic cartilage formation beneath a thickened perichondrium (Nakashima *et al.* 2002). Osterix appears to work cooperatively with nuclear factor for activated T-cells 2 (NFAT2) to regulate the expression of extra cellular-matrix proteins, such as type I collagen, by osteoblasts (Koga *et al.* 2005).

Other transcription factors are needed for osteoblast differentiation. Activating transcription factor 4 (ATF4) plays a role in the late stage differentiation of osteoblasts. ATF4 is a member of the basic leucine zipper domain transcription factor family and is a substrate of ribosomal S6 kinase 2 (RSK2), a growth factor regulated kinase. A missense mutation in *ATF4* is the cause of Coffin-Lowry syndrome, which is characterised by skeletal abnormalities (Yang *et al.* 2004). People with this syndrome have incomplete closure of the fontanelles, the “soft spot” of the skull and delayed bone development (Lowry *et al.* 1971). *ATF4*^{-/-} mice have defects in long bone mineralisation; however, knock out of *ATF4* does not affect *Runx2* or *Osx* expression (Reimold *et al.* 1996). So it can be seen that ATF4 acts as an important transcription factor downstream of *Runx2* and *Osx*. *ATF4*^{-/-} mice also have reduced expression of osteocalcin and receptor activator of nuclear factor kappaβ ligand (RANKL), two osteoblast associated factors (Reimold *et al.* 1996). ATF4 promotes amino acid uptake into the osteoblast, a process that may support the protein synthesis function of osteoblasts (Yang *et al.* 2004). *ATF4* also interacts with Forkhead box O (FoxO) proteins in osteoblasts to regulate glucose homeostasis (Kode *et al.* 2012). Factor inhibiting activating transcription factor 4 (FIAT) is a leucine zipper protein which is an inhibitor of ATF4. When over-expressed in transgenic mice FIAT reduced osteocalcin, bone mineral density, bone volume and trabecular thickness (Yu *et al.* 2005).

MSCs that differentiate down the osteoblast lineage *in vivo* can give rise to preosteoblasts, mature osteoblasts, osteocytes and bone-lining cells. Preosteoblasts may express some of the phenotypic markers of osteoblasts, for example ALP, but at lower levels. However, they lack many of the defining characteristics of mature osteoblasts, such as a well-developed endoplasmic reticulum, necessary for its matrix secretory role. On quiescent surfaces where bone remodelling is not taking place, the osteoblasts flatten to become lining cells. The bone-lining cells form a barrier between the extracellular fluid and the bone. It is thought that the lining cells may play a role in regulating the movement of calcium and phosphate in and out of the local bone environment (Miller & Jee 1987). The mature osteoblasts synthesise and secrete an extracellular matrix, osteoid, which provides the site for mineral deposition (Komori *et al.* 1997).

Osteocytes

Some osteoblasts differentiate into osteocytes. Osteocytes are the most numerous bone cell; they reside within lacunae, bathed in fluid, surrounded by mineralised matrix. These cells are dispersed throughout bone and are connected to each other by dendritic processes that pass down thin canals, called canaliculi. These dendrites allow the osteocytes to communicate with each other and with other cells on the surface of the bone. The canaliculi allow osteocytes to communicate in a paracrine and endocrine manner by enabling hormones, and other signalling molecules to reach the circulatory system. The canaliculi system also results in the osteocyte having a large surface area of interaction with the bone (reviewed in Bonewald 2011).

The process by which an osteoblast is converted into an embedded osteocyte is not fully understood. Osteoblasts have a slightly different gene expression profile based on their age and location within bone, this may affect their chance of becoming an osteocyte (Candelieri *et al.* 2001). It has been shown that mouse osteocytes release osteoblast stimulating factor-1 (OSF-1) / heparin binding growth associated molecule (HB-GAM), possibly to recruit and further differentiate osteoblasts into osteocytes (Imai *et al.* 2009). The first step of this differentiation

process involves the osteoblast becoming passively buried under the matrix that it, or a neighbouring osteoblast has produced (Franz-Odenaal *et al.* 2006). However, the whole process of osteocytogenesis is not a passive one, the collagen-lytic activity of matrix metalloproteinase I is required to help embed the osteoblast (Holmbeck *et al.* 2005). The change from an osteoblast to an osteocyte is a gradual process, starting with the down-regulation of osteoblast specific genes such as: ALP, type I collagen and osteocalcin, and with the concordant up-regulation of osteocyte specific genes including: dentine matrix protein-1 (*DMP1*), *E11*, sclerostin (*Sost*) and fibroblast growth factor 23 (*FGF23*) (Schulze *et al.* 1999; Toyosawa *et al.* 2001; Winkler *et al.* 2003; Ubaidus *et al.* 2009). It is not clear if these factors “make an osteocyte”, or “define an osteocyte”. As the cell progresses from being an osteoblast to an osteocyte, it reduces in volume by approximately 70% (Palumbo 1986). The osteocyte also loses some of the osteoblast defining intracellular characteristics, such as the well-developed endoplasmic reticulum and Golgi apparatus (Dudley & Spiro 1961).

The *E11* gene is expressed by early, immature osteocytes; it is a hydrophobic membrane protein that appears to play a role in the formation of dendrites. The addition of the *E11* protein to the osteocyte-like cell line MLO-Y4 resulted in the elongation of the cell’s dendrites (Zhang *et al.* 2006). It was also seen that MLO-A5 osteocyte-like cells increase their expression of *E11* when they are surrounded by mineralised extracellular matrix (Prideaux *et al.* 2012). *E11* is also expressed in endothelial cells, kidney and lung, where it is known as GP38, podoplanin and T1 α , respectively. Knockout of the *E11* gene resulted in mice that died at birth of respiratory failure (Ramirez *et al.* 2003). Fluid shear stress upon MLO-Y4 cells resulted in increased expression of *E11*; prevention of *E11* translation using small interfering RNA resulted in decreased dendrite length (Zhang *et al.* 2006).

Dentine matrix protein-1 (*DMP1*) is an extracellular matrix protein that was first discovered in rat teeth (George *et al.* 1993). In postnatal mammals, *DMP1* is predominantly expressed by osteocytes (Toyosawa *et al.* 2001); prenatally, *DMP1* is also expressed by hypertrophic chondrocytes and osteoblasts (Fen *et al.* 2002).

DMP1 knockout mice had defects in their osteocyte canaliculi system and delayed osteocyte development (Lu *et al.* 2011). One of the main functions of *DMP1* may be as a regulator of matrix mineralisation (He & George 2004). Knockout of *DMP1* in mice increased the concentration of circulating FGF23, and resulted in hypophosphatemia, osteomalacia, and rickets (Feng *et al.* 2006). Conversely, over expression of *DMP1* in mice was shown to have no effect in some models (Lu *et al.* 2011), but was seen to increase bone mineral density in other mice models (Bhatia *et al.* 2012). Humans with loss-of-function mutations in *DMP1* suffer from autosomal recessive hypophosphatemic rickets (Feng *et al.* 2006).

Osteocytes and osteoblasts are the main source of the circulating hormone FGF23 (Ubaidus *et al.* 2009). FGF23 acts via the FGF receptor (FGFR) on the cell surface, its affinity for this receptor is increased by the protein cofactor klotho (Martin *et al.* 2012). The main role of FGF23 is to inhibit renal phosphate reabsorption by sodium phosphate transporters (type 2a), therefore increasing urinary phosphate loss (Shimada *et al.* 2005). FGF23 also inhibits the formation of 1,25-dihydroxyvitamin D from 25-hydroxyvitamin D, by suppressing the enzyme CYP27B1 in the kidney; this reduces the amount of the active form of vitamin D in the circulation (Shimada *et al.* 2005). FGF23 may also increase the amount of 24,25-dihydroxyvitamin D formed from 25-hydroxyvitamin D by up-regulating CYP24 in the proximal tubule of the kidney, resulting in a higher circulating amount of the inactive form of vitamin D (Shimada *et al.* 2005). In both humans and mice, over production or gain-of-function mutations in *Fgf23* leads to autosomal dominant hypophosphatemic rickets (ADHR) (White *et al.* 2000; Shimada *et al.* 2002). Tumour-induced osteomalacia may have symptoms very similar to ADHR, FGF23 secreted by the tumour can be a cause of a low plasma phosphate concentration in these patients (Zimering *et al.* 2005).

Matrix extracellular phosphoglycoprotein (MEPE) is a member of the small integrin-binding ligand N-linked glycoprotein (SIBLING) family of proteins; these are proteins that seem to have no similarities when their amino acid sequences are compared, but are all located in the same chromosomal region (4q.21 in humans

and 5q in mice), all display an arg-gly-asp motif that mediates cell binding and attachment and all are associated with bone and dentine (Huq *et al.* 2005). The members of the SIBLING family are osteopontin (OPN), bone sialoprotein (BSP), DMP1, dentine sialophosphoprotein and MEPE. MEPE is expressed by both osteoblasts and osteocytes; it plays an important role in the mineralisation of bone. Knockout of MEPE leads to an increased bone mass in mice and increased osteoblast number and activity in culture (Gowen *et al.* 2003). Mice that over-express MEPE form osteoblasts normally but have a mineralisation defect due to decreased osteoblast activity (David *et al.* 2009). Administration of the MEPE protein to mice leads to phosphaturia and decreased plasma phosphate levels (Rowe *et al.* 2004). MEPE is cleaved to release a peptide that contains an acid serine and aspartic acid-rich motif (ASARM) which inhibits mineralisation (Martin *et al.* 2008). Phosphate regulating endopeptidase x-linked (PHEX) interacts with MEPE to prevent the release of the ASARM peptide. Humans with X-linked hypophosphatemic rickets (XLH) and the *Hyp* mouse, have defects in the *Phex* gene, this results in an inability to prevent the release of ASARM and leads to impaired mineralisation (Bresler *et al.* 2004; Rowe *et al.* 2005).

Along with the control of mineralisation, one of the main functions of osteocytes is thought to be the detection of mechanical force on the bone. Under normal conditions the rate of bone formation and loss is balanced. However, the skeleton is able to remodel and adapt to its mechanical environment by adding or removing bone. So, a bone under a high amount of mechanical force will adapt by increasing its mineralised tissue volume and / or density, a bone under little mechanical force will lose mineralised tissue volume and / or density (Skerry *et al.* 1989; Burr *et al.* 2002; Tatsumi *et al.* 2007; Klein-Nulend *et al.* 2013).

Various studies have shown that when force is applied to a bone the osteocytes respond by increasing the expression of DMP1 (Gluhak-Heinrich *et al.* 2003; Yang *et al.* 2005a) and E11 (Zhang *et al.* 2006), which may result in bone formation. Mechanical loading has been shown to down-regulate the expression of sclerostin, an inhibitor of WNT signalling and bone formation (Robling *et al.* 2006), whereas

unloading of the hind limbs of a mouse has been shown to up-regulate the expression of sclerostin, which would inhibit bone formation (Lin *et al.* 2009). Targeted deletion of all of the osteocytes within bone using diphtheria toxin resulted in mice that were resistant to unloading-induced bone loss (Tatsumi *et al.* 2007). It has also been shown that unloading of mouse hind legs increased the osteocytes' expression of RANKL, a potent cytokine required for osteoclast formation, which can lead to bone loss (Xiong *et al.* 2011).

It is believed that osteocytes are able to dissolve the non-organic matrix of the internal wall of their own lacunae by a process of osteolytic osteolysis (Belanger *et al.* 1967; Qing & Bonewald 2009; Atkins & Findlay 2012). In support of this theory, osteocyte-like cell lines have been reported to express the genes for tartrate-resistant acid phosphatase (TRAP) and a lysosomal proton pump, both are associated with bone resorption (Tazawa *et al.* 2004; Bivi *et al.* 2009). It has been reported that both PTH treatment and lactation result in enlargement of the osteocyte lacunae; it is believed that this dissolution of the lacunae may contribute to net circulating calcium and phosphate concentrations (Tazawa *et al.* 2004; Qing *et al.* 2012). However, the theory of osteolytic osteolysis is disputed (Parfitt 1977; Boyde & Jones 1979).

It has also been reported that osteocytes can actively replace the mineral and matrix within their lacunae (Baylink & Wergedal 1971; Zamboni *et al.* 1983). It has been reported that the osteocyte lacunae size increased in lactating mice and then returned back to the baseline size with weaning (Qing *et al.* 2012), suggesting that osteocytes are able to remodel their lacunae.

Osteoclasts

Osteoclasts are bone resorbing cells; they are motile and usually multinucleated. Osteoclasts are required for breaking down bone, so it can be reformed and remodelled during skeletal development and throughout adult life. Osteoclasts are formed from monocyte / macrophage precursors (reviewed in Arnett 2013a). Unlike osteoblasts and osteocytes, osteoclasts are formed from the haemopoietic

stem cell lineage. The source of osteoclasts was first elucidated in the 1970s when mice suffering from osteopetrosis, a condition resulting in excess bone, caused by an osteoclast defect, were cured by a bone marrow transplant, a spleen graft or a temporary parabiosis, allowing the blood of a wild type mouse to be shared with an affected mouse (Walker 1973; Walker 1975a; Walker 1975b).

The formation of monocytes and macrophages, from which osteoclasts are derived, requires the transcription factor PU.1 (Scott *et al.* 1994; Anderson *et al.* 1998). Knockout of PU.1 in mice results not only in the inability to produce monocytes and macrophages, but also osteopetrosis due to a lack of osteoclasts (Tondravi *et al.* 1997). This form of osteopetrosis was cured in PU.1^{-/-} mice by a bone marrow transplant. In the initial stages of monocyte and macrophage development from haemopoetic stem cells, PU.1 stimulates the expression of C-fms, the macrophage colony-stimulating factor (M-CSF) receptor (DeKoter *et al.* 1998).

M-CSF is a critical cytokine for the generation of osteoclasts. The osteopetrotic mouse strain *op/op* has an inactivating mutation in the M-CSF gene. This lack of M-CSF results in a severe reduction in osteoclasts and in osteopetrosis, which cannot be overcome by a bone marrow transplant (Yoshida *et al.* 1990). However, injection of these mice with M-CSF restores the osteoclast defect and treats the osteopetrosis (Felix *et al.* 1990).

RANKL is a member of the tumour necrosis factor (TNF) cytokine family; it is expressed by osteoblasts, osteocytes, stromal cells and activated T-cells (Yasuda *et al.* 1998; Kong *et al.* 1999b; Nakashima *et al.* 2011; Xiong *et al.* 2011). RANKL exists in both soluble and membrane bound forms, it acts on osteoclast precursor cells, via its receptor, RANK, and via the TNF receptor associated proteins, TRAF2, TRAF5 and TRAF6. This activates the nuclear transcription factor nuclear factor $\kappa\beta$ (NF $\kappa\beta$), which in turn activates the transcription factor of activated T-cells (NFATc1) (Franzoso *et al.* 1997; Iotsova *et al.* 1997). Activation of RANK signalling by RANKL leads to an increase in multinucleated osteoclasts. RANKL up-regulates the expression of the genes that cause the fusion of preosteoclast precursors: dendritic

cell stimulatory transmembrane protein (DC-STAMP) and osteoclast stimulatory transmembrane protein (OC-STAMP) (Miyamoto *et al.* 2012). Loss of function mutations in human RANK (Guerrini *et al.* 2008) or RANKL (Sobacchi *et al.* 2007), and knockout of RANK (Dougall *et al.* 1999) or RANKL (Kong *et al.* 1999a) in mice leads to an inability to form osteoclasts and therefore causes severe osteopetrosis and failure of tooth eruption. Over-expression of RANKL in humans, caused by factors such as tumours, resulted in extensive pathological osteolysis (Grimaud *et al.* 2003).

The actions of RANKL are inhibited by osteoprotegerin (OPG), a soluble decoy receptor that binds to RANKL and prevents its interaction with RANK (Simonet *et al.* 1997). Inactivating mutations in the OPG gene (*Tnfrs11b*) in humans results in Paget's disease due to excessive osteoclast activity (Whyte *et al.* 2002). In mice, over-expression of OPG, or injection with recombinant OPG, reduced osteoclast formation and caused osteopetrosis (Simonet *et al.* 1997).

The RANK-RANKL-OPG axis is one of the most important signalling pathways in the development of osteoclasts. NFATc1 is the transcription factor most strongly induced by RANKL and may represent the master gene in osteoclast formation (Takayanagi *et al.* 2002). Knock out of NFATc1 in mice caused them to die *in utero* because the pulmonary and aortic valves did not develop (de la Pompa *et al.* 1998; Ranger *et al.* 1998). Osteoblast targeted ablation of NFATc1 in mice, which prevented the lethal defects, resulted in a reduction in osteoclast number and size, and severe osteopetrosis (Winslow *et al.* 2006). Over expression of NFATc1 in mice led to a large increase in the number of osteoclasts formed *in vivo* (Winslow *et al.* 2006).

Osteoclasts have to attach to the bone surface in order to resorb it. The attachment and binding of the osteoclast to the bone surface occurs primarily through the $\alpha\beta3$ integrin (vitronectin receptor) (Davies *et al.* 1989; Nakamura *et al.* 1999). This integrin forms part of a structure known as a podosome that contains actin filaments, cortactin, Wiskott-Aldrich syndrome proteins and other attachment proteins such as vinculin and talin (Luxenburg *et al.* 2007). The $\alpha\beta3$

integrin recognises and binds to the arg-gly-asp (RGD) amino acid motif of proteins embedded in the matrix of bone (Horton *et al.* 1991). Knockout mice deficient in the $\beta 3$ integrin are able to form osteoclasts, yet these osteoclasts are not able to sufficiently bind to mineralised surfaces for resorption to occur (McHugh *et al.* 2000).

When an osteoclast attaches onto bone, the podosomes rearrange into a ring known as the sealing zone, which anchors the osteoclast onto the bone surface, and results in the formation of a compartment underneath the cell where bone resorption can take place. Podosome formation is under the control of c-Src, a tyrosine kinase and Rho (a GTPase) (Jurdic *et al.* 2006). Knock out of c-Src in mice resulted in osteopetrosis because the osteoclasts that these mice formed were unable to breakdown bone (Soriano *et al.* 1991). Src kinases phosphorylate many substrates, including cortactin and gelsolin, which regulate actin polymerisation and podosome turnover (De, V *et al.* 1997; Tehrani *et al.* 2007). Within the sealing zone, the cell membrane of the osteoclast develops the ruffled border. This highly convoluted folded membrane allows a large surface area of the osteoclast to interact with the bone.

The ruffled border of the osteoclast contains the vacuolar-type H^+ ATPase proton pump. This actively pumps protons out of the osteoclast, across the ruffled border, and into the sealed resorption compartment covering the surface of the bone (Blair *et al.* 1989). This acidifies the compartment and results in the “acid etching” of the bone. The protons secreted by the v-ATPase are mainly formed by the actions of carbonic anhydrase II; this also results in the formation of bicarbonate. This bicarbonate is passively exchanged for chloride ions at the basolateral membrane of the osteoclast (Blair *et al.* 1993). In order to maintain the intracellular pH and electrochemical charge of the osteoclast, these negatively charged chloride ions are expelled from the cells across the ruffled border into the resorption zone by the ClC-7 chloride channel (Brandt & Jentsch 1995). Knock out of ClC-7 channel in mice results in osteoclasts that are unable to resorb bone and severe osteopetrosis.

Defects in the CIC-7 Cl⁻ channel have been detected in humans, and shown to be a cause of malignant osteopetrosis (Kornak *et al.* 2001).

This acid environment that the osteoclast forms is able to dissolve hydroxyapatite, but for the complete destruction of bone, and the degradation of demineralised bone matrix, enzymatic digestion is required. Cathepsin K is an enzyme expressed by activated osteoclasts and secreted into the sealed resorption zone, where it cleaves all three chains of the type 1 collagen triple helix and the telopeptides (Costa *et al.* 2011a). A genetic defect in the gene encoding cathepsin K results in the rare condition pycnodysostosis. Humans with this loss of function mutation in cathepsin K have a short stature and skeletal malformations (Gelb *et al.* 1996). Cathepsin K knockout mice have increased bone mass due to impaired bone resorption; these mice formed osteoclasts which had little resorptive activity, resulting in impaired bone remodelling (Saftig *et al.* 1998; Li *et al.* 2006).

The matrix metalloproteinases (MMPs) are also secreted across the ruffled border of osteoclasts and degrade the organic component of bone (Delaisse *et al.* 2003). MMPs are generally regarded as contributing less to matrix degradation than cathepsin K; however, MMP knockout mice show skeletal defects. Knockout mice deficient in MMP9 and MMP13 have defects of the growth plate due to reduced osteoclast resorptive function (Vu *et al.* 1998; Inada *et al.* 2004). Osteoclasts express tartrate resistant acid phosphatase (TRAP). TRAP generates reactive oxygen species that may also aid in matrix degradation. TRAP knockout mice have reduced osteoclast activity and mild osteopetrosis (Hayman & Cox 2003). In histology and cell culture experiments, TRAP activity is used as a convenient marker for osteoclasts.

The activation of osteoclasts requires the up-regulation of key genes needed for resorption. The acidification of the osteoclast's extracellular environment is the key factor in the activation of resorption *in vitro* (Arnett & Dempster 1986). Extracellular acidification stimulates the formation of the podosome and the expression of the machinery needed for resorption: carbonic anhydrase II, v-type H⁺ ATPase, cathepsin K and TRAP (Teti *et al.* 1989; Murrills *et al.* 1993; Arnett

2010). After the initial activation by acidosis, other factors can influence the rate of resorption by osteoclasts, such as, PTH (Dempster *et al.* 2005), RANKL (Burgess *et al.* 1999), ATP / ADP (Morrison *et al.* 1998; Hoebertz *et al.* 2001). The key factor in the long term survival of the osteoclast is the RANKL / OPG ratio; reduction in RANKL or an increase in OPG leads to apoptosis (Lacey *et al.* 2000).

Bone matrix

Bone derives its strength by being a composite material of organic and non-organic factors. Type I collagen is the predominant structural protein in bone and provides the tensile strength (Vashishth 2007). This collagen provides the backbone for bone and is the site of initial mineral deposition. Osteogenesis imperfecta in humans and animals is caused by mutations in the genes encoding collagen; defective collagen is produced and results in brittle bones (Marini *et al.* 2007). Collagen is a trimeric molecule made up of three α -chain subunits. The amino acid sequence of each of these α -chains is made up of a repeating triplet sequence, gly-X-Y, where X is often proline and Y is often hydroxyproline. Collagenous proteins can be either homotrimeric, where all three α -chains are identical, or heterotrimeric, where the α -chains are different. These three chains coil together to form a triple helix. This structure is stabilised by hydrogen bonding between the OH groups of hydroxyproline (reviewed in Gordon & Hahn 2010). Collagen fibrils are formed by the collagen molecules lining up. Individual fibrils are aligned in a quarter-staggered way. As a result of this stagger there are gaps in the fibril structure; it is within these gaps that crystals of the bone mineral hydroxyapatite first appear in the extracellular environment (Traub *et al.* 1992).

Using proteomics and gene profiling it has shown that there are many thousands of different non-collagenous proteins in bone matrix, each with varying actions (Boskey 2013). Some of these proteins, such as albumin, are explanted into the bone from the blood plasma. Other proteins such as proteoglycans are assumed to become embedded during the bone formation process and may act to help stabilise the tissue integrity. Osteonectin is a phosphorylated glycoprotein found in bone that may regulate osteoblast proliferation and function. The SIBLING proteins may

play a role in the attachment of bone cells to the bone (reviewed in Robey & Boskey 2009).

The mineralisation of bone matrix

The inorganic mineral hydroxyapatite ($\text{Ca}_5(\text{PO}_4)_3\text{OH}$) provides the rigidity of bone. Unlike the naturally occurring geological form of this mineral, the biological form contains many other elements. These factors increase the solubility of the hydroxyapatite crystals, giving it an important role in Mg^{2+} , Ca^{2+} and phosphate homeostasis (McConnell *et al.* 1961; Hukins *et al.* 1986).

Collagen does not directly induce hydroxyapatite crystal deposition onto bone matrix. The first stages of mineralisation take place within matrix vesicles (Anderson 1969; Ali *et al.* 1970). These osteoblast organelles provide a site for Ca^{2+} and phosphate accumulation, which enables the formation of hydroxyapatite (Anderson *et al.* 1997). The matrix vesicle buds out from the osteoblast, next the matrix vesicle membrane is broken down releasing the hydroxyapatite into the extracellular matrix, where its crystal structure propagates further (Anderson *et al.* 2005a). A number of factors have been shown to be key regulators of mineralisation, these include three key enzymes: ecto-nucleotide pyrophosphatase / phosphodiesterase-1 (NPP1), alkaline phosphatase (ALP) and phosphatase orphan 1 (PHOSPHO1); the transport protein ANK and the pyrophosphate / phosphate ratio.

Ecto-nucleotide pyrophosphatase/phosphodiesterase-1 (NPP1) and mineralisation

The *Enpp1* gene, which encodes the membrane bound enzyme NPP1 is expressed in a wide variety of tissues including heart, kidney, vascular smooth muscle cells, osteoblasts and chondrocytes (Terkeltaub 2001; Johnson & Terkeltaub 2005; Johnson *et al.* 2005; Nitschke *et al.* 2011). NPP1 is a member of the NPP family of enzymes; it is highly expressed on the membrane of mineralising cells and within matrix vesicles. It acts to hydrolyse nucleotide triphosphates to their monophosphate form with the release of the inhibitor of mineralisation,

pyrophosphate (Fleisch & Bisaz 1962). For further details on its role in nucleotide hydrolysis see page 47.

NPP1 has been shown to have a role in extracellular matrix mineralisation (see section below on phosphate / pyrophosphate ratio) but there is also evidence to show that NPP1 plays a role in insulin signalling. Over expression of *Enpp1* in cultured fibroblasts inhibited insulin receptor tyrosine kinase, thereby reducing the actions of insulin on its receptor. Certain subpopulations of patients with non-insulin dependent diabetes mellitus have been shown to over express NPP1 (Maddux *et al.* 1995; Frittitta *et al.* 1998). Further studies revealed that the actions of NPP1 on insulin signalling are not mediated by its actions on nucleotide breakdown; the abolition of NPP1's nucleotidase activity did not affect its actions on insulin signalling (Grupe *et al.* 1995). Subsequent work has shown that NPP1 directly interacts with the α -subunit of the insulin receptor; antibodies against NPP1 can prevent this interaction and restore insulin receptor signalling in cells over-expressing *Enpp1 in vitro* (Maddux & Goldfine 2000). Transgenic mice with liver specific over expression of *Enpp1* show impaired glucose tolerance, but not overt diabetes. However, mice with targeted over-expression of *Enpp1* in both the liver and muscle show fed and fasting hyperglycaemia and hyperinsulinemia (Maddux *et al.* 2006). A short hairpin RNA adenovirus has been used to reduce *in vivo* hepatic *Enpp1* mRNA expression in a *db/db* mouse model of diabetes. Knockdown of *Enpp1* expression in this mouse led to a reduction in fasting and fed plasma glucose levels and an improvement in glucose tolerance (Zhou *et al.* 2009).

Alkaline phosphatase and mineralisation

In humans there are four ALP isoenzymes: tissue non-specific (TNAP), placental, germ cell and intestinal ALP (Millan 2013). Tissue non-specific ALP is expressed only in the bone, liver and kidney. There are slight differences in the post-translational modification of the tissue non-specific form depending on the tissue source of the enzyme; this results primarily in variations in the type and amount of glycosylation and differences in the number of sialic acid side-chains (Schreiber & Whitta 1986; Magnusson & Farley 2002; Halling *et al.* 2009). Mice also have four separate genes

that express ALP: the tissue non-specific form (*Akp2*), duodenum specific intestinal form, embryonic and the global intestinal form (Millan 2013). Rats have three different ALP genes, the tissue non-specific form and two isoenzymes of the intestinal form (Millan 2006). In both the human and the rodent, only the tissue non-specific form has been implicated in tissue mineralisation.

Tissue non-specific ALP is found anchored to the surface of osteoblasts by glycosylphosphatidylinositol (Fedde *et al.* 1988; Hooper 1997). ALP is also found on the surface of matrix vesicles (Anderson *et al.* 2004). It was first suggested in 1923 that ALP may be significant in bone mineralisation (Robison 1923). Later work showed that the key role of tissue non-specific ALP on osteoblasts and within matrix vesicles is to hydrolyse the inhibitor of mineralisation pyrophosphate, to produce phosphate (Hessle *et al.* 2002).

Phosphatase orphan 1 (PHOSPHO1)

PHOSPHO1, a phosphoethanolamine / phosphocholine phosphatase, is a member of the haloacid dehalogenase superfamily of enzymes (Houston *et al.* 1999; Stewart *et al.* 2003). Experiments in chicks and mice have shown that PHOSPHO1 is found inside the matrix vesicles of osteoblasts and hypertrophic chondrocytes (Stewart *et al.* 2006). The expression of *Phospho1* is up-regulated in mineralising cells by approximately 100 fold compared to non-mineralising cells (Houston *et al.* 1999). PHOSPHO1 is important in the initial stages of mineralisation. TNAP knockout mice still produce hydroxyapatite and calcium phosphate crystals within their matrix vesicles, despite having a bone mineralisation defect. It has been shown that PHOSPHO1 hydrolyses the phosphate groups from both phosphoethanolamine and phosphocholine to produce ethanolamine and choline (Roberts *et al.* 2004). Phosphoethanolamine and phosphocholine are found within the membrane of matrix vesicles, the phosphate groups that PHOSPHO1 hydrolyses from them contribute towards the initial formation of hydroxyapatite within matrix vesicles (Yadav *et al.* 2011).

Phospho1^{-/-} mice have hypomineralised bones that are prone to spontaneous fracture (Huesa *et al.* 2011; Yadav *et al.* 2011). Experiments using *Akp2 / Phospho1* double knockout mice have shown that PHOSPHO1 plays a key role in the generation of phosphate and formation of hydroxyapatite within matrix vesicles, whereas TNAP predominantly undertakes this role outside of the matrix vesicles (Yadav *et al.* 2011). All mineralisation is impaired in *Akp2 / Phospho1* mice; these mice die by day 18 post birth (Yadav *et al.* 2011). PHOSPHO1 has also been implicated in the regulation of insulin signalling in osteoblasts (Oldknow *et al.* 2012).

Phosphate / pyrophosphate ratio

Pyrophosphate (PPi) is produced from ATP in the extracellular environment by the actions of some members of the NPP family (Terkeltaub 2001); PPi inhibits mineralisation of the extracellular matrix (Meyer 1984; Russell 2011). PPi is the body's natural "water softener", it is a physicochemical inhibitor of mineralisation that is believed to work by reducing the dissolution of hydroxyapatite crystals, by lowering the equilibrium concentrations of calcium and phosphate (Fleisch *et al.* 1966), thereby preventing the precipitation of mineral out of solution and onto bone surfaces.

In the extracellular environment ALP plays a key role in hydrolysing PPi; this results not only in the reduction of this inhibitor of mineralisation, but also releases two phosphate molecules that can contribute to the formation of hydroxyapatite. The actions of NPP and ALP are antagonistic: NPP acts to inhibit mineralisation by increasing the concentration of PPi, whereas ALP acts to promote mineralisation by hydrolysing PPi and releasing Pi (Millan 2013).

Enpp1^{-/-} mice develop soft tissue calcification, calcification of the aorta and calcification of the joints (hyperostosis); this is because the loss of NPP1 leads to a reduction in the PPi concentration and therefore less inhibition of calcification (Sakamoto *et al.* 1994; Johnson *et al.* 2003; Zhu *et al.* 2011). *Enpp1*^{-/-} mice also have significant defects in long bone mineralisation, 22 week old mice have been

shown to have reduced trabecular bone volume. This seems a counterintuitive finding, when removing an inhibitor of mineralisation it would be expected that mineralisation is increased (Mackenzie *et al.* 2012b). In humans, mutations that decrease or knock out the expression of NPP1 lead to generalised arterial calcification during infancy; severe cases usually result in death before 6 months of age (Nitschke *et al.* 2012).

Akp2^{-/-} mice lack tissue non-specific alkaline phosphatase. *Akp2*^{-/-} mice begin to display skeletal hypomineralisation at around day 6 after birth, until they die at approximately day 20 (Narisawa *et al.* 1997). The lack of TNAP results in an inability to hydrolyse PPI; this results in both an excess of this mineralisation inhibitor and a deficit in phosphate, which is required for hydroxyapatite formation (Fedde *et al.* 1999; Anderson *et al.* 2004). Osteoblasts from *Akp2*^{-/-} mice form matrix vesicles which contain hydroxyapatite crystals; however, these crystals fail to propagate and spread outside of the matrix vesicle environment, resulting in poorly mineralised bone *in vivo* (Anderson *et al.* 1997; Anderson *et al.* 2004). Mouse osteoblasts did form from *Akp2*^{-/-} precursors *in vitro*, yet they were not able to deposit mineral onto the extracellular matrix that they produced (Wennberg *et al.* 2000). In humans, TNAP is encoded by the *ALPL* gene. Hypophosphatasia is an inherited metabolic disease caused by mutations in the *ALPL* gene that reduce the activity of TNAP, leading to rickets and osteomalacia. Approximately 200 mutations in the *ALPL* gene have been found so far; some are transmitted in an autosomal recessive way, others in an autosomal dominant way (Mornet *et al.* 1998; Whyte 2010). Hypophosphatasia is characterised by hypomineralisation of bone and teeth, the severity of which can vary between mild teeth defects to perinatal and infantile death (reviewed in Orimo 2010; Whyte 2010).

Akp2^{-/-} mice have defective mineralisation of the calvaria, spine and long bones; this phenotype can be rescued by knockout of the *Enpp1* gene creating an *Akp2/Enpp1* double knockout mouse (Hessle *et al.* 2002). These double knockout mice have a normalised PPI/Pi ratio, permissive for mineralisation of the long bones, yet sufficient to inhibit soft tissue mineralisation (Hessle *et al.* 2002; Harme

et al. 2004; Murshed *et al.* 2005). However deletion of the *Enpp1* gene does not completely compensate for the knockout of *Akp2*. Although double knockout of TNAP and NPP1 restored the level of mineralisation in the calvaria and spine to levels comparable with wild types, double knockout did not fully restore the hypomineralisation defects seen in the long bones. It is believed that this is due to differences in the local levels of expression of TNAP and NPP1. The axial skeleton has been shown to have higher levels of *Enpp1* expression compared to the appendicular skeleton (Anderson *et al.* 2005b).

In order to permit the mineralisation of bone, but prevent the mineralisation of soft tissues, the PPI/Pi ratio must be finely balanced. Organ cultures of foetal chick long bones have shown that PPI can have a bimodal effect. At physiological concentrations up to 1 μM , PPI is rapidly hydrolysed by TNAP to produce two phosphate molecules, which positively contribute towards mineralisation. Concentrations of PPI greater than 1 μM inhibit mineralisation, because the excess PPI is not hydrolysed by the pyrophosphatases (Anderson & Reynolds 1973; Anderson *et al.* 2005a).

ANK

PPI is produced within cells by a number of different metabolic processes, and the hydrolysis of nucleotides. The trans-membrane transport protein, ANK, transports PPI from the intracellular to the extracellular environment; it is encoded by the progressive ankylosis gene (*ank*) (Ho *et al.* 2000). Mice deficient in the PPI transport protein ANK show defects similar to NPP1 knockout mice. They have defects in bone mineralisation and have pathological soft tissue calcification (Ho *et al.* 2000; Kim *et al.* 2010).

Factors that regulate bone cells and bone formation

The vascular system

The vascular supply to the bone can have an effect on bone cells. In a typical long bone there are three major classes of blood vessels. The nutrient artery and vein are major vessels that invade the diaphysis (shaft) of the bone and extend down its

length. Metaphyseal vessels supply blood to the diaphyseal (inner) surface of the growth plate. Periosteal vessels are incorporated into the outer surface of growing bone (Martini 1998). The vascular system is important for supplying oxygen and nutrients to the bone and removing waste metabolic products; alterations in the vascular supply can lead to bone loss and changes in bone cell activity. Hypoxia due to a reduction in the vascular perfusion of bone can be caused by many factors; these include, fracture of the bone, infection, inflammation, cigarette smoking, pulmonary disease and sickle cell anaemia (reviewed in Arnett 2010).

Oxygen tension is a major regulator of osteoclast formation. Hypoxia stimulates mouse and human osteoclast formation *in vitro* (Arnett *et al.* 2003; Utting *et al.* 2010). Osteoclast formation may be mediated by hypoxia inducible factors (HIFs) 1 α and 2 α (Knowles & Athanasou 2009). Normal human bone marrow aspirates have a pO₂ of between 44 to 47 mmHg (Harrison *et al.* 2002), healthy mandible marrow has been shown to have a pO₂ of 61 mmHg. However, measurements have shown that diseased mandible and fracture haematomas have pO₂ levels of 11 to 6 mmHg (reviewed in Arnett 2010).

Chronic hypoxia inhibits the growth, differentiation and bone forming activity of rodent osteoblasts *in vitro*. ALP activity and collagen production were both decreased in osteoblasts cultured in a 2% pO₂ environment. The decreased collagen production may have been due to the decreased expression of the oxygen sensitive enzymes prolyl-hydroxylase and lysyl oxidase (Utting *et al.* 2006).

In contrast with osteoblasts, osteocytes reside in lacunae that may be hypoxic due to their distance away from the blood supply and closed structure. It has been suggested that bone loading may result in enhanced nutrient diffusion to the osteocytes; bone unloading has been reported to result in osteocyte hypoxia (Dodd *et al.* 1999; Gross *et al.* 2001).

Hydrogen ions

Impairment of the vascular supply to bone can result in an acidotic environment forming. The vascular system is required to transport acidic waste products such as

CO₂ and lactic acid to the kidneys and lungs for excretion. Failure to do so will result in a systemic acidosis. Under hypoxic conditions the mitochondria are unable to provide the ATP the body requires. This shortfall is compensated for by an increase in glycolysis. The pyruvate produced during glycolysis is converted to lactic acid; resulting in an acidosis. It has long been known that an acidosis, of any origin, can have detrimental effects on the bone (Goto 1918).

For many years it was thought that an acidosis resulted in a physicochemical etching of the bone, resulting in mineral release (Bushinsky *et al.* 1985; Barzel 1995). It was subsequently demonstrated that a reduction in the pH of the cell culture media was necessary to activate osteoclast resorptive activity *in vitro* (Arnett & Dempster 1986; Arnett & Dempster 1987; Brandao-Burch & Arnett 2004). Acidification was shown to increase the expression of mRNA for carbonic anhydrase II (Biskobing & Fan 2000) and increase cathepsin K activity (Muzylak *et al.* 2007) by osteoclasts. This indicated that osteoclasts are key mediators of the decrease in bone quality seen in acidosis.

In vitro experiments using mouse osteoblasts found that acidifying the growth media from pH 7.5 to pH 7.1 on day 8 of culture resulted in less mineralised bone nodule formation and prevented the normal developmental increase in the expression of matrix GLA protein and osteopontin mRNAs compared to non-acidified osteoblasts (Frick & Bushinsky 1998). However, work undertaken in the Arnett laboratory yielded slightly different results. They found that acidification of the culture media from pH 7.4 to pH 6.9 resulted in the inhibition of bone matrix mineralisation by rat osteoblasts, but these osteoblasts still formed collagenous extracellular matrix. They did not find that acidification led to a decrease in matrix GLA protein and osteopontin mRNA expression, but did observe an 8 fold decrease in ALP activity by osteoblasts when the pH was reduced to 6.9 (Brandao-Burch *et al.* 2005).

Glucocorticoids

High glucocorticoid levels, due to either administration as medicines or due to pathological conditions, cause bone loss (reviewed in Weinstein 2012). Mice administered with glucocorticoids for 28 days were reported to have a decrease in their bone vasculature (Weinstein *et al.* 2010). It has also been reported that glucocorticoids act directly on osteoclasts and inhibit their apoptosis, resulting in increased bone resorption (Jia *et al.* 2006). Additionally, glucocorticoids have been reported to suppress osteoblast activity by down regulating WNT signalling (WNT signalling is discussed below) (Ohnaka *et al.* 2004).

Endocrine and paracrine regulators of bone cells

The formation and resorption of bone, and the differentiation and activity of osteoblasts, osteocytes and osteoclasts needs to be tightly regulated; a number of signalling molecules fulfil this role.

Bone morphogenetic protein signalling

The family of bone morphogenetic proteins (BMPs), were originally identified as proteins with the ability to form ectopic bone when injected subcutaneously (Urist 1965). The 20 known BMPs are members of the transforming growth factor- β (TGF- β) super-family; the other members of the TGF β family are the activin / nodal proteins (reviewed in Sieber *et al.* 2009). There are two types of BMP receptors; each type is a serine-threonine kinase (reviewed in Rosen 2006). The main functions of BMP signalling are to initiate the differentiation of MSCs towards the osteoblast lineage and to promote osteoblast activity (Gitelman *et al.* 1995; Yamaguchi *et al.* 1996). BMPs also increase chondrocyte maturation and function, increasing the expression of type II and type X collagens (De *et al.* 2001; Grimsrud *et al.* 2001).

The antagonists, noggin, chordin and gremlin inhibit the interaction of BMP with its receptors, preventing BMP signalling (Piccolo *et al.* 1996; Brunet *et al.* 1998; Hsu *et al.* 1998). BMP3 can block signalling through the type II BMP receptor. Knock-in

mice that over-expressed BMP3 were more prone to fractures; BMP3 knockout mice formed more bone than wild types (Kokabu *et al.* 2012).

When BMP signalling is blocked in early chick limbs, the condensation of mesenchymal cells fails to occur, indicating that BMP signalling is necessary for endochondral ossification and chondrocyte generation (Pizette & Niswander 2000). Knock out of BMP2 and BMP4 in a mouse model leads to a complete failure of osteoblast differentiation from MSCs (Bandyopadhyay *et al.* 2006). The knockout of BMP2 within the post natal, formed limb of a mouse resulted in an inability to initiate fracture healing and repair after trauma, yet the limbs of the knockout mice formed normally (Tsuji *et al.* 2006).

Vitamin D

Vitamin D is a major regulator of calcium and phosphate homeostasis in the body. It exists in two different forms, vitamin D3 (cholecalciferol), the animal form, and vitamin D2 (ergocalciferol), the plant form. In mammalian skin, ultraviolet B rays convert 7-dehydrocholesterol into vitamin D (Holick *et al.* 1980). Vitamin D is transported to the liver where is converted by the enzyme 25-hydroxylase into 25-hydroxyvitamin D, the major form of vitamin D in the circulation. In the kidney 25-hydroxyvitamin D is converted into $1\alpha,25$ -dihydroxyvitamin D ($1,25(\text{OH})_2\text{vitD}$) by the actions of the enzyme 25(OH) vitamin D 1α -hydroxylase. $1\alpha,25$ -dihydroxyvitamin D, also known as calcitriol is the active form of vitamin D (reviewed in Haussler *et al.* 2011).

The classical actions of $1,25(\text{OH})_2\text{vitD}$ are to increase dietary calcium and phosphate absorption by the intestine, in order to maintain their plasma concentrations. $1,25(\text{OH})_2\text{vitD}$ can have a direct action on bone cells; it has been reported to decrease proliferation and increase differentiation of human osteoblast-like cells *in vitro* (Van Driel *et al.* 2006; Atkins *et al.* 2007). $1,25(\text{OH})_2\text{vitD}$ has also been reported to indirectly stimulate mouse osteoclastogenesis by up-regulating RANKL mRNA expression by osteoblasts *in vitro* (Takeda *et al.* 1999). Inadequate vitamin D production or intake, mutations in

the vitamin D receptor, or mutations in the enzymes required for $1,25(\text{OH})_2\text{vitD}$ production leads to rickets in children and osteomalacia in adults (reviewed in Bikle 2012).

Parathyroid hormone

Parathyroid hormone (PTH) has been known for many years to play a major role in the control of calcium and phosphate homeostasis (Collip 1925). The chief cells of the parathyroid gland secrete PTH in response to a decrease in the ionised calcium concentration in blood (Potts 2005). When rats were infused with PTH *in vivo* it was reported that the RANKL / OPG ratio was increased. The number of osteoclasts seen histologically was increased and an increase in blood serum calcium also was detected (Ma *et al.* 2001).

PTH reduces the reabsorption of phosphate in the proximal convoluted tubule of the kidney (Kempson *et al.* 1995; Keusch *et al.* 1998; Traebert *et al.* 2000) and also increases renal calcium reabsorption in the ascending limb of the loop of Henle and the distal convoluted tubule (Friedman & Gesek 1993), in doing so it is able to regulate their concentrations in blood.

PTH also increases the production of the enzyme $25(\text{OH})$ vitamin D- 1α -hydroxylase in the proximal tubule of the kidney, this leads to an increase in the production of $1,25(\text{OH})_2\text{vitD}$, which increases calcium absorption by the intestines (Fraser & Kodicek 1973; Kremer & Goltzman 1982).

Intermittent PTH administration to people or animals results in an increase in osteoblast numbers and anabolic bone formation. *In vitro* experiments on rodent and human osteoblasts and osteoblast-like cells have shown that intermittent low dose PTH administration increases the formation of osteoblasts from precursors (MacDonald *et al.* 1986; Ishizuya *et al.* 1997; Schiller *et al.* 1999). Intermittent PTH administration in rodents has also been shown to down-regulate two negative regulators of the WNT signalling pathway, dickkopf-1 (DKK-1) (Kulkarni *et al.* 2005) and sclerostin (Bellido *et al.* 2005; Keller & Kneissel 2005). Down-regulation of these two factors will result in increased bone formation. Intermittent PTH

administration has also been shown to decrease the rate of *in vitro* and *in vivo* osteoblast apoptosis in rodents (Jilka *et al.* 1999; Wang *et al.* 2007).

WNT signalling

The WNT acronym is derived from the combination of the gene names *wingless* type and *int-1* (Nusse *et al.* 1991). The WNT signalling pathways can be separated into two distinct groups; the canonical pathway, in which the actions are mediated by β -catenin; the non-canonical pathway, in which the effects are independent of β -catenin.

β -catenin is an intracellular signalling molecule. In the absence of any WNT proteins binding to their receptors, β -catenin is associated with an intracellular destruction complex. This destruction complex contains axin, adenomatous polyposis coli (APC), casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3); the ubiquitin-mediated proteolysis activity of this complex degrades β -catenin and prevents signalling (Aberle *et al.* 1997). The T-cell specific transcription factor / lymphoid enhancer-binding factor (Tcf/Lef) transcription factor in the nucleus remains bound to Groucho, a transcriptional co-repressor, so it cannot affect gene expression.

When WNT proteins bind to the Frizzled (FZD) and LRP5/6 cell surface receptors a FZD-LRP5/6 co-receptor complex recruits and activates the cytoplasmic signalling protein dishevelled (Dvl) (Bilic *et al.* 2007). Dvl recruits the axin-GSK3 complex; this complex phosphorylates the LRP5/6 receptor and leads to the inhibition of the β -catenin destruction complex, resulting in increased β -catenin levels in the cytoplasm (Zeng *et al.* 2005). β -catenin then translocates to the nucleus, where it displaces Groucho, freeing the Tcf/Lef transcription factors to activate target genes (Figure 1.1) (reviewed in Monroe *et al.* 2012).

Figure 1.1. The WNT β -catenin signalling pathway

(A) Inhibition of WNT signalling. The WNT protein is prevented from binding to the Frizzled / LRP co-receptor on the surface of the cell by sFZP or Wif-1 binding to it, or by sclerostin or DKK binding to the receptor. WNT signalling is inhibited, resulting in the formation of an intracellular destruction complex containing axin, APC, GSK3 and CK1. This destruction complex promotes phosphorylation and ubiquitin mediated breakdown of β -catenin. WNT related genes remain suppressed by Groucho. **(B)** Activation of WNT signalling. The binding of WNTs to the Frizzled / LRP co-receptor lead to the phosphorylation of the receptor and the recruitment of the Dvl proteins. Dvl inhibits the formation of the destruction complex and the phosphorylation of β -catenin. The un-phosphorylated β -catenin is free to translocate to the nucleus, where it displaces the inhibitor of transcription, Groucho, and interacts with Tcf/Lef to regulate gene expression.

APC = adenomatous polyposis coli, CK1 = casein kinase 1, Dvl = dishevelled, GSK3 = glycogen synthase kinase 3, P = phosphate, sFZP = secreted frizzled related proteins, Wif-1 = WNT inhibitory factor-1. (Adapted from (Goltzman 2011; Monroe *et al.* 2012).

The non-canonical WNT signalling pathways include: the WNT/Ca²⁺ pathway, the WNT/planar cell polarity (PCP) pathway, the WNT/JNK pathway, WNT/Rho-Rac and the WNT/Ror pathways. In non-canonical signalling, WNT proteins bind to FZD or co-receptor complexes of FZD/Ror2 or Ryk. WNT proteins bind to the FZD receptors and activate Dvl, Dvl acts independently of β -catenin to affect gene transcription. WNT proteins may also bind to Ror2 and Ryk receptors on the surface of the cell. Within these pathways, WNT signalling occurs independently of Dvl and β -catenin (Monroe *et al.* 2012).

WNTs are a family of 19 secreted glycoproteins, they are classified as either canonical or non-canonical depending on their ability to mobilise β -catenin. There is a considerable amount of functional overlap between the two groups. Wnt3a is considered to be a canonical WNT, yet may also activate WNT/G-protein coupled receptors (Tu *et al.* 2007), Wnt5a is considered to be predominantly a non-canonical WNT, but under specific circumstances it may activate canonical signalling (Mikels & Nusse 2006).

The name Frizzled was used to describe the tightly coiled hairs of the *FZD*^{-/-} *Drosophila Melanogaster* fly, before the receptor was discovered (Gubb & Garcia-Bellido 1982). There are currently 10 known FZD receptors; all are 7-transmembrane domain receptors and each different FZD receptor regulates a different intracellular signalling cascade depending upon the nature of the co-receptor (Schulte 2010). LRP5 and LRP6 are low-density-lipoprotein receptors (Rey & Ellies 2010). LRP5 and LRP6 have distinct functions, LRP6^{-/-} mice die at birth, yet LRP5^{-/-} mice do not (Pinson *et al.* 2000; Kato *et al.* 2002).

WNT signalling can be antagonised in a number of different ways. Sclerostin, DKK and Wise all down-regulate WNT signalling by interacting with the LRP5/6 co-receptor complex. There are four DKK proteins; DKK1 and DKK4 always act as antagonists, DKK2 may act as an antagonist or an agonist depending on whether it is bound to kremen, a co-receptor for antagonism (Zorn 2001; Mao & Niehrs 2003; Semenov *et al.* 2008). Sclerostin is encoded by the *Sost* gene; it is produced

primarily by osteocytes (Van Bezooijen *et al.* 2004). However, sclerostin may also be produced by hypertrophic chondrocytes in the growth plate and cementocytes in teeth (Van Bezooijen *et al.* 2009; Chan *et al.* 2011). Sclerostin is a member of the Dan family of glycoproteins; like many members of this family, sclerostin can inhibit BMP signalling (Winkler *et al.* 2003). However, sclerostin's main mechanism of action is by binding to the LPR5/6 receptors and preventing WNT signalling (Semenov *et al.* 2005; Li *et al.* 2005b). Wise also belongs to the Dan family of proteins; it acts to inhibit WNTs binding to LRP5/6 and down-regulates WNT signalling (Itasaki *et al.* 2003; Ahn *et al.* 2010). Wise can also bind BMPs and inhibit their signalling (Laurikkala *et al.* 2003; Yanagita *et al.* 2004).

WNT signalling may also be antagonised by factors that bind to the WNT ligands directly, interfering with their ability to bind to receptors. The secreted frizzled related proteins (sFZPs) family has five members that have a high structural similarity with FZD, allowing them to bind to WNTs (Bovolenta *et al.* 2008). WNT inhibitory factor-1 (Wif-1) has a high structural similarity with the WNT receptor Ryk, this means that like sFZPs, it is able to bind to and inhibit WNTs (Malinauskas *et al.* 2011).

Both canonical and non-canonical WNT signalling plays a key role in the development of bone and bone cells. WNTs are required for both osteoblast and chondrocyte differentiation in the developing bone, and for the maintenance of mature bone. Knockout of *Wnt5a* is embryonically lethal in mice, *Wnt5a*^{+/-} mice have reduced bone mass. *Wnt5a* was found to down-regulate *PPAR γ* expression; this repressed the conversion of MSCs to adipocytes, so MSCs in *Wnt5a*^{+/-} mice favoured an osteoblastic lineage (Takada *et al.* 2007). β -Catenin has been shown to down regulate *Sox9* expression, the transcription factor necessary for the differentiation of MSCs into chondrocytes; WNT signalling therefore pushes MSCs towards a more osteoblastic phenotype (Akiyama *et al.* 2004). In mice and human cell cultures *Wnt3a* has been shown to increase MSC proliferation, increase the differentiation of MSCs into osteoblasts and prevent osteoblast apoptosis (Boland *et al.* 2004; Almeida *et al.* 2005; Tu *et al.* 2007).

Inactivating mutations in the LRP5 gene in humans leads to osteoporosis pseudoglioma syndrome, an autosomal recessive disorder characterised by reduced bone mass and early onset osteoporosis (Gong *et al.* 2001). Mutations in LRP5 resulting in a high bone mass have also been reported. Mutations to the LRP5 co-receptor can result in a decreased binding affinity of the WNT inhibitors DKK and sclerostin, resulting in less WNT inhibition and a greater bone mass (Boyden *et al.* 2002; Little *et al.* 2002). Mouse models with loss of function and gain of function mutations in LRP5 have similar bone phenotypes to affected people. Overall the animal studies show that LRP5 regulates bone formation by affecting osteoblast proliferation, apoptosis and the bone formation rate (Kato *et al.* 2002; Babij *et al.* 2003).

WNT signalling increases osteoblast formation and has an anabolic effect on bone; however, it may also decrease osteoclast formation via an indirect mechanism. Wnt3a was seen to act on osteoblasts *in vitro* to down regulate RANKL expression; this decrease in RANKL concentration led to a reduction in the formation of osteoclasts from precursors in osteoblast / osteoclast co-cultures *in vitro* (Spencer *et al.* 2006).

Extracellular nucleotide signalling

The nucleotide adenosine triphosphate (ATP) is a well-known unit of energy currency; however, its ability to act as an extracellular signalling molecule is less well known. The P2 receptors are located on the surface of most cells and are activated by the nucleotides: ATP, adenosine diphosphate (ADP), uridine triphosphate (UTP), uridine diphosphate (UDP) and their synthetic derivatives. The P2 receptors are subdivided into the P2X ligand gated ion channel receptors and the P2Y G-protein coupled receptors. The P2X receptors are trimeric ion channels assembled as homo- or heteromers from seven different gene products (P2X₁₋₇) (Kaczmarek-Hajek *et al.* 2012). There are eight (P2Y_{1,2,4,6,11-14}) genes encoding the P2Y receptors. Of the naturally occurring P2 receptor ligands, only ATP acts through the P2X receptors; ATP, ADP, UTP, UDP and UDP-glucose all activate one or all of the P2Y receptors. Purinergic signalling has been shown to have physiological

and pathological effects in numerous tissues (Kennedy & Burnstock 1985; Abbracchio & Burnstock 1994; Burnstock 2007). Purinergic signalling also plays a significant role in the regulation of bone cell function.

ATP may be released from cells in a number of different ways. ATP may be packaged into secretory granules along with neurotransmitters or other extracellular signal mediators and released by vesicular exocytosis. ATP is packaged into these granules from the cytoplasm by vesicular nucleotide transporter (VNUT). Alternatively ATP may be released from cells by large conductive anion channels such as the volume regulated anion channel. Other transmembrane channels such as connexins and pannexins may also facilitate release ATP. P2X7 receptor stimulation results in a large pore formation that allows ATP release (reviewed in Lazarowski 2012).

Purinoceptor signalling and osteoblasts

The expression of the P2Y receptors by osteoblasts has been studied by many groups. It has been shown that primary rat osteoblasts in culture express mRNAs for the P2Y_{1,2,4,6,12-14} receptors in a differentiation dependent manner (Hoebertz *et al.* 2000; Orriss *et al.* 2006; Orriss *et al.* 2010). In contrast, the rat osteoblast-like cell lines ROS17/2.8 and UMR 106 do not express the P2Y₂ receptor (Jorgensen *et al.* 1997; Buckley *et al.* 2001). Primary rat osteoblasts in culture have been shown to express mRNA for all seven P2X receptors (Orriss *et al.* 2010). The human osteosarcoma cells lines Mg-63, SaOS-2, OHS-4, SaM-1 have between them been shown to express P2Y_{1,2,4,6,12}, and the P2X_{2,4-7} receptors (Bowler *et al.* 1995; Maier *et al.* 1997; Nakamura *et al.* 2000; Gartland *et al.* 2001; Ihara *et al.* 2005; Alqallaf *et al.* 2009). There can be considerable variation in the expression of the P2 receptors by osteoblasts depending on the source of cells and stage of maturation (Orriss *et al.* 2006).

ATP is found in the cytoplasm of osteoblasts, and other mammalian cells, at concentrations between 2 - 5mM (Orriss *et al.* 2010; Rumney *et al.* 2012). This ATP is released by the cell into the extracellular compartment via a number of different

mechanisms. ATP may be released due to cell membrane damage or necrosis; alternatively, the ATP may exit the cell in a controlled way (Buckley *et al.* 2003; Orriss *et al.* 2013). Fluid shear stress upon *in vitro* osteoblasts has been shown to increase the rate of ATP release (Genetos *et al.* 2005). Other factors that have been shown to increase ATP release from osteoblasts *in vitro* are hypoxia (Orriss *et al.* 2009) and 1,25(OH)₂vitD3 (Biswas & Zanello 2009). ATP can be released by vesicles; when osteoblast-like cells were subjected to fluid shear stress, ATP release was inhibited by the presence of the vesicle inhibitors brefeldin A, monensin and n-ethylmaleimide (Genetos *et al.* 2005). There is also evidence that the P2X₇ receptor may mediate ATP release. HEK293 cells that were made to overexpress the P2X₇ receptors showed increased ATP release (Pellegatti *et al.* 2005). Primary rat osteoblasts grown *in vitro* released less ATP into their extracellular environment in the presence of P2X₇ inhibitors (Brandao-Burch *et al.* 2012). However, osteoblasts from P2X₇^{-/-} mice did not show any difference in amplitude or timing of ATP release compared to wild type cells *in vitro* (Li *et al.* 2005a).

Osteoblasts respond to extracellular nucleotides with a prompt increase in intracellular calcium (Kumagai *et al.* 1989; Schofl *et al.* 1992; Orriss *et al.* 2006; Orriss *et al.* 2012a). Importantly, exogenous ATP, UTP and other nucleotide analogues also potently inhibit mineralisation of bone nodules formed by osteoblasts in culture (Hoebertz *et al.* 2002; Orriss *et al.* 2007; Orriss *et al.* 2010; Orriss *et al.* 2012a). Moreover, endogenous ATP also appears to act as a significant local inhibitor of mineralisation by osteoblasts (Orriss *et al.* 2013).

It has been shown that ATP and UTP act via the P2Y₂ receptor on osteoblasts to inhibit mineralisation of deposited osteoid *in vitro*. ATP and UTP elicit this inhibitory effect by inhibiting ALP activity (Orriss *et al.* 2007). P2Y₂ receptor knockout mice skeletons have increased trabecular and cortical bone mineral content, most notably in the hind limbs (Orriss *et al.* 2007). Later work showed that ATP signalling through the P2Y₂ receptor on osteoblast-like cells increased ERK1/2, P38 mitogen activated protein kinase and JNK1 signalling (Katz *et al.* 2006; Katz *et al.* 2008).

Stimulation of the P2X receptors has also been shown to inhibit mineralisation by osteoblasts *in vitro*. Used in conjunction with receptor antagonists, the P2X₁ receptor agonist α,β -methylene adenosine 5'-triphosphate was found to inhibit mineralisation by primary rat osteoblasts *in vitro*, as did the P2X₃ agonist β,γ -methylene adenosine 5'-triphosphate and the P2X₇ agonist 2'(3')-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate (Bz-ATP) (Orriss *et al.* 2012a).

Clopidogrel is a P2Y₁₂ receptor antagonist used in the treatment of myocardial infarction and stroke; it works by preventing platelet aggregation. Exposure of primary rat osteoblasts *in vitro* to clopidogrel resulted in decreased ALP expression, decreased collagen production and inhibited the formation of mineralised bone nodules. Mice dosed with clopidogrel for four weeks had reduced trabecular bone volume in the tibia and femur compared to controls (Syberg *et al.* 2012).

Knockout of the P2Y₁₃ receptor in mice led to a decrease in both osteoblasts and osteoclasts *in vivo*; this resulted in a reduced rate of bone turnover (Wang *et al.* 2012). Follow-on work showed that the P2Y₁₃ receptor is important for the development of osteoblasts and adipocytes from MSCs. *In vitro* stimulation of the P2Y₁₃ receptor with ADP resulted in a greater number of osteoblasts forming from MSCs, knockout of P2Y₁₃ led to a greater number of adipocytes forming (Biver *et al.* 2013).

In addition, ATP acting via the P2X₅ receptors has also been shown to increase proliferation of human osteoblast-like cells *in vitro* (Nakamura *et al.* 2000). It has also been reported that ATP increased the expression of RANKL by human osteoblast-like cells *in vitro*, this elevated RANKL in turn led to increased osteoclast formation within a cell co-culture system (Buckley *et al.* 2002).

Purinoceptor signalling and osteoclasts

Nucleotides also have a direct effect on osteoclasts. It has been shown that *in vitro* mouse osteoclasts express mRNAs for the P2X_{1-5,7} receptors and the P2Y_{1,2,6,12-14} receptors (Orriss *et al.* 2010). *In vitro* human osteoclasts have been shown to express mRNAs for the P2X_{1,4,7} and the P2Y_{1,2,4,6,11} receptors (Bowler *et al.* 1995;

Buckley *et al.* 2002; Gartland *et al.* 2003a). Like osteoblasts, osteoclasts constitutively release ATP into their extracellular environment. Also like osteoblasts it has been shown that P2X₇ receptor antagonists reduce the rate of ATP release per cell, however, unlike osteoblasts, vesicle inhibitors do not affect the rate of ATP release from primary rodent osteoclasts *in vitro* (Brandao-Burch *et al.* 2012).

ATP and ADP have been shown to stimulate the formation of rodent osteoclasts from precursors *in vitro* and increase the rate of resorption per osteoclast. Using a selective agonist (2-methylthioADP) and antagonist (MRS2179), it was determined that the P2Y₁ receptor mediates the response of osteoclasts to ATP and ADP (Hoebertz *et al.* 2001).

The P2Y₆ receptor has also been shown to play a role in osteoclast function. A selective P2Y₆ agonist was shown to induce the translocation of NFκβ from the cytoplasm to the nucleus of *in vitro* rodent osteoclasts (Korcok *et al.* 2005). This increase in NFκβ caused by P2Y₆ signalling suppressed apoptosis and increased the survival time of the osteoclasts in culture (Korcok *et al.* 2005).

Stimulation of the P2X₇ receptor in macrophages has been shown to promote multinuclear giant cell formation (Chiozzi *et al.* 1997). This led to the idea that it may be important in the formation of multinuclear osteoclasts. It has been shown that the formation of osteoclasts from human peripheral blood monocytes was inhibited by an antibody blocking the P2X₇ receptor (Gartland *et al.* 2003a). In support of this observation, RAW 264.7 cells which lacked the P2X₇ receptor failed to form multinucleated osteoclast-like cells when exposed to RANKL (Hiken & Steinberg 2004). P2X₇ antagonists have also been shown to induce apoptosis in human osteoclasts *in vitro* (Penolazzi *et al.* 2005). However, in contradiction to these results, P2X₇ receptor knockout mice have been histologically shown to form osteoclasts *in vivo*, and precursors from these mice have been shown to develop into viable osteoclasts *in vitro* (Ke *et al.* 2003; Gartland *et al.* 2003b).

Ecto-nucleotidases

Ecto-nucleotidases are cell surface enzymes that hydrolyse nucleotides, these enzymes act to reduce the concentration of ATP in the extracellular compartment, and therefore modulate purinergic signalling. There are four major families: ecto-nucleoside triphosphate diphosphohydrolase (NTPdase), ALP, NPP and ecto-5'nucleotidase (eN). There are also a number of other enzymes that may metabolise nucleotides. These include prostatic acid phosphatase (PAP) (Zylka *et al.* 2008), TRAP (Mitic *et al.* 2005), the calcium activated nucleotidase (CAN) (Smith & Kirley 2006), α -sarcoglycan (Sandona *et al.* 2004) and the neural cell adhesion molecule (NCAM) (Dzhandzhugazyan & Bock 1993).

Ecto-nucleoside triphosphate diphosphohydrolase (NTPdase)

NTPdases hydrolyse ATP to ADP and then AMP with the release of Pi at each stage (Figure 1.2). NTPdases represent one of the major classes of nucleotidases; however, they are unable to hydrolyse the dinucleoside polyphosphates, ADP-ribose or AMP.

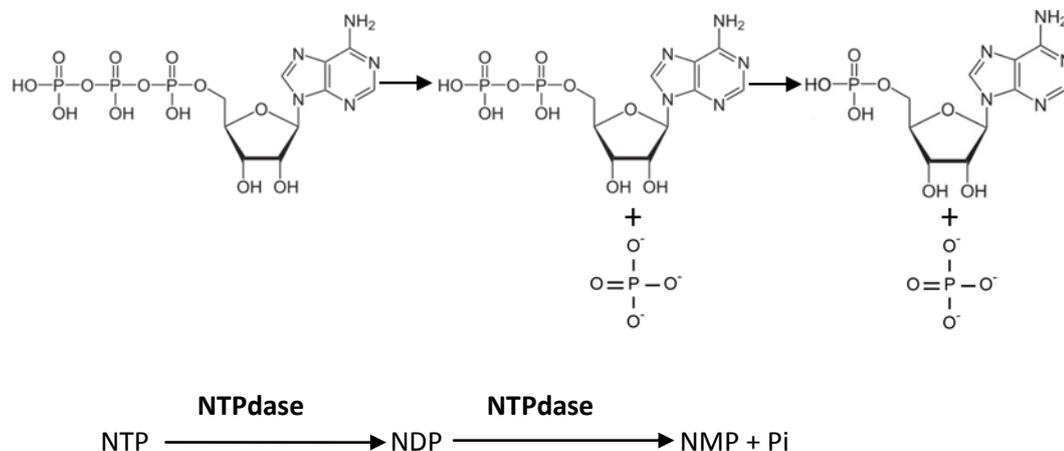


Figure 1.2. The actions of ecto-nucleotidase triphosphate diphosphohydrolase (NTPdase)

NTPdase hydrolyses nucleoside triphosphates (NTP) to nucleoside diphosphates (NDP) and nucleoside monophosphates (NMP) with the release of phosphate (Pi).

There are currently eight known NTPdases, four of these enzymes: NTPdase1-3 and NTPdase8 are cell surface bound, NTPdase4-7 are located on the membranes of intracellular organelles, NTPdase5 and 6 are found in the cytosol and in a secreted form (Grinthal & Guidotti 2006; Robson *et al.* 2006; Knowles 2011). NTPdase1-3

and NTPdase8 hydrolyse nucleoside triphosphonucleosides and diphosphates; the other NTPdases do not show activity for the full possible range of triphosphonucleosides and diphosphonucleosides; for example, NTPdase6 shows activity against UDP but not ATP (Zimmermann *et al.* 2012).

NTPdases are expressed in most tissues (Zimmermann *et al.* 2012). NTPdase1, also known as CD39, was first characterised on the surface of B-cells and activated natural killer cells (Maliszewski *et al.* 1994). An NTPdase1 knockout mouse model has been developed. The major and most notable defect in these mice is their prolonged coagulation / bleeding times (Enjoji *et al.* 1999; Pinsky *et al.* 2002). ADP increases platelet aggregation, in wild type mice NTPDase1 will break down ADP and prevent clotting. In *NTPdase*^{-/-} mice the coagulation time was prolonged because the P2Y₁ receptor, which is involved in the regulation of platelet function, became desensitised to the excess nucleotides. *NTPdase*^{-/-} mice also show disordered cellular migration of monocytes and macrophages and defective angiogenesis. This is believed to be due to impaired breakdown of nucleotides and P2 receptor desensitisation (Goepfert *et al.* 2001).

In vitro, mouse osteoblasts and the mesenchymal stem cells from which they form have been shown to express mRNA for *NTPdase1* (Roszek *et al.* 2013). Primary mouse osteoblasts have been cultured from the bone marrow of *NTPdase*^{-/-} mice *in vitro*. No difference was seen between knockout and wild type osteoblasts in the amount of mineralised bone formed and the ALP activity *in vitro*, although it was not explicitly shown that the wild type form of these cells expressed NTPdase1, (He *et al.* 2013a).

Ecto-nucleotide pyrophosphatase/phosphodiesterase (NPP)

There are seven structurally related members of the NPP, of which NPP1 is a member (Stefan *et al.* 2005). These seven isoenzymes can be divided into two families based on their structural domains and orientation within the cell membrane. Most of the NPPs are membrane bound. NPPs4-7 have a C-terminal transmembrane domain and are referred to as type 1 enzymes; NPP1 and 3 are

type 2 enzymes and have an N-terminal transmembrane domain (Nishimasu *et al.* 2012). NPP2 is different to the other NPPs in that it is secreted as a pre-pro-enzyme and only exists in the secreted form; it is not membrane bound (Jansen *et al.* 2005; Nishimasu *et al.* 2012).

The NPP1-3 enzymes contain between 863–925 amino acid residues and have a molecular mass of approximately 115 to 125 kDa. At the protein level they have approximately 40–50 % similarity (Zimmermann *et al.* 2012). NPP1, previously named PC-1, was first discovered on the plasma cell membrane of B-lymphocytes (Takahashi *et al.* 1970). Its structure was further characterised (Goding & Shen 1982) and it was then purified from murine cells (Stearne *et al.* 1985) and human cDNA libraries (Buckley *et al.* 1990).

NPPs hydrolyse nucleoside triphosphates, nucleoside diphosphates, dinucleoside polyphosphates, ADP ribose, NAD^+ , but not AMP; some NPPs hydrolyse phospholipids (Figure 1.3 -1.9) (Umezu-Goto *et al.* 2002; Zimmermann *et al.* 2012). All of the NPP enzymes possess a similar catalytic domain, but only NPP1-3 have been shown to have nucleotidase activity (Stefan *et al.* 2005). Further studies have shown that molecules other than nucleotides with a pyrophosphate or a phosphodiester bond may be substrates for NPPs (Umezu-Goto *et al.* 2002). NPP1-3 can hydrolyse ATP to AMP, with the release of pyrophosphate, or ADP to AMP with the release of Pi. NPP1-3 can also hydrolyse PPi to release two Pi molecules (Clair *et al.* 1997; Ciancaglini *et al.* 2010).

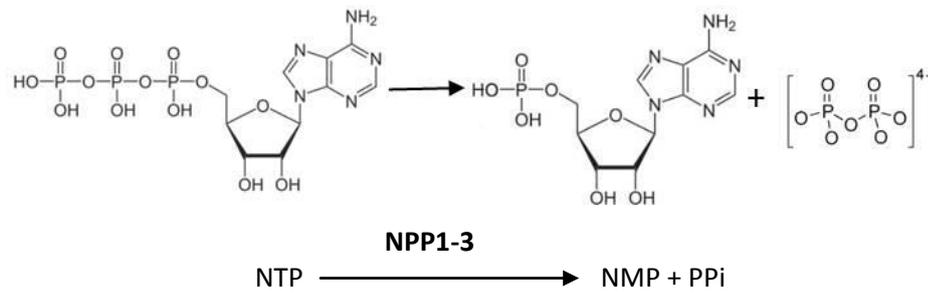


Figure 1.3. The actions of ecto-nucleotide pyrophosphatase / phosphodiesterase (NPPs) on nucleoside triphosphates

NPPs hydrolyse nucleoside triphosphates (NTP) to nucleoside monophosphates (NMP) with the release of pyrophosphate (PPi).

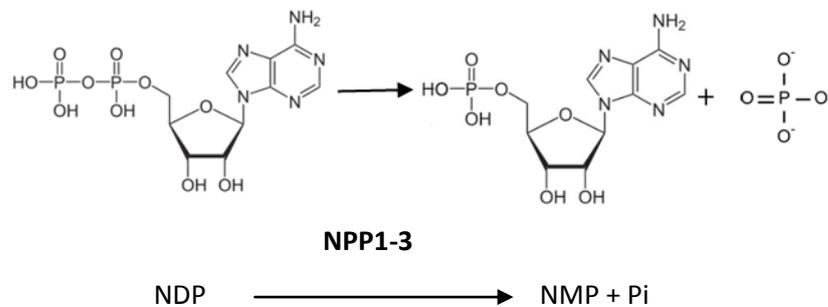


Figure 1.4. The actions of ecto-nucleotide pyrophosphatase / phosphodiesterase (NPPs) on nucleoside diphosphates

NPPs hydrolyse nucleoside diphosphates (NDP) to nucleoside monophosphates (NMP) with the release of phosphate (Pi).

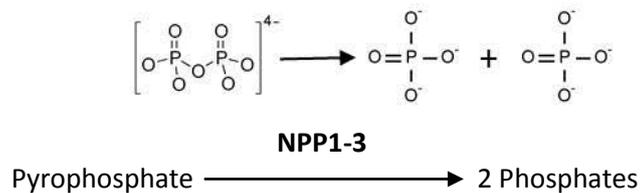
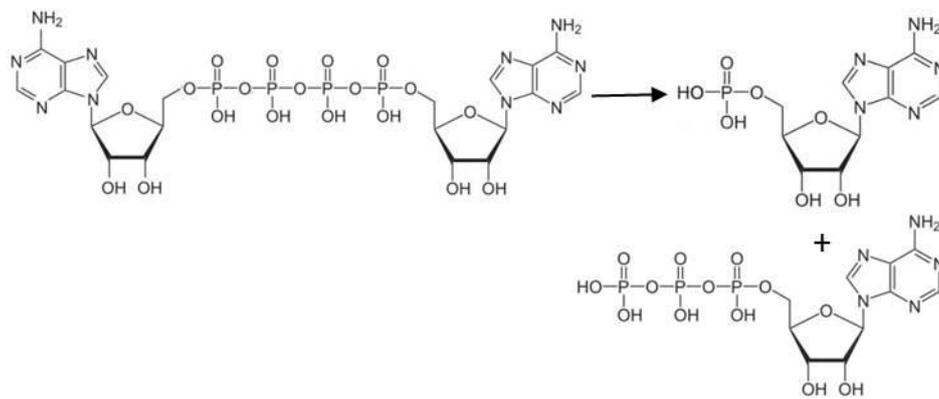


Figure 1.5. The actions ecto-nucleotide pyrophosphatase / phosphodiesterase (NPPs) on pyrophosphate

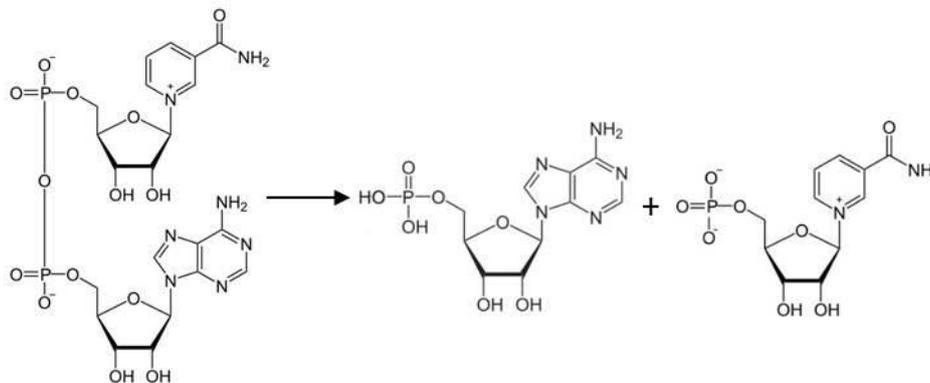
NPPs can hydrolyse pyrophosphate, releasing two phosphate molecules.

**NPP1-3**

Dinucleoside polyphosphate \longrightarrow NMP + nucleoside 5'(N-1) polyphosphate ($N_{p_{n-1}}$)

Figure 1.6. The actions of ecto-nucleotide pyrophosphatase / phosphodiesterase (NPPs) on dinucleoside polyphosphates

NPPs hydrolyse dinucleoside polyphosphates with the release of nucleoside monophosphates (NMP) and a nucleoside with the remaining phosphates attached, in this case, three phosphates (NTP).

**NPP1-3**

NAD+ \longrightarrow AMP + nicotinamide mononucleotide

Figure 1.7. The actions of ecto-nucleotide pyrophosphatase / phosphodiesterase (NPPs) on nicotinamide adenine dinucleotide

NPPs hydrolyse nicotinamide adenine dinucleotide to adenosine monophosphate (AMP) and nicotinamide mononucleotide.

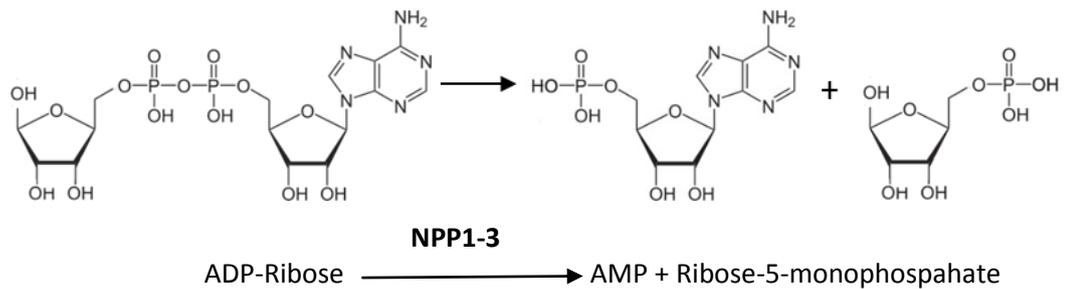


Figure 1.8. The actions of ecto-nucleotide pyrophosphatase/phosphodiesterase (NPPs) on adenosine diphosphate - ribose

NPPs hydrolyse adenosine diphosphate ribose to adenosine monophosphate (AMP) and ribose-5-monophosphate.

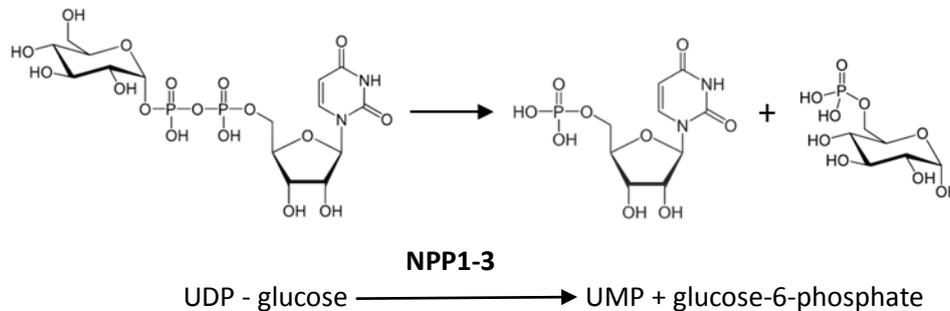


Figure 1.9. The actions of ecto-nucleotide pyrophosphatase / phosphodiesterase (NPPs) on uridine diphosphate glucose

NPPs hydrolyse uridine diphosphate (UDP) glucose to uridine monophosphate (UMP) and glucose-6-phosphate.

NPP2, also known as autotaxin, has nucleotidase activity and is a lysophospholipase-D that hydrolyses albumin-bound or membrane-bound lysophosphatidylcholine, to produce lysophosphatidic acid and choline. Lysophosphatidic acid can then act on G-protein coupled receptors (LPA1-6) to produce a cellular response (Noguchi *et al.* 2009). It has been shown *in vitro* that lysophosphatidic acid can increase tumour cell growth; *in vivo*, lysophosphatidic acid dysregulation has been shown to affect the differentiation and proliferation of neural cells and cause craniofacial dysmorphism (Umezu-Goto *et al.* 2002; Noguchi *et al.* 2009). NPP2 also hydrolyses sphingosylphosphorylcholine to produce sphingosine-1-phosphate (S1P), which has been shown to regulate angiogenesis and cell motility *in vitro* (Clair *et al.* 2003). S1P has been reported to induce mouse

osteoclast chemotaxis *in vitro* and *in vivo* by acting via G-protein coupled receptors (Ishii *et al.* 2009). It has also been reported that S1P increases human and rat osteoblast proliferation and induces heat shock protein 27 expression *in vitro* (Kozawa *et al.* 1999). *Enpp2*^{-/-} mice are not viable past day 10 of gestation; it has been assumed that this was due to a defect in lipid signalling, related to blood vessel formation (Tanaka *et al.* 2006). Although NPP2 is a weak nucleotidase compared to NPP1 and NPP3, there is evidence that it plays a greater role in the hydrolysis of phospholipids than nucleotides (Gijbers *et al.* 2003).

NPP6 and NPP7 are both choline phosphate esterases (Duan *et al.* 2003; Sakagami *et al.* 2005). NPP6 hydrolyses p-nitrophenyl phosphorylcholine but not p-nitrophenyl thymidine 5'-monophosphate, indicating it has phospholipase C activity but not nucleotide phosphodiesterase activity. NPP6 has lysophospholipase-C activity; unlike NPP2 when it hydrolyses lysophosphatidylcholine it produces monoacylglycerol and phosphorylcholine (Sakagami *et al.* 2005).

NPP7 has been shown to possess alkaline sphingomyelin phosphodiesterase (sphingomyelinase) activity, generating ceramide from sphingomyelin in the intestinal tract (Duan *et al.* 2003). It is believed that NPP7 may play a role in the pathogenesis of inflammatory bowel disease by affecting the activity of platelet activating factor with its phospholipase activity (Wu *et al.* 2006).

Ecto-5'nucleotidase (eN)

Ecto-5'nucleotidase (eN), also referred to as CD73, is a glycosylphosphatidylinositol (GPI) cell surface anchored enzyme that hydrolyses the remaining phosphate group from nucleoside monophosphate to produce phosphate and a nucleoside. EN hydrolyses ribonucleoside 5'-monophosphates and deoxyribonucleoside 5'-monophosphates including AMP, CMP, UMP, IMP, and GMP (Figure 1.10). AMP is the most effectively hydrolysed nucleotide by eN, it has much lower activity with deoxyribonucleotides as substrates (Zimmermann *et al.* 2012). ATP and ADP are competitive inhibitors of eN. These nucleotides bind to the catalytic site of eN, but

apparently without being hydrolysed, thereby blocking the binding for AMP and preventing its hydrolysis (Grondal & Zimmermann 1987).

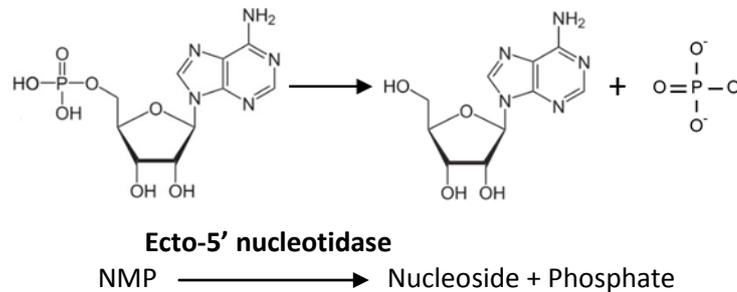


Figure 1.10. The actions of ecto-5' nucleotidase (eN)

Ecto-5' nucleotidase hydrolyses nucleoside monophosphates (NMP) to their constituent nucleoside and phosphate. Shown here is the conversion of AMP to adenosine and phosphate.

The production of adenosine from AMP is considered to be one of the key roles of eN. Dependent upon the supply of the substrate AMP, eN activity could have a significant effect on the extracellular adenosine concentration. An ecto 5'-nucleotidase knockout mouse ($eN^{-/-}$) has been developed. $eN^{-/-}$ mice were reported to be healthy, gain weight normally and have a normal immune system; however, when subjected to hypoxia, $eN^{-/-}$ mice developed vascular leakage, perivascular oedema and inflammatory infiltrates (Thompson *et al.* 2004). These symptoms were considered to be due to the lack of adenosine, the addition of adenosine receptor agonists and soluble eN partially rescued this phenotype. Further experiments showed that the $eN^{-/-}$ mice had a defective renal response to NaCl at the glomerulus due to a presumed lack of adenosine in the kidney (Castrop *et al.* 2004). It has also been shown that $eN^{-/-}$ mice have increased platelet aggregation, increased adhesion of leucocytes to the vascular endothelium and a decrease in vascular tone (Koszalka *et al.* 2004).

Alkaline phosphatase (ALP)

The alkaline phosphatase isoenzymes and their roles in P_i metabolism and mineralisation of the extracellular matrix have been discussed on page 29. ALP also plays a role in the hydrolysis of nucleotides that is intimately related to the regulation of mineralisation. ALP can hydrolyse NTP to NDP and NMP (Figure 1.11); unlike the NPPs and the NTPdases, ALP may also remove the phosphate group from NMP to produce a nucleoside and a phosphate (Figure 1.12) (Ciancaglini *et al.* 2010; Simao *et al.* 2010).

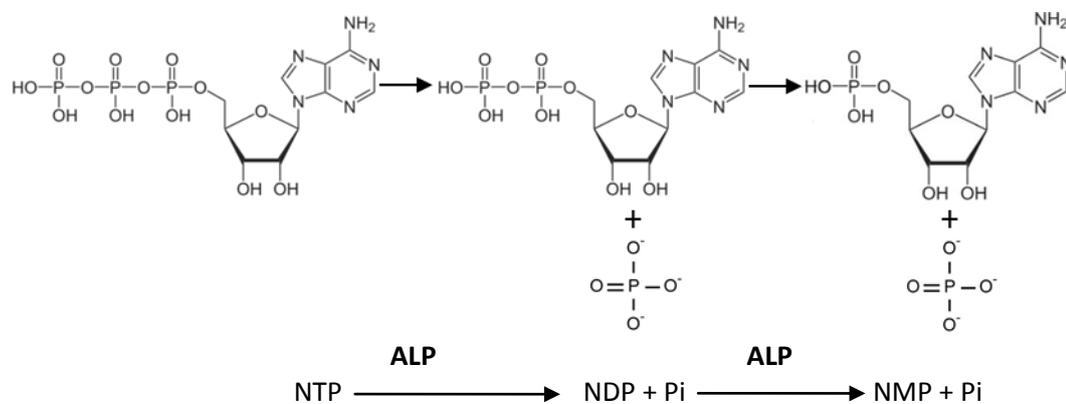


Figure 1.11. The actions of alkaline phosphatase on nucleotides

ALP hydrolyses nucleoside triphosphates (NTP) to nucleoside diphosphates (NDP) and nucleoside monophosphates (NMP) with the release of phosphate (Pi).

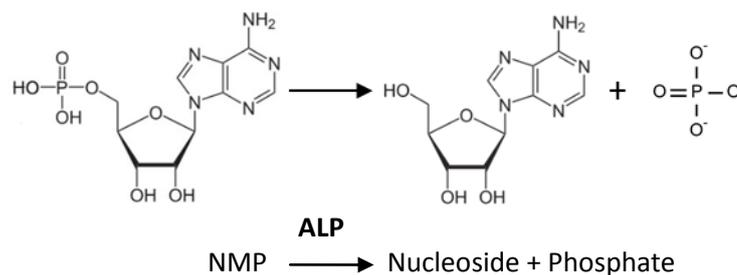


Figure 1.12. The actions of alkaline phosphatase on nucleoside monophosphates

Alkaline phosphatase (ALP) hydrolyses nucleoside monophosphates (NMP) to their constituent nucleoside and phosphate.

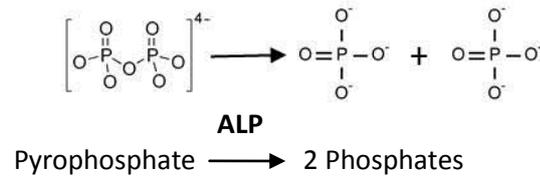


Figure 1.13. The actions of alkaline phosphatase on pyrophosphate

Alkaline phosphatase (ALP) hydrolyses pyrophosphate to produce two phosphate molecules.

The ability of ALP to hydrolyse ATP and AMP means that it can modulate P2 signalling by hydrolysing ATP, and it can influence adenosine signalling by affecting the rate of adenosine formed from AMP. It has been shown in the airway tissues, that where both eN and ATP are expressed in one tissue, eN may predominate in the breakdown of AMP at low concentrations, and ALP may predominate at high concentrations (Picher *et al.* 2003). The ALPs have a pH optimum of approximately 8; TNAP has been shown to hydrolyse ATP at both pH 7.4 and 9.4 (Demenis & Leone 2000). The neuronal like cells NG108-15 are able to hydrolyse AMP at pH 8.5, this nucleotidase activity is markedly decreased at pH 8.5. In the presence of the non-competitive ALP inhibitor levamisole, NG108-15 cells are unable to hydrolyse AMP (Ohkubo *et al.* 2000).

Nucleoside mono/di/tri-phosphate inter-conversion

Hydrolysis to adenosine is not the only fate that may befall nucleotides; they may also be re-phosphorylated to produce ATP. ATP, ADP or GDP may also be used in the production of dinucleoside polyphosphates. These molecules consist of two nucleosides joined by a chain of between two and seven phosphate molecules between their 5' carbon molecules (McLennan 2000). The enzyme glycyl-tRNA synthetase can cause the condensation of two ATP molecules, with the release of P_i to produce diadenosine 5',5'''P¹,P⁴-tetrphosphate (Ap4A) (Guo *et al.* 2009). The function of Ap4A is not truly known, but it has been implicated in a number of functions, including: regulation of the cell cycle in mouse liver cell lines (Rapaport & Zamecnik 1976), as an extracellular signalling molecule in the cardiovascular system (Stavrou 2003) and as a neurotransmitter (Pintor *et al.* 2000)

Adenosine kinase

Adenosine kinase phosphorylates adenosine to produce AMP (Figure 1.14). When the adenosine concentration in the extracellular environment is high it is transported by the ENT transporters into the intracellular environment, where adenosine kinase is located (Lloyd & Fredholm 1995).

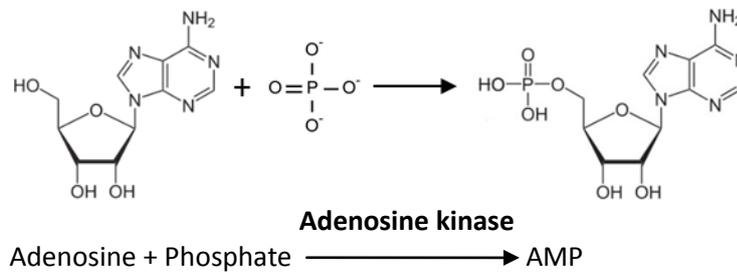


Figure 1.14. The actions of adenosine kinase

Adenosine kinase adds a phosphate onto adenosine to produce adenosine monophosphate.

Adenosine kinase knockout mice showed a delay in development from day 3 after birth, and die within approximately one week of birth of a grossly fatty liver and vascular stenosis. These mice also had reduced levels of all the adenine nucleotides and elevated levels of S-adenosyl-homocysteine. It is believed that the formation of a fatty liver in these mice is due to a decrease in transmethylation reactions, caused by a disruption in the conversion of S-adenosylmethionine to S-adenosylhomocysteine (Boison *et al.* 2002).

Adenylate kinase

Adenylate kinase is expressed in both the intracellular and extracellular compartments. In the intracellular compartment, adenylate kinase has been found in the cytosol, the mitochondria and the nucleus, it is believed to play a key role in energy transfer and distribution (reviewed in Yegutkin 2008). In the extracellular environment adenylate kinase has been shown to be expressed on: the vascular endothelium cells (Yegutkin *et al.* 2001), lymphocytes and leukemic cell lines (Yegutkin *et al.* 2002), hepatocytes and hepatic cell lines (Fabre *et al.* 2006), airway epithelia (Donaldson *et al.* 2002; Picher & Boucher 2003) and keratinocytes (Burrell

et al. 2005). Adenylate kinase transfers a phosphoryl group from ATP to AMP to produce two ADP nucleotides, by regulating AMP and ADP levels, adenylate kinase may play a role in purinergic signalling.

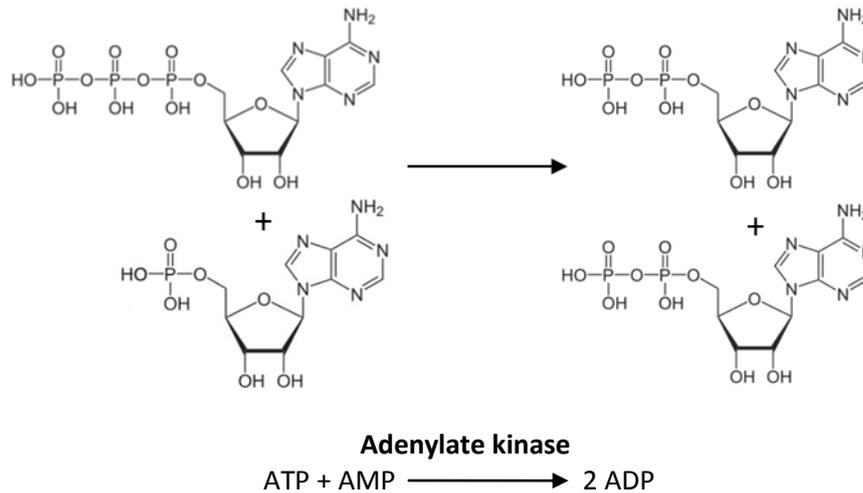


Figure 1.15. The actions of adenylate kinase

Adenylate kinase is a phosphotransferase that catalyses the conversion of ATP and AMP into two ADP molecules.

Adenylate kinase-1 knockout mice had reduced energy efficiency in their muscles. Enzyme kinetics show that these mice used ATP in a less efficient way, resulting in a greater *de novo* synthesis of ATP needed per muscle contraction because it could not be synthesised from other nucleotides efficiently (Janssen *et al.* 2000).

Nucleoside diphosphate kinase

Nucleoside diphosphate kinase (NDPK) catalyses the transfer of a phosphate group from a nucleoside triphosphate to a nucleoside diphosphate (Figure 1.16) (Yegutkin 2008).

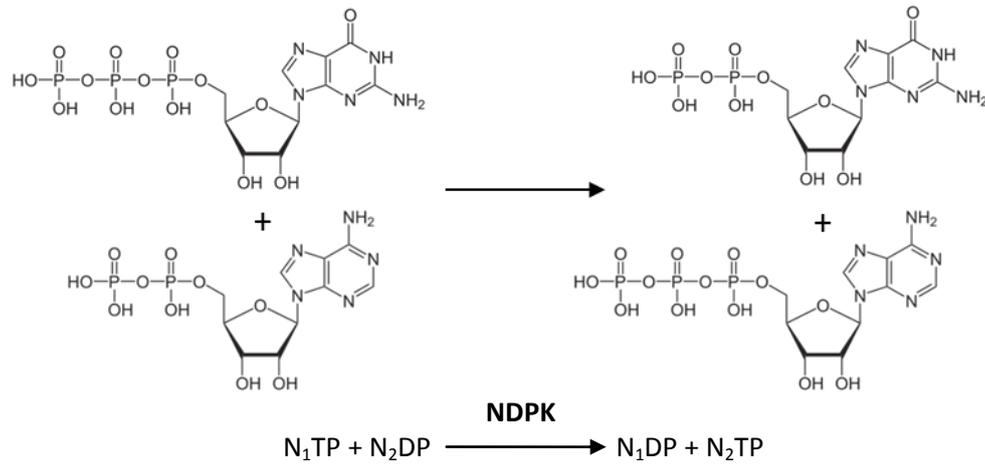


Figure 1.16. The actions of nucleoside diphosphate kinase (NDPK)

NDPK catalyses the transfer of a phosphate group from one nucleotide to another. Here NDPK transfers a phosphate group from nucleoside triphosphate 1 (N_1TP), here represented by GTP, to nucleoside diphosphate 2 (N_2DP), represented by ADP. Nucleotide 1 loses a phosphate group and nucleotide 2 gains a phosphate group, resulting in the formation of GDP and ATP.

Extracellular ATP synthesis via the NDPK enzyme has been reported (Ronquist 1968; Agren *et al.* 1974). This may involve the transfer of a phosphate group from GTP onto ADP to produce ATP (Figure 1.16). Expression of NDPK has been shown on the cell surface of a number of cell types, including, erythrocytes (Ronquist 1968), glioma and glia cell lines (Agren *et al.* 1974), astrocytoma cells (Lazarowski *et al.* 1997), vascular endothelium cells (Yegutkin *et al.* 2001), lymphocytes (Yegutkin *et al.* 2002) and hepatocytes (Fabre *et al.* 2006). NDPK plays a role in maintaining the balance of ATP within the cell; it is also involved in growth and developmental control, signal transduction and tumour metastasis suppression (Otero 2000; Okabe-Kado & Kasukabe 2003).

Adenosine triphosphate synthase

ATP synthase (also known as F_1F_0 ATP synthase) catalyses the formation of ATP from ADP and P_i . Located within the mitochondria, ATP synthase consists of two regions, the F_0 portion is within the membrane of the mitochondria, the F_1 portion (also called H^+ ATPase) is located in the matrix of the mitochondria (Yoshida *et al.* 2001).

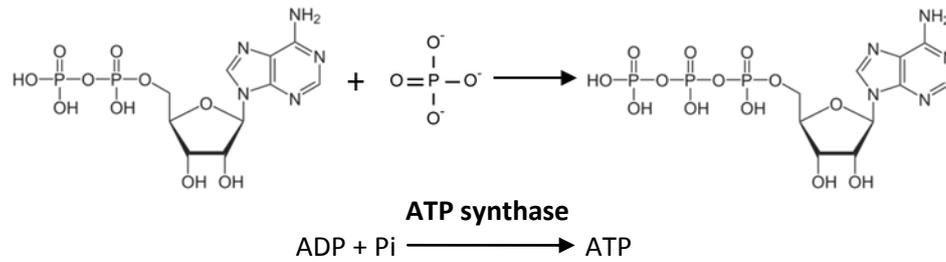


Figure 1.17. The actions of adenosine triphosphate synthase

Adenosine triphosphate synthase catalyses the formation of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and phosphate (Pi).

Although generally expressed in mitochondria, there is evidence that ATP synthase may also be an ecto-enzyme expressed on the outer surface of the plasma membrane (Das *et al.* 1994). It has been shown that at least some of the catalytic subunits of the enzyme are expressed on the cell surface of: vascular endothelial cells (Yamamoto *et al.* 2007), adipocytes (Kim *et al.* 2004), keratinocytes (Burrell *et al.* 2005) and various tumour cell lines (Chi & Pizzo 2006). However, it is not clear if this ecto-ATP synthase is enzymatically active, or if the metabolic effects seen using cells *in vitro* could be due to adenylate kinase and NDPK (Yegutkin 2008).

Adenosine and adenosine receptors

All of the above mentioned ecto-nucleotidases, ALP, NPP, NTPdase and eN act together to hydrolyse extracellular ATP and produce adenosine. Adenosine is an endogenous nucleoside widely distributed in all body fluids and tissues. It is continuously formed in both the intra- and extracellular compartments of most cells (Schubert *et al.* 1979; Zimmermann 2000). The intracellular production of adenosine is by either de-phosphorylation of AMP by ecto-5' nucleotidase / ALP or by hydrolysis of S-adenosyl-homocysteine (Broch & Ueland 1980).

Intracellular adenosine is transported to the extracellular compartment by an equilibrative nucleoside transporter, of which there are four (ENT1-4), or by a concentrative nucleoside transporter, of which there are three (CNT1-3) (Baldwin *et al.* 1999; Young *et al.* 2013). Adenosine cannot freely cross the cell membrane because nucleosides are hydrophilic molecules (Baldwin *et al.* 1999). Under normal conditions the extracellular concentration of adenosine in the human body is 30 -

300 nM (Ballarin *et al.* 1991; Fredholm *et al.* 2001); under physiological stress, extracellular adenosine can rise to concentrations of approximately 1 μ M (Fredholm & Sollevi 1981; Zetterstrom *et al.* 1982).

The physiological actions of adenosine have been studied for over 80 years after its cardiovascular actions were first demonstrated by Drury & Szent-Gyorgyi (1929). Adenosine acts via the G-protein coupled P1-receptors, found on the surface of many cell types. The P1 receptor family can be further subdivided into the A₁, A_{2A}, A_{2B} and A₃ receptors (Fredholm *et al.* 2001; Fredholm *et al.* 2011). The A_{2A} and A_{2B} adenosine receptors are predominantly stimulatory and are coupled to G_s to stimulate cAMP signalling, the A₁ and A₃ receptors are predominantly G_i coupled and act to inhibit cAMP signalling (Freissmuth *et al.* 1991; Pierce *et al.* 1992; Palmer *et al.* 1995; Olah 1997).

Adenosine plays a key role in many tissue types. In the heart, adenosine has been shown to have a key role in the control of coronary blood flow. Decreased coronary blood flow, hypoxia, or increased oxygen utilisation by the myocardial cells leads to a drop in myocardial oxygen tension (pO₂). Decreased pO₂ causes myocardial cells to release adenine nucleotides; adenosine is formed from these nucleotides in the extracellular compartment. Adenosine then acts on the coronary arterioles, causing them to dilate. This dilation results in a greater coronary blood flow and a normalisation of the pO₂ level, thereby reducing the release of nucleotides. This feedback mechanism allows adenosine to control the blood flow and pO₂ level in the heart (Berne 1963; Gerlach & Deuticke 1966). It has been shown using *in vivo* animal models that the coronary vasodilatory effect of adenosine is mediated predominantly by the A_{2A} and A_{2B} adenosine receptors (Morrison *et al.* 2002; Frobert *et al.* 2006).

The administration of adenosine either prior to myocardial ischemia or during reperfusion has been shown to reduce both the reversible and irreversible tissue damage and apoptosis this condition may cause. Adenosine A₁ receptor agonists, or receptor over-expression, reduced myocardial tissue damage and contractile dysfunction in rat hearts when they were subjected to hypoxia *in vitro* (Matherne *et*

al. 1997; Cerniway *et al.* 2002). It has also been reported that infusion of adenosine A_{2A} receptor agonists into rats *in vivo* during reperfusion decreases myocardial infarct size (Norton *et al.* 1992; Yang *et al.* 2005b).

Adenosine has been shown to have potent anti-inflammatory and pro-inflammatory functions. Adenosine reduces neutrophil mediated injury to the vascular endothelium during inflammation by inhibiting the adhesion of neutrophils to the endothelium (Cronstein *et al.* 1986). Adenosine acting via the A_{2A} receptor reduced phagocytosis by neutrophils and also reduced the production of potentially pathogenic oxygen radicals *in vitro* (Taylor *et al.* 2005). Adenosine has also been shown to reduce the inflammatory effects of macrophages by suppressing the production of pro-inflammatory chemokines and cytokines such as IL-12, nitric oxide and TNF α (Hasko *et al.* 1996; Ryzhov *et al.* 2008). Adenosine acting via the A_{2B} receptor can increase the release of the anti-inflammatory cytokine IL-10 from mice cells *in vitro* (Nemeth *et al.* 2005).

VEGF is released by macrophages; it is a potent stimulator of angiogenesis, it causes the differentiation of endothelium cells, and promotes the growth of new capillaries from existing blood vessels. It has been shown that adenosine acting via the A_{2A} receptor stimulates *in vitro* human and rodent macrophage production of VEGF, and therefore it may promote angiogenesis (Ramanathan *et al.* 2007; Ernens *et al.* 2010; Gessi *et al.* 2010).

Adenosine is neither stored nor released as a classical neurotransmitter, yet it may influence synaptic transmissions. Early work showed that adenosine inhibited neuromuscular transmission *in vitro*, as a consequence of its inhibition of acetylcholine release from the presynaptic nerves (Ginsborg & Hirst 1971). Adenosine is also implicated in the regulation of glycogen metabolism, glutamate transporters, cell proliferation and cellular swelling in astrocytes and glial cells (Dare *et al.* 2007). Adenosine has a key role in sleep homeostasis; experiments performed in cats showed that adenosine levels in the basal forebrain rise during wakeful periods, and leads to a decrease in neuronal activity, after which sleep is induced (Porkka-Heiskanen *et al.* 1997). Caffeine and other adenosine

receptor antagonists increase wakefulness (Landolt *et al.* 1995). In mice and humans the actions of adenosine and adenosine receptor antagonists on sleep are mediated through the A_1 and A_{2A} receptors (Elmenhorst *et al.* 2007; Retey *et al.* 2007).

Although adenosine is found ubiquitously throughout the human body, pharmacological intervention with adenosine is mainly directed towards the cardiovascular system, such as treatment of supraventricular arrhythmia, congestive heart failure, controlling blood pressure and attenuating reperfusion injury (Neubauer 2007; Peart & Headrick 2007).

Adenosine deaminase

Adenosine deaminase (ADA) catalyses the deamination of adenosine to inosine (Figure 1.18). It is found in the extracellular and intracellular environment (Lloyd & Fredholm 1995). ADA has a K_m of 2-100 μM , meaning it has a lower affinity for adenosine than adenosine kinase (K_m , 100nM) (Arch & Newsholme 1978; Lloyd & Fredholm 1995; Sychala *et al.* 1996).

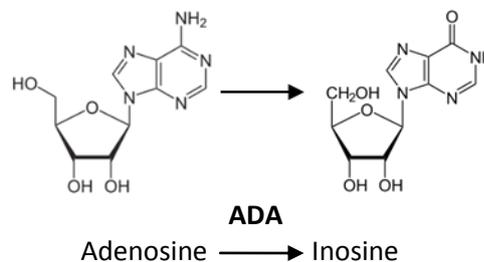


Figure 1.18. The actions of adenosine deaminase (ADA)

ADA irreversibly removes an amine group from adenosine and replaces it with a hydroxyl group, forming inosine.

ADA knockout mice died of hepatocellular impairment within 3 weeks of birth (Wakamiya *et al.* 1995). This liver phenotype was attributed to the formation of toxic 2-deoxyadenosine metabolites (Hershfield 1979); these can inhibit S-adenosylhomocysteine hydrolase and alter the ratio of S-adenosylmethionine to S-adenosylhomocysteine (Migchielsen *et al.* 1995; Wakamiya *et al.* 1995).

$ADA^{-/-}$ mice were studied at day 19 post birth and found to have a bone defect. $ADA^{-/-}$ mice had shorter femora and tibiae, and a reduced volume of trabecular bone. $ADA^{-/-}$ mice also have reduced levels of circulating RANKL and normal levels of OPG, this may lead to reduced osteoclast formation. $ADA^{-/-}$ osteoblasts *in vitro* proliferated significantly less than wild types and the expression of collagen type I and osteocalcin was reduced (Sauer *et al.* 2009).

In humans, ADA deficiency is a major cause of severe combined immunodeficiency (ADA-SCID). In patients who have ADA-SCID, the main symptoms are related to immune defects such as: lymphopenia, severely impaired cellular and humoral immunity, failure to thrive, and recurrent infections (Sauer *et al.* 2012). People with ADA-SCID may also suffer from skeletal, hepatic, renal, lung, and neurologic abnormalities (Ratech *et al.* 1985). Patients with ADA-SCID may have a short stature, femora bowing and disorganised chondrocyte arrangement in the growth plates, however, symptoms can be variable (Cederbaum *et al.* 1976; MacDermot *et al.* 1991).

Adenine and adenine receptors

Although it has long been known that nucleotides and nucleosides act as extracellular signalling molecules, it is only recently that it has been shown that the nucleobase adenine can act in this capacity. Adenine was shown to be an agonist of the Mas-related gene receptor A (MrgA) in rats, this receptor was soon renamed the rat adenine receptor (Bender *et al.* 2002), it has been suggested that adenine receptors could form the P0 class of purinergic receptors (Thimm *et al.* 2013). This would result in a family of purinoceptors as shown in Table 1. Two mouse adenine receptors have been identified, mAde1R and mAde2R, these have been shown to be activated by nano-molar concentrations of adenine (von Kugelgen *et al.* 2008). Analysis of the sequences of these rodent adenine receptors has not identified an equivalent receptor in humans. However, there is pharmacological evidence to suggest that a human adenine receptor may exist (Slominska *et al.* 2002; Gorzalka *et al.* 2005; Borrmann *et al.* 2009; Knospe *et al.* 2013).

| Receptor | Natural agonist | References |
|--|--------------------|---|
| P0 | Adenine | (Thimm <i>et al.</i> 2013) |
| P1 (A _{1A} , A _{2A} , A _{2B} and A ₃) | Adenosine | (Fredholm <i>et al.</i> 2011) |
| P2 (P2X ₁₋₇ & P2Y _{1,2,4,6,11-14}) | ATP, ADP, UTP, UDP | (Abbracchio & Burnstock 1994) |
| P3 | Adenosine / ATP | (Smith <i>et al.</i> 1997; King <i>et al.</i> 1998) |
| P4 | Dinucleotides | (Pintor & Miras-Portugal 1995) |

Table 1. The proposed purinoceptor family

More recent work casts doubt on the existence of a separate P3 receptor and suggests the actions seen *in vivo* are due to ATP sensitive P1 receptors or adenosine sensitive P2 receptors (Morikawa *et al.* 2007; Tautenhahn *et al.* 2012).

Purine salvage pathway

Intracellular ADA converts adenosine into inosine, which may then have its ribose group removed by purine nucleoside phosphorylase, converting it into hypoxanthine. Xanthine oxidase converts hypoxanthine to xanthine, the enzyme then further adds oxygen to xanthine, forming uric acid. This uric acid is then excreted by the kidneys.

An alternative fate may await purines; they can be salvaged and reused. Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) forms inosine monophosphate from hypoxanthine. HGPRT also catalyses the conversion of guanine to guanine monophosphate. Adenine phosphoribosyltransferase (APRT) is an enzyme that is functionally similar to HGPRT. APRT catalyses the formation of AMP from adenine. This AMP may be deaminated to form inosine monophosphate or converted to adenosine by eN (Figure 1.19). During *de novo* synthesis of purines, IMP is the first nucleotide formed (Berg *et al.* 2002).

Mild deficiency of HGPRT in humans leads to an over production of uric acid, kidney stones and gout. Total loss of HGPRT function in people results in Lesch-Nyhan syndrome, the symptoms of which are: mental retardation, self-harm, and the muscle conditions of dystonia (sustained torsion), choreoathetosis (contractions, twisting, writhing) and ballismus (rapid irregular movements) (Torres & Puig 2007).

Aims

The work in this thesis relates to the topic of tissue mineralisation and its control, with particular emphasis placed on the role of the hydrolysis products of ATP. I investigated what effects the products of ATP breakdown have on bone and tissue mineralisation, independent of nucleotide signalling through P2 receptors.

- In Chapter 3, I address the question of what role do NPP1, an enzyme that hydrolyses ATP, and PPi, the hydrolysis product, play in the maintenance of cortical bone structure, osteocyte lacunae size and endocrine function; I also investigated their roles in preventing soft tissue mineralisation.
- In Chapter 4, I investigated the role NPP1 plays in regulating osteoblast and osteoclast function and formation, with particular emphasis on the latter cell.
- In Chapter 5, I studied the effects of adenosine, a hydrolysis product of ATP, on rodent osteoblasts and osteoclasts *in vitro*.
- In Chapter 6, I examined the actions of sclerostin on rodent osteoclasts and osteoblasts *in vitro*, and show a potential link with NPP1, nucleotide signalling and nucleotide hydrolysis.

Chapter 2

Materials and methods

Reagents

All tissue culture and molecular biology reagents were purchased from Life Technologies (Paisley, UK), unless stated otherwise. Chemical reagents and adenosine were purchased from Sigma Aldrich (Pool, Dorset, UK). 2-chloroadenosine and ATP were purchased from Tocris (Bristol, UK). Nucleotides and nucleosides were stored protected from light as per the manufactures instructions and solubilised in PBS. Sclerostin and an anti-sclerostin antibody were provided by Amgen (Thousand Oaks, California, US).

Transgenic animals

Enpp1^{-/-} mice were generated by Dr José Luis Millán and colleagues (Sandford Burnham Institute, La Jolla, US) (Sali *et al.* 1999). A breeding colony of *Enpp1*^{+/-} was maintained within the UCL Biological Services animal facility. Genotyping was performed by Mr Stuart Martin at the UCL genotyping service. *Sost*^{-/-} bone marrow was provided by Amgen (Thousand Oaks, California, US).

Cell culture

Rat and mouse calvarial osteoblast culture

Primary rat or mouse osteoblasts of calvarial origin were obtained by sequential digestion of the calvarial bones dissected from 2 day old Sprague-Dawley rats or C57BL/6 - 129/SvTerJ crossed mice. In this three-step process, calvariae were digested using 0.25% trypsin for 10 minutes, followed by 0.2% collagenase in Hank's buffered salt solution (HBSS) for 30 minutes and finally 0.2% collagenase in HBSS for 60 minutes all at 37°C. The first two digests were discarded and the remaining rat cells were suspended in Dulbecco's modified essential medium supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml

streptomycin, 0.25 µg/ml amphotericin (mixture abbreviated to DMEM). Mouse cells were obtained by an identical digestion procedure and then suspended in α -modified essential medium supplemented with 10% FCS, 70 µg/ml gentamicin, 50 U/ml penicillin, 50 µg/ml streptomycin, 0.125 µg/ml amphotericin (mixture abbreviated to α -MEM). Rat and mouse cells were cultured for 4 days in 75 cm² or 25 cm² flasks in a humidified atmosphere of 5% CO₂ at a temperature of 37°C until confluent. Upon confluence rat cells were cultured into 6, 12 or 24 well trays in DMEM further supplemented with 2 mM β -glycerophosphate, 50 µg/ml ascorbate and 10 nM dexamethasone (mixture abbreviated to supplemented DMEM) (Orriss *et al.* 2012b) at a cell density of 1 x 10⁵, 5 x 10⁴, or 2.5 x 10⁴ cells per well respectively. When confluent, mouse cells were cultured into 6 well trays in α -MEM further supplemented with 2 mM β -glycerophosphate and 50 µg/ml ascorbate (mixture abbreviated to supplemented α -MEM) at a density of 1 x 10⁵ cells per well. Half media changes were performed every third day of culture. The culture media was supplemented with adenosine, 2-chloroadenosine, ATP, sclerostin, anti-sclerostin antibody or phosphate buffered saline (PBS) (for controls) when the cells were cultured into plates and at each media change. Experiments were terminated by fixing the cells in 2% glutaraldehyde buffered in PBS for 5 minutes. α -MEM contains phenol red; the effects this has on oestrogen and P2 receptors was considered.

Rat bone marrow osteoblast culture

Primary rat osteoblasts of bone marrow / stromal cell origin were obtained by dissecting the long bones from 6 week old Sprague-Dawley rats. The epiphyses were cut across and the marrow was flushed out of the bones using PBS. The collected cells were suspended in α -MEM within a 75 cm² flask at 37°C and 5% CO₂. After 24 hours the α -MEM was removed and all cells that had not adhered to the wall of the flask were discarded; the adherent stromal cells were cultured for a further 2 days in fresh α -MEM until confluent. Upon confluence cells were cultured into 6 well trays with supplemented α -MEM at a density of 1 x 10⁵ cells per well. Experiments were terminated by fixing the cells in 2% glutaraldehyde for 5 minutes.

Mouse osteoclast culture

Primary mouse osteoclasts were formed from precursors obtained from the bone marrow of 8 and 15 week old mice. The precursor cells were incubated in a 75cm² flask containing modified essential medium (MEM) supplemented with 10% FCS, 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin, 100 nM prostaglandin E₂ and 50 ng/ml macrophage colony stimulating factor (M-CSF) within a humidified atmosphere of 5% CO₂ at 37°C. After 24 hours the non-adherent cells were collected from the flask, the stromal cells which had adhered to the flask were discarded. The cells were re-suspended in MEM supplemented with: 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin, 100 nM prostaglandin E₂ (PGE₂), 150 ng/mL M-CSF, and 3 ng/mL receptor activator of NF-κB (RANKL). Cells were seeded (10⁶) onto 5 mm-diameter ivory discs in a 96 well tray. After a further 24 hours the ivory discs were transferred into 6 well trays for a further 6 days. For the final 2 days of the culture the media was acidified to pH 6.9 by the addition of HCl to activate osteoclast resorption (Orriss & Arnett 2012). At the end of the culture cells were fixed in 2% glutaraldehyde and treated to detect the presence of TRAP activity. Cells were deemed to be osteoclasts if they were multinucleated (≥3 nuclei) and stained positive for TRAP. The number of osteoclasts per dentine disc was manually counted “blind” using transmitted light microscopy and the total plan area of resorption per disc was quantified “blind” using reflective light microscopy and dot-counting morphometry.

In vitro osteoclasts used in the *Sost*^{-/-} experiments, and the wild type controls to which they were compared, were grown from frozen bone marrow, stored at -80°C, provided by Amgen (Thousand Oaks, California, US). Bone marrow was defrosted in MEM supplemented with 20% FCS at 37°C, the cells solution was centrifuged and the cells were seeded onto dentine discs, the experiments then proceeded as per the osteoclast experiments stated above.

Mouse osteocyte-like cell culture

This primary mouse osteocyte isolation method is based on previously published methods (Van Der Plas & Nijweide 1992; Stern *et al.* 2012). Primary mouse osteocytes were obtained from the long bones of 15 week old C57BL/6 - 129/SvTerJ crossed *Enpp^{-/-}* and wild type mice. The long bones were dissected out of the mice, the soft tissues were removed and the marrow was flushed out from them. The remaining bones were cut into small pieces using a scalpel. This bone was digested using 0.2% collagenase for 30 min at 37°C, this process was repeated three times, the cells and digested materials obtained were discarded; the bones were washed in HBSS between each digestion step. Next, the bones were digested using 5 mM EDTA in a 1% BSA solution for 30 minutes; they were then washed in HBSS, and then digested in collagenase for 30 min as previously. The collagenase and EDTA digestion steps were repeated alternately and the bones were washed in HBSS between each digestion step. After the first eight digestion steps the cell solution and digested material obtained was discarded, these were considered to contain osteoblasts, fibroblasts and other unwanted cell types. After the ninth digestion step the cells obtained were retained and washed in HBSS. These cells were seeded into collagen coated 6 well trays at a density of 2×10^5 cells per well with α MEM. Half media changes were performed on every third day; the cells were used for experimental procedures on day 7. The first eight digestion steps were intended to remove any cells resident on the surface of the bones and hydrolyse the collagen bonds within the bone, to reveal the inner structure of the bone. The ninth digestion was intended to release osteocytes from the bone.

Quantification of *in vitro* bone nodule formation

The cell layers in osteoblast cell culture experiments at time points from 4 - 29 days were fixed in 2.5% glutaraldehyde for 5 min, then washed with 70% ethanol and allowed to air dry. The cell culture plates were imaged at 800dpi using a flatbed scanner (Epson, Hemel Hempstead, UK) in reflected light mode. The images so generated were converted to binary form using Adobe Photoshop (San Jose,

California, US) and the plan surface area of bone nodules (now appearing as black areas) were quantified by automated analysis using Image J (NIH, USA) (**Figure 2.1**).

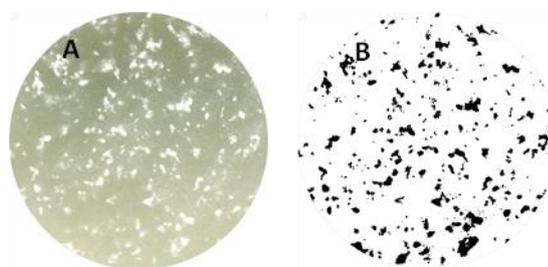


Figure 2.1. The quantification of the total area of bone formed by rodent osteoblasts *in vitro*

Images of the cell layers within the culture plates underwent an automated multistep process which allows the quantification of the area of mineralised bone formed. **(A)** Original image of bone within the cell culture plate; **(B)** final binary image used for bone quantification.

Biochemical assays

Alkaline phosphatase (ALP) activity measurement

The ALP activity of cell lysates was determined colorimetrically using a commercially available kit (Biotron Diagnostics, California, USA) and a Bio-Tek EL x800 plate reader (Fisher Scientific, Loughborough, UK). This assay measures the hydrolysis by cell lysates of a p-nitrophenyl phosphate substrate to p-nitrophenol, a yellow dye, the absorbance of which is measured at 405 nm. ALP activity was calculated using the molar absorption coefficient and a p-nitrophenol standard curve. ALP activity was normalised to the cell protein content (see below).

Total NPP activity measurement

Total NPP activity was measured spectrophotometrically using the method first described by Razzell & Khorana (1959); this method measures total NPP activity, not NPP1 activity. Cells were lysed in a buffer containing 1% triton X100 and 1.6 mM MgCl in a 0.2 M Tris base at pH 8.1. After centrifugation at 500 g for 5 minutes, the NPP activity of the supernatant was measured by its ability to hydrolyse p-nitrophenyl-thymidine 5'-monophosphate, again yielding a yellow dye

which was quantified at 405 nm. NPP activity was normalised to the protein concentration.

Measurement of intra- and extracellular ATP

Cell culture media were aspirated from the plates and cell layers were washed with PBS. Next 1 ml of serum-free DMEM was added to each well of the cell culture plate. For the experiments measuring the breakdown of ATP, this serum-free DMEM was spiked with exogenous ATP (100 nM – 1 μ M). In the experiments examining the effects of adenosine on ATP release the serum-free medium was spiked with adenosine (1 - 100 μ M). Extracellular ATP released into the serum-free medium by the cells was measured using a commercially available luciferin-luciferase based kit ('Cell Titre Glo', Promega, Southampton, UK). Luminescence was measured in a luminometer (Promega GloMax 2020) and values were normalised to cell number, quantified using an assay which measures lactate dehydrogenase (LDH) release from lysed cells. To determine the intracellular ATP concentration, cells were lysed using a 1% solution of Triton X-100. Standard curves were constructed by spiking cell culture media with ATP (100pM – 1 μ M). The coefficient of variation of the ATP assay was found to be less than 2.5% regardless of ATP concentration measured. PPI up to a concentration of 1 μ M was found to have little interfering effect on the measurement of ATP.

Protein measurement (Bradford assay)

The Bradford assay (Bradford 1976; Compton & Jones 1985) was used to measure protein levels within cell lysates against a bovine serum albumin standard, according to the manufacturer's instructions (Sigma-Aldrich, Gillingham, UK).

Cell number and viability assays

Osteoblast cell number and viability were measured using the CytoTox 96 nonradioactive cytotoxicity assay (Promega UK, Southampton UK). This assay measures the activity of LDH, a cytosolic enzyme that is released on cell lysis. LDH in the cell lysate oxidises lactate into pyruvate, generating NADH from NAD⁺, which

is then used to convert a tetrazolium salt into a red formazan product, which was quantified photometrically at 490 nm.

To determine cell viability, the LDH activity in the cell culture media was measured. All of the culture medium was removed and the cells were washed with PBS. Next 1 ml of serum free DMEM was added to each well of the cell culture plate and LDH activity measured. The cells were then lysed using 1% Triton X-100 in water and the LDH activity determined. A standard curve for the determination of cell numbers was constructed using cells seeded at 10^3 to 10^6 /well. Measurement of the total cellular LDH allows us to calculate the total cell number. The ratio of LDH in the cell supernatant to total cellular LDH allows us to calculate the cell viability.

Serum sclerostin measurement

Blood was collected from 8 and 15 week old *Enpp1*^{-/-} and wild type mice by cardiac puncture immediately after killing by cervical dislocation. Blood from 22 week old *Enpp1*^{-/-} and wild type mice was provided by Dr. Vicky MacRae (Roslin Institute, University of Edinburgh) because the development of *Enpp1*^{-/-} mice beyond 15 weeks was not permitted under UCL veterinary advice. Blood was collected into plain tubes and allowed to clot; samples were then centrifuged at 500 g for 25 min, the serum was separated and frozen at -20°C until analysis. Serum sclerostin was measured using a commercially available ELISA kit (R&D Systems, Abingdon, UK), according to the manufacturer's instructions.

Molecular Biology

Total RNA extraction, DNase treatment and complimentary DNA synthesis

Osteoblasts were cultured in 6 well trays for up to 28 days; total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. Osteoclasts were cultured on dentine discs for up to 10 days before mRNA was extracted using TRIzol. Extracted RNA was treated with RNase-free DNase I (Promega, Southampton, UK) for 30 min at 37°C to remove contaminating genomic DNA. The

reaction was terminated by heat inactivation at 65°C for 10 min. Total RNA was quantified spectrophotometrically by measuring absorbance at 260 nm. cDNA was synthesised from approximately 1µg of mRNA using Superscript 3 reverse transcriptase, oligo dT, RNasin and a deoxyribo-nucleotide mix.

RT-PCR

The cDNA produced from osteoblast and osteoclast mRNA was amplified by PCR using 1U GoTaq DNA polymerase, 1.5 mM MgCl₂, 0.8 µM nucleotide mix (Promega, Southampton, UK) and 0.5 µM primers (MWG Biotech, Ebersberg, Germany). The primer sequences used for rat and mouse RT-PCR are shown in Appendix 1. This mixture was placed in a thermal cycler and heated to 94°C for 5 minutes. Next the sample was cycled through a three different temperatures approximately 30 times (primer dependent cycle number). The temperatures cycled through were 94°C for 30 seconds, approximately 53°C for 30 seconds (the exact temperature is primer specific) and 72°C for 45 seconds. After the sample had passed through this cycling process the desired number of times it was heated to 72°C for 5 minutes. Next, electrophoresis was used to quantify the amount of cDNA present in the sample in relation to standards of known weight. 10µl of the amplified cDNA solution was placed into a 1.5% agarose gel containing 10 mg/ml ethidium bromide and electrophoresis was performed for 25 minutes at 100v. The cDNA was visualised under UV light.

Imaging techniques

Computed tomography

Micro-computed tomography (microCT) was performed on the left humerus, head and lungs of *Enpp1*^{-/-} and wild type mice. All scans were performed using a Skyscan 1172 microCT scanner (Bruker, Kontich, Belgium), and all data analyses were performed using Skyscan proprietary software.

The left humerus bone was dissected from 8 and 15 week old male and female *Enpp1*^{-/-} and wild type mice. Humerus bones from 22 week old female *Enpp1*^{-/-} and wild type mice were provided by Dr. Vicky MacRae (Roslin Institute, University of

Edinburgh) for reasons stated above. All outer soft tissues were removed from the bones; the bones were then fixed in formaldehyde for 24 hours and then transferred to 75% ethanol. After 1 week the bones were removed from the ethanol and allowed to air dry. The humerus bones were scanned using the following microCT parameters: the x-ray generation energy was 50 kv and 200 μ A; a 0.25 mm aluminium filter was placed in the X-ray path; the image pixel size was 0.9 μ m; the sample was rotated 0.3 degrees between images; and each image was averaged from two separate exposures. To analyse the cortical bone a 0.25 mm region of interest was selected 0.5 mm below the deltoid tuberosity. This was to ensure that the same region of cortical bone in all the samples was compared.

The heads were dissected from wild type and *Enpp1*^{-/-} mice, fixed in formaldehyde for 24 hours and then transferred to 75% ethanol. All soft tissues were left attached *in situ*. MicroCT scans were performed on hydrated samples with the following scan parameters: the x-ray generation energy was 50 kv and 200 μ A; a 0.5 mm aluminium filter was placed in the x-ray path; the image pixel size was 10 μ m; the sample was rotated 0.4 degrees between images; and each image was averaged from two separate exposures. All skull measurements were performed on a region of interest 4 mm in height, 2 mm from the back of the skull.

Scanning electron microscopy

The femur from the right leg was dissected out from wild type and knockout mice; the soft tissues were removed using a scalpel. The bones were then cut along their longitudinal axis using a low-speed diamond saw (Isomet, Buehler, Düsseldorf, Germany). The bone marrow and any remaining soft tissues were then digested using a protease based detergent, Tergazyme (Alconox, New York, US) at a concentration of 6% in water; this solution had a pH of 8.0. After three weeks the bones were removed from the Tergazyme solution and placed in 50 and 70% ethanol solutions for 2 hours each, and then transferred to 100% ethanol and left overnight. Finally the bones were left to air dry. Images from 22 week old animals, both wild type and knockout, were generated using a JEOL 7401 scanning electron microscope (Tokyo, Japan) at UCL, with the kind assistance of Mr Mark Turmaine.

Images of bones from 15 week old animals were kindly generated by Prof Alan Boyde using a Zeiss EVO MA10 SEM (Oberkochen, Germany) at Queen Mary, University of London. Bones from 22 week old animals were gold coated before imaging using an ion beam coater; bones from 15 week old animals were not gold coated. Image analysis of all bones was performed using Image J.

Histology

Histological analysis of the lungs, ear and the muzzle / whisker follicles of *Enpp1*^{-/-} and wild type mice was performed. The lungs were removed and fixed using the procedure detailed above in the microCT section. After microCT analysis, the lungs were processed for histology. For whisker follicle analysis, mice were culled and the muzzle was dissected away from the head and fixed in 10% neutral buffered formalin. An automated tissue processor (Leica microsystems, Wetzlar, Germany) was used to prepare all of the samples. The samples were passed through an increasing series of ethanol baths until dehydrated in 100% ethanol; they were then infiltrated with paraffin wax. The samples were then manually set within paraffin wax blocks. The set paraffin wax block was next placed face down on an ice block, when cool, 3µm sections were cut using a microtome and mounted onto microscope slides coated with poly-l-lysine. Before staining, the slides were deparaffinised using xylene, then rehydrated through a series of decreasing ethanol solutions and finally water. After staining, the samples were covered with a glass cover slip. Ear tissues were prepared for histological examination as per the muzzle / whisker tissues. The kind assistance of Dr Chris Scotton (Exeter University) is gratefully acknowledged.

Staining of tissues and cells

Alizarin red staining

The Alizarin red S stain binds to calcium. Histological sections were prepared as stated above and then immersed in a 1% w/v solution of Alizarin red S in glass Coplin jars for 5 minutes to demonstrate the presence of calcium deposits. After 5

minutes the slides were washed three times in deionised water and then counter-stained for 5 minutes with 1% w/v Fast green.

Osteoblasts cultured *in vitro* were fixed in glutaraldehyde as stated above and then washed three times with 70% ethanol. The cell monolayer was covered with a 1% Alizarin red S solution for 5 minutes. After 5 minutes the Alizarin red S was discarded and each well was washed three times with 70% ethanol and left to air dry.

ALP activity

Cell associated ALP activity was measured using a commercially-available leukocyte alkaline phosphatase kit (Sigma-Aldrich, Gillingham, UK) according to the manufacturer's instructions. Osteoblast cell layers were fixed in glutaraldehyde, as described above, washed with PBS and deionised water and incubated with the ALP reagent in the dark for 30 minutes, followed by washing with deionised water and air drying. This reaction involves the liberation of naphthol AS-BI from its phosphate ester by cell surface ALP, leading to the formation of an insoluble blue diazonium salt.

Tartrate resistant acid phosphatase (TRAP) activity

Cell-associated TRAP activity was measured using a commercially-available leukocyte tartrate resistant acid phosphatase kit (Sigma-Aldrich, Gillingham, UK) according to the manufacturer's instructions. Osteoclast-forming mouse marrow cells, grown on dentine discs were fixed in glutaraldehyde as described above and then washed with 70% ethanol. Each dentine disc was covered with the TRAP reagent and incubated in the dark at 37°C for 30 minutes. After 30 minutes the TRAP reagent was discarded and the dentine discs were washed with 70% ethanol. Cells that have TRAP activity are able to hydrolyse phosphoric acid from naphthol AS-BI; this produces a maroon / purple coloured dye deposit.

Haematoxylin and eosin staining

All histological samples were stained and examined using haematoxylin and eosin (H&E) staining, as well as other specialist stains. Haematoxylin is a basic dye that stains acidic structures purple / blue; eosin is an acidic dye that stains basic structures pink.

Statistics

Statistical comparisons were made using one-way analysis of variance (ANOVA) and adjusted using the Bonferroni method. Calculations were performed using In Stat 3 software (GraphPad, San Diego, CA). All data are presented as means \pm SEM for between 6 - 12 replicates. Results are representative of experiments performed at least three times, unless otherwise stated.

Chapter 3

***Enpp1* is important for the prevention of soft tissue mineralisation and for regulating the size of osteocyte lacunae in mice**

Introduction

Ecto-nucleotide pyrophosphatase/phosphodiesterase-1 (NPP1)

There are seven structurally related members of the NPP family, of which NPP1 is a member (Stefan *et al.* 2005). NPP1 (previously called PC-1), encoded by the *Enpp1* gene, was first discovered during the study of surface antigens on plasma cells (Takahashi *et al.* 1970), it was later purified and its gene sequence determined (Stearne *et al.* 1985; Van Driel & Goding 1987). NPP1 is a trans-membrane enzyme orientated with its amino terminal within the cytoplasm of the cell, and its carboxyl terminal and active site in the extracellular environment (Singer *et al.* 1987; Van Driel & Goding 1987). Crystal structure analysis of the NPP1 enzyme showed that its preferred substrate is ATP, and that unlike some of its family members, it is unable to hydrolyse lipids (Kato *et al.* 2012).

The development of an *Enpp1* knockout mouse was prompted by observations in the pathological condition of ossification of the posterior longitudinal ligament (OPLL) (Sali *et al.* 1999). In OPLL, the ligaments surrounding the spinal cord calcify; OPLL is commonly found in elderly Asian men (Saetia *et al.* 2011). The tiptoe walking mouse has a naturally occurring autosomal recessive mutation that results in OPLL; it also has an unusual gait when walking due joint calcification (Hosoda *et al.* 1981; Okawa *et al.* 1998). The mutation in the tiptoe walking mouse was eventually found to be due to a solitary G to T nucleobase substitution, resulting in

a nonsense stop mutation in the *Enpp1* gene (Okawa *et al.* 1998). This discovery prompted the development of the *Enpp1* knockout mouse.

NPP1 has been shown to be expressed in a wide variety of tissues including the heart, kidney, vascular smooth muscle cells, osteoblasts and chondrocytes (Terkeltaub 2001; Johnson & Terkeltaub 2005; Johnson *et al.* 2005; Nitschke *et al.* 2011). NPP1 hydrolyses ATP to produce AMP and PPI (Terkeltaub *et al.* 1994), a potent inhibitor of mineralisation (Fleisch & Bisaz 1962; Addison *et al.* 2007). Extracellular PPI is hydrolysed by ALP to produce phosphate (Pi), thereby removing the inhibitor of mineralisation (Hessle *et al.* 2002).

NPP1 and soft tissue mineralisation

In children, mutations in the *Enpp1* gene have been shown to lead to the autosomal recessive condition of generalised arterial calcification of infancy (GACI); infants with this condition often die before they are 6 months old (Rutsch *et al.* 2003). GACI has been suggested to be due to reduced circulating levels of PPI and thus decreased inhibition of aortic calcification (Rutsch *et al.* 2000; Ruf *et al.* 2005).

It has been shown that *Enpp1*^{-/-} mice have severe calcification of the aortic arch by the time they are 5 weeks old (Johnson *et al.* 2005; Zhu *et al.* 2011). Using *in vitro* mouse vascular smooth muscle cells, it was also seen that calcifying vascular smooth muscle cells up-regulate the osteocyte associated genes for sclerostin, DMP1 and E11 (Zhu *et al.* 2011).

Glycosylated end-products that accumulate in diabetic and aged tissues are detected by the receptor for advanced glycation end products (RAGE). Double knockout of *Rage*^{-/-} and *Enpp1*^{-/-} in mice reportedly reduced the *in vivo* arterial calcification seen compared to *Enpp1*^{-/-} alone, but did not restore the defects seen in the skeleton of *Enpp1*^{-/-} mice. This suggests that the *Rage-Enpp1* axis may only have effects in vascular smooth muscle cells (Cecil & Terkeltaub 2011). RAGE promotes atherosclerosis, osteoclastogenesis, calcification of smooth muscle cells, and is a key mediator of inflammation (Zhou *et al.* 2006; Ramasamy *et al.* 2008; Soro-Paavonen *et al.* 2008; Yan *et al.* 2008; Basta *et al.* 2010). Aortic explants from

Enpp1^{-/-} released less of the RAGE inhibitor, sRAGE, when stimulated by phosphate (Cecil & Terkeltaub 2011). *Ex vivo* aortas from *Enpp1*^{-/-} mice are therefore more prone to the damage caused by RAGE signalling.

Mutations in *Enpp1* have also been detected in some patients with pseudoxanthoma elasticum, an autosomal recessive disorder associated with soft tissue mineralisation of the eyes, kidneys and skin (Li *et al.* 2012; Nitschke *et al.* 2012). Pseudoxanthoma elasticum has previously been linked to defects in the ATP-binding cassette subfamily-C member 6 gene (*ABCC6*), encoding the MRP6 transport protein (Le *et al.* 2000; Ringpfeil *et al.* 2000). *ABCC6*^{-/-} mice have ectopic mineralisation in the kidneys, skin, and mineralisation of whisker follicles (Klement *et al.* 2005). Mutations in *ABCC6* have been detected in patients with GACI (Nitschke *et al.* 2012). This suggests a close genetic relationship, and common downstream mediators of calcification in these two diseases (Rutsch *et al.* 2011). Recent work has additionally described a reduction in the serum PPI concentration of *ABCC6*^{-/-} mice compared to wild types (Jansen *et al.* 2013).

Genome-wide analysis in a highly consanguineous family found that mutations in *eN* resulted in arterial and joint calcification (St Hilaire *et al.* 2011). Cultured *eN*^{-/-} fibroblasts were reported to have reduced extracellular adenosine levels, increased TNAP activity and increased PPI hydrolysis. Adenosine supplementation was reported to suppress TNAP activity in *eN*^{-/-} cells (St Hilaire *et al.* 2011).

Osteopontin is also, like PPI, a direct inhibitor of bone mineralisation, vascular smooth muscle cell mineralisation and hydroxyapatite crystal formation (Wada *et al.* 1999; Boskey *et al.* 2002). The PPI generated by NPP1 is believed to increase the expression of OPN by rat calvarial osteoblasts *in vitro* (Johnson *et al.* 2003). *In vitro* osteoblasts from *Enpp1*^{-/-} mice expressed less osteopontin than wild type osteoblasts (Johnson *et al.* 2003). Supplementing the media in which these *Enpp1*^{-/-} osteoblasts were grown with PPI restored the osteopontin expression back to levels analogous with the wild type cells (Johnson *et al.* 2003). Later work showed that *Enpp1*^{-/-} mice have a decreased serum osteopontin concentration, *Akp2*^{-/-} mice have an increased serum OPN concentration, and *Akp2*^{-/-} *Enpp1*^{-/-} double knockout mice

have levels of serum osteopontin similar to wild type mice (Harmey *et al.* 2004). Osteopontin also promotes bone resorption by osteoclast, by acting as a binding site for $\alpha v\beta 3$ integrins (Yoshitake *et al.* 1999; Ihara *et al.* 2001).

NPP1 and bone mineralisation

Hessle and colleagues studied the effects of gene deletion on the calvariae of 20 day old mice using histology and staining techniques. They found that knockout of *Akp2*, the mouse tissue non-specific ALP gene, led to hypomineralisation of the calvariae, compared to wild type mice (Hessle *et al.* 2002). Double knockout of *Akp2* and *Enpp1* in mice rescued the calvarial hypomineralisation phenotype. Knockout of *Enpp1* alone did not have any effect on the mineralisation of the calvariae in 20 day old mice (Hessle *et al.* 2002). Hessle *et al.* also studied the spines of the 20 day old knockout mice. They found that knockout of *Enpp1* led to pathological mineral deposits in the vertebrae, which were again corrected by double knockout with the *Akp2* gene. Knockout of *Akp2* alone led to decreased mineralisation of the vertebrae (Hessle *et al.* 2002). Hessle *et al.* found that osteoblasts grown in culture from *Enpp1*^{-/-} mice produced less PPI than wild type cells. Extracellular PPI levels in osteoblast cultures from *Akp2*^{-/-} mice were greater than in wild type cultures but this difference was eliminated in cultures from *Enpp1/Akp2* double knockout mice (Hessle *et al.* 2002). These results indicated that NPP1 and TNAP have antagonistic actions that are central to the control of mineralisation. Similar experiments were performed on the metatarsals and tibia bones; however, knockout of *Enpp1* did not completely rescue the hypomineralisation phenotype of the bone, as it did in the calvaria (Anderson *et al.* 2005b). It was hypothesised that these site-specific differences in mineralisation were due to local differences in TNAP and NPP1 expression.

Using microCT analysis it was reported that *Enpp1*^{-/-} mice have reduced mineralised bone volume in the tibiae and femora at 6 and 22 weeks. *Enpp1*^{-/-} mice were also reported to have a lower body weight and shorter femurs (Mackenzie *et al.* 2012b). It was surprising that the loss of NPP1 and a reduction in PPI, which is an inhibitor of mineralisation, would lead to a reduction in mineralised bone

volume. It was hypothesised that the knockout of NPP1 resulted in a reduction in the PPi concentration, which cumulated in there not being enough PPi to provide the Pi required for normal mineralisation (Anderson *et al.* 2005b; Mackenzie *et al.* 2012a). However, both the tiptoe walking mouse and the *Enpp1* knockout mouse have been shown to have hyperostosis of the spine and joints (Okawa *et al.* 1998; Sali *et al.* 1999).

NPP1 and osteocyte lacunae

It is widely accepted that osteocytes play an active role in regulating mineral accretion and removal from lacunae (Bonewald 2011; Atkins & Findlay 2012; Arnett 2013b). This topic and the wider biology of osteocytes were covered in detail in the introduction.

Aims

Taken together, the previous studies discussed above all indicate that NPP1 and PPi are important for the maintenance and formation of bone, and in preventing soft tissue mineralisation. In order to further elucidate this role; the aims of the work in this chapter were to:

- Investigate any previously unreported soft tissue mineralisation in mice due to *Enpp1*^{-/-}.
- Determine if cultured primary osteocyte-like cells express *Enpp1* and release ATP.
- Examine the cortical bone of *Enpp1*^{-/-} mice in greater detail than has been previously reported and determine if NPP1 and PPi are important in maintaining the size of the osteocyte lacunae. I hypothesise that PPi produced by NPP1 on osteocytes is involved in maintaining the lacunae.
- Investigated whether the circulating sclerostin concentration in mice, which is determined by osteocytes, is influenced by the knockout of *Enpp1*.
- Examine the skulls of *Enpp1*^{-/-} mice to determine if a reduction in PPi has any effect on intramembranous bone formation.

Results

Enpp1^{-/-} mice weigh less than wild type mice

Female *Enpp1*^{-/-} mice weighed approximately 12% less than age and sex matched wild types by 8 weeks. However, wild type and *Enpp1*^{-/-} mice increased in weight by approximately 18% between 8 and 15 weeks of age.

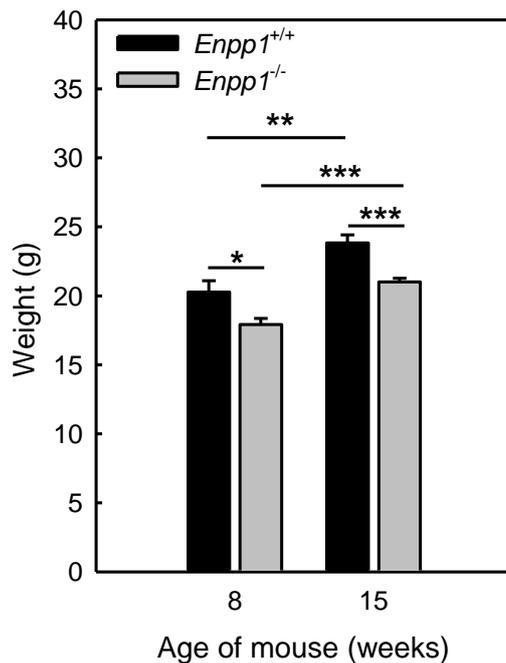


Figure 3.1. *Enpp1*^{-/-} mice have a lower body weight than wild type mice

Results shown are from female mice. Male wild type and *Enpp1*^{-/-} mice show a similar trend in weight. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; $n = 9$).

Enpp1^{-/-} mice exhibit ectopic mineralisation and hyperostosis in the spine, knee joints and paws

As previously observed, *Enpp1*^{-/-} mice have defective mineral deposition (Hessle *et al.* 2002; Anderson *et al.* 2005b; Mackenzie *et al.* 2012b). In the present study microCT was used to visualise the aberrant mineralisation in these mice in greater detail. In *Enpp1*^{-/-} mice pathological mineralisation was observed between the vertebra (**Figure 3.2A & B**), within the knee joint capsule (**Figure 3.2C & D**), and in the capsule surrounding the joints of each digit on all four paws (**Figure 3.2E & F**). *Enpp1*^{-/-} mice were unable to grip the bars of their cages due to inflexibility in their fingers and toes caused by ectopic mineralisation.

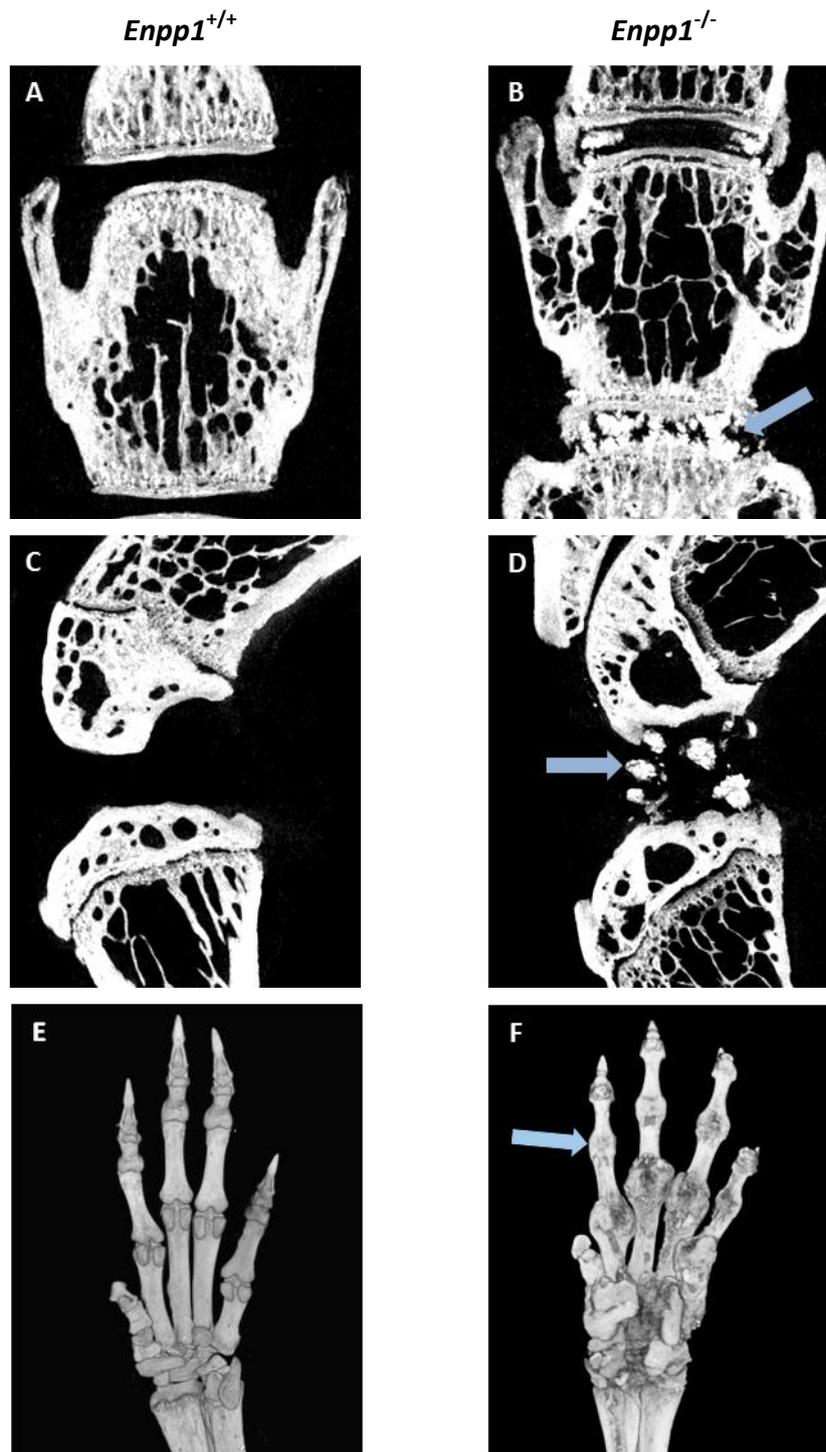


Figure 3.2. Pathological mineralisation of the vertebra, knee and paw of *Enpp1*^{-/-} mice
 MicroCT images of the 3rd lumbar vertebra (A, B), knee joints (C, D) and paws (E, F) of wild type and *Enpp1*^{-/-} mice. Images are transverse sections (A, B, C, D) and 3D models (E, F). Dystrophic mineralisation is evident between the vertebrae (B), within the knee joint (D) and surrounding the joints of the paw (F) in *Enpp1*^{-/-} mice (blue arrow). Note: inflexibility of *Enpp1*^{-/-} toe joints.

Mineralisation of whisker follicles in *Enpp1*^{-/-} mice

The heads were dissected from 8, 15 and 22 week old *Enpp1*^{-/-} and wild type mice. The heads, including all attached soft tissues were fixed in neutral buffered formalin (NBF) and scanned by microCT. Surprisingly, microCT analysis showed that from week 8 onwards, all *Enpp1*^{-/-} mice examined exhibited a striking x-ray opaque substance around their whisker follicles. In the configuration used for this investigation, the x-ray energy of the microCT scanner used does not allow the visualisation of soft tissues, only hard mineral deposits are detected. This indicates that the whisker follicle was mineralised. This mineralisation was sub-dermal and not visible to the naked eye. No whisker follicle mineralisation was evident in *Enpp1*^{+/+} mice (**Figure 3.3**).

22 week old *Enpp1*^{-/-} and wild type mice had the tissue across their left premaxilla bone of the skull dissected away and fixed in neutral buffered formalin (this is the tissue from which most of the whiskers protrude). Alizarin red histological staining showed that the collagen rings surrounding the whisker follicles in *Enpp1*^{-/-} mice contained a calcium mineral. There was no other mineralisation detected in this tissue, the smaller hair follicles that do not have a surrounding collagen ring were not mineralised (**Figure 3.4**).

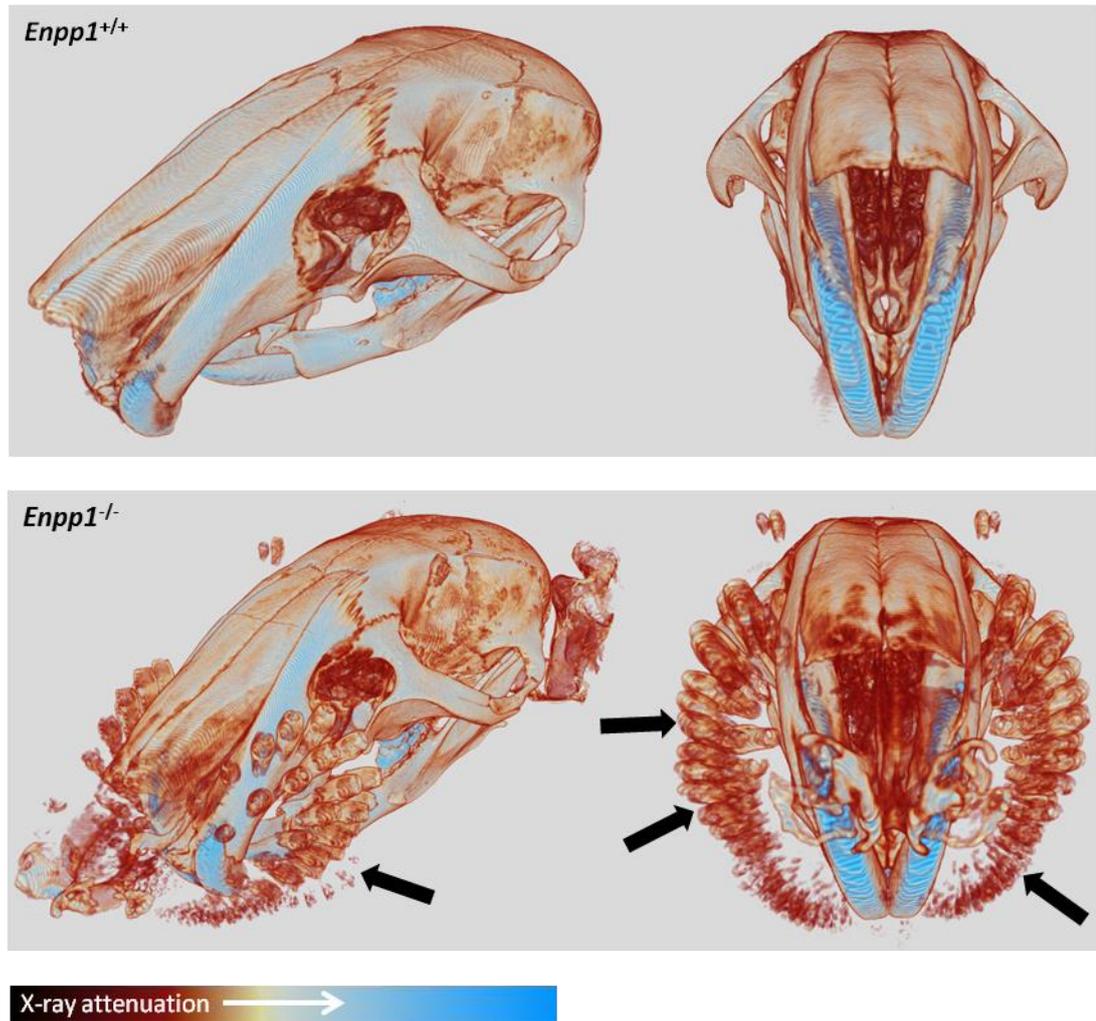


Figure 3.3. MicroCT imaging of mineralised whisker follicles of *Enpp1*^{-/-} mice

Coronal microCT images of the non-embedded hydrated heads with all of the soft tissues still attached; 3D reconstructed images shown are representative of 22 week old animals and have false colour added based on x-ray attenuation (see scale). Mineralised whisker follicles on the *Enpp1*^{-/-} mouse are indicated by the arrows. The enamel on the incisors is dense and appears blue; the mineralisation around the whisker follicle is of a similar density to bone.

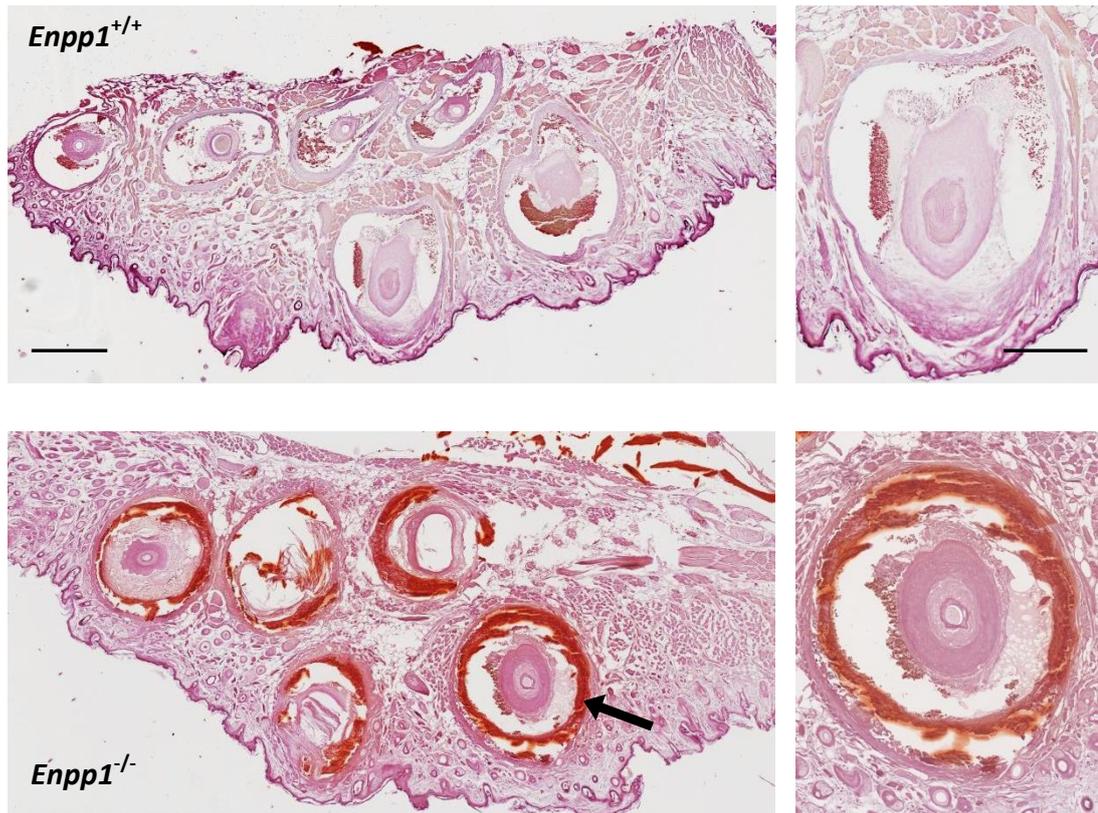


Figure 3.4. Mineralised whisker follicles in *Enpp1*^{-/-} mice

Sections (3 μm) across the soft tissue over the premaxilla of wild type and *Enpp1*^{-/-} mice (22 weeks), stained with alizarin red and fast green. Mineralisation of the collagenous sheath around the large whisker follicles of *Enpp1*^{-/-} animals is indicated by arrows. (Scale bars; left, 0.5 mm; right, 0.25mm)

***Enpp1*^{-/-} mice show tracheal mineralisation**

The tracheas of *Enpp1*^{-/-} mice were examined to determine if this large collagen containing structure was mineralised. The tracheas were dissected from 15 week old wild type and *Enpp1*^{-/-} mice and fixed in NBF. MicroCT analysis showed that the cartilage rings of the tracheas from *Enpp1*^{-/-} mice had greater x-ray attenuation than the cartilage rings of the tracheas from wild type mice. This suggested that the tracheal rings of *Enpp1*^{-/-} mice were mineralised (**Figure 3.5**).

Histological examination using alizarin red staining showed that the cartilage rings of the tracheas of *Enpp1*^{-/-} mice contained a calcium containing mineral; wild type mice tracheas were not mineralised (**Figure 3.5**).

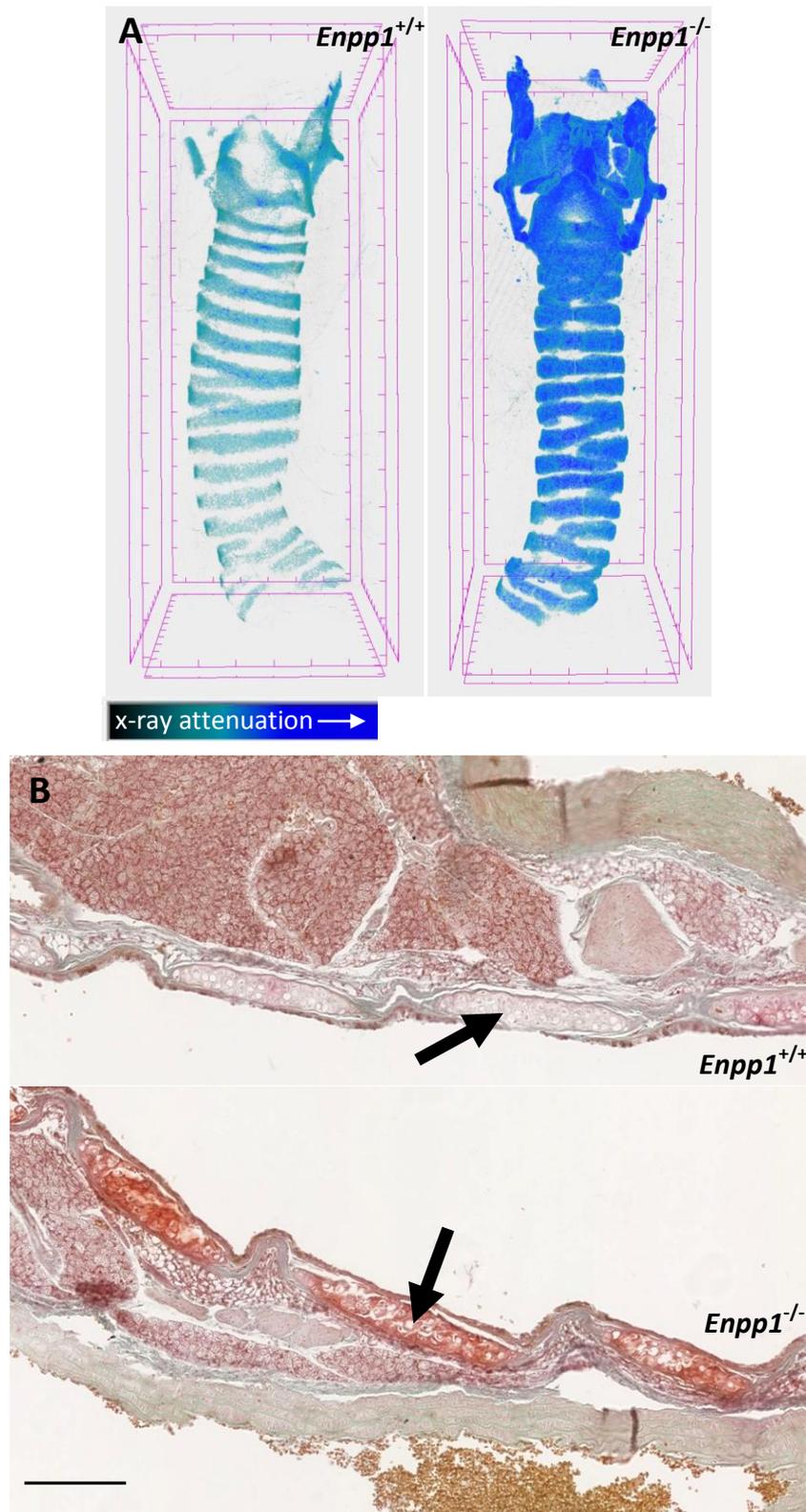


Figure 3.5. Tracheal mineralisation in *Enpp1*^{-/-} mice

Tracheas were dissected out of wild type and *Enpp1*^{-/-} mice and fixed in NBF. **(A)** The tracheas were scanned by microCT. Scale bar A = 500 μ m per mark. **(B)** Alizarin red stained histological sections (3 μ m). MicroCT shows that the tracheas of *Enpp1*^{-/-} mice attenuate x-rays more. The arrow points to the cartilage rings of the trachea in cross section, which have stained red for calcium in the *Enpp1*^{-/-} mouse. Scale bar B = 250 μ m.

Ear pinna mineralisation in *Enpp1*^{-/-} mice

The heads from 8, 15 and 22 week old *Enpp1*^{-/-} and wild type mice were scanned using microCT with all soft tissues still attached. MicroCT analysis showed that from 8 weeks onwards, all *Enpp1*^{-/-} mice had greater x-ray attenuation in the pinna of the ear (**Figure 3.6A**). Histological examination using alizarin red staining showed that the hyaline cartilage within the pinna of the ears of *Enpp1*^{-/-} but not wild-type mice contained a calcium mineral (**Figure 3.6B**).

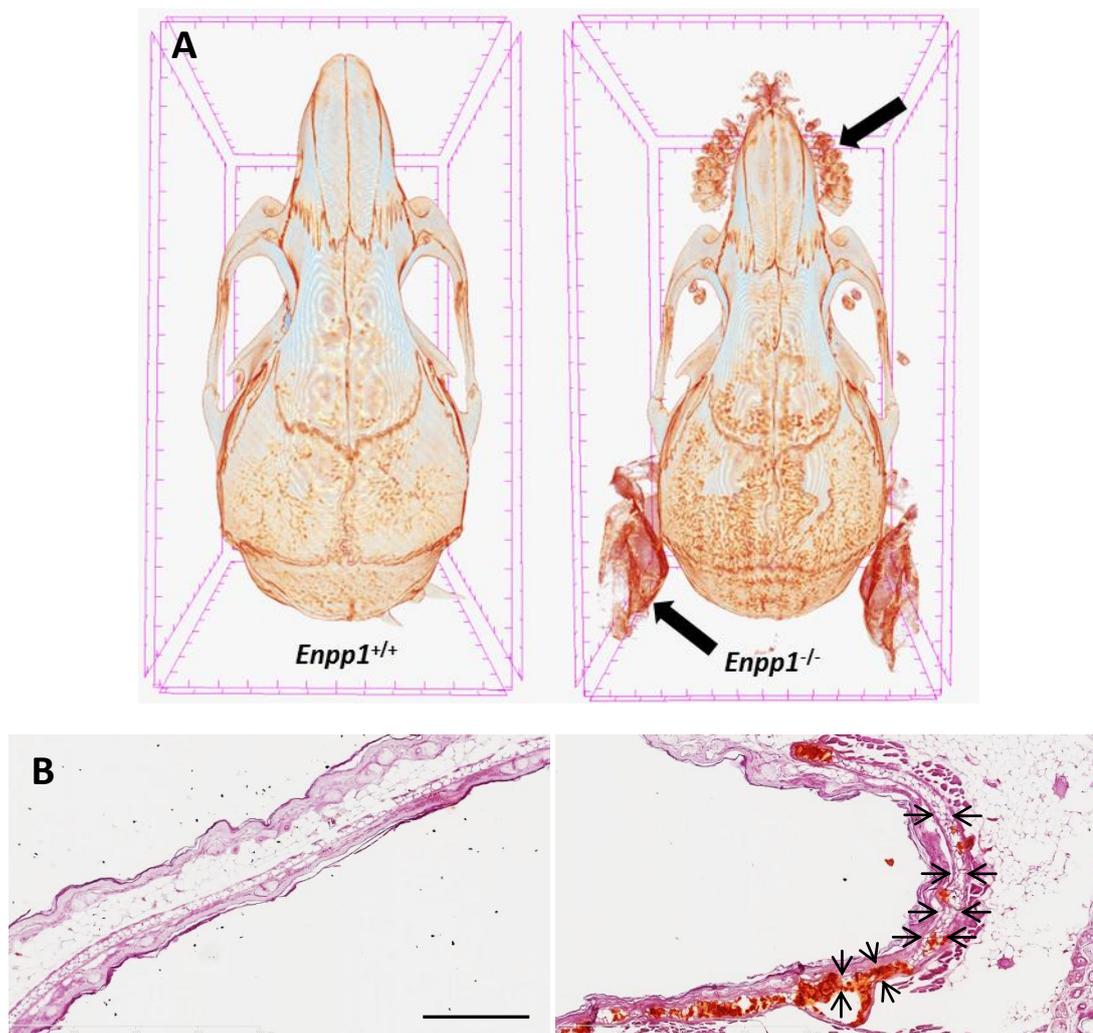


Figure 3.6. Mineralised ear pinnas in *Enpp1*^{-/-} mice

Wild type and *Enpp1*^{-/-} mice heads were fixed in NBF. **(A)** The heads were scanned by microCT. The arrows point to the mineralised ears and whiskers. Scale bar = 1 mm. **(B)** The ears were then sectioned for histology (3 μ m), and stained with alizarin red. The arrows point to the cartilage within the ear pinna, which has partially stained red, indicating the presence of calcium deposits in the *Enpp1*^{-/-} mouse ear. Scale bar B = 250 μ m.

Mouse primary osteocyte-like cells express mRNA for *Enpp1* *in vitro*

Mouse osteocyte-like cells were extracted from the long bones of 15 week old wild type mice using collagenase and EDTA, and grown *in vitro* for 7 days within a collagen coated plate. Microscopic examination revealed that these cells had the characteristic dendritic processes of osteocytes (**Figure 3.7A**). RT-PCR showed that these cells express mRNA for the osteocyte specific gene *DMP1* and mRNA for *Enpp1* (**Figure 3.7B**).

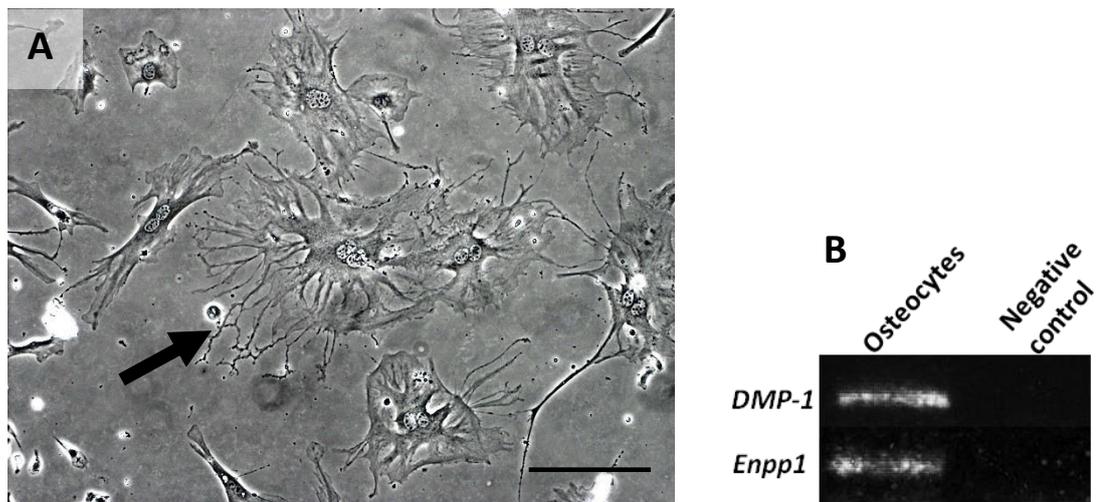


Figure 3.7. Primary osteocyte-like cells express mRNA for *DMP-1* and *Enpp1*

(A) Osteocyte-like cells were extracted from the long bones of wild type mice using repeated digestion by collagenase EDTA solutions and seeded onto collagen-coated plates. The cells can be seen to have dendritic processes, characteristic of osteocytes (arrow); scale bar = 100 μ m. **(B)** RT-PCR shows that these cells express *DMP-1* and *Enpp1*.

Osteocyte-like cells cultured from *Enpp1*^{-/-} mice show reduced viability and release less ATP than wild types

Mouse osteocyte-like cells were seeded at 1×10^5 cells per well in a collagen-coated 6 well plate and cultured for 7 days *in vitro*. There was no significant difference in the number of cells initially obtained from wild type and *Enpp1*^{-/-} bones, but after 7 days in culture, there were 48% fewer cells in *Enpp1*^{-/-} cultures, compared to wild types, as determined by manual counting ($p < 0.05$; $n = 6$) (**Figure 3.8A**).

On day 7 of culture the ATP release per osteocyte-like cell was measured by luminescence. It was found that *Enpp1*^{-/-} osteocyte-like cells release less ATP per cell than wild type cells ($p < 0.05$; $n = 6$) (Figure 3.8B).

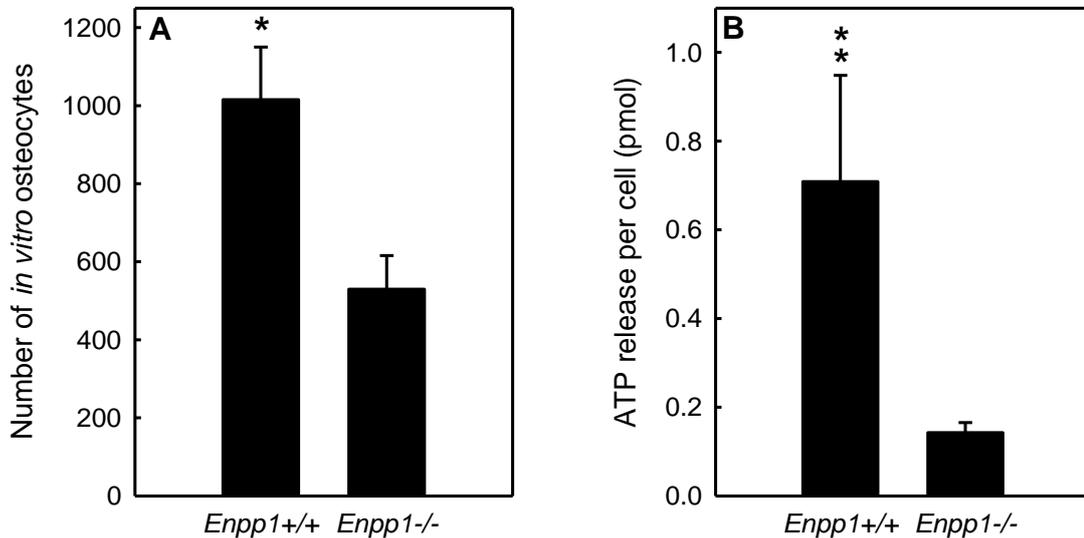


Figure 3.8. *Enpp1*^{-/-} mouse long bone osteocyte-like cells are less viable than wild type cells *in vitro* and release less ATP

Osteocyte-like cells were extracted from the long bones of wild type mice using collagenase and EDTA, they were then seeded onto collagen coated plates. On day 7 of culture the number of cells was counted (A), and the mean ATP release per cell was measured (B). (*, $p < 0.05$; **, $p < 0.01$; data are means \pm SEM; $n = 6$)

***Enpp1*^{-/-} mice have less porous cortical bone than wild type mice**

MicroCT image analysis of a specific region of interest, 0.25 mm long, 0.5 mm below the deltoid tuberosity in the cortical bone of the left humerus from 8, 15 and 22 week old *Enpp1*^{-/-} and wild type mice was performed. The total porosity of *Enpp1*^{-/-} mice bones was found to be reduced by 30% ($p < 0.001$) at 15 weeks and by 60% ($p < 0.001$) at 22 weeks, compared to wild types (Figure 3.9 & 3.10).

22 week old wild type bones were also 34% ($p < 0.001$) less porous than 15 week old bones. Total porosity is a measurement of all of the space within the cortical bone not filled by mineral, for example, a blood vessel channel, a large osteocyte lacuna or a crack. This pore space may contain a soft tissue or cell, but it is not detectable by microCT when used with these settings.

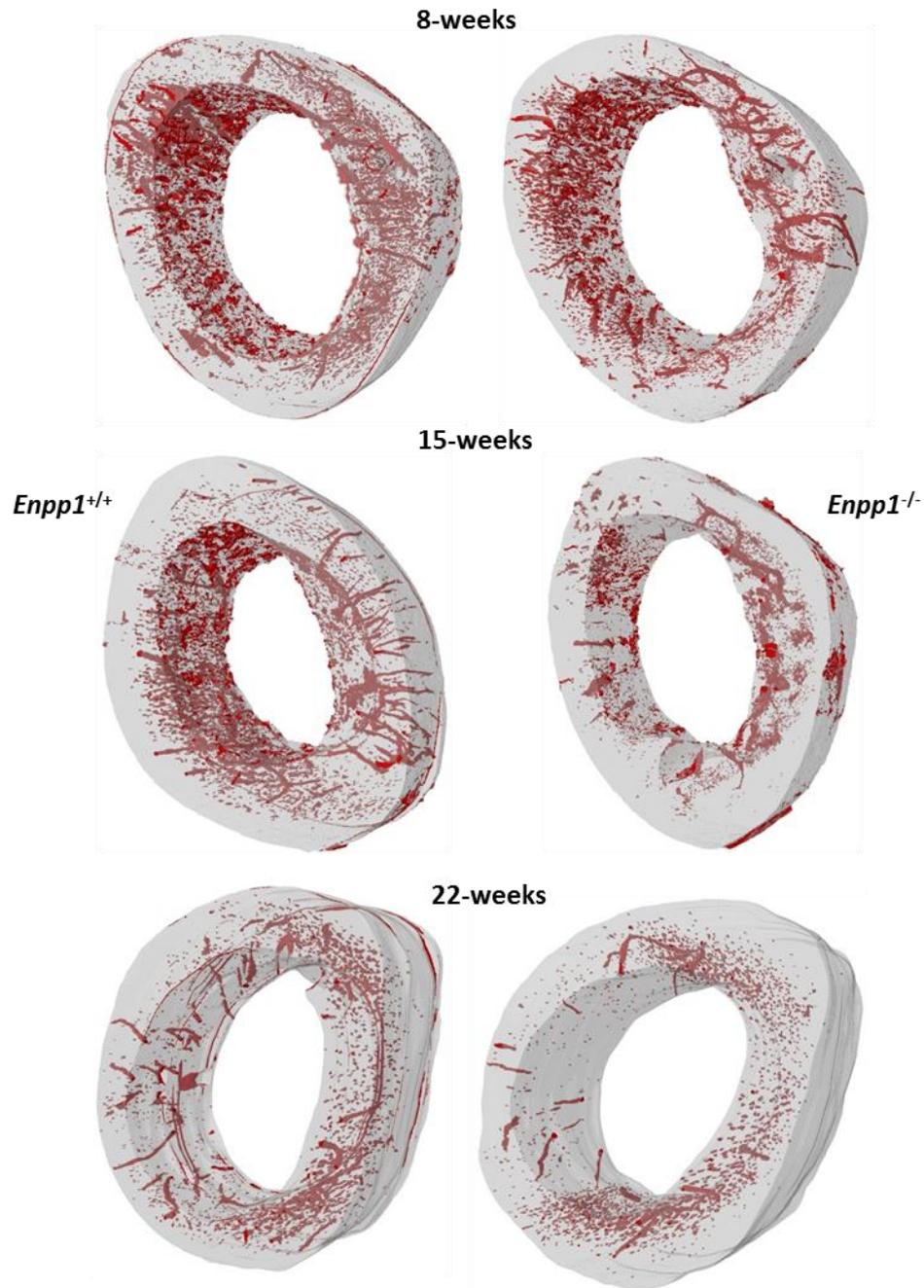


Figure 3.9. MicroCT cross sections of the diaphysis of the humerus bones of *Enpp1*^{-/-} and wild type mice

Humerus bones from 8, 15 and 22 week old mice were scanned by microCT. The images shown represent a region 0.25 mm in length, 0.5 mm below the deltoid tuberosity. Red = empty space within the cortical bone, grey = bone. *Enpp1*^{-/-} mice show reduced porosity (space) within their cortical bone compared to age and sex matched wild types (see Figure 3.10).

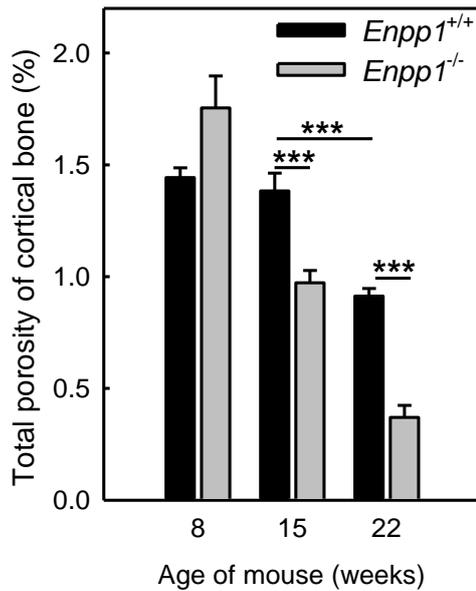


Figure 3.10. The total porosity of *Enpp1*^{-/-} mouse humerus bone is reduced

Ex vivo humerus bones from 8, 15 and 22 week old female mice were scanned by microCT and the region of interest was analysed. “Total porosity” is a composite measurement that will include any space within the cortical bone (blood vessel channels, large osteocyte lacunae, cracks). (Data are means \pm SEM; ***, $p < 0.001$; $n = 5$).

***Enpp1*^{-/-} mice have fewer and smaller “closed pores” in their cortical bone compared to wild types**

The microCT data was further analysed to determine what factors contribute to the decrease in total porosity of the cortical bone in the humerus of *Enpp1*^{-/-} mice. Each individual pore within the region of interest in the cortical bone was classified as either a “closed pore” or an “open pore”. “Open pores” were spaces within the bone which opened onto the periosteal or endosteal surface, or are pores that were bisected by the region of interest. A blood vessel channel running longitudinally within the bone from the proximal to the distal end would be bisected by the region of interest, so would be classified as an open pore. A crack that runs laterally from either the endosteal surface to the periosteal surface would also be classified as an “open pore”. A “closed pore” was classified as a space within the cortical bone than was fully enclosed by mineral. A crack within the bone that does not reach the surface, or get bisected by the perimeter of the region of interest would be classified as a closed pore. The osteocyte canaliculi are too small to be detected by this method of microCT; this means that larger sized osteocyte lacunae may be one of the contributing factors to the “closed pore” measurements within the cortical bone.

All “open pore” results from the data sets were discarded. Any “closed pore” with a total volume of less than $22 \mu\text{m}^3$ was discarded as it was deemed to be below the accurate limit of detection of the microCT. This value represents 30 individual voxels (3D pixels). Any closed pore with a volume greater than $950 \mu\text{m}^3$ was also excluded.

MicroCT analysis of the *ex vivo* humerus bones from 8 week old *Enpp1*^{-/-} and wild type mice showed that there was no difference between the two groups in the number and volume of closed pores within the cortical bone (**Figure 3.11**). MicroCT analysis showed that at 15 weeks, the cortical bone of the humeri in *Enpp1*^{-/-} mice contained 50% ($p < 0.05$ $n=5$) fewer closed pores than wild type mice, had a 41% ($p < 0.001$) reduction in the total closed pore volume and each individual pore was reduced in diameter by an average of 10% ($p < 0.05$) (**Figure 3.11**). At 22 weeks of age, microCT analysis showed that the cortical bone of the humerus in *Enpp1*^{-/-} mice contained 55% ($p < 0.001$ $n=5$) fewer closed pores than wild type mice, had a 59% reduction in closed pore volume ($p < 0.05$) and each individual pore was reduced in diameter by an average of 15% ($p < 0.001$) (**Figure 3.11**).

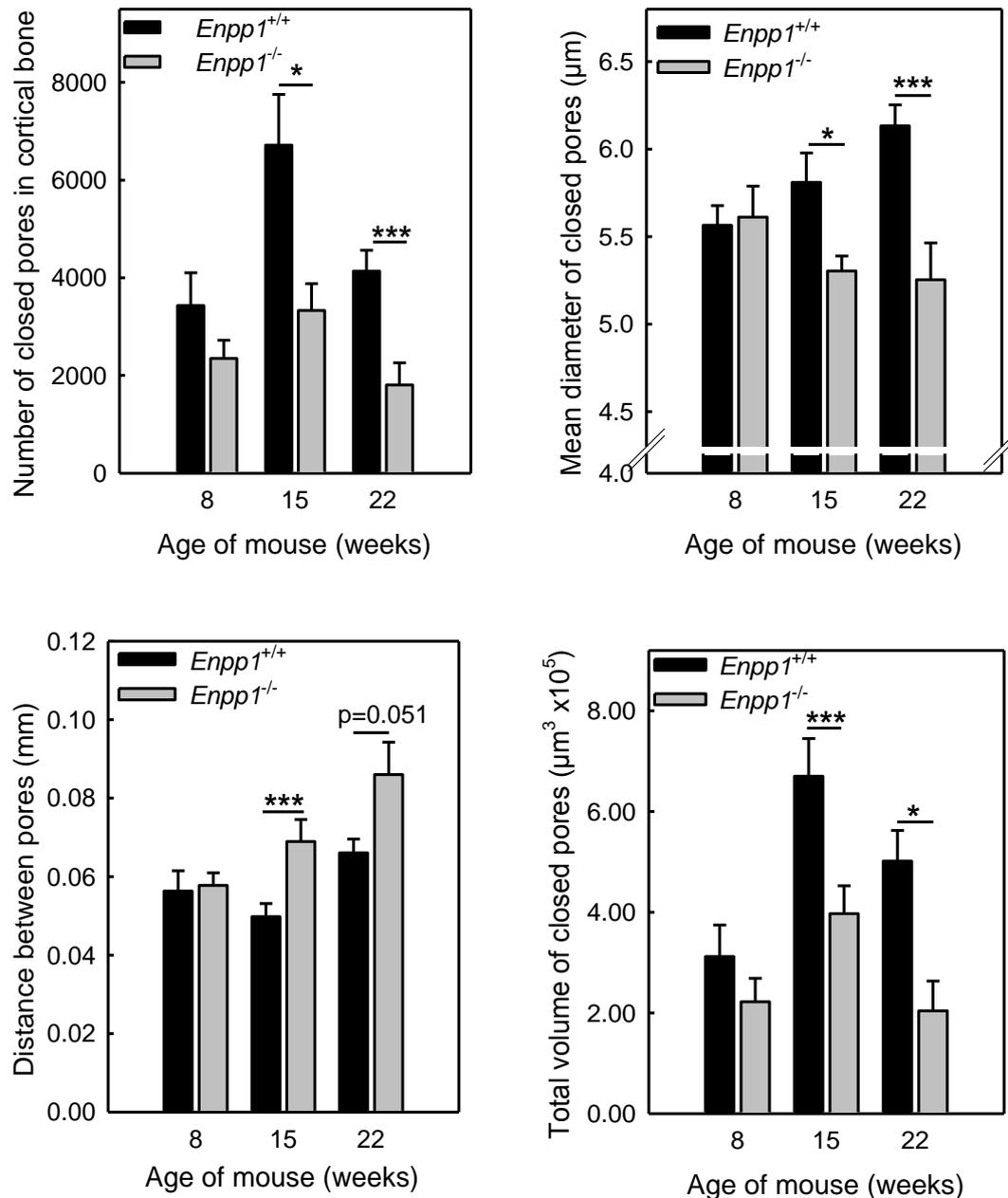


Figure 3.11. *Enpp1*^{-/-} mouse humerus cortical bones have a reduced number of ‘closed pores’, reduced closed pore diameter and volume compared to wild type bone

Ex vivo humerus bones from 8, 15 and 22 week old mice were scanned by microCT and the region of interest was analysed. A “closed pore” is a space within the cortical bone that is completely encapsulated by bone when imaged by microCT. (Data are means ± SEM; n = 5 bones, individual pore values are based on calculations on all pores in each bone; *, p<0.05; ***, p<0.001).

Osteocyte lacunae are smaller in the femurs of *Enpp1*^{-/-} mice compared to wild types

The left femur was dissected from the legs of 15 and 22 week old *Enpp1*^{-/-} and wild type mice. The bones had all of the soft tissues digested from them before being dehydrated and air dried. The diameter of the osteocyte lacunae along its longest axis and the plan surface area were measured using SEM imaging.

SEM showed that the osteocyte lacunae of 15 week old *Enpp1*^{-/-} mice were 25% shorter than wild type osteocyte lacunae ($p < 0.001$) and had a 35% ($p < 0.001$) reduction in their plan surface area. SEM also revealed that 22 week old *Enpp1*^{-/-} mice had osteocyte lacunae that were 22% ($p < 0.001$) shorter than wild type osteocyte lacunae, with a 39% ($p < 0.001$) reduction in their plan surface area (Figure 3.13).

SEM analysis also showed 27% and 23% reductions in the length of wild type and *Enpp1*^{-/-} mouse osteocyte lacunae, respectively between the ages of 15 and 22 weeks ($p < 0.001$ in each case) (Figure 3.12).

***Enpp1*^{-/-} mice have fewer open blood vessel channels on the endosteal surface of their cortical bone**

Bone samples for SEM were prepared as detailed above. Low resolution SEM (x 16 magnification) showed that *ex vivo* femur bones from 15 week old *Enpp1*^{-/-} mice appear to have fewer blood vessel channels opening at the endosteal surface than wild type mice (Figure 3.13). However, this methodology does not allow quantification of this difference.

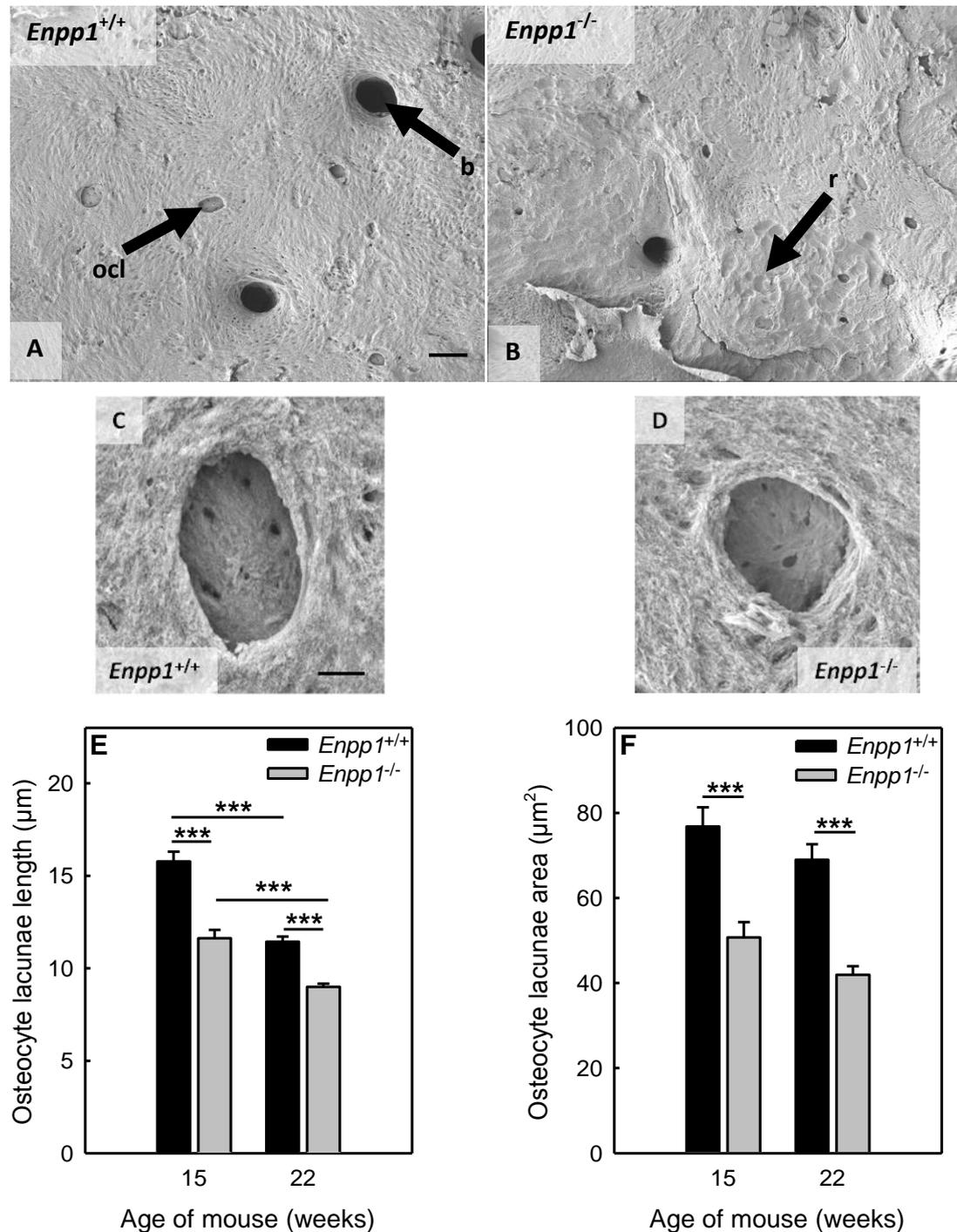


Figure 3.12. The osteocyte lacunae in 15 and 22 week old *Enpp1*^{-/-} mouse femurs are shorter and have a reduced plan surface area compared to wild types

(A, B) Representative SEM images of the endosteal surface of mouse femurs from 22 week old mice at x400 magnification. The arrows point to (ocl) an osteocyte lacuna, (b) a blood vessel channel and (r) resorption pits on the surface of bone. (C, D) Images of an osteocyte lacunae. Lacunae diameter was measured along the longest axis. Quantitative analysis of osteocyte lacunae SEM images based on n = 60 measurements per group (E, F). Scale bar A = 20 μm, C = 5 μm; data are means ± SEM ***, p<0.001.

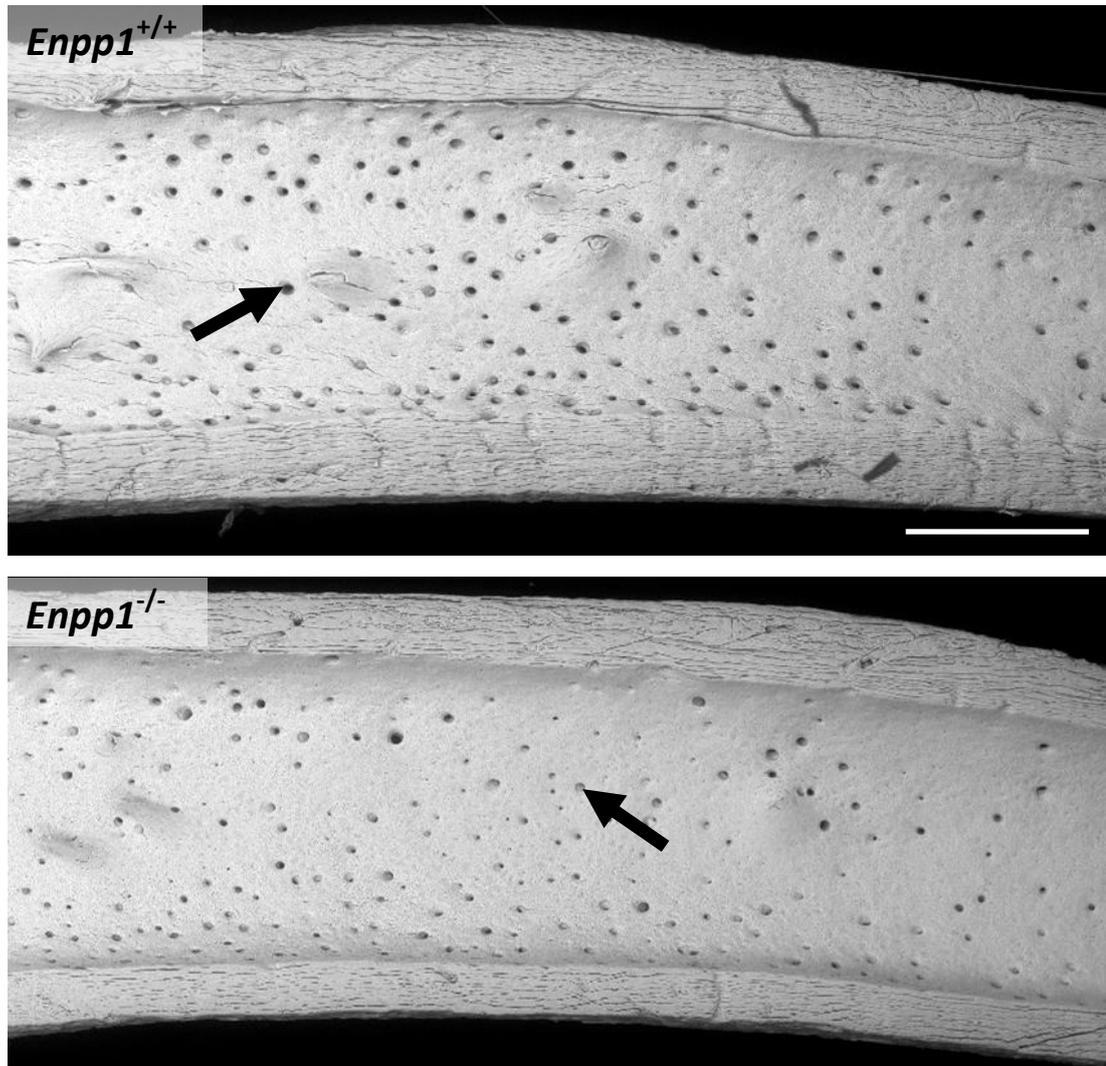


Figure 3.13. SEM shows that the endosteal bone surface of 15 week old *Enpp1*^{-/-} mouse femurs contains fewer open blood vessel channels than wild type bone

The arrows point to blood vessel channels; scale bar = 0.5 mm. These images were generated by Prof Alan Boyde, QMUL.

Increased serum sclerostin in *Enpp1*^{-/-} mice

Blood was collected by terminal cardiac puncture from 8, 15 and 22 week old wild type and *Enpp1*^{-/-} mice. The serum sclerostin concentration, measured by ELISA, of wild type mice decreased by 37% ($P < 0.01$) between the ages of 8 and 15 weeks, and 62% ($p < 0.001$) between 8 and 22 weeks (**Figure 3.14**). There was no difference in the serum concentration of sclerostin between wild type and *Enpp1*^{-/-} mice at 8 weeks. At 15 and 22 weeks, however, serum sclerostin was 75% ($p < 0.001$) and 52% ($p < 0.01$) higher in *Enpp1*^{-/-} mice, compared to wild types (**Figure 3.14**).

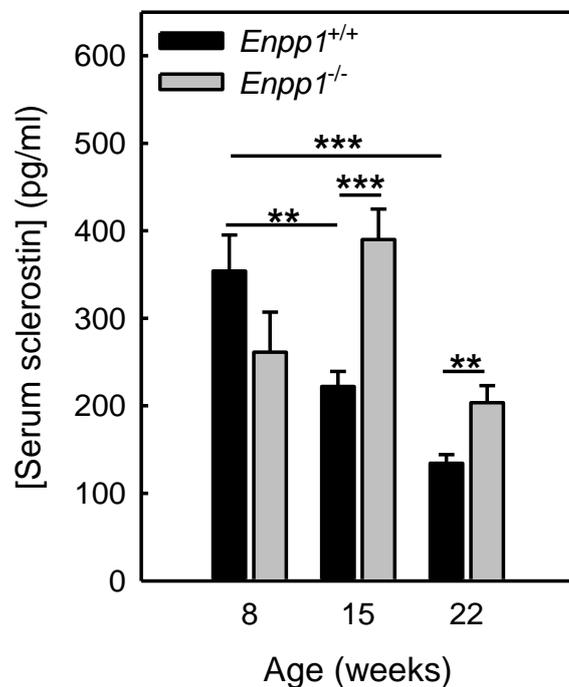


Figure 3.14. Knockout of *Enpp1* leads to an increase in serum sclerostin

Sclerostin was measured by ELISA in serum collected from 8, 15 and 22 week old *Enpp1*^{-/-} and wild type mice. (**, $p < 0.01$; ***, $p < 0.001$; data are means \pm SEM; $n = 5$).

***Enpp1*^{-/-} humerus bones have reduced cortical bone thickness and a wider bone marrow cavity**

Outer soft tissues were removed from 8, 15 and 22 week old *Enpp1*^{-/-} and wild type mice left humerus bones; the bones were fixed and then left to air dry. MicroCT measurements of the cortical bone width (thickness), bone marrow cavity diameter (endosteal diameter) and bone diameter (periosteal diameter) were undertaken in a specific region of interest, 0.25 mm long, 0.5 mm below the deltoid tuberosity (**Figure 3.15**).

There was no difference in any of these measured parameters between *Enpp1*^{-/-} and wild type mice at 8 weeks. There was no difference in the periosteal diameter between *Enpp1*^{-/-} and wild type mice at any time point. In wild type mice the thickness of the cortical bone increased with age. The cortical bone thickness of 15 and 22 week old wild type mice was 34% ($p < 0.001$) and 57% ($p < 0.001$) greater than that of 8 week old bone respectively. *Enpp1*^{-/-} mice showed no increase in cortical bone thickness with age (**Figure 3.15**).

15 week old *Enpp1*^{-/-} mice had a 16% ($p < 0.05$) thinner cortical bone thickness and a 22% ($p < 0.001$) larger endosteal diameter compared to wild type mice. 22 week old *Enpp1*^{-/-} mice had a 35% ($p < 0.001$) reduction in their cortical bone thickness and a 23% ($p < 0.05$) increase in their endosteal diameter compared to wild type mice. When combined, these results indicate that from 15 weeks onwards, the humerus bones of *Enpp1*^{-/-} mice have a similar total diameter to wild type mice, but their bone marrow cavity has a greater diameter and their cortical bone is thinner (**Figure 3.15**).

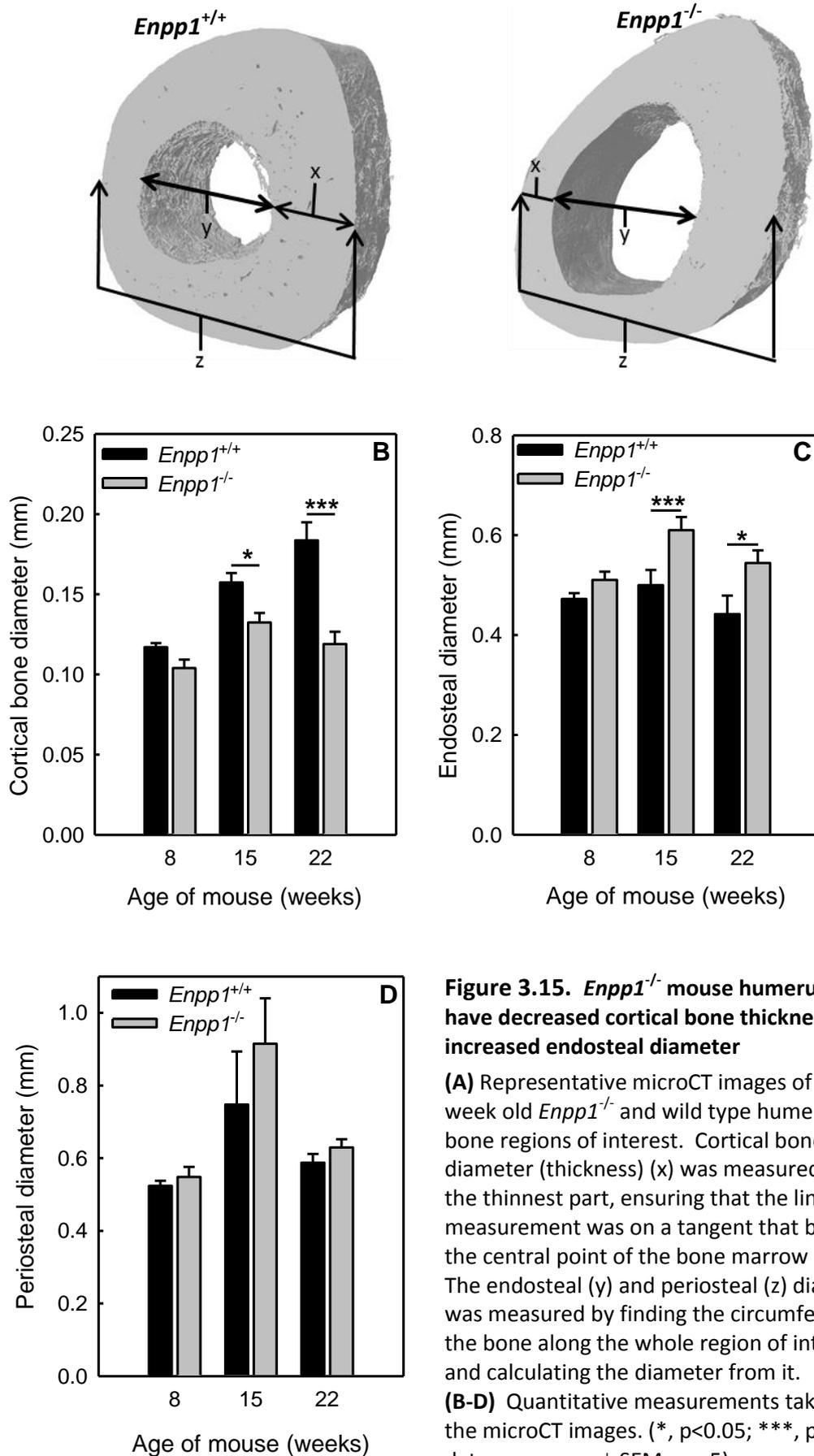


Figure 3.15. *Enpp1*^{-/-} mouse humerus bones have decreased cortical bone thickness and increased endosteal diameter

(A) Representative microCT images of 22 week old *Enpp1*^{-/-} and wild type humerus bone regions of interest. Cortical bone diameter (thickness) (x) was measured across the thinnest part, ensuring that the line of measurement was on a tangent that bisected the central point of the bone marrow cavity. The endosteal (y) and periosteal (z) diameter was measured by finding the circumference of the bone along the whole region of interest and calculating the diameter from it.

(B-D) Quantitative measurements taken from the microCT images. (*, p<0.05; ***, p<0.001; data are means ± SEM; n = 5).

***Enpp1*^{-/-} mouse skulls are similar in size and shape to wild type skulls**

Humerus bones are made by the process of endochondral ossification. The skull, which is made by intramembranous ossification, was examined to detect differences between *Enpp1*^{-/-} and wild type mice.

The heads from 8, 15 and 22 week old *Enpp1*^{-/-} and wild type mice were scanned by microCT. The lengths of the skulls were measured from the tip of the nasal plate to the occipital condyle along the medial axis (**Figure 3.16a**). The widths of the skulls were measured at a point 6 mm forward from the back of the skull, at an axis point spanning the parietal bones (**Figure 3.16b**). The height from the most dorsal point of the parietal bone (top) to the most distal point (bottom) was measured at a site exactly 6 mm forwards from the back of the skulls, to determine the depth of the calvariae (**Figure 3.16c**). A 4 mm wide strip of bone, 2 mm from the back of the skull, across the whole calvaria was analysed to determine calvarial bone volume in *Enpp1*^{-/-} and wild type mice (**Figure 3.16d**).

At 22 weeks, the skulls of *Enpp1*^{-/-} mice were 10% longer than those of wild type mice ($p < 0.05$). *Enpp1*^{-/-} mice were also found to have 10% ($p < 0.05$) less calvarial bone compared to wild type mice at 15 weeks. No other significant differences were observed (**Figure 3.17**).

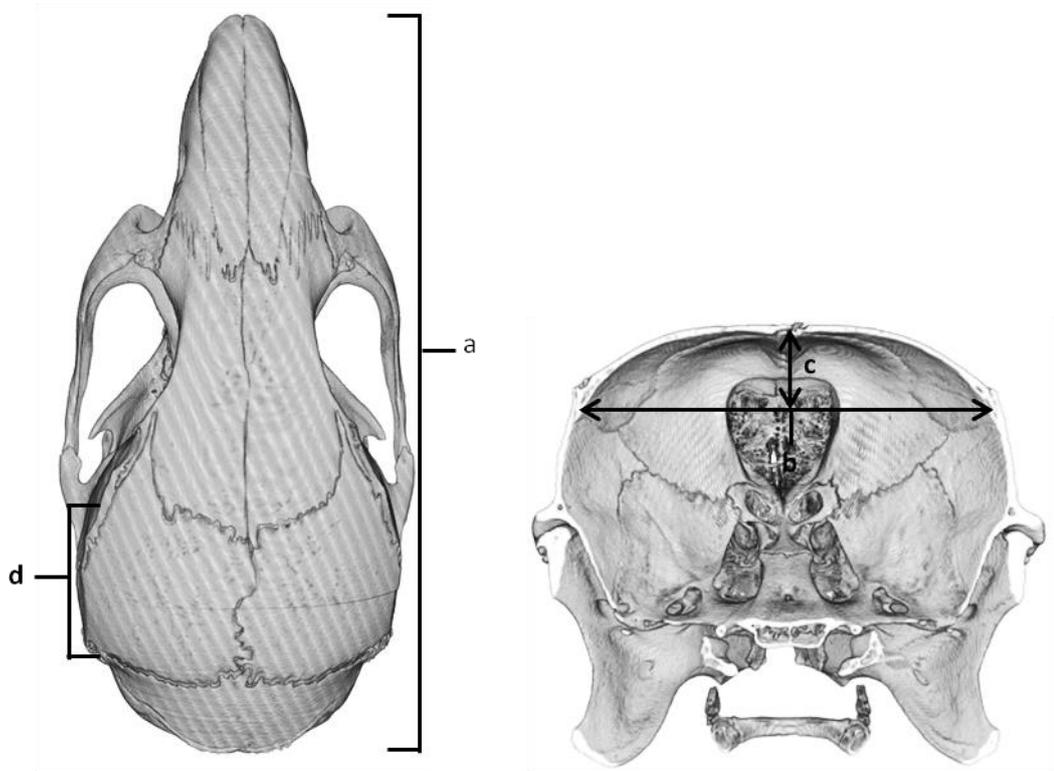


Figure 3.16. MicroCT images of a mouse skull showing the parameters examined as part of the morphological examination of skull dimensions

MicroCT images were evaluated to determine if there were any skull morphological differences between wild type and *Enpp1*^{-/-} mice. See figure 3.18 for results. **(a)** Length of skull, **(b)** diameter of skull, **(c)** depth of calvaria and **(d)** calvarial volume, were measured.

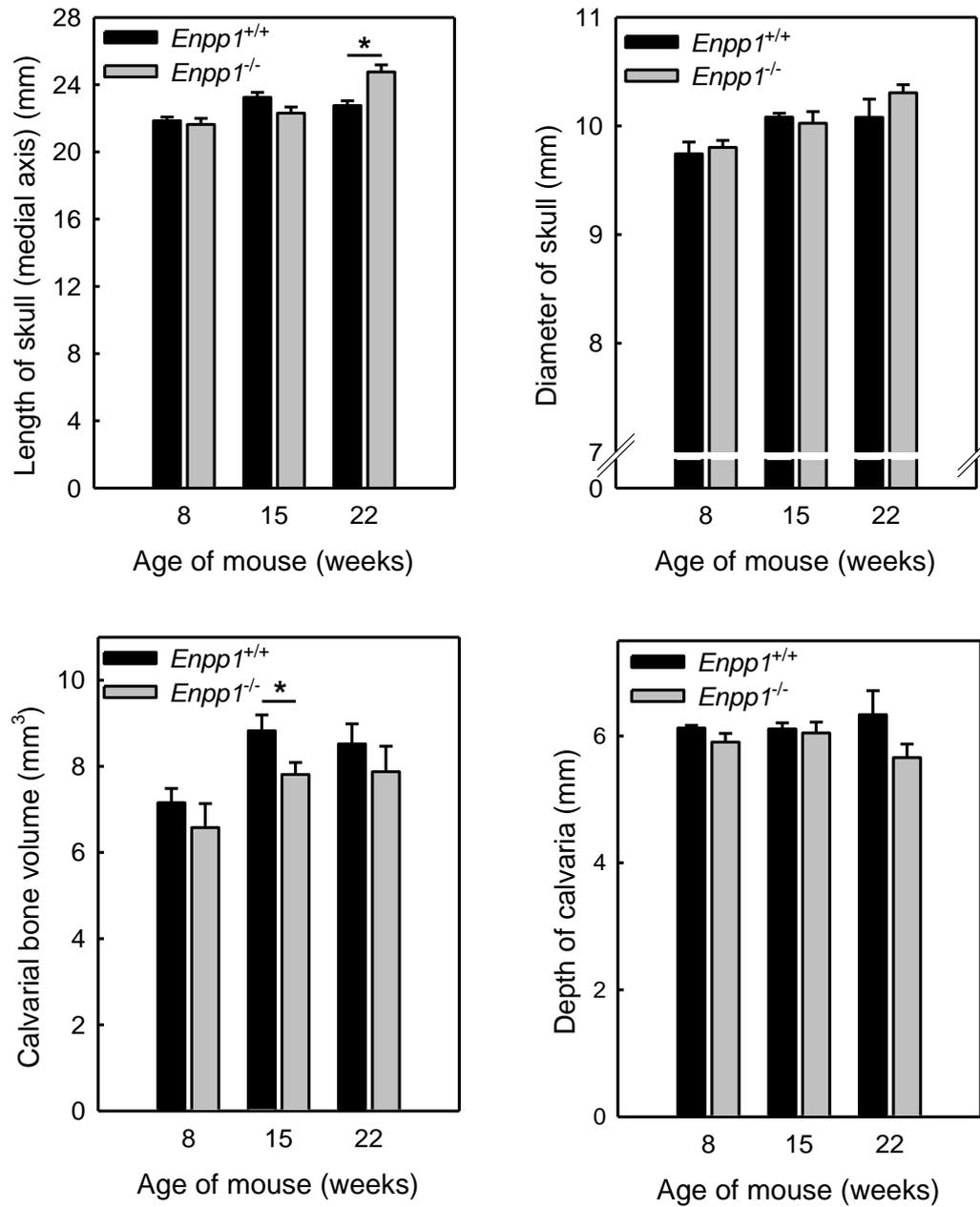


Figure 3.17. *Enpp1*^{-/-} mouse skulls are morphometrically similar to wild type skulls
Data are derived from microCT scans (Data are means ± SEM; *, p < 0.05; n = 5).

Discussion

This work showed that NPP1 is vital to prevent soft tissue calcification in the whisker follicles, the ear pinna and the trachea. The strikingly high levels of mineralisation seen in these tissues have not been previously reported. SEM analysis demonstrated for the first time that *Enpp1*^{-/-} mice have fewer open blood vessel channels on the endosteal surface of their femurs, compared to wild type mice. SEM analysis also revealed that *Enpp1*^{-/-} osteocyte lacunae are reduced in size compared to wild types. MicroCT analysis demonstrated that the knockout of *Enpp1* results in thinner, less porous cortical bone; however, no effects on the skull bones were noted. Long bone porosity and osteocyte lacunar size also decreased with age in control mice. It has also been shown here for the first time that primary mouse osteocyte-like cells release ATP and express mRNA for *Enpp1* *in vitro*. Moreover, it was demonstrated here that *Enpp1*^{-/-} osteocytes are less viable and release less ATP in culture. Furthermore, *Enpp1*^{-/-} mice have a greater circulating sclerostin concentration than wild type mice.

Histological and x-ray studies more than 40 years ago showed that hypercalcaemic rats developed hair follicle mineralisation when their skin was subjected to a mild crush injury (Pearce *et al.* 1972). Rat hair follicles grown in a high calcium and phosphate media *in vitro* also showed spontaneous mineralisation (Pearce & Smillie 1973). In the present study, whisker follicles in *Enpp1*^{-/-} mice may have mineralised before the much smaller hair follicles because the large collagen sheath around the whisker follicle acted as a preferential nucleation site for mineral deposition. These results suggest that *Enpp1* hydrolysis of ATP could be involved in the prevention of inappropriate mineralisation of the hair follicle. Most cell types release ATP; although there has been no specific evidence of ATP release from hair follicles. However, hair follicles grown *in vitro* have been shown to express P2X₅, P2X₇, P2Y₁ and P2Y₂ receptors on which local ATP may act (Greig *et al.* 2008).

Calcification of the hyaline cartilage of the ear pinna is rare; however, it occurs in Primrose syndrome (Dalal *et al.* 2010; Carvalho & Speck-Martins 2011) and very occasionally it is seen in cases of frost bite (Lautenschlager *et al.* 1994; Stites *et al.*

2003), Addison's disease (Cohen *et al.* 1991), inflammatory states (Chopra *et al.* 2013), pituitary insufficiency (Gogate *et al.* 2012), diabetes (Strumia *et al.* 1997) and trauma (Gordon 1964). Anecdotal evidence suggests that calcification of the ears is more frequent in older people, especially those who had worked out-doors (Bowers & Gould 1998). My results suggest the possibility that alterations in *Enpp1* expression may play a role in calcification of ear pinna cartilage.

Histological and microCT analysis showed that the hyaline cartilage rings were also mineralised along the entire length of the trachea and the primary and secondary bronchi in *Enpp1*^{-/-} mice. No mineralisation of adjacent soft tissues such as arterioles, bronchioles or smooth muscle was detected. It has been reported that in healthy human subjects with a mean age of 70, approximately 50% showed signs of tracheal cartilage mineralisation (Kusafuka *et al.* 2001). The present results suggest the possibility that decreased expression of *Enpp1*, leading to a reduction in the production of PPi may play a role in the mineralisation of the tracheal cartilage with age. Cartilaginous mineralisation of the trachea has been reported in patients on long term warfarin anticoagulant therapy (Moncada *et al.* 1992; Thoongsuwan & Stern 2003). The effect of warfarin on vascular calcification has been suggested to be mediated by its actions on the vitamin K-dependent proteins matrix gla protein and osteocalcin (Gundberg *et al.* 2012; Kruger *et al.* 2013), but could also be due to a NPP1 mediated mechanism.

An important finding in this chapter is that the osteocyte lacunae in the cortical bone of *Enpp1*^{-/-} mice had a surface area that was 35 - 39% smaller than wild types. Further extrapolation of this data suggests that the volume of the prolate spheroid shape of the *Enpp1*^{-/-} osteocyte lacunae could be approximately 60% less than the wild type lacunae. The "closed pore" data generated by microCT suggests that there may also be a reduction in the total number of osteocyte lacunae, because large osteocyte lacunae could be one of the factors contributing to this parameter. The most obvious explanation for this reduced lacunae size is related to PPi formation by osteocytes. Osteocytes release ATP; this ATP may be broken down by NPP1 to produce PPi; this PPi then inhibits the further mineralisation of the

osteocyte lacunae. Knockout of *Enpp1* would decrease this process and result in greater lacunar mineralisation and decreased lacunar size. In support of this theory it was shown that primary mouse osteocyte-like cells release ATP and express mRNA for *Enpp1* *in vitro*, so may be capable of generating P_{Pi}. It has previously been shown that the MLO-Y4 osteocyte-like cells release ATP (Genetos *et al.* 2007; Kringelbach *et al.* 2013). It should be noted, however, that the methods used in this study cannot distinguish between mineral deposition and true bone formation (which would also involve collagenous matrix deposition by osteocytes).

It was also observed that *Enpp1*^{-/-} osteocyte-like cells release less ATP *in vitro* than wild type cells; this is potentially a second factor contributing towards a reduction in P_{Pi} concentrations within the osteocyte lacunae *in vivo*. Mouse osteocyte-like cell lines have been shown to express mRNA for some of the P_{2Y} receptors (Kringelbach *et al.* 2013); although it is unknown what, if any effects purinergic signalling has on osteocytes. The reduction in ATP release found here may lead to changes in autocrine purinergic signalling.

The simplest interpretation of my results is that P_{Pi} is acting in a mainly physicochemical manner on the inner surface of the osteocyte lacunae to prevent mineral encroachment. This mechanism could also be seen to be involved in the still-controversial process of 'osteocytic osteolysis' (which is thus being reduced in *Enpp1*^{-/-} mice). Increased osteocyte lacunae size has been reported in rats infused with PTH (Tazawa *et al.* 2004), as well as during lactation (Qing *et al.* 2012). Humans with *Enpp1* gene defects have been found to have normal serum PTH and calcium concentrations and a low phosphate concentration (Lorenz-Depiereux *et al.* 2010). *Enpp1*^{-/-} mice have previously been shown to have low blood serum calcium and phosphate concentrations and a high blood serum FGF23 concentration (Mackenzie *et al.* 2012b). Circulating PTH was not measured in the present study, but these results suggest that it could be elevated in *Enpp1*^{-/-} mice, as part of a homeostatic response to normalise the blood calcium concentration. The change in osteocyte lacunae size I found in *Enpp1*^{-/-} mice is unlikely to be due to a high PTH

concentration because that would result in an increased lacunae size not decreased.

SEM measurements also revealed that the osteocyte lacunae of 22 week old wild type mice were smaller than those of 15 week old mice. A number of reports have also shown a reduction in osteocyte lacunae size with age in human and rodent bones (Mullender *et al.* 1996; Mori *et al.* 1997; Power *et al.* 2002; Qiu *et al.* 2002; Busse *et al.* 2010; Torres-Lagares *et al.* 2010; Carter *et al.* 2013), presumably as a consequence of continuing secondary mineralisation. It is not known what effect ageing has on the activity of NPP1 in humans and mice. A reduction of NPP1 activity with age may permit the secondary mineralisation seen.

In young adults, exercise and hypoxia lead to increased ATP release from red blood cells (RBCs); this ATP release has been reported to be attenuated with age (Kirby *et al.* 2012). This released ATP is believed to cause vasodilation and relieve the hypoxia (Sprague *et al.* 2011). Reduced ATP release with ageing might thus be expected to result in decreased relief of hypoxia. Reduced ATP release from RBCs might also lead to decreased circulating PPI, which could contribute to the secondary mineralisation seen with age.

From 15 weeks onwards, *Enpp1*^{-/-} mice had thinner and less porous cortical bone in the humerus than wild types. The *Enpp1*^{-/-} bones had the same diameter as those of wild type mice, but a bigger marrow cavity. This increase in the endosteal / periosteal diameter ratio is indicative of increased osteoclast activity in the bone. Mackenzie and colleagues saw histological evidence of increased *in vivo* osteoclast activity on the bones of *Enpp1*^{-/-} mice (Mackenzie *et al.* 2012b). A possible explanation as to why *Enpp1*^{-/-} mice have altered bone structure may be related to acidosis and hypoxia. The knockout of NPP1 leads to a reduction in PPI; this reduction in PPI has been shown to lead to arterial blood vessel calcification (Villa-Bellosta *et al.* 2011). These vessels may have reduced capacity for transporting oxygenated blood; this may result in tissue hypoxia and acidosis. Results presented here show a reduction in the number of open blood vessels channels in *Enpp1*^{-/-} bone (**Figure 3.13**), and a reduction in the total pore / channel space in bone

(Figure 3.10), which may result in hypoxia and acidosis in the bone environment. Acidosis and hypoxia have been shown to increase osteoclast formation and resorption rate (Arnett & Dempster 1986; Arnett *et al.* 2003) and inhibit bone mineralisation (Brandao-Burch *et al.* 2005; Utting *et al.* 2006). This increase in osteoclast activity may be responsible for the changes seen in the diameter of the cortical bone of *Enpp1*^{-/-} mice. *Enpp1*^{-/-} mice reportedly have an increased serum K⁺ concentration (Mackenzie *et al.* 2012b); increased serum K⁺ may be an indicator of an acidosis (Nyirenda *et al.* 2009).

No large changes in skull dimensions were detected at 8, 15 and 22 weeks in *Enpp1*^{-/-} mice compared to wild types. This finding aligns with previous work which found that 20 day old *Enpp1*^{-/-} mice did not have any calvarial defects (Hessle *et al.* 2002). The skull is different to the long bones in that it is formed by intramembranous ossification and develops from the neural crest cells (Santagati & Rijli 2003); the long bones (axial skeleton) are formed by the process of endochondral ossification from the sclerotome compartment of the somite (Fan & Tessier-Lavigne 1994). This may be a reason why *Enpp1*^{-/-} had no effect on the skull bones, but a dramatic effect on the long bones.

Enpp1^{-/-} mice had a higher serum sclerostin concentration than wild type mice. A possible explanation for this may be found in their decreased mobility (Okawa *et al.* 1998; Sali *et al.* 1999). It has previously been reported that *Enpp1*^{-/-} mice have a raised blood serum creatine kinase concentration (Mackenzie *et al.* 2012b), this indicates that they have muscle damage. MicroCT data presented here shows mineralisation of the knees and toes; coupled with the reported muscle damage, the *Enpp1*^{-/-} mouse may be attempting to put as little force as possible through their limbs when moving, this would result in unloading of the long bones. Unloading of the bones can result in increased expression of sclerostin by osteocytes (Lin *et al.* 2009; Macias *et al.* 2013; Spatz *et al.* 2013) and may be a reason why *Enpp1*^{-/-} mice have increased blood serum sclerostin.

A further explanation for the elevated sclerostin concentration seen in *Enpp1*^{-/-} mice may be related to their reduced PPI production (Hessle *et al.* 2002). It is

possible that the increased sclerostin concentration may be a homeostatic response by the osteocyte to the hypermineralisation of their lacunae caused by the decrease in PPI. It has been reported that sclerostin increased the expression of carbonic anhydrase 2 in osteocyte-like cell lines *in vitro*. It has been suggested that this carbonic anhydrase 2, through its production of carbonic acid, enables the osteocyte to dissolve the mineralised lacunae wall and increase the lacunae size (Kogawa *et al.* 2013). The increased blood sclerostin concentration seen in *Enpp1*^{-/-} mice may be due to the osteocytes' attempts to reduce the mineral encroachment into the lacunae.

Increased differentiation of osteoblasts to osteocytes may also be reason for the increased sclerostin concentration detected in *Enpp1*^{-/-} mice. Increased mineralisation of the extracellular matrix has been shown to up-regulate the osteocytic differentiation of MLO-A5 osteocyte-like cells and increase their expression of E11 *in vitro* (Prideaux *et al.* 2012). This suggests the possibility that increased mineralisation in *Enpp1*^{-/-} mice bones may also act to increase osteocyte differentiation.

Increased sclerostin could provide another explanation for the increased osteoclast function seen *in vivo*. Sclerostin has been shown to increase the expression of RANKL by osteoblasts and osteocytes, and therefore increase osteoclast formation (Wijenayaka *et al.* 2011). Along with hypoxia discussed above, the increased blood sclerostin levels seen in *Enpp1*^{-/-} mice may stimulate osteoclast formation and bone resorption, and be responsible for some of the changes seen in *Enpp1*^{-/-} mouse bones. My observation that blood sclerostin decreased with age in wild type mice contrasts with the results of a number of studies in humans showing that circulating sclerostin increases with age (Modder *et al.* 2011; Bhattoa *et al.* 2013; Roforth *et al.* 2014). The reason for this difference is not clear.

In summary, the work presented in this chapter provides significant new evidence of the important role of *Enpp1* and PPI in regulating the mineralisation of soft tissues and bone.

Chapter 4

Effects of *Enpp1* knockout on osteoclasts and osteoblasts *in vitro*

Introduction

Enpp1^{-/-} osteoclasts

The previous chapter showed that the bone marrow cavity is enlarged in *Enpp1*^{-/-} mice (**Figure 3.16**), with prominent resorption pits on the endosteal surface (**Figure 3.12B**), suggesting that these mice have increased osteoclastic resorptive activity. Mackenzie and colleagues saw an increased number of osteoclasts on the *ex vivo* bone surfaces of *Enpp1*^{-/-} mice, compared to age-matched, wild type mouse bones (Mackenzie *et al.* 2012b). They also found that the blood serum concentration of C-terminal telopeptides of type I collagen (CTX), a marker of osteoclast activity, was increased by 364% in 22 week old *Enpp1*^{-/-} mice compared to wild types. They observed that the concentration of blood serum CTx decreases with age in wild type but not *Enpp1*^{-/-} mice. It was suggested that *Enpp1*^{-/-} mice failed to appropriately down-regulate osteoclast numbers and activity with age. Mackenzie *et al.* hypothesised that the increased number of osteoclasts in *Enpp1*^{-/-} mouse bones may be due to the increased circulating creatine kinase activity they found in these animals. It has been reported that brain-type creatine kinase (CK-BB) is up-regulated during osteoclastogenesis and that knockout of CK-BB reduced osteoclast formation *in vitro*, and decreased bone loss in ovariectomised mice and rats *in vivo* (Chang *et al.* 2008).

One of the main biological functions of calcitonin is to suppress osteoclast resorptive activity by binding to receptors on their surface (Chambers & Moore 1983; Shyu *et al.* 2007; Hamdy & Daley 2012). Administration of calcitonin for 4 weeks to the tiptoe walking mouse partially corrected the low bone volume seen in the cervical vertebrae (Okawa *et al.* 1999). This suggests that tiptoe walking mice,

have increased osteoclast activity; it also suggests that the *Enpp1*^{-/-} mice, which have a similar genetic abnormality, may have an osteoclast related defect. However, calcitonin has not been shown to reduce the number of osteoclasts in bone (Ikegame *et al.* 2004; Karsdal *et al.* 2008; Hamdy & Daley 2012).

ATP and ADP have been shown to increase the formation rate and resorptive activity of mouse osteoclasts formed from precursors *in vitro*, by signalling through the P2Y₁ and P2Y₆ receptors; whereas AMP has no effect (see chapter 1, for a detailed review). This suggests that an additional potential reason for the increased number or resorptive activity of osteoclasts in *Enpp1*^{-/-} mice may be due to a decrease in the rate of extracellular nucleotide hydrolysis, leading to increased extracellular ATP and ADP (and reduced AMP).

***Enpp1*^{-/-} osteoblasts**

Osteoblasts cultured from precursor cells obtained from *Enpp1*^{-/-} mice calvariae produced more mineralised bone nodules than wild types. Conversely, osteoblasts formed from precursor cells from *Enpp1*^{-/-} bone marrow produced less mineralised bone nodules than wild types *in vitro*. No differences were detected in the rate of cell proliferation between wild type and *Enpp1*^{-/-} osteoblasts in culture (Anderson *et al.* 2005b).

It has been reported that when *Enpp1* expression was suppressed by shRNA in the mouse osteoblast-like cell line MC3T3, these cells were unable to mineralise the matrix they deposited. Confusingly, it was also reported that *Enpp1*^{-/-} primary mouse calvarial osteoblasts, when grown from precursors *in vitro*, were less differentiated than *Enpp1*^{+/+} cells, resulting in decreased ALP and OCN expression and produced less mineralised bone than wild type cells (Nam *et al.* 2011). These results are not consistent with those reported by (Anderson *et al.* 2005b). It was also reported that catalytically inactive NPP1 enhanced the differentiation of precursor cells into osteoblasts and increased mineral production (Nam *et al.* 2011). It has also been proposed that NPP1 may modulate insulin signalling. NPP1 may be able to bind to the insulin receptor (Maddux & Goldfine 2000) and block osteoblast

bone formation by preventing insulin from suppressing the *Runx2* inhibitor *Twist2* (Fulzele *et al.* 2010).

Previous reports have shown that over-expression of *Enpp1* inhibited adipogenesis and the expression of adipocyte associated genes, *PPAR γ* , *adipsin* and *C/EBP β* in the adipocyte-like cell line 3T3-L1. Knockout of *Enpp1* reportedly increased adipogenesis in primary mesenchymal precursor cells, and increase expression of the adipocyte associated genes *in vitro* (Liang *et al.* 2007). This suggests that *Enpp1* may play a role in the differentiation of MSCs to osteoblasts and adipocytes. Increased adipose tissue expression of *Enpp1* in humans has been shown to be linked with decreased expression of adiponectin, a hormone secreted by adipocytes that can lead to increased liver triglyceride deposition (Chandalia *et al.* 2012).

The PPI generated by NPP1 reportedly increased the expression of OPN by rodent *in vitro* osteoblasts in a phosphate independent way (Boskey *et al.* 2002; Johnson *et al.* 2003; Addison *et al.* 2007). OPN, like PPI, is a direct inhibitor of mineralisation (Wada *et al.* 1999; Boskey *et al.* 2002). *In vitro* osteoblasts from *Enpp1*^{-/-} mice expressed less OPN than wild type cells (Johnson *et al.* 2003), the addition of PPI or soluble NPP1 to the cell culture media increased OPN expression (Boskey *et al.* 2002; Johnson *et al.* 2003; Addison *et al.* 2007; Nam *et al.* 2011). This indicated that *Enpp1* can regulate the mineralisation process in a dual inhibitory way by either a PPI or OPN mediated mechanisms.

PPI has been shown to inhibit mineralisation by osteoblasts *in vitro* by either a physicochemical method or increasing the expression of OPN. In addition, PPI has also been reported to decrease the expression of ALP by the osteoblast-like cell line MC3T3-E1. This decrease in ALP activity resulted in the reduced hydrolysis of phosphate from β -glycerophosphate *in vitro*, therefore resulting in less available phosphate for the formation of mineral (Addison *et al.* 2007).

Aims

The aim of the experiments in this chapter was to determine if the knockout of *Enpp1* affects the function of osteoclasts and osteoblasts *in vitro*.

Results

Enpp1 and osteoclasts

Osteoclasts express mRNA for ecto-nucleotidases and related proteins *in vitro*

On ivory discs, primary mouse osteoclasts were formed from precursors flushed from the bone marrow of wild type mice (see chapter 2). On day 8 the cells were acidified to pH 6.90 to activate resorption. TRIZol was used to collect mRNA from the osteoclasts on days 3, 6, 8 and 10 of culture, these represent: immature, maturing, mature and mature resorbing osteoclasts respectively (**Figure 4.1**). RT-PCR showed that osteoclasts express mRNAs for *Enpp1*, *Enpp3*, *NTPdase1*, *NTPdase3* and the PPI transport protein *Ank*. These *in vitro* mouse osteoclasts did not to express mRNAs for *Enpp2* and *NTPdase2* (**Figure 4.2**).

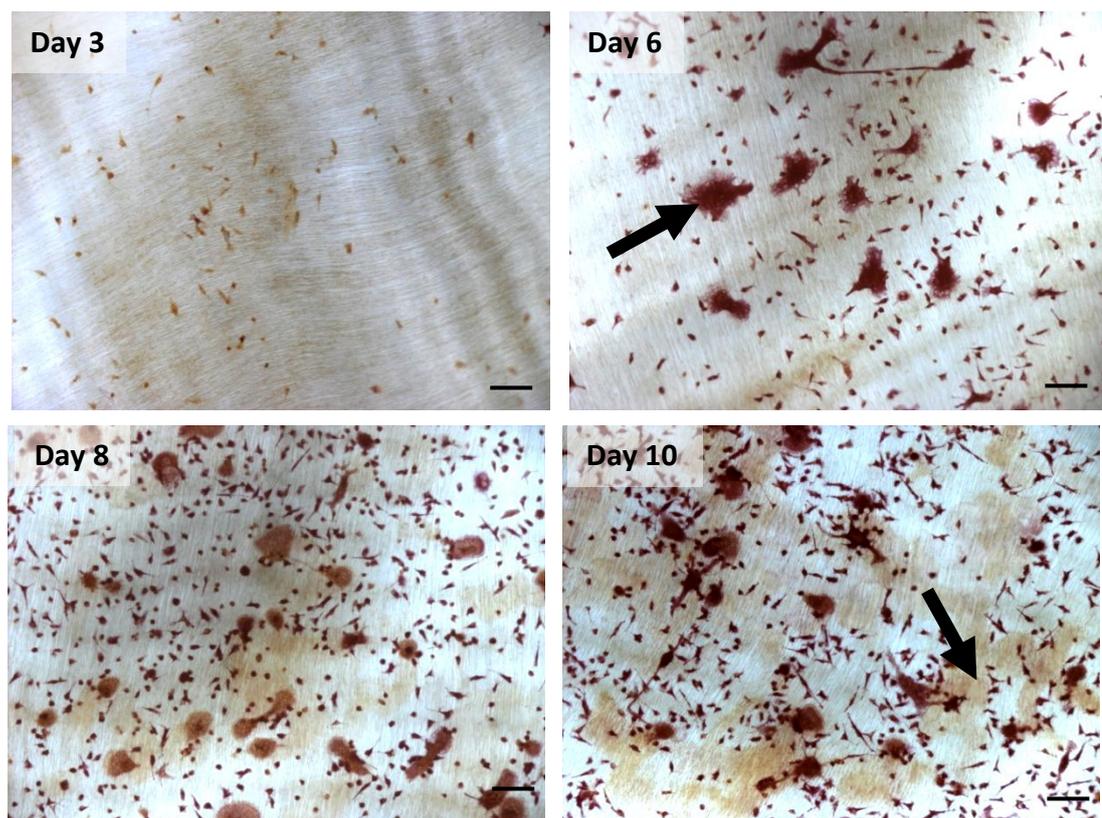


Figure 4.1. Transmitted light microscopy images of mouse osteoclasts grown *in vitro*

Osteoclast precursors were seeded onto dentine discs on day 2. The culture medium was acidified to pH 6.9 on day 8 to activate resorption; no resorption was visible on day 6. TRAP-stained osteoclasts (red) and resorption pits (tan); scale bar = 50 μ m.

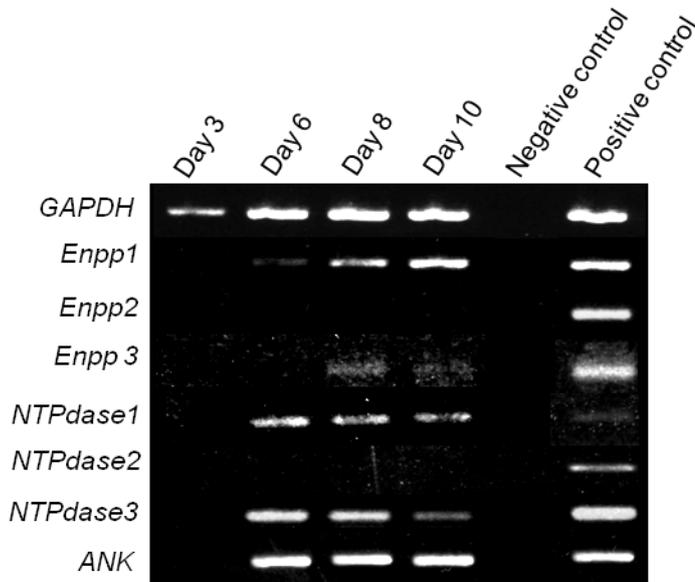


Figure 4.2. Ecto-nucleotidases expression by differentiating mouse osteoclasts *in vitro*

RT-PCR showed that mouse osteoclasts expressed mRNAs for the nucleotidases *Enpp1*, *Enpp3*, *NTPdase1*, *NTPdase3* and the *PPI* transport protein *ANK* from day 6 of culture onwards. mRNAs for *Enpp2* and *NTPdase2* were not detected. *Enpp1* expression increased throughout the culture; *NTPdase3* expression decreased in mature resorbing osteoclasts; positive control = mouse brain.

Mouse osteoclasts have NPP activity *in vitro*

Osteoclasts were grown from precursors obtained from the bone marrow of *Enpp1*^{-/-} and wild type mice on ivory discs for 10 days. On day 8 the cells were acidified to pH 6.90 to activate resorption. The total NPP activity of the cells was measured photometrically using p-nitrophenyl-thymidine 5'-monophosphate on days 6, 8 and 10. It was found that osteoclasts have functional NPP enzyme activity, which decreased throughout the duration of the experiment. *Enpp1* knockout resulted in a 25% reduction in the total NPP activity of osteoclasts at days 6 and 8 of culture, and a 70% decrease in the total NPP activity on day 10 of culture (Figure 4.3).

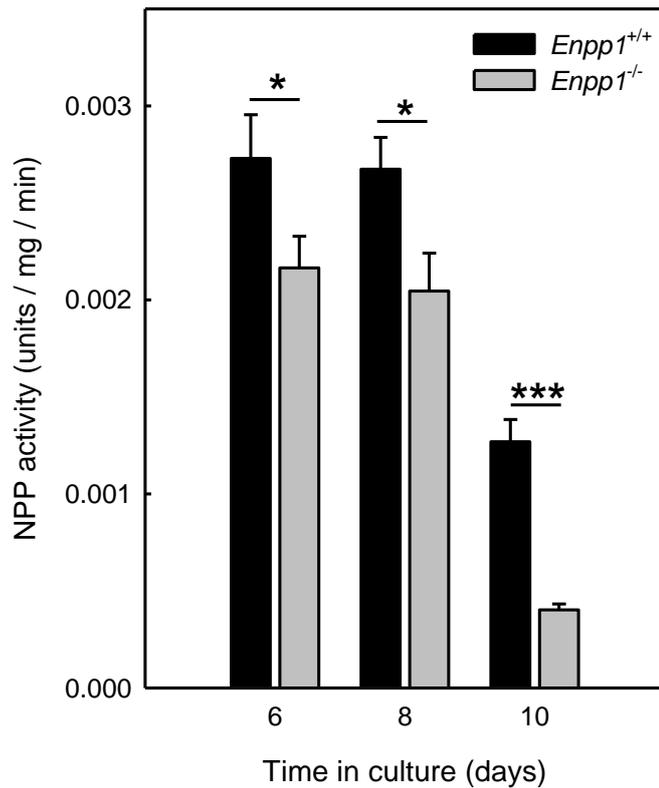


Figure 4.3. NPP activity of mouse osteoclasts *in vitro*

Osteoclasts were grown from precursors on ivory discs. Total NPP activity was determined by the osteoclasts' ability to hydrolyse p-nitrophenyl-thymidine 5'-monophosphate. (Data are means \pm SEM; *, $p < 0.05$; ***, $p < 0.001$; $n = 12$).

Acid activation of mouse osteoclasts increases NPP activity and *Enpp1* mRNA expression

Wild type mouse osteoclasts were grown from precursors, on ivory discs for 10 days. On day 8 of culture one group of cells was acidified to pH 6.90 ($n = 12$ discs), a second group was maintained at pH 7.30. On day 10 the experiment was terminated, NPP activity was measured and mRNA was collected. Osteoclasts which were acidified had increased mRNA expression of *Enpp1* and greater total NPP activity compared to non-acidified cells (**Figure 4.4**).

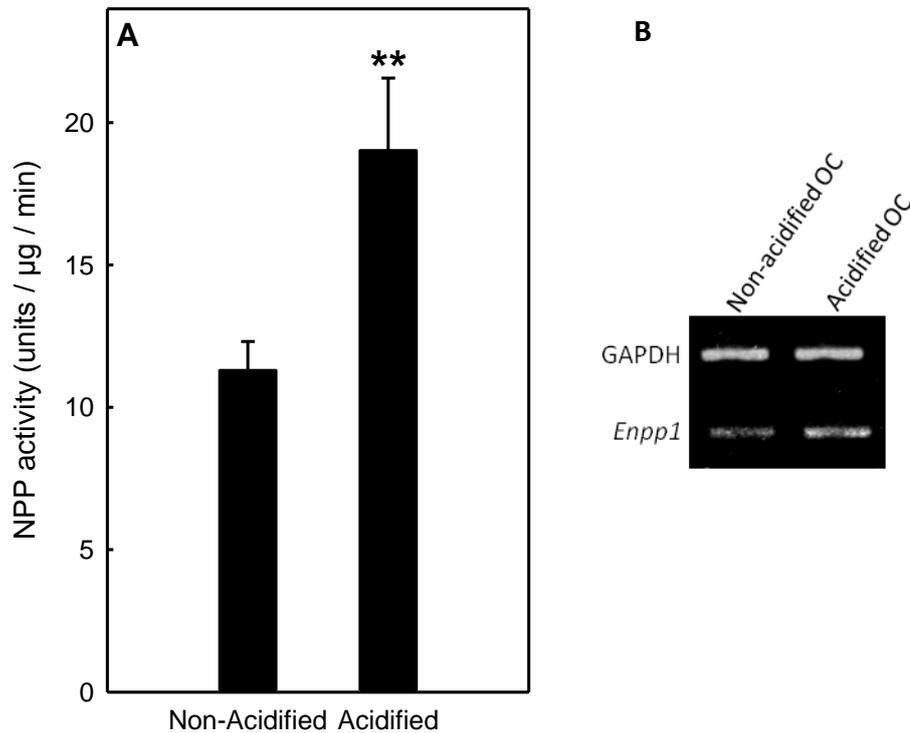


Figure 4.4. Increased *Enpp1* mRNA expression and NPP activity in acid-activated mouse osteoclasts *in vitro*

Osteoclasts were grown from precursors on ivory discs. On day 8 the test group osteoclasts were acidified to pH 6.90, the control group was maintained at pH 7.30. On day 10 of culture **(A)** the total NPP activity was measured in cell lysates; **(B)** mRNA was collected for RT-PCR analysis. (Data are means \pm SEM; **, $p < 0.01$; $n = 12$).

The effect of *Enpp1*^{-/-} on osteoclast mRNA expression

Osteoclasts were grown from precursors obtained from the bone marrow of *Enpp1*^{-/-} and wild type mice on ivory discs for 8 days. On day 8 of culture TRIzol was used to collect mRNA from the osteoclasts. RT-PCR demonstrated that *Enpp1*^{-/-} osteoclasts have increased expression of mRNAs for *NTPdase1*, *Enpp3* and *Ank* compared to wild type cells **(Figure 4.5)**.

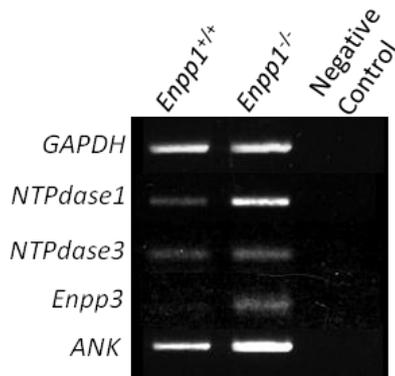


Figure 4.5. Altered expression of mRNAs for nucleotidase-related genes in *Enpp1*^{-/-} mouse osteoclasts *in vitro*

Mouse osteoclasts were grown from precursors on ivory discs. On day 8 of culture mRNA was collected for RT-PCR analysis.

Reduced ATP release and increased intracellular ATP in *Enpp1*^{-/-} osteoclasts

As described previously, osteoclasts were formed from precursor cells on ivory discs that were obtained from the bone marrow of 8 and 15 week old *Enpp1*^{-/-} and wild type mice. The amount of ATP released per cell, and the intracellular ATP concentration were measured on days 6, 8 and 10 of culture. It was found that *Enpp1*^{-/-} osteoclasts formed from precursors obtained from both 8 and 15 week old mice released less ATP per cell than wild type cells (**Figure 4.6A & B**). It was also found that *Enpp1*^{-/-} osteoclasts grown from precursors from 8 week old mice had a higher intracellular ATP concentration than wild type cells from day 6 onwards (**Figure 4.6C**). *Enpp1*^{-/-} osteoclasts grown from precursors from 15 week old mice had a higher intracellular ATP concentration than wild type cells from day 8 onwards (**Figure 4.6D**).

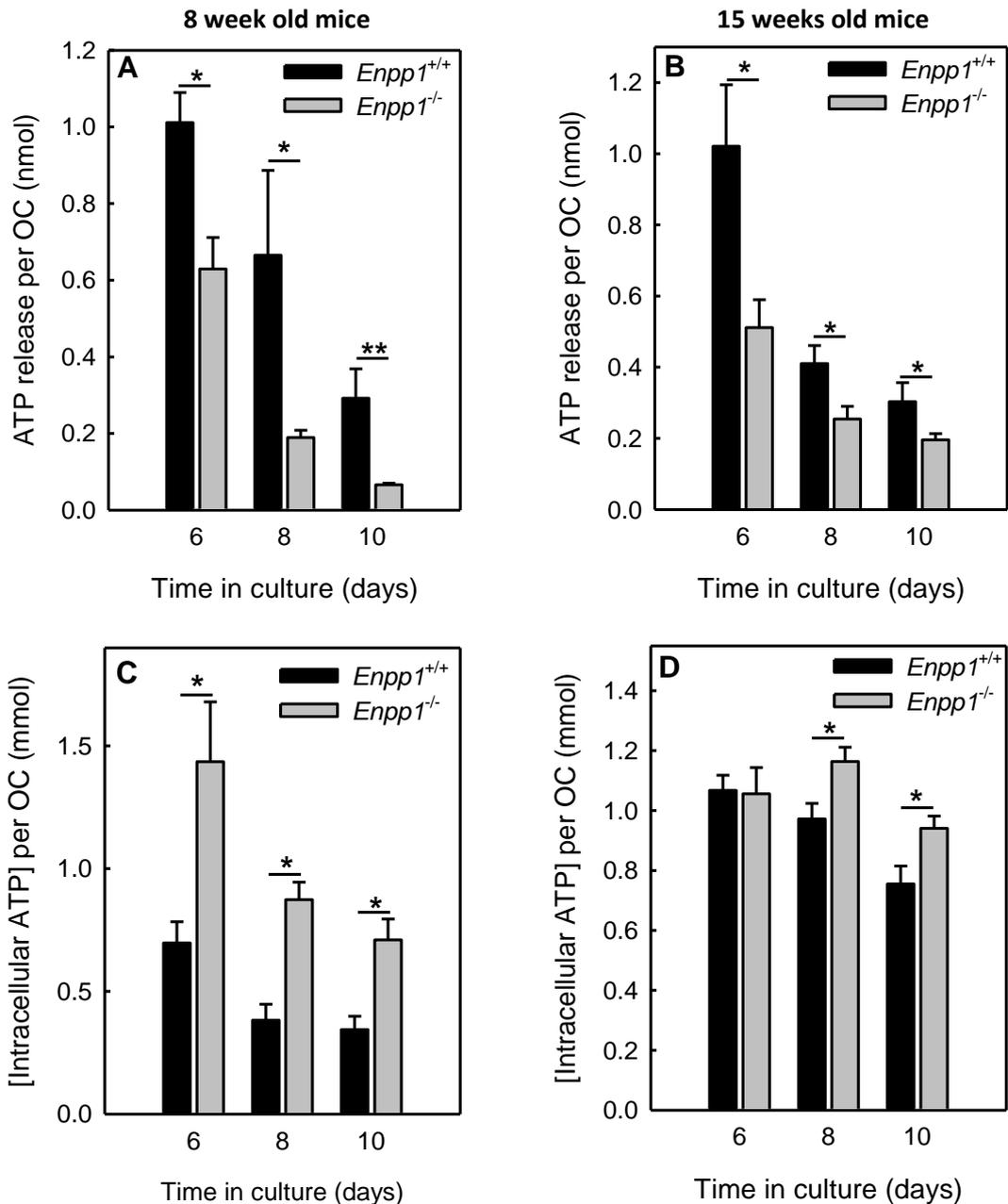


Figure 4.6. *Enpp1*^{-/-} mouse osteoclasts release less ATP and have a higher intracellular ATP concentration compared to wild types *in vitro*

Osteoclasts were grown from precursors obtained from the bone marrow of 8 (A, C) and 15 (B, D) week old mice. To determine the rate of ATP release, medium was replaced with fresh serum free medium, after 1 hour the ATP concentration in the medium was measured. To determine the intracellular ATP concentration the osteoclasts were lysed using Triton X-100 before the ATP concentration was measured. Cell viability was found to be above 90% in all groups, with no significant differences seen. (**, $p < 0.01$; *, $P < 0.05$; data are means \pm SEM; $n = 12$).

***Enpp1* knockout does not affect extracellular ATP hydrolysis by cultured osteoclasts**

Experiments were performed to determine if the knockout of *Enpp1* resulted in a decreased rate of ATP breakdown. On ivory discs, osteoclasts were grown from precursor cells obtained from the bone marrow of 8 and 15 week old *Enpp1*^{-/-} and wild type mice. On day 8, all of the cell culture media were removed from the cells and replaced with serum free media containing 100 nM ATP. The rate of ATP hydrolysis in the cell culture media was measured using a luminescent method described on page 72. Using precursors from both 8 and 15 week old mice, it was found that knockout of *Enpp1* in osteoclasts did not reduce the rate of extracellular ATP hydrolysis (**Figure 4.7**).

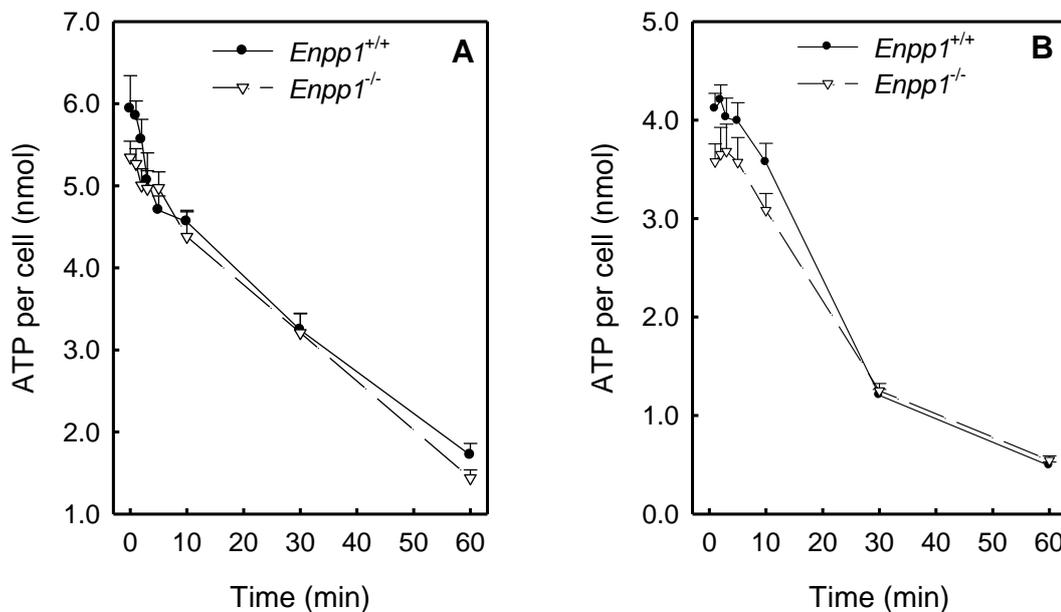


Figure 4.7. Knockout of *Enpp1* does not affect the rate of extracellular ATP hydrolysis by mouse osteoclasts *in vitro*

In vitro osteoclasts were cultured from the bone marrow of 8 (**A**) and 15 (**B**) week old mice. On day 8, all of the culture media were removed and replaced with fresh media containing 100 nM ATP (t = 0). The concentrations of ATP in the culture media were measured using a luminescent method from when the media was added. Cell viability was measured using LDH and found to be above 90%, with no significant differences seen; data are means \pm SEM; n = 12.

Reduced ATP release from *Enpp1*^{-/-} osteoclasts stimulated by fluid flow

Mechanical stimulation of osteoblasts and osteocyte-like cells *in vitro* by the flow of fluid across their surface increases ATP release (Romanello *et al.* 2001; Genetos *et al.* 2005; Genetos *et al.* 2007). However, there are currently no published papers showing ATP release by primary osteoclasts in response to fluid flow. Because it was found that *Enpp1*^{-/-} osteoclasts release less ATP compared to wild types, and have a greater intracellular ATP concentration, their response to fluid flow *in vitro* was investigated.

Osteoclasts were cultured as described above. On day 8, cell culture medium was removed from the ivory discs and replaced with serum free medium, the cells were then left undisturbed. After 1 hour the cells were stimulated by removing 85% of the cell culture media, tilting the culture plate to 45° and running the same culture media over the cells with a Gilson pipette at a fixed rate of 50µl per second until the entire medium was reintroduced to the well. This procedure was carried out twice in total. The concentrations of ATP and LDH (to determine cell viability) in the cell culture media were measured for 30 minutes. Osteoclasts released ATP in response to fluid flow. It was also found that *Enpp1*^{-/-} osteoclasts formed from precursors from 8 and 15 week old mice released less ATP when stimulated by fluid flow than wild types (**Figure 4.8**).

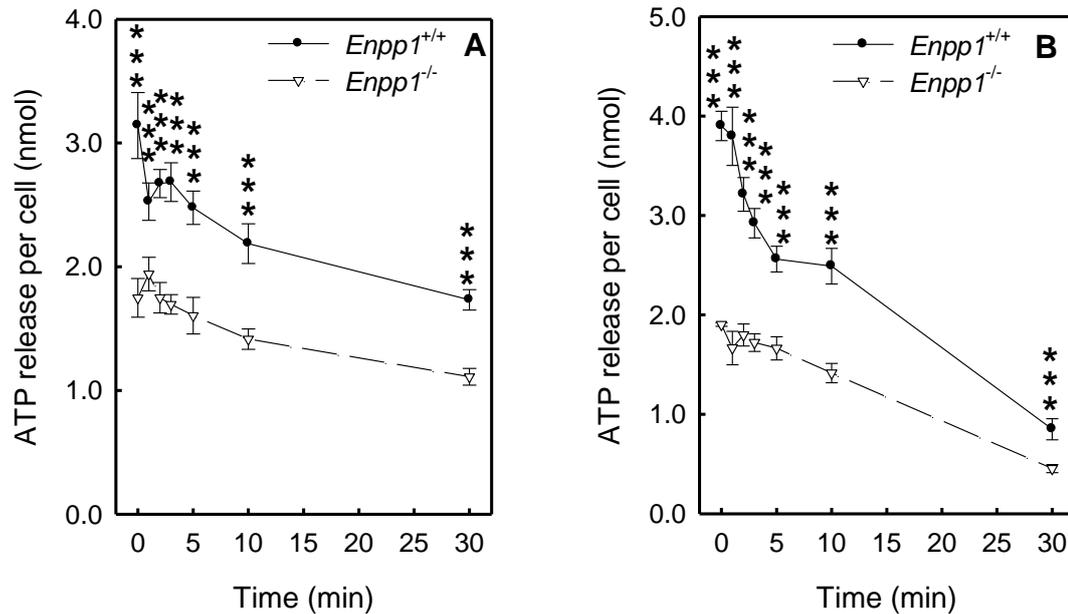


Figure 4.8. *Enpp1*^{-/-} mouse osteoclasts released less ATP under fluid flow stimulation than wild types *in vitro*

Osteoclasts were generated from the bone marrow of 8 (A) and 15 (B) week old *Enpp1*^{-/-} and wild type mice. Osteoclasts were subjected to flow fluid and their ATP release into serum free medium was measured by luminometry. Cell viability determined by LDH assay was $\geq 85\%$ with no significant differences seen. (***, $p < 0.001$; data are means \pm SEM; $n = 12$.)

Knockout of *Enpp1* has no effect on the formation or resorptive activity of mouse osteoclasts *in vitro*

Osteoclasts were cultured on ivory discs from precursors that were collected from the bone marrow of 8 and 15 week old *Enpp1*^{-/-} and wild type mice. On day 8 of culture the cells were acidified to pH 6.90 to activate resorption. It was found that knockout of *Enpp1*^{-/-} had no effect on the formation, or the rate of resorption, of mouse osteoclasts *in vitro* (Figure 4.9).

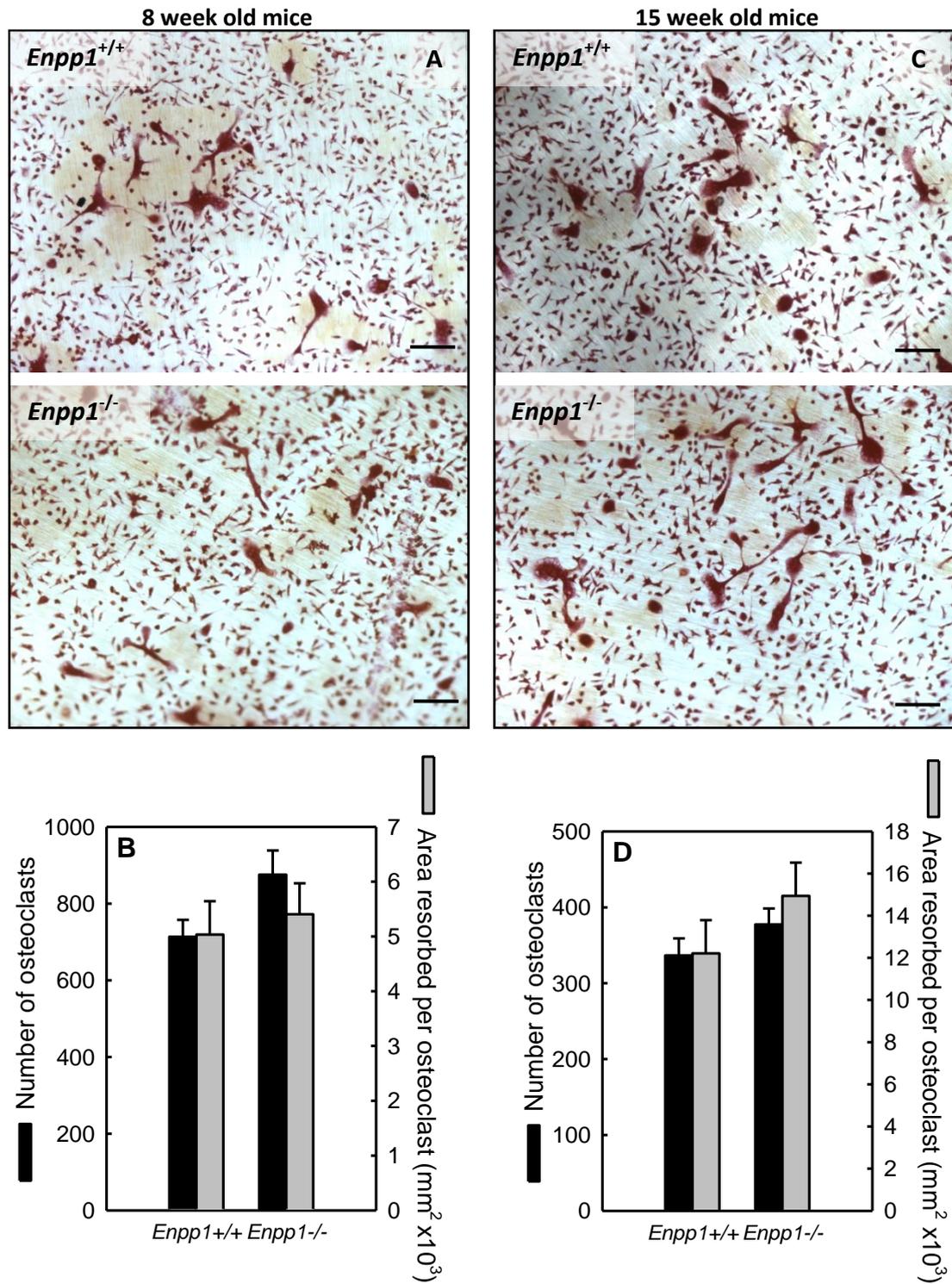


Figure 4.9. Knockout of *Enpp1* has no effect on the formation or resorptive activity of mouse osteoclasts *in vitro*

Osteoclasts were formed from the bone marrow of 8 (**A, B**) and 15 (**C, D**) week old mice and cultured on ivory discs for 10 days; scale bar = 50 μ m. On day 8 of culture the osteoclasts were acidified to pH 6.90 to activate resorption. (Data are means \pm SEM; n = 8)

***Enpp1* and osteoblasts**

***Enpp1*^{-/-} mouse osteoblasts formed more mineralised bone *in vitro* than wild type osteoblasts**

Wild type and *Enpp1*^{-/-} mouse calvarial osteoblasts were cultured for up to 28 days in supplemented α MEM. The experiments were terminated and the amount of mineralised bone formed in the cell culture plates was quantified by automated image analysis. It was found that cultured mouse *Enpp1*^{-/-} osteoblasts produce approximately 32% ($p < 0.001$) more bone than wild type cells (**Figure 4.10A-C**).

***Enpp1*^{-/-} mouse osteoblasts proliferated at the same rate as wild type osteoblasts**

To determine if the knockout of *Enpp1* affects the rate of osteoblast proliferation *in vitro*, wild type and *Enpp1*^{-/-} mouse calvarial osteoblasts were cultured for up to 14 days in α MEM. 10^5 cells were seeded into each well of a six well cell culture plate, the number of cells within each well was determined spectrophotometrically at specific time points using an LDH method. *Enpp1*^{-/-} osteoblasts proliferated at the same rate as wild type osteoblasts, resulting in no significant difference in the number of osteoblasts formed at each time point (**Figure 4.10D**).

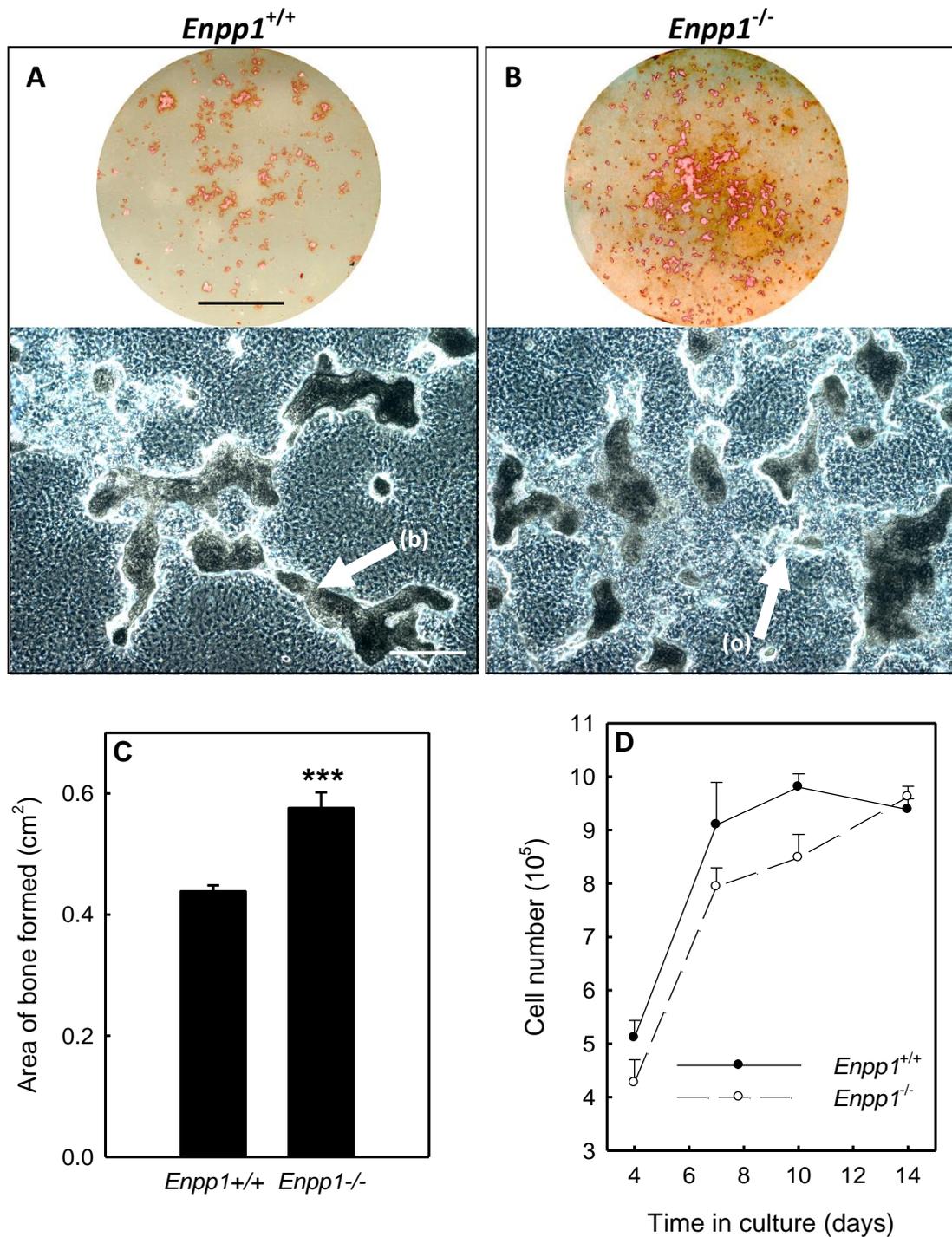


Figure 4.10. *Enpp1*^{-/-} mouse osteoblasts form more mineralised bone than wild types *in vitro*, but proliferate at the same rate

Osteoblast precursors from the calvariae of wild type and *Enpp1*^{-/-} mice were seeded at 10⁵ cells / well into 6 well trays. Images of the bone formed by **(A)** wild type and **(B)** *Enpp1*^{-/-} osteoblasts by day 28; top image, scanned well stained with alizarin red; lower image, phase contrast microscopy of unstained bone nodules (b). Note: *Enpp1*^{-/-} cultures appear to contain more unmineralised matrix than wild types (o); Scale bar top = 1 cm, bottom = 500 μ m. **(C)** Bone formation was quantified using 28 day unstained cultures. **(D)** Cell number was determined using an LDH based method. (***, $p < 0.001$; data are means \pm SEM; $n = 6$.)

Enpp1*^{-/-} osteoblasts had decreased NPP activity, but not ALP activity compared to wild types *in vitro

Wild type and *Enpp1*^{-/-} mouse calvarial osteoblasts were cultured for up to 28 days in supplemented α MEM. The total NPP and ALP enzymatic activity of the osteoblasts were measured by spectrophotometry at 4 time points during the experiment. It was found that *Enpp1*^{-/-} osteoblasts had approximately 50% ($p < 0.001$) less total NPP activity than wild type osteoblasts (**Figure 4.11A**), with no change in ALP activity (**Figure 4.11B**).

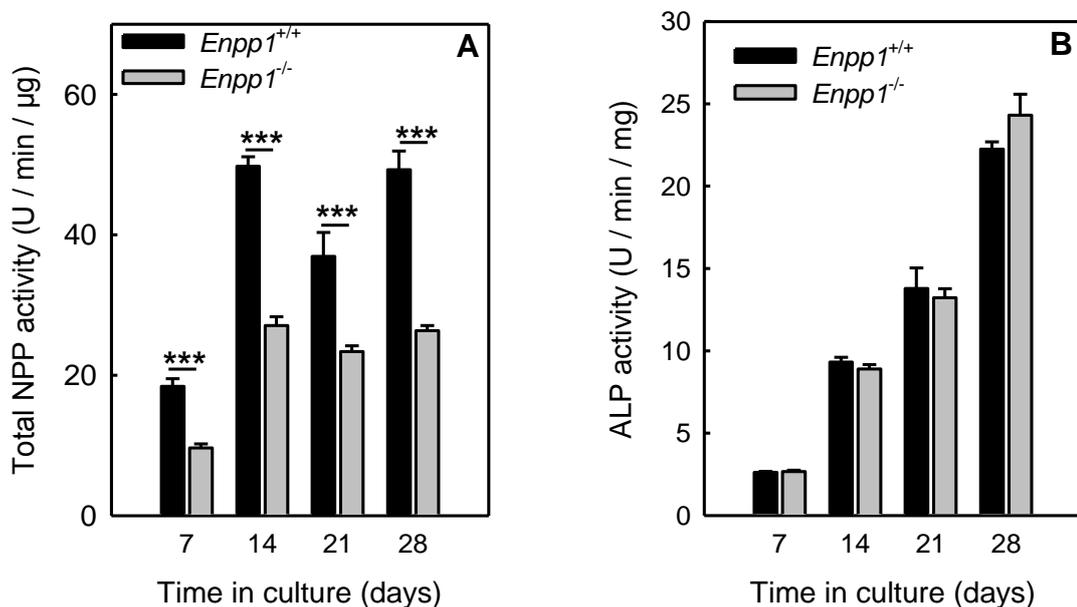


Figure 4.11. *Enpp1*^{-/-} osteoblasts had reduced total NPP activity, but unchanged ALP activity compared to wild types *in vitro*

Osteoblasts precursors for the calvariae of wild type and *Enpp1*^{-/-} mice were seeded at 10^5 cells / well into 6 well trays. **(A)** The total NPP and **(B)** ALP activity of the cells was normalised to the cell protein concentration. (Data are means \pm SEM; $n = 6$; ***, $p < 0.001$).

Exogenous ATP inhibited mineralised bone formation by *Enpp1*^{-/-} and wild type mouse osteoblasts *in vitro*

Wild type and *Enpp1*^{-/-} mouse calvarial osteoblasts were cultured for up to 28 days in supplemented α MEM with 10 and 100 μ M ATP. The experiments were terminated, and automated image analysis revealed that ATP inhibited the mineralisation of deposited matrix by both *Enpp1*^{-/-} and wild type osteoblasts (**Figure 4.12**).

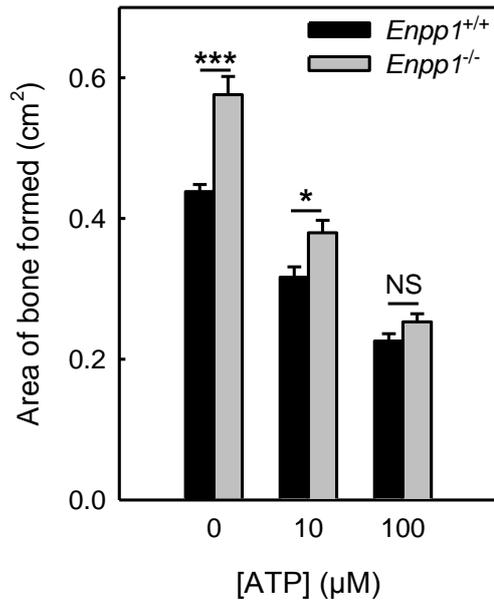


Figure 4.12. Exogenous ATP inhibited bone formation by *Enpp1*^{-/-} and wild type mouse osteoblasts *in vitro*

Osteoblasts precursors for the calvariae of wild type and *Enpp1*^{-/-} mice were seeded at 10⁵ cells / well into 6 well trays. ATP was added from the start of the culture and at each medium change. (Data are means ± SEM; n = 6; NS, not significant; *, p<0.05; ***, p<0.001.)

Enpp1*^{-/-} mouse osteoblasts had lower ATP release and higher intracellular ATP concentration than wild types *In vitro

As described above, wild type and *Enpp1*^{-/-} mouse calvarial osteoblasts were cultured for 28 days. The amount of ATP released per cell, and the intracellular ATP concentration were measured using a luciferase based method on days 7, 14 and 21 of culture. The cell number and cell viability were measured using an LDH assay. It was found that *Enpp1*^{-/-} osteoblasts released between 50 – 70 % (p<0.001) less ATP per cell than wild type cells (**Figure 4.13A**). *Enpp1*^{-/-} osteoblasts also had intracellular ATP levels that were 60 – 350% (p<0.001) greater than wild types (**Figure 4.13B**).

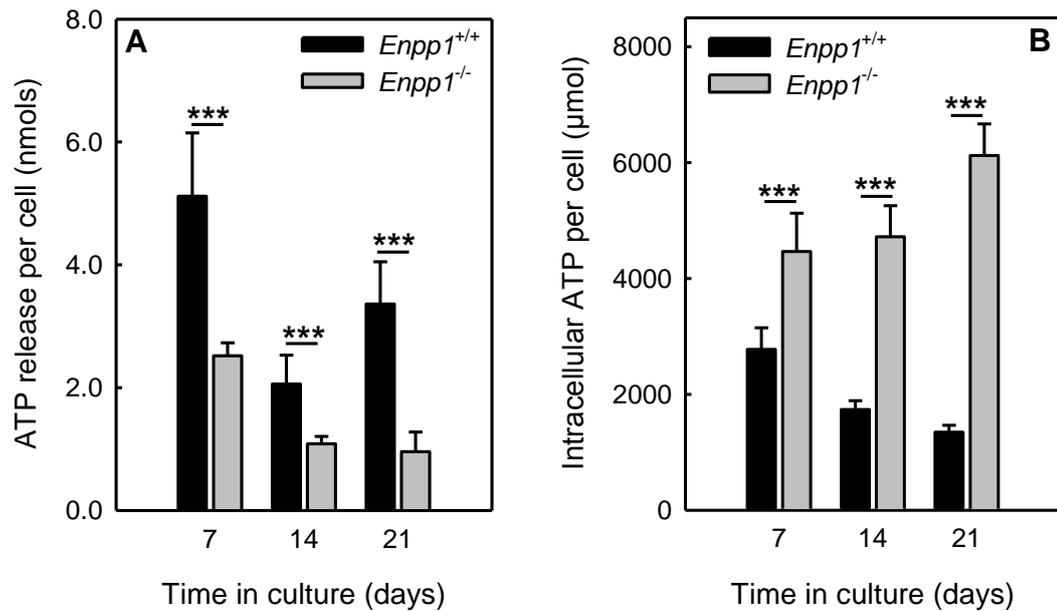


Figure 4.13. *In vitro* *Enpp1*^{-/-} mouse osteoblasts had an increased intracellular ATP concentration and decreased basal ATP release compared to wild types

Osteoblasts precursors for the calvariae of wild type and *Enpp1*^{-/-} mice were seeded at 10⁵ cells / well into 6 well trays. To determine basal ATP release (A), media was removed from the cells and replaced with fresh serum free media, after 1 hour the ATP concentration in the media was measured. To determine the intracellular ATP concentration (B) the osteoclasts were lysed before the ATP concentration was measured. All of the cells were found to be 95% viable by LDH measurement. (Data are means ± SEM; ***, p<0.001.)

***In vitro* *Enpp1*^{-/-} osteoblasts released less ATP per cell when stimulated compared to wild type osteoblasts**

Similar to the *Enpp1*^{-/-} osteoclasts, it was found that *Enpp1*^{-/-} osteoblasts released less ATP compared to wild types, and had a greater intracellular ATP concentration than wild type. The response of *Enpp1*^{-/-} and wild type mouse osteoblasts to fluid flow across their surface *in vitro* was investigated.

Enpp1^{-/-} and wild type osteoblasts, developed from calvarial precursors, were cultured in 6 well trays. On day 14, all of the cell culture media was removed from the culture plate and replaced with serum free media, the cells were then left undisturbed. After 1 hour the cells were stimulated by removing 85% of the cell culture media, tilting the cell culture plate to 45° and running the same culture media over the surface of the cells at a fixed rate using a graduated pipette until all of the culture media was reintroduced. The concentration of ATP in the cell culture

media was measured at fixed time points for 30 minutes. The LDH concentration was also measured to determine if the viability of the cells was affected by the fluid flow, and to calculate the number of cells. It was found that *Enpp1*^{-/-} osteoblasts released less ATP when stimulated by fluid flow than wild type osteoblasts (Figure 4.14A).

Knockout of *Enpp1* does not affect the rate of extracellular ATP hydrolysis by osteoblasts *in vitro*

Osteoblasts were cultured in 6 well trays as described. On day 14 all of the cell culture media was removed and replaced with serum free media containing 1 μM ATP. The rate of ATP hydrolysis of the in the cell culture media was measured using a luciferase method as described. The LDH concentration was also measured to determine the viability of the osteoblasts, and to calculate the number of cells. There was no significant difference in the rate of extracellular ATP hydrolysis between cultured *Enpp1*^{-/-} and wild type mouse osteoblasts (Figure 4.14B).

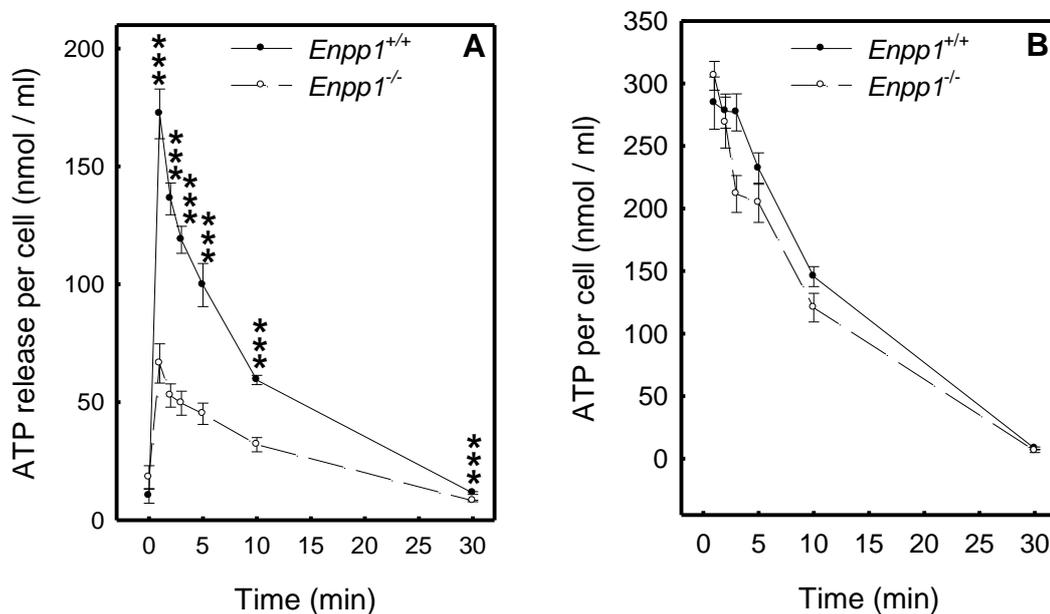


Figure 4.14. Cultured *Enpp1*^{-/-} osteoblasts release less ATP in response to fluid flow than wild type cells, but hydrolyse extracellular ATP at a similar rate

Osteoblasts precursors were seeded at 10⁵ cells / well into 6 well trays. **(A)** Culture media was removed and replaced with serum free media. After 1 hour cells were stimulated by fluid flow. **(B)** Culture media was replaced with media containing 1 μM ATP (t = 0) and the ATP concentration determined. Cell viability was found to be >85% by LDH measurement for all groups. (***, p<0.001; n = 12).

Discussion

The work presented in this chapter found that cultured *Enpp1*^{-/-} osteoclasts differentiate from precursor cells, and resorbed mineralised tissue at the same rate as wild types. *Enpp1*^{-/-} osteoclasts showed reduced ATP release and increased intracellular ATP compared to wild types, but with no change in the rate of extracellular ATP hydrolysis. *Enpp1*^{-/-} osteoblasts were found to have 50% less NPP activity compared to wild types and produce approximately 30% more bone *in vitro*. *Enpp1*^{-/-} osteoblasts were also found to have a higher intracellular ATP concentration and a lower rate of ATP release than wild types, with no change in the rate of extracellular ATP hydrolysis.

This study of *Enpp1*^{-/-} osteoclasts was undertaken because evidence of increased activity was seen *in vivo* (see chapter 3). This work showed for the first time that cultured osteoclasts expressed mRNA for ecto-nucleotidases and had NPP activity. *Enpp1*^{-/-} osteoclasts had greater mRNAs expression for *Enpp3* and *NTPdase1* than wild types; this may be a compensatory mechanism to counteract the loss of NPP1 and may be a reason why extracellular ATP hydrolysis was unchanged in *Enpp1*^{-/-} cells.

Extracellular ATP and ADP, signalling through the P2 receptors, stimulate osteoclasts to resorb mineralised tissues *in vitro* (Hoebertz *et al.* 2001). It was therefore expected that *Enpp1*^{-/-} osteoclasts might resorb fewer pits *in vitro* due to their reduced release of ATP into the extracellular environment. However, no difference was observed between *Enpp1*^{-/-} and wild type osteoclasts. *Enpp1*^{-/-} osteoclasts were found to release approximately 50% less ATP than wild types; this is equivalent to approximately 0.5 nmol / cell (**Figure 4.6**). Each ivory disc had approximately 700 individual cultured osteoclasts on it; therefore the total difference in ATP released was 350 nmol per replicate. Work within Chapter 5 of this thesis and previous work has shown that an exogenous ATP concentration of 2 - 10 μ M is required to stimulate formation and resorption by osteoclasts (Morrison *et al.* 1998). Taken together, these data indicates that the difference in ATP release

between wild type and *Enpp1*^{-/-} osteoclasts is 5 - 28 times lower than what would be required to stimulate an observable effect *in vitro*.

ATP and P_i both inhibit the formation of bone by rodent osteoblasts *in vitro* (Hoebertz *et al.* 2002; Orriss *et al.* 2007). *Enpp1*^{-/-} osteoblasts produced more mineralised bone than wild type cells *in vivo*. The most obvious explanations for this are the 50% reduction in NPP activity, leading to reduced P_i formation, and the 50 – 70% reduction in endogenous ATP release seen (Orriss *et al.* 2013). *Enpp1*^{-/-} osteoblasts released approximately 1 - 2 nmol / cell less ATP than wild types *in vitro*. The large number of cells within each well of the cell culture plate means that differences of up to 1µM could arise; which is the concentration needed to significantly reduce matrix mineralisation (Orriss *et al.* 2007). Wild type and *Enpp1*^{-/-} osteoblast cultures were found to have equal ALP rates of activity; therefore any P_i that is formed will be hydrolysed at similar rates.

A higher concentration of intracellular ATP was seen in both *Enpp1*^{-/-} osteoblasts and osteoclasts compared to wild types. This may be due to the decreased release of ATP by these cells. However it is not clear if the rate of ATP synthesis by *Enpp1*^{-/-} cells is altered compared to wild types. The P_{2X}₇ receptor has been implicated as a mechanism for the release of ATP from osteoblasts (Romanello *et al.* 2001; Buckley *et al.* 2003; Genetos *et al.* 2005) and osteoclasts (Suadicani *et al.* 2006; Pellegatti *et al.* 2011). P_{2X}₇ receptor antagonists have been shown to reduce the rate of ATP release by osteoblasts and osteoclasts (Brandao-Burch *et al.* 2012). Reduced stimulation of the P_{2X}₇ receptor on the surface of *Enpp1*^{-/-} osteoblasts and osteoclasts, due to the lower extracellular concentration of ATP, may lead to a feedback loop resulting in lower ATP release. Also, inhibitors of vesicular exocytosis can reduce ATP release from osteoblasts by up to 90% (Orriss *et al.* 2009). Knockout of *Enpp1*^{-/-} in osteoblasts *in vitro* may interfere with the vesicular release of ATP.

Enpp1^{-/-} osteoblasts released less ATP than wild type cells when stimulated by fluid flow. In the previous chapter, *Enpp1*^{-/-} primary osteocyte-like cells were shown to release less ATP than wild types. Together, these results suggest the

possibility that *Enpp1*^{-/-} mice may be less sensitive to the fluid flow induced by mechanical strain upon bones *in vivo*. It has been suggested that fluid flow through the osteocyte canaliculi and lacunae is important in load sensing (Bonewald 2011; Price *et al.* 2011).

In summary, cultured *Enpp1*^{-/-} osteoclasts are not more active and do not form faster than wild type osteoclasts. This indicates that the increased osteoclast activity seen *in vivo* (Chapter 3) is due to an undetermined factor, such as a hormone, cytokine or physiological parameter acting upon the osteoclasts. *Enpp1*^{-/-} mouse osteoblasts produced more bone than wild type osteoblasts, but differentiate from precursors at the same rate as wild type cells. Surprisingly, *Enpp1*^{-/-} mouse osteoblasts and osteoclasts had a higher intracellular ATP concentration and a reduced rate of ATP release compared to wild type cells *in vitro*.

Chapter 5

Lack of effect of adenosine on rodent osteoblasts and osteoclasts *in vitro*

Introduction

Adenosine

Adenosine is a hydrolysis product of ATP. It is formed in both the intra and extracellular compartments by the orchestrated actions of multiple enzymes (see page 60). Adenosine acts via the G-protein coupled P1-receptors, found on the surface of many cell types. Both osteoblasts and osteoclasts have been reported to express all four P1 receptor subtypes (Kara *et al.* 2010a; Gharibi *et al.* 2011; Pellegatti *et al.* 2011; Vincenzi *et al.* 2013). However, the actions of extracellular adenosine on bone cells are not clear.

Osteoblasts and adenosine

Synthetic adenosine analogues were shown to elicit a receptor-mediated rise in cAMP levels in calvarial osteoblast-like cells (Lerner *et al.* 1987) but adenosine itself had no effect on intracellular calcium levels in these cells (Orriss *et al.* 2006). Two independent groups failed to find an effect of adenosine on the formation of mineralised bone nodules by rat calvarial osteoblasts *in vitro* (Jones *et al.* 1997; Hoebertz *et al.* 2002). However, a more recent study has indicated that adenosine, acting via the A_{2B} receptor, may increase the osteogenic differentiation of rat long bone mesenchymal stem cells *in vitro* (Gharibi *et al.* 2011). Bone nodule formation by osteoblasts cultured from the bone marrow of A_{2B} receptor knockout mice has also been shown to be reduced (Carroll *et al.* 2012); the same authors also found that a synthetic adenosine receptor agonist increased bone nodule formation by wild type osteoblasts. It has been reported that adding the adenosine A₁ or A_{2B} receptor agonists to cultures increased human osteoblast ALP activity, but A_{2A} agonists decreased it and A₃ agonists were without effect (Costa *et al.* 2011b).

Bone nodule formation by osteoblasts from mice lacking ecto-5′nucleotidase (eN) (which may result in reduced endogenous adenosine) also showed reductions, along with decreased ALP expression (Takedachi *et al.* 2012). Conversely, others have reported that adenosine decreases alkaline phosphatase activity and mineralised bone produced by *eN*^{-/-} human fibroblasts *in vitro* (St Hilaire *et al.* 2011).

Osteoclasts and adenosine

The study of Lerner and colleagues (1987) found that adenosine analogues had no effect on the resorption of cultured mouse calvarial bones. Adenosine was subsequently reported to be without effect on the formation or resorptive activity of primary rodent osteoclasts *in vitro* (Morrison *et al.* 1998; Hoebertz *et al.* 2001). However, more recent work has indicated that adenosine, acting through the A_{2A} receptor may stimulate the formation of osteoclasts from human peripheral blood cells (Pellegatti *et al.* 2011). In contrast, Mediero *et al.* (2012) found that A_{2A} receptor agonists inhibited mouse osteoclast formation *in vitro*. Blockade or deletion of the A₁ receptor has additionally been reported to reduce the formation of mouse osteoclasts in culture (Kara *et al.* 2010b); however, the same group also found that stimulation of the A₁ receptor had no effect on mouse osteoclasts (He & Cronstein 2012). In the *eN*^{-/-} mouse no change in the circulating levels of the osteoclast markers TRAP5b and CTX were observed (Takedachi *et al.* 2012), although osteoclastogenesis *in vitro* was reduced (He *et al.* 2013b).

Caffeine

Caffeine is a non-specific adenosine receptor antagonist (Degubareff & Sleator 1965; Fredholm 1982). It has been reported that caffeine increased rodent osteoclast differentiation and formation in both a direct (Choi *et al.* 2013), and osteoblast mediated way *in vitro* (Liu *et al.* 2011) and inhibited the formation of rodent osteoblasts, and osteoblast like cells *in vitro* (Tsuang *et al.* 2006; Su *et al.* 2013). A number of clinical trials have investigated the effect of caffeine on fracture risk. Some studies demonstrated a mild risk of fracture in certain specific bones due to caffeine (Kiel *et al.* 1990; Hernandez-Avila *et al.* 1991; Hansen *et al.*

2000; Hallstrom *et al.* 2006); others found no association (Holbrook *et al.* 1988; Fujiwara *et al.* 1997; Huopio *et al.* 2000). It has been suggested that this risk highlights an important role of adenosine receptors in bone formation (Ham & Evans 2012).

Aims

The aim of the work in this chapter was to determine the direct actions of adenosine on normal osteoblasts and osteoclasts, using well-characterised assays that measure the accepted physiological functions (i.e., bone formation and bone resorption) of these cells. This work also examined the effects of the synthetic universal adenosine receptor agonist 2-chloroadenosine, which is more resistant to hydrolysis, on osteoblasts and osteoclasts *in vitro*.

Results

Rodent osteoblasts and osteoclasts express P1 receptors *in vitro*

On days 14, 16 and 28 of culture mRNA was collected from rat calvaria, rat bone marrow and mouse calvarial osteoblasts respectively using TRIzol. RT-PCR showed that rat calvarial osteoblasts expressed mRNA for the A₁ and A_{2B} adenosine receptors. Rat bone marrow osteoblasts were shown to express mRNAs for the A₁, A_{2A}, A_{2B} and A₃ adenosine receptors. When normalised to β -actin, RT-PCR showed that rat bone marrow osteoblasts expressed less A₁ and A_{2B} receptor mRNA compared to rat calvarial osteoblasts (**Figure 5.1A**). Mouse calvarial OB expressed mRNA *in vitro* for A₁, A_{2A} and A_{2B} adenosine receptors, but not mRNA for the A₃ receptor (**Figure 5.1B**).

On day 10 of culture, mRNA was collected from mouse osteoclasts *in vitro*. RT-PCR showed that mouse osteoclasts express A_{2A}, A_{2B} and A₃ adenosine receptor mRNA *in vitro* (**Figure 5.1C**).

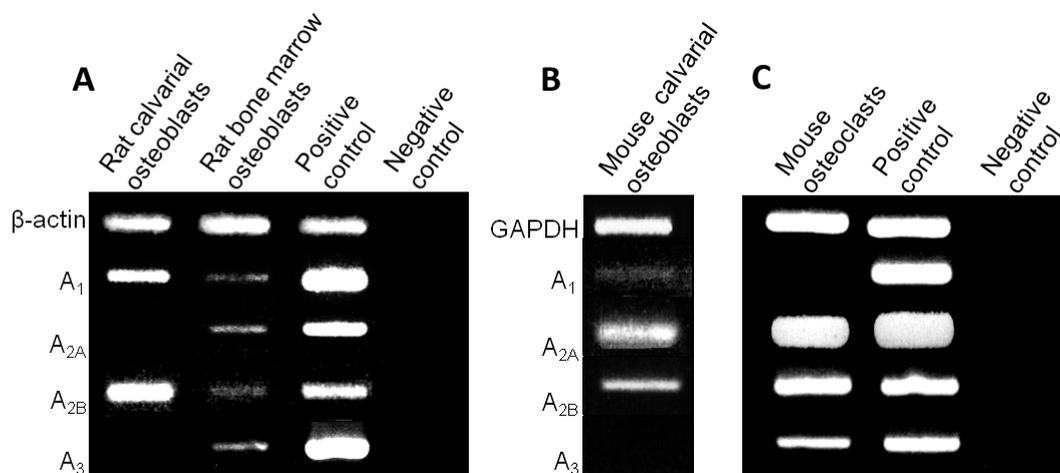


Figure 5.1. Expression of mRNAs for adenosine receptors by rodent bone cells *in vitro*

(A) Rat calvarial osteoblasts expressed mRNAs for the A₁ and A_{2B} adenosine receptors. Rat bone marrow osteoblasts showed weak expression of mRNAs for all of the adenosine receptors. **(B)** Mouse calvarial osteoblasts expressed mRNA for the A₁, A_{2A} and A_{2B} adenosine receptors. **(C)** Mouse osteoclasts strongly expressed mRNA for the A_{2A}, A_{2B} and A₃ adenosine receptors. Positive control: rat / mouse brain.

Adenosine and 2-chloroadenosine have no effect on bone formation by mouse or rat calvarial osteoblasts *in vitro*

Rat calvarial osteoblasts were cultured for up to 14 days in the presence of 1 nM – 100 µM adenosine or 100 pM – 10 µM 2-chloroadenosine *in vitro*. Mouse calvarial osteoblasts were cultured for up to 28 days in the presence of 1 nM – 100 µM adenosine or 1 nM – 1 µM 2-chloroadenosine. No differences were seen in the amount of bone formed between the control and the adenosine or 2-chloroadenosine treated groups (**Figures 5.2 & 5.3**).

2-Chloroadenosine, but not adenosine, increases bone formation by rat bone marrow osteoblasts *in vitro*

Rat bone marrow osteoblasts were cultured with 1 nM – 1 µM 2-chloroadenosine or 1 nM – 100 µM adenosine for up to 16 days. 1 µM 2-chloroadenosine increased the total amount of bone formed by rat bone marrow osteoblasts *in vitro* by approximately 50% ($p < 0.001$) (**Figure 5.2 & 5.3**). Concentrations of 10 µM 2-chloroadenosine and above were toxic; concentrations of less than 1 µM had no effect on the amount of bone formed. Adenosine had no effect on the amount of bone formed by rat bone marrow osteoblasts compared to the control group (**Figure 5.2 & 5.3**).

ATP inhibits bone formation *in vitro* by rodent osteoblasts

Rat osteoblasts were cultured for up to 16 days in the presence of 10 and 100 µM ATP. Mouse calvarial osteoblasts were cultured for up to 28 days in the presence of 10 and 100 µM ATP. ATP was found to inhibit mineralisation of deposited collagen by >50% ($p < 0.001$) in both rat and mouse osteoblasts *in vitro* when at a concentration of 100 µM (**Figure 5.3**).

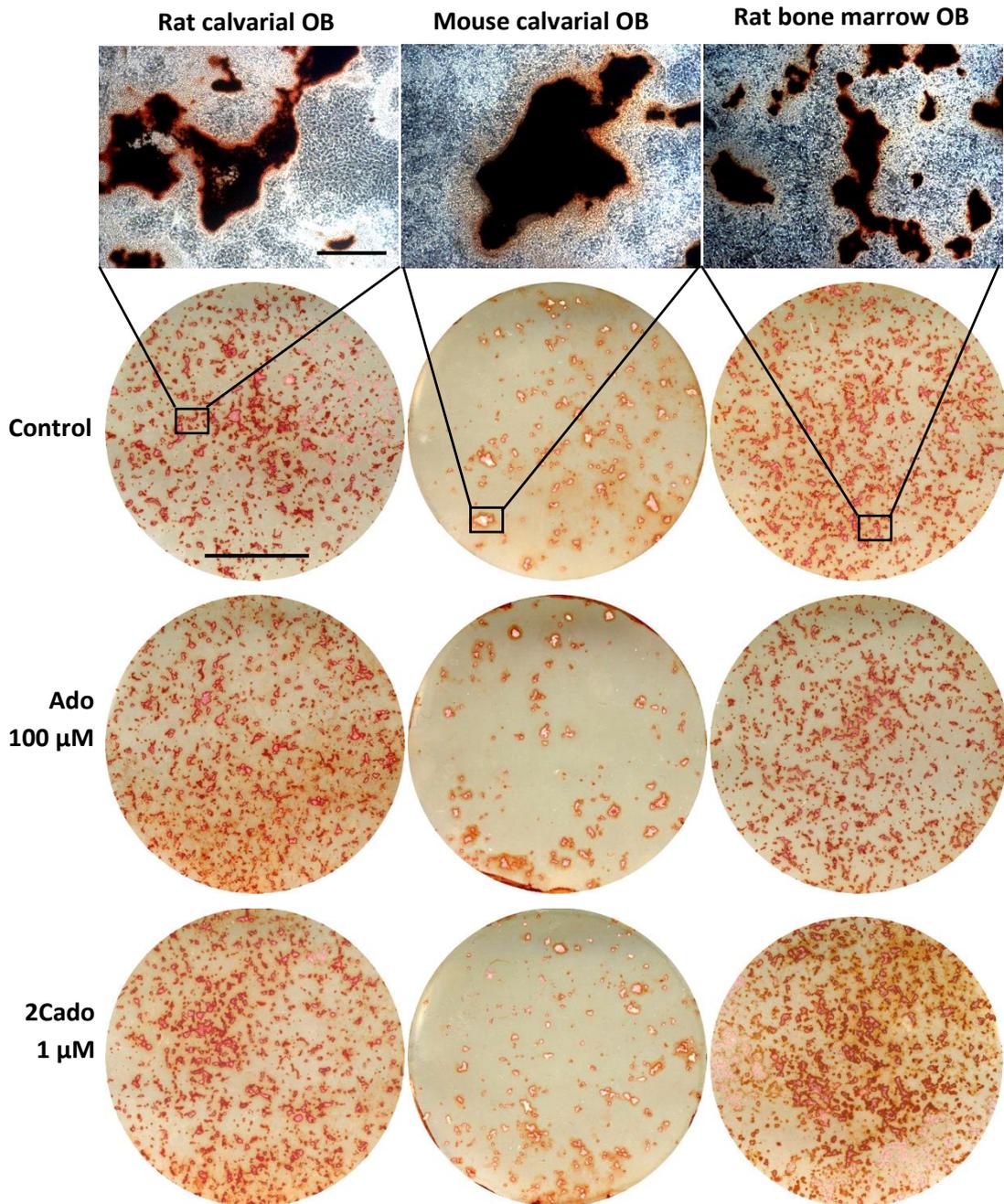


Figure 5.2. Effects of adenosine and 2-chloroadenosine on mineralised bone nodule formation by rodent osteoblasts *in vitro*

Images show alizarin red-stained mineralised bone nodules, viewed by phase contrast microscopy (top) and low power reflected light scans. Adenosine, at a concentrations of 100 μM had no effect on bone formation by rat calvarial, mouse calvarial or rat bone marrow osteoblasts (cultured on plastic for 14, 28 and 16 days, respectively). 2-chloroadenosine at a concentration of 1 μM appeared to cause a modest increase in bone formation by rat bone marrow osteoblasts. Scale bar top = 100 μm , bottom = 1cm; Ado, adenosine; 2Cado, 2-chloroadenosine.

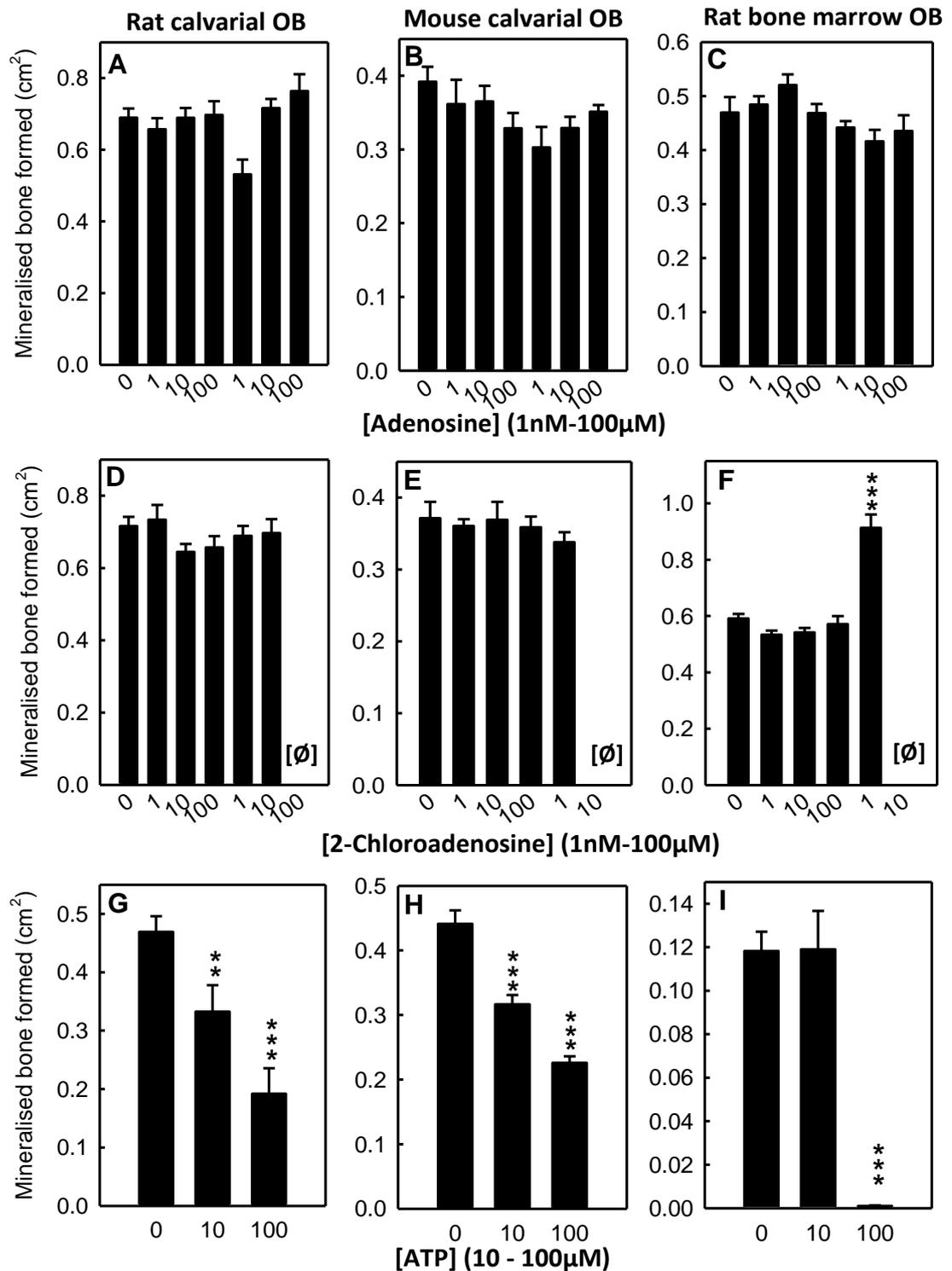


Figure 5.3. Lack of effect of adenosine on the formation of mineralised bone nodules by cultured rodent osteoblasts; modest stimulatory action of 2-chloroadenosine on rat bone marrow osteoblasts

Adenosine and 2-chloroadenosine had no effect on rat calvarial (A, D) or mouse calvarial (B, E) osteoblasts. 1 µM 2-chloroadenosine increased bone nodule formation by rat bone marrow osteoblasts ($p < 0.001$) (F); note toxicity [∅] of 2-chloroadenosine at 10 µM (E, F) and 100 µM (D). ATP inhibited bone formation by rat calvarial (G), mouse calvarial (H) and rat bone marrow (I) osteoblasts (**, $p < 0.01$; ***, $p < 0.001$). Data are means \pm SEM for 6 replicate determinations.

Adenosine and 2-chloroadenosine do not affect the number of osteoblasts formed from precursors *in vitro*

Rat calvarial osteoblast precursors were seeded into 24 well trays, rat bone marrow osteoblast precursor cells were seeded into 12 well trays and mouse calvarial osteoblast precursors were seeded into 6 well trays. Osteoblasts were cultured with 1 – 100 μM adenosine or 100 nM – 10 μM 2-chloroadenosine for 14, 17 or 28 days respectively. Using an LDH assay as described, it was found that adenosine and 2-chloroadenosine had no effect on the number of mouse or rat calvarial osteoblasts formed from precursors *in vitro* (**Figure 5.4**).

2-chloroadenosine increases the ALP activity of *in vitro* rat bone marrow osteoblasts, but not *in vitro* calvarial osteoblasts

Rat bone marrow osteoblast precursor cells were seeded into 12 well trays with 10 μM adenosine or 1 μM 2-chloroadenosine. 1 μM of 2-chloroadenosine increased the ALP activity of *in vitro* rat bone marrow osteoblasts by approximately 48% ($p < 0.01$) from day 10 of culture and was seen to have an effect until day 16, when the experiment was terminated (**Figure 5.5D**). Adenosine had no effect on rat bone marrow osteoblasts *in vitro* (**Figure 5.5D**).

Rat calvarial osteoblast precursors were seeded into 24 well trays with 1 μM - 100 μM adenosine or 10 nM - 1 μM 2-chloroadenosine. Mouse calvarial osteoblast precursors were seeded into 6 well trays, in the presence of 10 μM adenosine or 1 μM 2-chloroadenosine. Adenosine and 2-chloroadenosine had no effect on the ALP activity of rat or mouse calvarial osteoblasts *in vitro* (**Figure 5.5**).

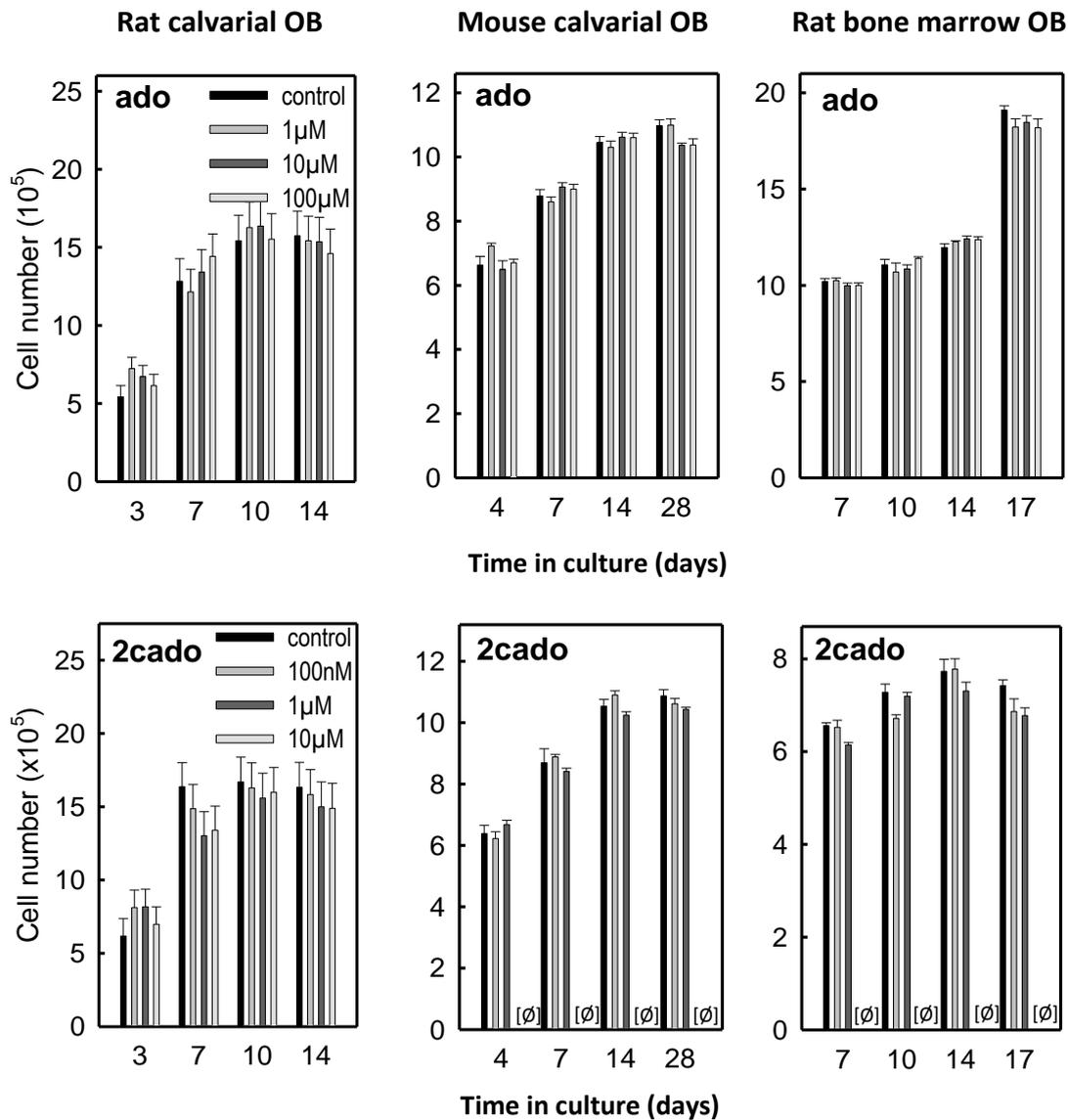


Figure 5.4. The number of rodent osteoblasts formed from precursors *in vitro* is not affected by adenosine or 2-chloroadenosine

Adenosine (ado) and 2-chloroadenosine (2cado) had no effect on numbers of rodent calvarial osteoblasts or rat marrow osteoblasts in culture. Cell numbers were estimated using a LDH assay. ∅ symbol indicates cell toxicity of 10 μM 2cado. Data are means ± SEM for 6 replicate determinations.

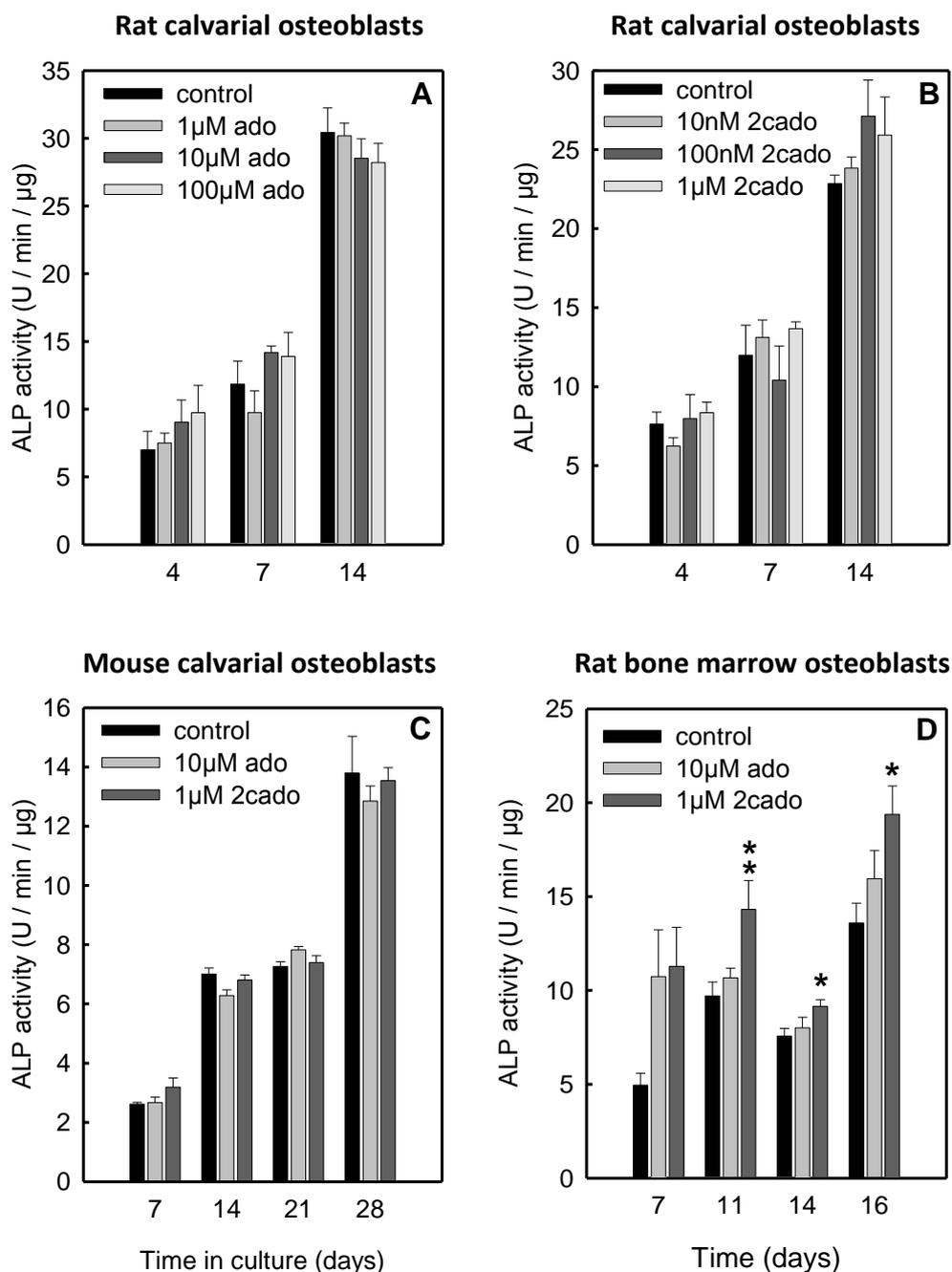


Figure 5.5. Effects of adenosine and 2-chloroadenosine on alkaline phosphatase (ALP) activity of rodent osteoblasts

Adenosine had no effect on the ALP activity of rodent osteoblasts (A, C, D). 2-chloroadenosine also was without effect on rat and mouse calvarial osteoblasts (B, C) but caused mild stimulation of rat bone marrow osteoblast ALP activity (D). Data are means \pm SEM for 6 replicate determinations; *, $p < 0.05$; **, $p < 0.01$; Ado, adenosine; 2Cado, 2-chloroadenosine.

Adenosine and 2-chloroadenosine do not affect mouse osteoclast formation or resorptive activity *in vitro*.

Mouse mononuclear cells from the bone marrow of 8 week old mice were cultured in osteoclastogenic media on ivory discs for 10 days. Adenosine (10 nM – 100 μ M), 2-chloroadenosine (10 nM- 1 μ M) or ATP (1 μ M – 100 μ M) was added to the cell culture media from day 3 onwards. Cells were acidified to pH 6.90 on day 8 of the culture to activate osteoclastic resorption. Neither adenosine nor 2-chloroadenosine affected the number of multinucleated osteoclasts formed from precursors by day 10 of culture or the amount of resorption per osteoclast (**Figures 5.6 & 5.7**). ATP was seen to increase the number of osteoclasts and resorption (**Figures 5.6D & 5.7C**).

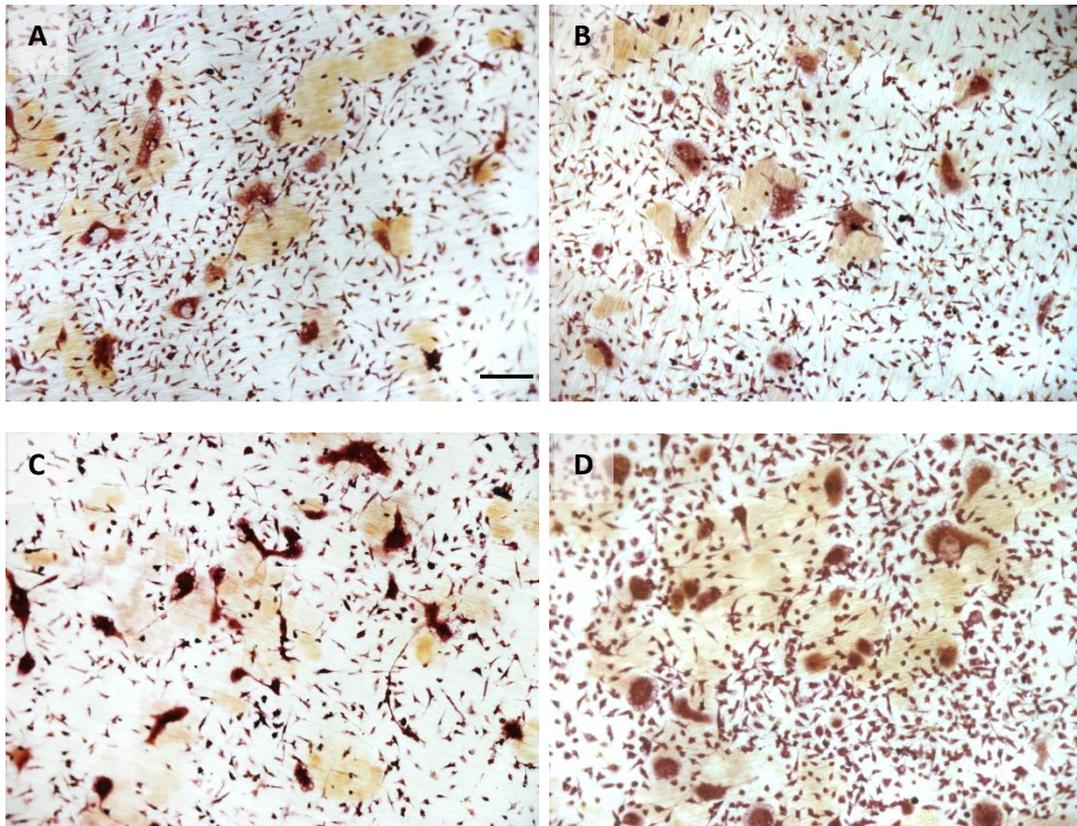


Figure 5.6. Effect of P1 and P2 receptor agonists on osteoclasts

Osteoclasts were generated in 10 day cultures of mouse marrow cells on ivory discs. There was no difference in osteoclast number or resorptive activity between **(A)** control **(B)** adenosine, **(C)** 2-chloroadenosine or **(D)** ATP. Cells were acidified to pH 6.90 on day 8 of culture to activate resorption. Representative transmitted light images of cultures, showing TRAP-positive multinucleated osteoclasts (large red cells) and resorption pits (tan areas); scale bar, 50 μ m.

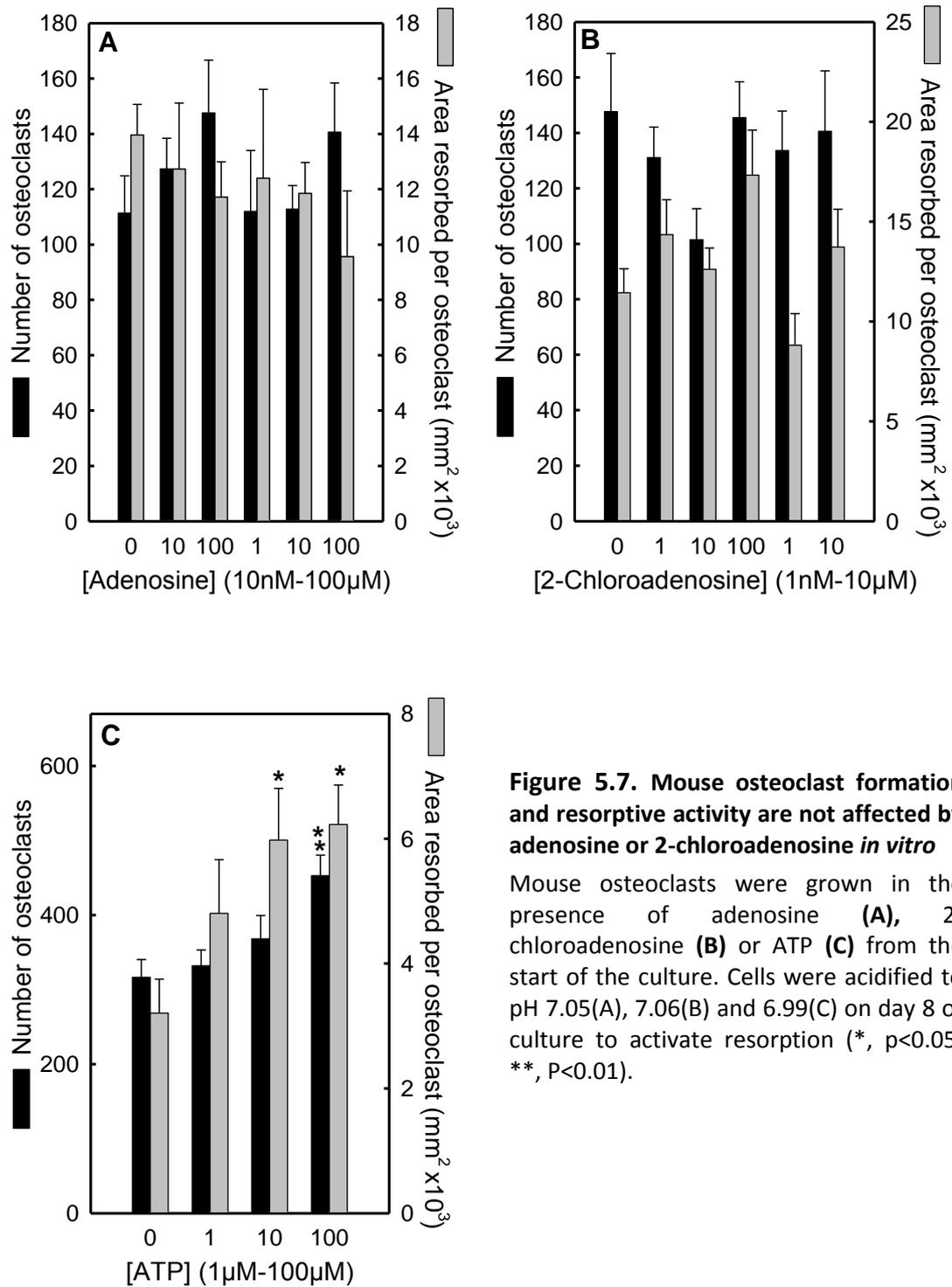


Figure 5.7. Mouse osteoclast formation and resorptive activity are not affected by adenosine or 2-chloroadenosine *in vitro*

Mouse osteoclasts were grown in the presence of adenosine (A), 2-chloroadenosine (B) or ATP (C) from the start of the culture. Cells were acidified to pH 7.05(A), 7.06(B) and 6.99(C) on day 8 of culture to activate resorption (*, p<0.05; **, P<0.01).

Discussion

The results presented in this chapter show that adenosine had no effect on rat and mouse osteoblasts or mouse osteoclasts *in vitro*. However, the synthetic universal adenosine receptor agonist, 2-chloroadenosine, modestly increased ALP activity and the amount of bone formed by rat bone marrow osteoblasts *in vitro*, but did not affect rat and mouse calvarial osteoblasts or mouse osteoclasts. In contrast, the established osteogenic inhibitory effects and osteoclastic stimulatory effects of ATP (Orriss *et al.* 2010) were observed.

It is possible that the stimulatory effects of 2-chloroadenosine on rat bone marrow osteoblasts may be related to the greater potency of this synthetic analogue compared to adenosine (Daly *et al.* 1993; Van Galen *et al.* 1994; Yan *et al.* 2003); moreover, it is not as quickly hydrolysed (Abdel-Hamid *et al.* 2000). One explanation why 2-chloroadenosine had no effect on rat and mouse calvarial osteoblasts may be due to the observed differences in adenosine receptor mRNA expression (**Figure 5.1**). Rat bone marrow osteoblasts expressed mRNA for all four P1 receptors; rat calvarial osteoblasts lacked A_{2A} and A_3 mRNA expression, but expressed A_1 and A_{2B} at higher concentrations than rat bone marrow osteoblasts; mouse calvarial osteoblasts expressed the A_1 , A_{2A} and A_{2B} receptors, but not the A_3 . The A_{2A} and A_{2B} adenosine receptors are predominantly linked to Gs and stimulate cAMP signalling; A_1 and A_3 are predominantly Gi associated and act to inhibit cAMP (Freissmuth *et al.* 1991; Pierce *et al.* 1992; Palmer *et al.* 1995; Olah 1997). There is evidence to suggest that adenosine receptors can form homomers between two A_1 receptors (Ciruela *et al.* 1995), or between two A_{2A} receptors (Canals *et al.* 2004). Adenosine receptor A_1 - A_{2A} heteromers have been reported (Ciruela *et al.* 2006), along with A_1 -P2Y₁ and A_1 -P2Y₂ adenosine-receptor-ATP-receptor G-protein heteromers (Yoshioka *et al.* 2001; Suzuki *et al.* 2006). This dimerisation of receptors may lead to alterations in the response of osteoblasts to P1 receptor agonists and could be a reason for the differences seen between cell types.

It is possible that the differences in responsiveness of osteoblasts to 2-chloroadenosine between rat calvarial and bone marrow cells could be related to

the age of the animals. Rat bone marrow osteoblasts were obtained from 6 week old animals, whereas the rodent calvarial osteoblasts were collected from 2 day old animals. It has been reported that MSCs from older mice have a much higher expression of ecto-5'-nucleotidase, an enzyme responsible for converting AMP to adenosine, than younger mice (Katsara *et al.* 2011) and reduced viability and differentiation potential (Kretlow *et al.* 2008; Choudhery *et al.* 2014).

In the results reported here, no expression of mRNA encoding the A₁ receptor by mouse osteoclasts was detected. Pellagatti *et al.* (2011) reported very weak mRNA expression for the A₁ receptor by human osteoclasts *in vitro*, but suggested that it did not play a significant role in regulating osteoclast formation. However, other workers have reported the A₁ receptor to be vital (Merrill *et al.* 1997; Kara *et al.* 2010b; He & Cronstein 2012). My own results suggest that the adenosine A₁ receptor is unlikely to be of importance in regulating osteoclast function.

The expression of ecto-nucleotidases by osteoblasts and osteoclasts may also be an additional factor in determining the actions of adenosine on these cells. These enzymes could alter the rates of ATP hydrolysis and adenosine formation in the experiments reported here (Zimmermann *et al.* 2012). In chapter 4, some factors that affect the expression of ecto-nucleotidases by osteoclasts were investigated (**Figures 4.2 - 4.5**). The expression of adenosine deaminase, which converts adenosine to inosine, may play a key role in these experiments; a previously unknown factor regulating its expression is reported in chapter 6 (**Figure 6.5**). Future work should examine the potential role of ecto-nucleotidases on adenosine signalling. The possibility that these cultures contained saturating concentrations of adenosine cannot be ruled out.

Knockout mouse models for each of the adenosine receptors exist. Knockout of the adenosine A₁ receptor reportedly increased cortical and trabecular bone volume of in the femurs of 6 month old mice (Kara *et al.* 2010b). Four month old mice with the A_{2A} receptor knocked-out were also reported to have decreased cortical and trabecular bone in the femur (Mediero *et al.* 2012). Based on three samples, knockout of the A_{2B} receptor resulted in a decrease in the cortical bone

volume, with no change in the trabecular bone volume of the femurs of mice, but only after 15 weeks (Carroll *et al.* 2012). The effects of adenosine A₃ receptor knockout on the bones of mice have not been specifically investigated; however, no overt changes have been noted (Salvatore *et al.* 2000). Mice with the adenosine transport protein ENT1 knocked out reportedly had reduced trabecular bone in the femur and increased trabecular bone in the cervical and upper thoracic vertebrae at 7 months (Hinton *et al.* 2014). Mouse models have shown that adenosine acting via the A_{2A} and A_{2B} receptors induced coronary vasodilation (Morrison *et al.* 2002; Frobert *et al.* 2006). Adenosine has also been reported to stimulate human and rodent macrophage production of VEGF *in vitro*, and could thereby promote angiogenesis (Ramanathan *et al.* 2007; Ernens *et al.* 2010; Gessi *et al.* 2010). Therefore, the global knockout of adenosine receptors in mouse models *in vivo* could affect bone indirectly by inducing a hypoxic / acidotic state which can result in increased osteoclast formation and activity, and decreased osteoblast activity (Arnett *et al.* 2003; Brandao-Burch *et al.* 2005; Arnett 2010; Utting *et al.* 2010). It is suggested that further histomorphometric examination of mice with targeted conditional P1 receptor knockout in osteoblasts and osteoclasts should be performed.

In conclusion, supraphysiological concentrations of adenosine did not have an effect on rodent osteoblasts or mouse osteoclasts *in vitro*. 2-Chloroadenosine did not have any effect on mouse osteoclasts or rodent osteoblasts obtained from the calvaria, but did increase ALP expression and bone formation by rat bone marrow osteoblasts *in vitro*, but only when added to cell cultures in extremely high concentrations. Using state of the art assays for measuring the accepted cell functions, these data suggest that adenosine has very little effect on osteoblasts and osteoclasts.

Chapter 6

The actions of sclerostin on osteoblasts and osteoclasts *in vitro*

Introduction

The discovery of sclerostin

Sclerosteosis is a rare autosomal recessive condition that mainly affects Afrikaners. Syndactyly, the fusion of the second and third fingers, is a common symptom of the condition, and one of the few that occurs prenatally (Beighton 1988). All of the symptoms of sclerosteosis that develop throughout the life of affected humans are due to the over production of bone (Hamersma *et al.* 2003). Radiographs show that sufferers have abnormally large and dense bones (Beighton *et al.* 1976). Bone constricting the cranial nerves leads to facial palsy and deafness (Robinson *et al.* 2013), and sufferers usually die in their mid-30's due to complications related to high intracranial pressure caused by over-growth of the calvaria (Hamersma *et al.* 2003; Robinson *et al.* 2013). Van Buchem disease is also a condition characterised by an over-production of bone, but its symptoms are less severe than those of sclerosteosis. Most cases of Van Buchem disease are found localised to a small Dutch fishing village. It was later shown that sclerosteosis was due to a defect in the *Sost* gene (Brunkow *et al.* 2001) and Van Buchem disease was due to a deletion in one of the promoter elements that drive expression of the *Sost* gene (Van Hul *et al.* 1998; Balemans *et al.* 1999; Staehling-Hampton *et al.* 2002).

Sclerostin and WNT signalling

Sclerostin, the product of the *Sost* gene, inhibits bone formation. It is produced primarily by osteocytes (Van Bezooijen *et al.* 2004), but may also be produced by hypertrophic chondrocytes in the growth plate and cementocytes in teeth (Van Bezooijen *et al.* 2009; Chan *et al.* 2011). Sclerostin is a member of the Dan family of

glycoproteins, many members of which inhibit BMP signalling (Winkler *et al.* 2003). However, the main mechanism of action for sclerostin is by binding to the LPR5/6 cell surface receptors and blocking WNT signalling (Semenov *et al.* 2005; Li *et al.* 2005b). WNT signalling is of vital importance for bone development and maintenance (see introduction).

Knock out models designed to mimic sclerosteosis and Van Buchem disease have been developed; histological analysis of their bones indicated that the high bone mass was due to increased osteoblast activity (Li *et al.* 2008). Transgenic mice over-expressing human sclerostin have been produced. These mice were reported to have an osteopenic phenotype; histological analysis revealed these mice had reduced cortical and trabecular bone volume (Winkler *et al.* 2003). Transgenic over expression of sclerostin in human osteoblast-like cells *in vitro* decreased the expression of ALP and reduced mineralised bone nodule formation (Winkler *et al.* 2003).

Loading of bones and sclerostin

Using immunohistochemistry, it was shown that mechanically loading the limbs of mice inhibits sclerostin expression by osteocytes *in vivo* and resulted in increased bone formation. Mechanical unloading of the limbs resulted in a slight increase in sclerostin expression and the loss of bone volume (Robling *et al.* 2008). *Sost*^{-/-} mice that were suspended by their tails so that their hind limbs could not touch the floor did not display the trabecular bone loss in the femora typically seen with unloading experiments (Lin *et al.* 2009). Mice under general anaesthesia that were forced to exercise by electrical muscle stimulation had a greater tibiae cortical bone volume and reduced sclerostin expression (Macias *et al.* 2012). Along with other factors such as nitric oxide (Pitsillides *et al.* 1995), prostacyclin (Rawlinson *et al.* 1993) and prostaglandin E₂ (Thorsen *et al.* 1996), sclerostin may be important in mediating the response of bone to mechanical stress.

Sclerostin is produced by mature osteocytes

In situ hybridisation has been used to demonstrate that rat osteocytes only start secreting sclerostin when they are mature and buried within mineralised matrix (Irie *et al.* 2008). Using human iliac bone samples and anti-sclerostin antibodies, it has been reported that the deeper an osteocyte is buried within the bone, the greater its probability of actively secreting sclerostin. It was hypothesised that this is a mechanism by which bone that is well mineralised prevents further osteoblast activity (Poole *et al.* 2005).

Anti-sclerostin antibodies

The apparent role that sclerostin has as a regulator of bone mass made it a clear therapeutic target. A number of anti-sclerostin antibodies have been developed: AMG785 (Amgen/UCB), AMG167 (Amgen/UCB), BPS804 (Novartis) and LY2541546 (also known as blosozumab, Eli Lilly) (Paszty *et al.* 2010; Robinson *et al.* 2013). Aged, ovariectomised rats were treated with the anti-sclerostin antibody in the first *in vivo* experiments. Ovariectomy of rodents mimics postmenopausal osteoporosis and is a standard *in vivo* model that results in bone loss (Iwaniec *et al.* 2006). Administration of the anti-sclerostin antibody resulted in increased bone mass and bone strength (Li *et al.* 2009). Histological analysis of the bones from these rats revealed evidence of increased osteoblast activity, and decreased osteoclast activity (Li *et al.* 2009); this is similar in phenotype to *Sost*^{-/-} mice, they also displayed histological evidence of increased osteoblast activity and decreased osteoclast activity (Li *et al.* 2008). Unlike other antiresorptive agents such as bisphosphonates, which decrease osteoclast and osteoblast activity, anti-sclerostin antibodies increase the “anabolic window” by decreasing osteoclast activity and increasing osteoblast activity (Li *et al.* 2009; McClung *et al.* 2014).

In the first clinical trial, a single dose of the anti-sclerostin antibody AMG785/CDP785, now called romosozumab, led to an increase in the bone formation markers P1NP, bone specific ALP, and osteocalcin; and a decrease in the marker of bone breakdown, CTx (Padhi *et al.* 2011). A larger, follow-on study confirmed these results (McClung *et al.* 2014). Based on the concentrations of

P1NP and CTx in the blood, this year-long clinical trial found that the initial response to the anti-sclerostin antibody was mediated predominantly by up-regulation of osteoblast function, after 6 months any further improvements in bone quality were mediated mainly by down-regulation of osteoclasts (McClung *et al.* 2014). It is not clear if the changes observed in osteoclast activity in this *in vivo* study were due to a direct or indirect action of sclerostin.

The direct effects of sclerostin on bone cells

There is little doubt about the effects that sclerostin has on bone mass in both rodents and humans; however its effects at the cellular level are less well defined. *In vitro* experiments have shown that sclerostin prevents the incorporation of calcium into the matrix produced by human osteoblasts and increases the expression of the mineralisation inhibitors MEPE and osteopontin (Atkins *et al.* 2011). Sclerostin has also been shown to decrease the expression of osteocalcin and *Runx2*, a key osteogenesis transcription factor, in human osteoblast-like cell lines (Vincent *et al.* 2009) and decrease ALP expression in a mouse osteoblast-like cell line (Winkler *et al.* 2003).

Further *in vitro* experiments using primary human osteoblasts have shown that sclerostin increases the expression of RANKL, and has no effect on the expression of OPG (Wijenayaka *et al.* 2011). Similar effects were also seen when sclerostin was added to cultures of mouse osteocyte-like MLO-Y4 cells *in vitro* (Wijenayaka *et al.* 2011). This alteration in the RANKL / OPG ratio *in vivo* would lead to an increase in the formation of osteoclasts.

Sclerostin and PTH

Intermittent dosing of mice with PTH, either directly onto the calvaria or infused into the blood, led to a decrease in *Sost* expression by osteocytes in bone (Bellido *et al.* 2005; Keller & Kneissel 2005). Intermittent PTH administration has potent anabolic effects on bone *in vivo*. Administration of PTH caused a significant increase in mouse vertebrae bone mineral density and femoral cortical and trabecular bone volume; however, sclerostin over-expressing mice had a blunted

response when PTH was administered to them (Kramer *et al.* 2010). Transgenic mice engineered to express a constitutionally active form of the PTH receptor on their osteocytes have reduced expression of *Sost*, and increased bone mass (O'Brien *et al.* 2008).

PTH reportedly reduces the expression of *Sost* by the MLO-A5 osteocyte-like cell-line (Bellido *et al.* 2005). In osteoblast-like cells PTH seemingly acts to suppress MEF2, and in doing so it down regulates *Sost* expression (Leupin *et al.* 2007). PTH may induce the PTH receptor, PTH1R, to interact with the LRP6 WNT receptor; this complex then activates canonical WNT signalling, leading to an increase in bone formation, irrespective of sclerostin (Wan *et al.* 2008). Further reports show PTH still retains its normal anabolic actions in LRP5 knockout mice (Sawakami *et al.* 2006; Iwaniec *et al.* 2007).

Tcf/lef transcription factor

The Tcf/Lef transcription factors play an important role in the WNT / β -catenin signalling pathway. Tcf/Lef is functionally inactive unless bound to β -catenin (reviewed in Brantjes *et al.* 2002). When β -catenin is bound to Tcf/Lef they form a bipartite transcription factor which activates target genes.

The enzymes ecto 5'-nucleotidase (eN) and adenosine deaminase (ADA) have been previously discussed. The expression of the genes for both eN and ADA has been shown to be under the control of the regulatory transcription factor Tcf/Lef in mammalian cell lines (Aronow *et al.* 1992; Spychala & Kitajewski 2004). Using mammalian tumour cell lines, it has been shown that WNT signalling can up-regulate eN expression and down regulate ADA expression. These results indicate that WNT signalling can play a role in the hydrolysis of nucleotides and the formation and breakdown of adenosine.

Aims

The aim of the experiments in this chapter was to investigate for the first time the direct effects of sclerostin and an anti-sclerostin antibody on osteoblast function and osteoclast formation and resorption *in vitro* using assays of true bone formation and resorption[§].

§ NOTE: The bone formation assays predominantly used precursor cells from neonatal rat calvariae; the osteoclast resorption assay used precursor cells from juvenile mouse bone marrow. These are the most efficient and best validated cell culture systems presently available for bone formation and resorption studies *in vitro*.

Results

Sclerostin and an anti-sclerostin antibody have no effect on the formation and resorptive activity of mouse osteoclasts *in vitro*

As described, mouse mononuclear cells from the bone marrow were cultured in osteoclastogenic media on ivory discs for 9 days. Either sclerostin (10 pg/ml – 500 ng/ml) or an anti-sclerostin antibody (10 pg/ml – 5000 ng/ml) was added to the cell culture media at each media change, from day 3 onwards. Cells were acidified to pH 6.90 on day 7 of the culture to activate osteoclastic resorption. No difference was seen in the number of osteoclasts formed or the amount of mineralised tissue resorbed per osteoclast compared to controls (**Figure 6.1**).

Knockout of *Sost* has no effect on mouse osteoclast formation or resorptive activity *in vitro*

Osteoclasts were formed *in vitro* from precursors obtained from wild type and *Sost*^{-/-} mouse bone marrow. Cells were cultured on elephant ivory for 9 days; on day 7 cells were acidified to pH 6.90 to activate resorption. No difference was seen in the number of osteoclasts formed from precursors, or the amount of mineral resorbed per osteoclast, between wild type and *Sost*^{-/-} cells (**Figure 6.2**).

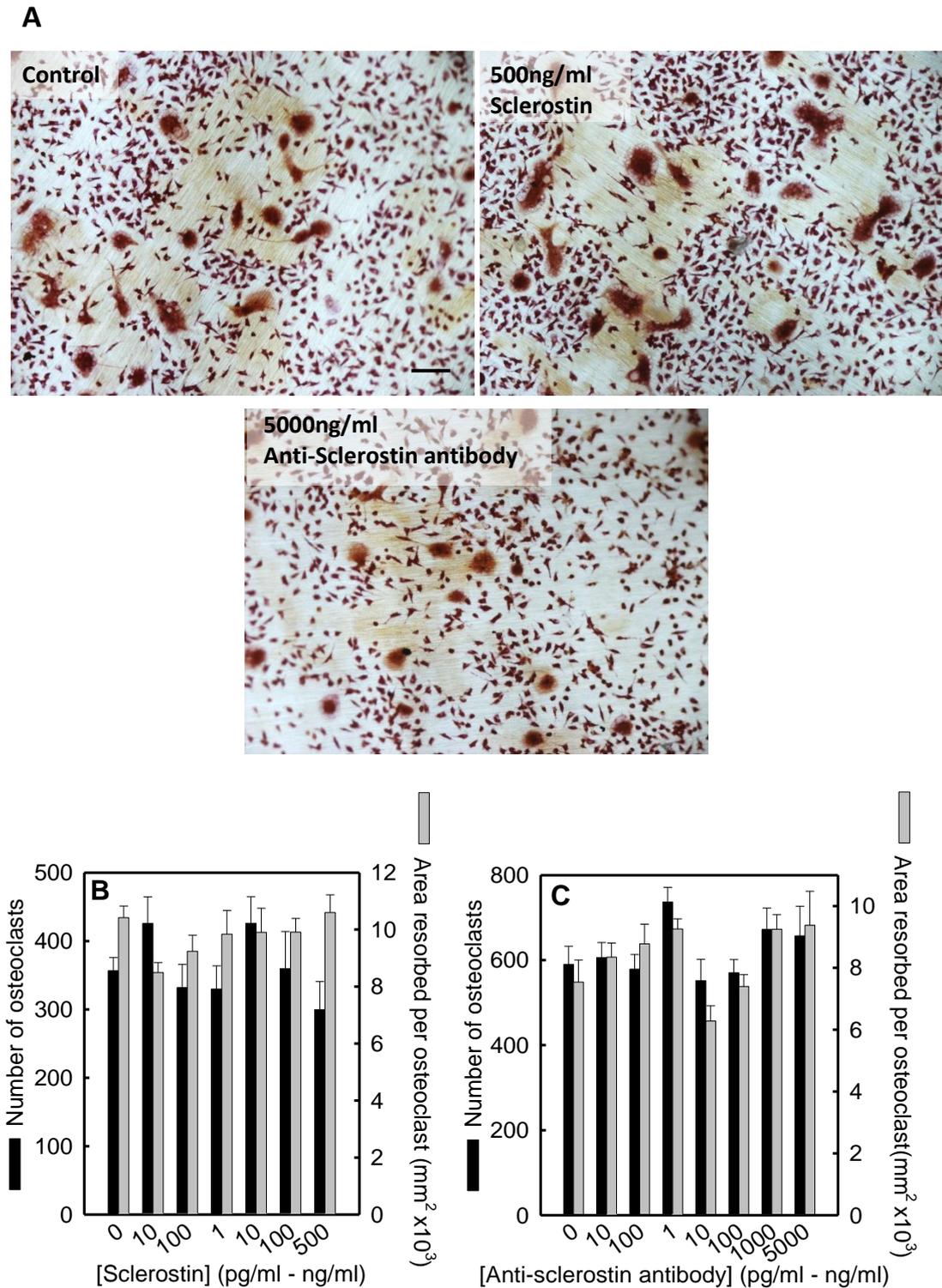


Figure 6.1. Sclerostin and an anti-sclerostin antibody do not affect mouse osteoclast formation or resorption *in vitro*

(A) Transmitted light images of TRAP-stained osteoclasts (red) and resorption pits (tan). (Scale bar = 50 μ m). Osteoclasts were cultured in the presence of (B) sclerostin or (C) an anti-sclerostin antibody. On day 7 of culture the cells were acidified to pH 6.90; n = 8.

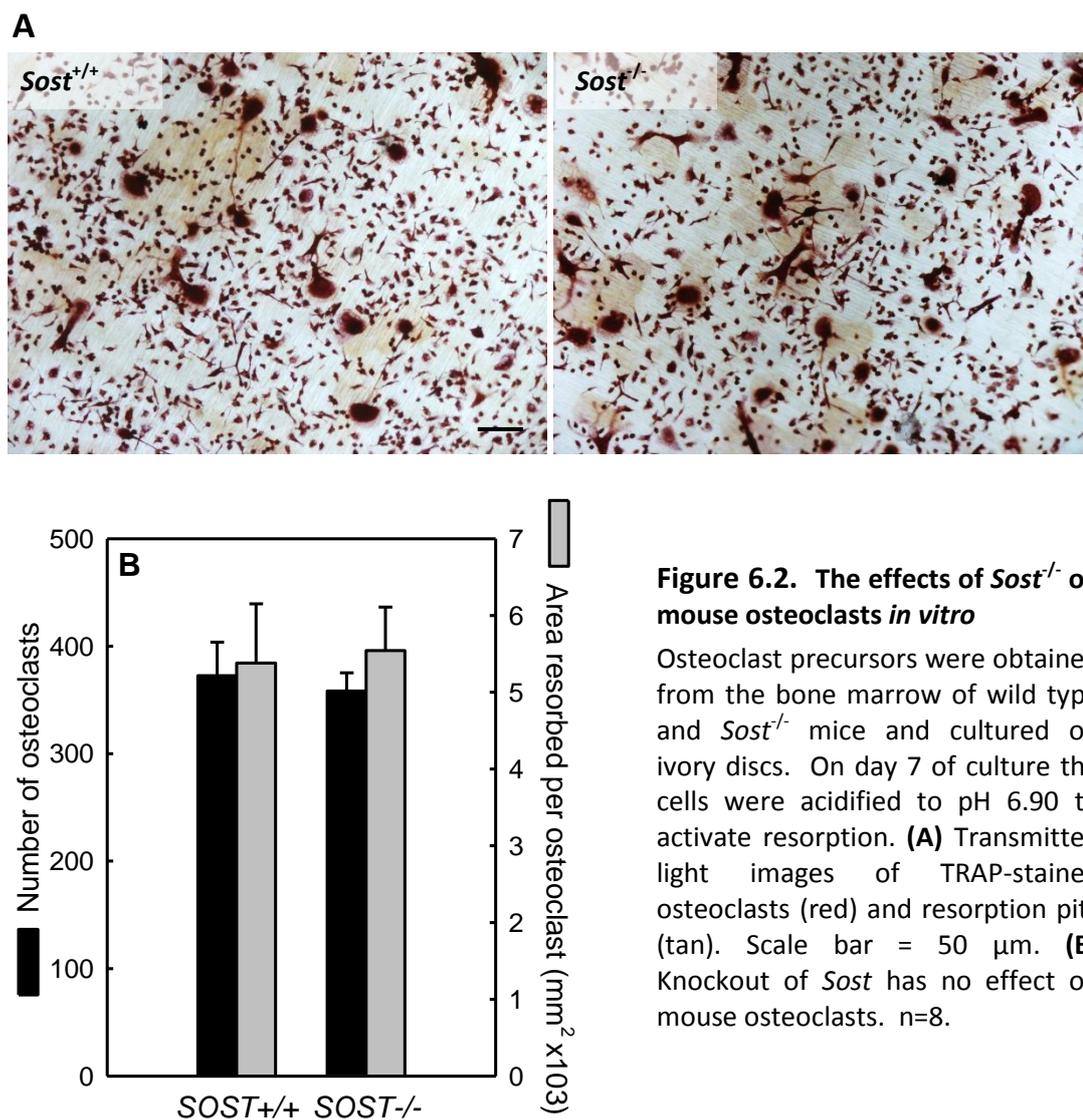


Figure 6.2. The effects of *Sost*^{-/-} on mouse osteoclasts *in vitro*

Osteoclast precursors were obtained from the bone marrow of wild type and *Sost*^{-/-} mice and cultured on ivory discs. On day 7 of culture the cells were acidified to pH 6.90 to activate resorption. **(A)** Transmitted light images of TRAP-stained osteoclasts (red) and resorption pits (tan). Scale bar = 50 μ m. **(B)** Knockout of *Sost* has no effect on mouse osteoclasts. n=8.

Sclerostin reduces bone formation by rat osteoblasts *in vitro*

Bone forming osteoblasts were cultured from rat calvarial precursor cells for 14 days with sclerostin (10 – 500 ng/ml). Sclerostin at concentrations greater than 100 ng/ml inhibited the amount of mineralised bone formed by up to 100% compared to controls in a dose dependent manner ($p < 0.001$) (**Figures 6.3, 6.4A**).

Anti-sclerostin antibody has no effect on mineral formation by rat osteoblasts *in vitro*

Rat calvarial osteoblasts were cultured for 14 days with anti-sclerostin antibody (10 – 500 ng/ml). Anti-sclerostin antibody had no effect on the amount of bone formed compared to controls (**Figures 6.3, 6.4B**).

An anti-sclerostin antibody inhibited the effects of sclerostin on rat osteoblasts *in vitro*

Rat calvarial osteoblasts were grown in the presence of either 100 ng/ml sclerostin, 100 ng/ml sclerostin + 500 ng/ml anti-sclerostin antibody, or neither (control) for 14 days. Sclerostin at a concentration of 100 ng/ml reduced the amount of mineralised bone nodules formed by rodent osteoblasts *in vitro* ($p < 0.01$); 500 ng/ml of the anti-sclerostin antibody inhibited the effects of 100 ng/ml of sclerostin and restored the amount of mineralised bone formed (Figures 6.3, 6.4C).

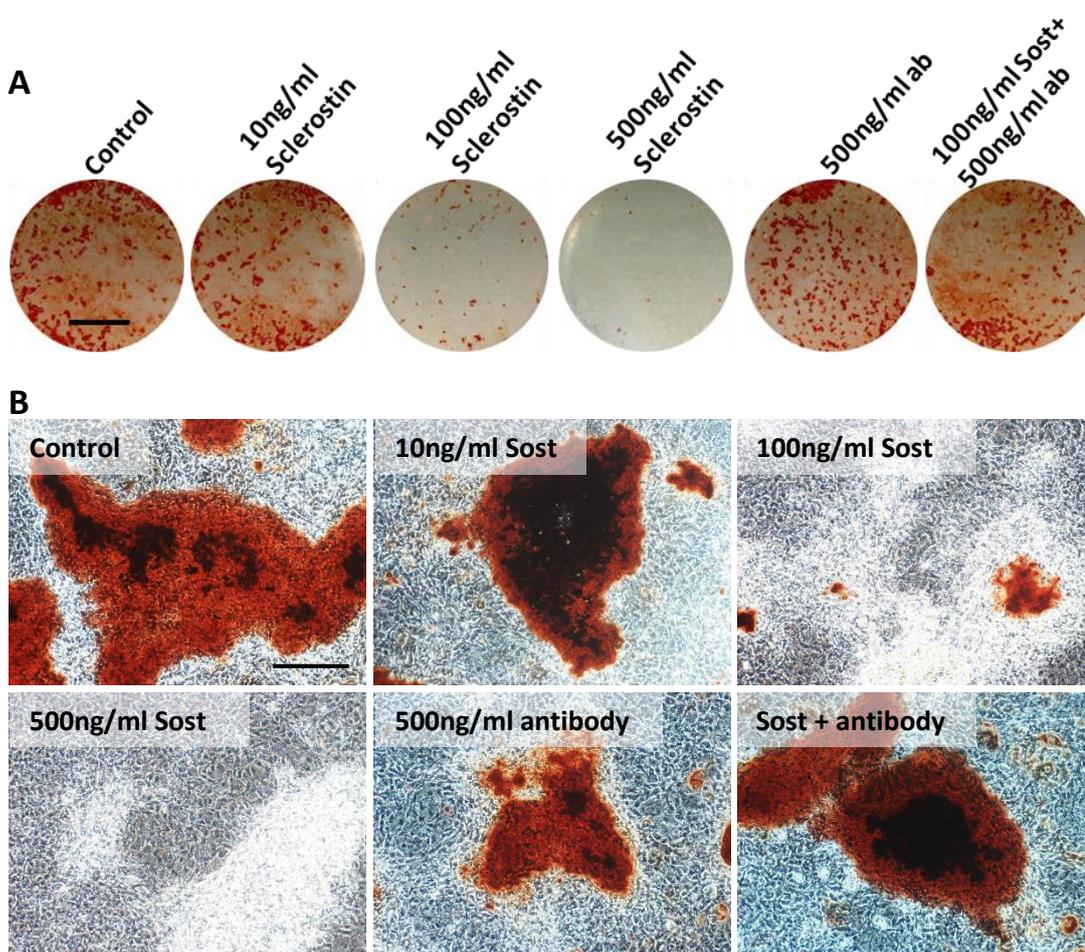


Figure 6.3. Images of the bone formed by rat osteoblasts cultured in the presence of sclerostin, anti-sclerostin antibody, or both

Rat osteoblasts were cultured in 24 well trays. On day 14 cultures were terminated and the bone nodules were stained with alizarin red. **(A)** Scanned images of the wells of the cell culture plates. **(B)** Transmitted light images of bone nodules. Sclerostin inhibits bone formation; anti-sclerostin antibody alone has no effect on the amount of bone formed per well, the anti-sclerostin antibody inhibits the actions of sclerostin. (Scale bar = 0.5 cm (A), 100 μ m (B).) (Sost = sclerostin, ab = antibody.)

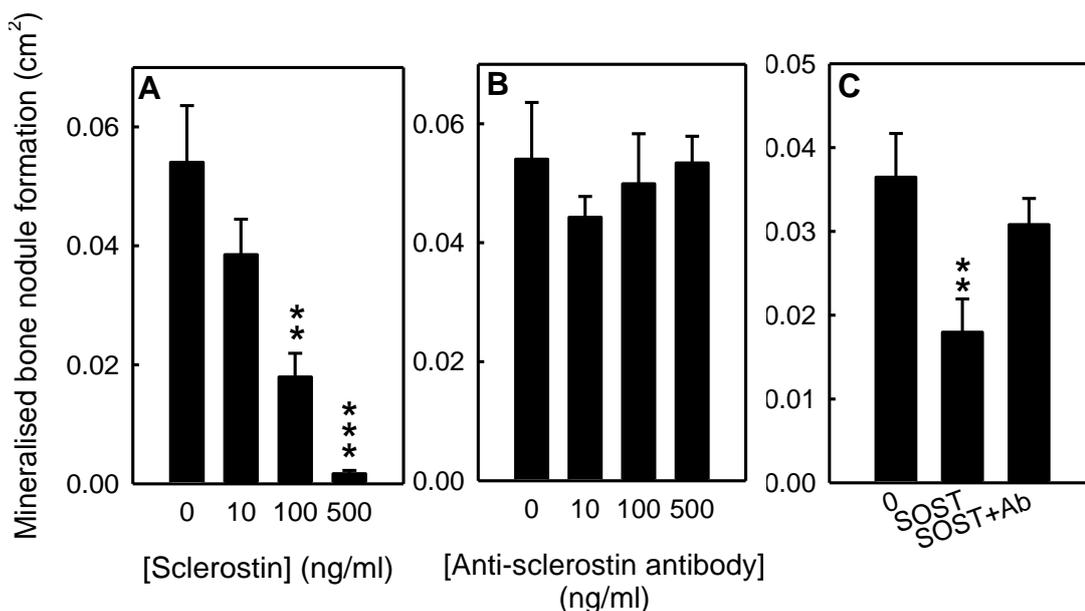


Figure 6.4. Sclerostin inhibits mineralised bone formation by rat osteoblasts *in vitro*, anti-sclerostin antibody prevents this effect

On day 14 the cultures were terminated and the total area of unstained mineralised bone formed was quantified. **(A)** Sclerostin added from the start of the culture reduced the amount of mineralised bone formed. **(B)** An anti-sclerostin antibody had no direct effect on osteoblasts. **(C)** 500 ng/ml anti-sclerostin antibody blocked the effect of 100 ng/ml sclerostin on osteoblasts. (**, $p < 0.01$; ***, $p < 0.001$, $n = 6$).

Sclerostin affected the expression of ecto-nucleotidase related mRNAs by rat osteoblasts *in vitro*

Rat osteoblasts were grown *in vitro* with or without 500 ng/ml sclerostin. On day 14 the cultures were terminated and mRNA was collected. RT-PCR showed that sclerostin up-regulated *Enpp1*, *NTPdase1*, *ADA*, *eN* and *ANK* gene expression but had no effect on the expression of *ALP* (**Figure 6.5**).

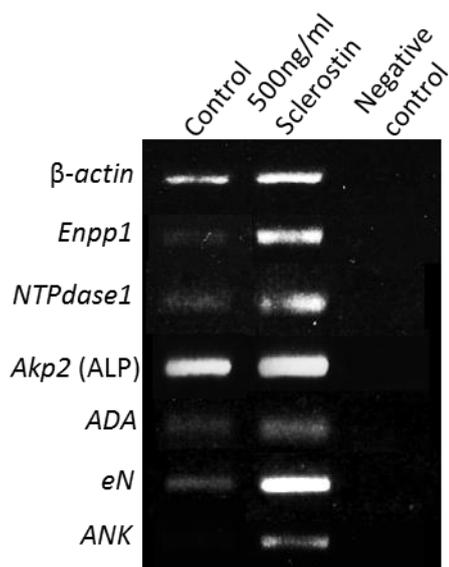


Figure 6.5. Sclerostin affects ecto-nucleotidase and ecto-nucleotidase related mRNAs expression by rat osteoblasts *in vitro*

Rat calvarial osteoblasts, grown from precursors, were cultured for 14 days with or without 500 ng/ml sclerostin. RT-PCR showed that 500 ng/ml sclerostin increased the expression of mRNAs for *Enpp1*, *NTPdase1*, *ADA*, *eN* and *ANK* by rat osteoblasts.

Sclerostin increased the total NPP activity of rat osteoblasts *in vitro*

Rat calvarial osteoblasts were cultured with sclerostin (500 ng/ml) or an anti-sclerostin antibody (1 μ g/ml). On day 14 the cultures were terminated and the total NPP activity of the osteoblasts was measured. Sclerostin increased the NPP activity of the osteoblasts by approximately 150% ($p < 0.001$); the anti-sclerostin antibody had no direct effect on osteoblast NPP activity (**Figure 6.6A**).

Sclerostin has no effect on the ALP activity of rat osteoblasts *in vitro*

Rat calvarial osteoblasts were grown in culture for 14 days with sclerostin (500 ng/ml) or an anti-sclerostin antibody (1 μ g/ml). Neither sclerostin, nor the anti-sclerostin antibody, had an effect on the *in vitro* ALP activity of rat osteoblasts (**Figure 6.6B**).

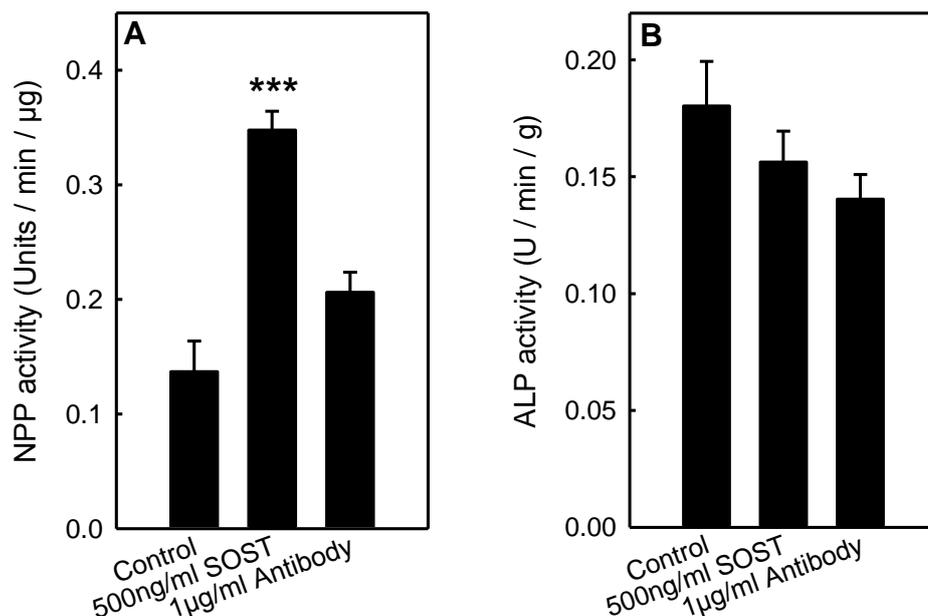


Figure 6.6. Sclerostin increases total NPP activity of rat osteoblasts *in vitro*, but has no effect on ALP activity

On day 14 of culture, the NPP and ALP enzyme activity of rat osteoblasts was measured. **(A)** NPP activity of *in vitro* rat osteoblasts. **(B)** ALP activity of *in vitro* rat osteoblasts. (***, $p < 0.001$ $n=6$).

Sclerostin inhibits mineralisation by *in vitro* *Enpp1*^{-/-} mouse osteoblasts

In order to determine if the actions of sclerostin are mediated by NPP1, wild type and *Enpp1*^{-/-} mouse calvarial osteoblasts were grown in culture for 28 days in the presence of 100 - 500 ng/ml sclerostin. Cultured *Enpp1*^{-/-} osteoblasts produced more bone than wild type osteoblasts (see chapter 4). Sclerostin inhibited mineralised bone nodule formation by both wild type and *Enpp1*^{-/-} osteoblasts *in vitro* (**Figure 6.7**).

Effects of sclerostin on expression of RANKL and OPG by rat osteoblasts *in vitro*

It was investigated whether sclerostin could act indirectly through osteoblasts to affect osteoclasts. Rat osteoblasts were cultured with or without 500 ng/ml sclerostin. On day 14 cultures were terminated and the osteoblast mRNA was collected. RT-PCR showed that sclerostin moderately increased the expression of

RANKL (*TNFSF11*) mRNA by rat calvarial osteoblasts *in vitro*, but had little effect on the expression of *OPG* (*TNFRSF11B*) mRNA (Figure 6.8).

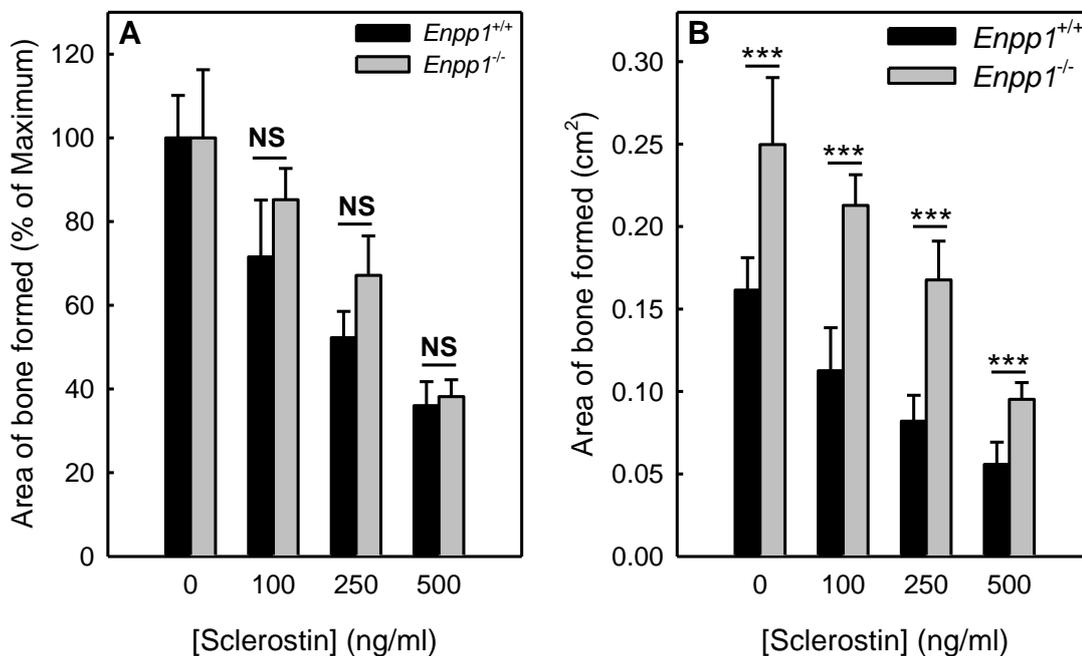


Figure 6.7. Sclerostin decreased the amount of mineralised bone formed by wild type and *Enpp1*^{-/-} mouse osteoblasts *in vitro*

Wild type and *Enpp1*^{-/-} mouse calvarial osteoblasts were cultured for 28 days with sclerostin. Sclerostin inhibited the formation of mineralised bone by wild type and *Enpp1*^{-/-} osteoblasts. (A) Results expressed as percentage of maximum bone formed. (B) Total area of bone formed. (NS = not significant; ***, $p < 0.001$).

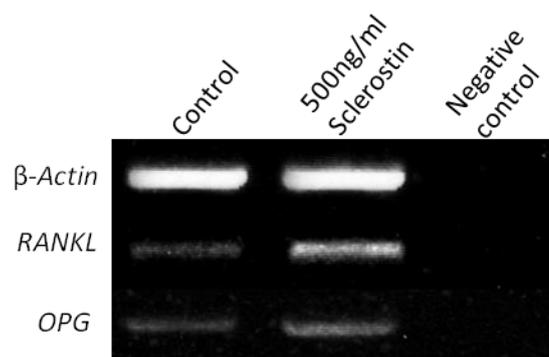


Figure 6.8. Sclerostin affects osteoclast related mRNAs expression by rat osteoblasts *in vitro*

Rat calvarial osteoblasts grown from precursors, were cultured for 14 days with or without 500 ng/ml sclerostin. RT-PCR shows that when normalised against β -actin, 500 ng/ml sclerostin increased the expression of mRNA for *RANKL* by rat osteoblasts but did not affect *OPG* expression.

Discussion

Data presented in this chapter show that exogenous sclerostin had no direct effect on the formation or resorptive activity of mouse osteoclasts *in vitro*. It was also shown that the knockout of the *Sost* gene, or the blocking of potential sclerostin activity using an antibody had no effect on osteoclast formation and activity. However, the same preparation of sclerostin elicited a strong, dose-dependent inhibition of bone formation by rodent osteoblasts *in vitro*; this inhibition was abrogated by an anti-sclerostin antibody. The anti-sclerostin antibody alone was without effect on osteoblast function, indicated that within this cell culture system there is little or no sclerostin inhibiting mineralisation. Sclerostin was also seen to effect the expression of genes related to ATP and adenosine hydrolysis by osteoblasts.

The primary action of sclerostin is to inhibit WNT signalling (Semenov *et al.* 2005; Li *et al.* 2005b). The lack of effect of sclerostin on osteoclasts observed here is consistent with Spencer *et al.* (2006), who showed that *Wnt3a* has no direct inhibitory or stimulatory effects on the formation of osteoclasts from human peripheral blood cells. Gain of function mutations in LRP5, a key receptor in the WNT signalling pathway, also have no effects on human and rodent osteoclast function *in vitro* and *in vivo* (Boyden *et al.* 2002; Babij *et al.* 2003), as is the case for loss of function mutations in LRP5 (Gong *et al.* 2001; Yadav *et al.* 2008). It was not determined if the targets of sclerostin, the LRP5/6 receptors, were expressed by osteoclasts in these experiments, but previous works have only found their expression on osteoblasts (Gong *et al.* 2001; Kato *et al.* 2002; Williams & Insogna 2009). Clinical trials have indicated that anti-sclerostin antibodies decrease resorption *in vivo*, as shown by a decreased circulating concentration of CTx (McClung *et al.* 2014). My own results show that sclerostin does not act directly on osteoclast formation and activity, indicating that the *in vivo* effects of sclerostin depletion on osteoclast function are likely to occur via indirect mechanisms.

RT-PCR showed that sclerostin increased the expression of RANKL mRNA by rat osteoblasts *in vitro*, but had no effect on OPG mRNA expression. These results

demonstrate a mechanism by which sclerostin may affect osteoclast formation and activity indirectly via osteoblasts. In support of this, previous studies using co-cultures have shown that WNT signalling affects osteoclasts indirectly via osteoblasts (Spencer *et al.* 2006; Wijenayaka *et al.* 2011).

Exogenous sclerostin increased not only the expression of *Enpp1* mRNA but also the total NPP enzymatic activity of rat osteoblasts *in vitro*, without affecting ALP mRNA expression or activity. An increase in NPP without a corresponding increase in ALP may lead to an up-regulation in the formation of PPi without affecting its rate of hydrolysis; this will produce an environment that is inhibitory to mineralisation (Millan 2013). This may be one of the mechanisms by which sclerostin inhibits bone nodule formation. Sclerostin also increased the expression of mRNA for the PPi transport protein ANK. However, it should be noted that knockout of the *Enpp1* gene did not significantly affect the inhibitory action of sclerostin on mineralised bone formation by osteoblasts *in vitro*, indicating that ENPP1 is unlikely to be a primary target for the action of sclerostin.

Sclerostin also increased rat osteoblast expression of mRNAs for *NTPdase1*, *ADA* and *eN* *in vitro*. This suggests that since both NPP1 and NTPdase1 hydrolyse ATP (Zimmermann *et al.* 2012); sclerostin may act to increase the rate of ATP hydrolysis and AMP formation by osteoblasts. Increased expression of eN could lead to an increase in the rate of adenosine formation from AMP (Zimmermann *et al.* 2012) and by increasing the expression of ADA by rat osteoblasts *in vitro*, sclerostin may also increase the rate of adenosine conversion to inosine (Lloyd & Fredholm 1995). These data indicate that sclerostin and WNT signalling may have the potential to interact with purinergic and adenosine signalling.

Rat NPP1 has a Km value of 0.281mM when acting on ATP, and requires Mg²⁺ and Zn²⁺ as co-factors; it is active between pH 6.5 – 11.0, with an optimum working pH around pH 9.5. NTPdase1 has a Km value of 0.234mM and requires Mg²⁺ and Ca²⁺ as co-factors; its optimum pH at around 7.7 is less alkaline than that for NPP1 (International Union of Biochemistry and Molecular Biology Database, accessed 02/06/2014). The very similar Km values of these enzymes suggest that the biggest

determinants of their relative ability to hydrolyse ATP will be their levels of expression and the pH of the environment they are working in. Sclerostin increased the expression of both NPP1 and NTPdase1 (**Figure 6.5**), although it is not clear if this affected the relative activity ratio between the two enzymes.

In conclusion, sclerostin (and anti-sclerostin antibodies) had no direct effect on osteoclast formation or resorption. It was seen that sclerostin could affect osteoclasts *in vivo* by altering mRNA expression of RANKL by osteoblasts. Sclerostin also inhibited mineral formation by osteoblasts and increased total NPP activity. Sclerostin may also regulate PPi levels around osteoblasts and could potentially have effects on purinergic signalling by altering the expression of ecto-nucleotidases.

Chapter 7

General discussion and future work

Nucleotide signalling has been known for many years to affect the *in vitro* function of osteoblasts (Kumagai *et al.* 1989; Schofl *et al.* 1992; Orriss *et al.* 2007; Orriss *et al.* 2012a) and osteoclasts (Hoebertz *et al.* 2001; Korcok *et al.* 2005). The work in this thesis focused on PPi and adenosine, the hydrolysis products of ATP. I also investigated the effects of sclerostin on osteoclasts and osteoblasts, and the potential links between the WNT signalling pathway and ATP hydrolysis. The findings of this work suggest further important questions.

The work presented here has shown that it is possible using the established SEM and CT technologies to quantify osteocyte lacunar size and mineralisation. This approach can now be applied to a whole range of pathologies and knockout models. Advances in bench-top microCT technologies also make it possible to study osteocyte lacunae in whole specimens. A study of 100 randomly selected mouse genes found that 10% had effects on the bone when knocked-out (Bassett *et al.* 2012). It is unknown what effect many of these genes have on osteocytes. Although osteocytes themselves are difficult to investigate, the changes that they make to their lacunae are relatively easy to study. The techniques I have demonstrated here could provide a practical basis for future studies of osteocyte function in experimental animals or humans – for example, in diverse settings such as ageing, menopause, renal disease, respiratory disease and vitamin D deficiency, as well as in response to therapeutic interventions such as bisphosphonate treatment.

Further work using synchrotron radiation-based CT or nanoCT could also be used to image osteocyte lacunae in 3-dimensions and overcome the methodological problems discussed in Chapter 3. Lacunae and possibly canaliculi could be imaged at resolutions down to 100 nm. However, both of these methods present practical

problems. Synchrotron CT is relatively difficult to gain access to, and nanoCT is currently only able to image a very small volume of bone ($\sim 200 \text{ mm}^3$). A drawback of CT imaging systems in general is that they allow only the visualisation of mineralised bone, not unmineralised collagenous matrix. This is a potentially important shortcoming because it is possible that a thin layer of demineralised matrix lining osteocyte lacunae could act as a site for rapid remineralisation (Arnett 2013b). SEM in backscattered mode, which provides high-resolution information about mineral density, could offer one approach to studying the more subtle changes in mineralisation around osteocytes.

I found that *Enpp1*^{-/-} osteocytes were less viable than wild types *in vitro*. Fluid flow stimulation has been reported to increase WNT signalling and prevent osteocyte apoptosis (Bakker *et al.* 2004; Santos *et al.* 2009). I detected an increased circulating level of sclerostin, an inhibitor of WNT signalling, and an alteration in ATP release from osteoblasts and osteoclasts in response to fluid flow stimulation in *Enpp1*^{-/-} mice. It will clearly be of interest to determine whether osteocyte viability or apoptosis *in vivo* is different between *Enpp1*^{-/-} (or indeed other ecto-nucleotidase knockout mouse models such as eN, NTPdase1 that might affect hydrolysis of extracellular ATP) and wild type mice. Reductions in osteocyte viability *in vivo*, either due to an inherent cellular defect or to increased mineral encroachment might be expected to impact on the sensing of mechanical strain and on the production of key paracrine / endocrine factors such as sclerostin, FGF23 and RANKL.

I reported in Chapter 3 that the bones of *Enpp1*^{-/-} mice showed an increased endosteal diameter and an increase in resorption pits on their endosteal surfaces. These signs of increased osteoclast activity *Enpp1*^{-/-} mice are consistent with other reports (Okawa *et al.* 1999; Mackenzie *et al.* 2012b). However, I found that *Enpp1*^{-/-} mouse osteoclasts did not resorb more bone than wild type osteoclasts *in vitro*. I hypothesised that the increased osteoclast activity seen *in vivo* in knockouts may be due to either the increased sclerostin concentration I detected, or hypoxia and acidosis in the bone environment due to vascular calcification (Rutsch *et al.* 2003;

Villa-Bellosta *et al.* 2011; Mackenzie *et al.* 2012b) and thus impaired blood flow. Doppler ultrasound imaging could be used to determine if significant reductions in bone perfusion are indeed occurring in *Enpp1*^{-/-} mice. It would also be useful to measure the pH and the partial pressure of O₂ and CO₂ in the arterial blood of these mice would to determine whether systemic acidosis or hypoxia were present.

I found *Enpp1*^{-/-} mice had a greater bone marrow cavity volume than wild types. It has previously been reported that *Enpp1*^{-/-} mice do not have a different number of platelets or red blood cells compared to wild type mice (Mackenzie *et al.* 2012b), suggesting that *Enpp1*^{-/-} mouse bone is not haematopoietically more active. This suggests the possibility that the increased bone marrow cavity volume in *Enpp1*^{-/-} mice may have been primarily occupied with adipose tissue. It has previously been reported that the knockout of *Enpp1* induces MSCs to differentiate into adipocytes (Liang *et al.* 2007; Nam *et al.* 2011). Changes in the bone marrow fat volume have been linked with ageing (Hardouin *et al.* 2014). Further analysis of the bone marrow composition of *Enpp1*^{-/-} mice is now clearly warranted.

The present work has emphasised the key role played by NPP1 and PPi in soft tissue mineralisation such as the ear pinna and whisker follicles. This can be demonstrated by microCT and relatively simple histological methods. NPP1 has already been shown to play a role in the calcification of the kidney and the aortic arch (Mackenzie *et al.* 2012a). The formation of kidney or salivary duct stones may also be potentially affected by NPP1 or PPi (Moochhala *et al.* 2008; Pradeep *et al.* 2011). Further investigation is clearly now needed on the role of NPP1 in in the pathological calcification of soft tissues. For example, mineralisation of nodules within the lungs is common in patients who have lung cancer (Khan *et al.* 2010). It is known that lung cancer patients may have increased NTPdase activity in their platelets, with no change in NPP activity (Zanini *et al.* 2012). This suggest the possibility that a higher percentage of ATP would be broken down by NTPdase than NPPs, resulting in less PPi being formed and less inhibition of mineralisation. NPP1 may also play key roles in preventing the calcification of tissues which normal express high levels of ALP such as the liver and bile duct (Millan 2013).

As discussed previously, calcification of the cartilage of the knee and trachea has been reported to increase with age (Teale *et al.* 1989; Kusafuka *et al.* 2001; Mitsuyama *et al.* 2007), as has the hyper-mineralisation of osteocyte lacunae (Busse *et al.* 2010; Carter *et al.* 2013). *Enpp1*^{-/-} mice consistently showed these symptoms, raising the possibility that these mice may represent a model of accelerated ageing. The tide mark zone of mineralised articular cartilage, which advances with age (Goldring & Goldring 2010), should also be examined in *Enpp1*^{-/-} mice. It could be of interest to determine whether NPP activity in circulating blood changes (decreases?) with age in human patients.

Unexpectedly, I found that *Enpp1*^{-/-} osteoblasts and osteoclasts had a higher concentration of intracellular ATP, and a lower rate of ATP release. Further work should be carried out to elucidate the mechanism behind these differences. ATP release from osteoblasts may occur via vesicular exocytosis (Orriss *et al.* 2009). The P2X₇ receptor has been implicated as a mechanism for the release of ATP from osteoblasts and osteoclasts (Romanello *et al.* 2001; Buckley *et al.* 2003; Genetos *et al.* 2005; Suadicani *et al.* 2006; Pellegatti *et al.* 2011). Initial experiments should determine if *Enpp1* can influence the P2X₇ receptor or vesicular exocytosis. Further experiments should be performed to determine if the knockout of other ecto-nucleotidases also effects ATP release and the intracellular ATP concentration.

Very little is known about the environmental, chemical or physiological factors that may affect NPP1 activity. Although the phenotype of the *Enpp1* knockout mouse represents an extreme example, it highlights the importance of NPP1 for the healthy functioning of many tissues. It is conceivable that even moderate chronic reductions in NPP1 activity could eventually cause significant disturbances.

In conclusion, the work presented here revealed a number of important new findings relating to role of NPP1 in the mineralisation of osteocyte lacunae and soft tissues. It also highlighted a potential new link between sclerostin and ecto-nucleotidases and further clarified the role that adenosine and sclerostin have on bone cells *in vitro*. Additionally, this work emphasised the greater role that

extracellular nucleotides play in the control of *in vitro* bone cells compared to nucleosides.

References

- Abbracchio MP & Burnstock G 1994 Purinoceptors: are there families of P2X and P2Y purinoceptors? *Pharmacol. Ther.* **64** 445-475.
- Abdel-Hamid M, Novotny L & Hamza H 2000 Stability study of selected adenosine nucleosides using LC and LC/MS analyses. *J.Pharm.Biomed.Anal.* **22** 745-755.
- Aberle H, Bauer A, Stappert J, Kispert A & Kemler R 1997 beta-catenin is a target for the ubiquitin-proteasome pathway. *EMBO J.* **16** 3797-3804.
- Addison WN, Azari F, Sorensen ES, Kaartinen MT & McKee MD 2007 Pyrophosphate inhibits mineralization of osteoblast cultures by binding to mineral, up-regulating osteopontin, and inhibiting alkaline phosphatase activity. *J.Biol.Chem.* **282** 15872-15883.
- Agren G, Ponten J, Ronquist G & Westermarck B 1974 Nucleoside diphosphate kinase at the cell surface of neoplastic human cells in culture. *J.Cell Physiol* **83** 91-101.
- Ahn Y, Sanderson BW, Klein OD & Krumlauf R 2010 Inhibition of Wnt signaling by Wise (Sostdc1) and negative feedback from Shh controls tooth number and patterning. *Development* **137** 3221-3231.
- Akiyama H, Lyons JP, Mori-Akiyama Y, Yang X, Zhang R, Zhang Z, Deng JM, Taketo MM, Nakamura T, Behringer RR, McCrea PD & de Crombrugge B 2004 Interactions between Sox9 and beta-catenin control chondrocyte differentiation. *Genes Dev.* **18** 1072-1087.
- Ali SY, Sajdera SW & Anderson HC 1970 Isolation and characterization of calcifying matrix vesicles from epiphyseal cartilage. *Proc.Natl.Acad.Sci.U.S.A* **67** 1513-1520.
- Almeida M, Han L, Bellido T, Manolagas SC & Kousteni S 2005 Wnt proteins prevent apoptosis of both uncommitted osteoblast progenitors and differentiated osteoblasts by beta-catenin-dependent and -independent signaling cascades involving Src/ERK and phosphatidylinositol 3-kinase/AKT. *J.Biol.Chem.* **280** 41342-41351.
- Alqallaf SM, Evans BA & Kidd EJ 2009 Atypical P2X receptor pharmacology in two human osteoblast-like cell lines. *Br.J.Pharmacol.* **156** 1124-1135.
- Anderson HC 1969 Vesicles associated with calcification in the matrix of epiphyseal cartilage. *J.Cell Biol.* **41** 59-72.
- Anderson HC, Garimella R & Tague SE 2005a The role of matrix vesicles in growth plate development and biomineralization. *Front Biosci.* **10** 822-837.

- Anderson HC, Harmey D, Camacho NP, Garimella R, Sipe JB, Tague S, Bi X, Johnson K, Terkeltaub R & Millan JL 2005b Sustained osteomalacia of long bones despite major improvement in other hypophosphatasia-related mineral deficits in tissue nonspecific alkaline phosphatase/nucleotide pyrophosphatase phosphodiesterase 1 double-deficient mice. *Am.J.Pathol.* **166** 1711-1720.
- Anderson HC, Hsu HH, Morris DC, Fedde KN & Whyte MP 1997 Matrix vesicles in osteomalacic hypophosphatasia bone contain apatite-like mineral crystals. *Am.J.Pathol.* **151** 1555-1561.
- Anderson HC & Reynolds JJ 1973 Pyrophosphate stimulation of calcium uptake into cultured embryonic bones. Fine structure of matrix vesicles and their role in calcification. *Dev.Biol.* **34** 211-227.
- Anderson HC, Sipe JB, Hessle L, Dhanyamraju R, Atti E, Camacho NP & Millan JL 2004 Impaired calcification around matrix vesicles of growth plate and bone in alkaline phosphatase-deficient mice. *Am.J.Pathol.* **164** 841-847.
- Anderson KL, Smith KA, Conners K, McKercher SR, Maki RA & Torbett BE 1998 Myeloid development is selectively disrupted in PU.1 null mice. *Blood* **91** 3702-3710.
- Arch JR & Newsholme EA 1978 Activities and some properties of 5'-nucleotidase, adenosine kinase and adenosine deaminase in tissues from vertebrates and invertebrates in relation to the control of the concentration and the physiological role of adenosine. *Biochem.J.* **174** 965-977.
- Arnett TR 2010 Acidosis, hypoxia and bone. *Arch.Biochem.Biophys.* **503** 103-109.
- Arnett TR 2013a Osteoclast biology. In *Osteoporosis*, Eds R Marcus, D Feldman, DW Dempster, M Luckey & J Cauley. Elsevier.
- Arnett TR 2013b Osteocytes: regulating the mineral reserves? *J.Bone Miner.Res.* **28** 2433-2435.
- Arnett TR & Dempster DW 1986 Effect of pH on bone resorption by rat osteoclasts *in vitro*. *Endocrinology* **119** 119-124.
- Arnett TR & Dempster DW 1987 A comparative study of disaggregated chick and rat osteoclasts *in vitro*: effects of calcitonin and prostaglandins. *Endocrinology* **120** 602-608.
- Arnett TR, Gibbons DC, Utting JC, Orriss IR, Hoebertz A, Rosendaal M & Meghji S 2003 Hypoxia is a major stimulator of osteoclast formation and bone resorption. *J.Cell Physiol* **196** 2-8.

- Aronow BJ, Silbiger RN, Dusing MR, Stock JL, Yager KL, Potter SS, Hutton JJ & Wiginton DA 1992 Functional analysis of the human adenosine deaminase gene thymic regulatory region and its ability to generate position-independent transgene expression. *Mol.Cell Biol.* **12** 4170-4185.
- Atkins GJ, Anderson PH, Findlay DM, Welldon KJ, Vincent C, Zannettino AC, O'Loughlin PD & Morris HA 2007 Metabolism of vitamin D3 in human osteoblasts: evidence for autocrine and paracrine activities of 1 alpha,25-dihydroxyvitamin D3. *Bone* **40** 1517-1528.
- Atkins GJ & Findlay DM 2012 Osteocyte regulation of bone mineral: a little give and take. *Osteoporos.Int.* **23** 2067-2079.
- Atkins GJ, Rowe PS, Lim HP, Welldon KJ, Ormsby R, Wijenayaka AR, Zelenchuk L, Evdokiou A & Findlay DM 2011 Sclerostin is a locally acting regulator of late-osteoblast/preosteocyte differentiation and regulates mineralization through a MEPE-ASARM-dependent mechanism. *J.Bone Miner.Res.* **26** 1425-1436.
- Babij P, Zhao W, Small C, Kharode Y, Yaworsky PJ, Bouxsein ML, Reddy PS, Bodine PV, Robinson JA, Bhat B, Marzolf J, Moran RA & Bex F 2003 High bone mass in mice expressing a mutant LRP5 gene. *J.Bone Miner.Res.* **18** 960-974.
- Bakker A, Klein-Nulend J & Burger E 2004 Shear stress inhibits while disuse promotes osteocyte apoptosis. *Biochem.Biophys.Res.Commun.* **320** 1163-1168.
- Baldwin SA, Mackey JR, Cass CE & Young JD 1999 Nucleoside transporters: molecular biology and implications for therapeutic development. *Mol.Med.Today* **5** 216-224.
- Balemans W, van Den Ende J, Freire Paes-Alves A, Dijkers FG, Willems PJ, Vanhoenacker F, de Almeida-Melo N, Alves CF, Stratakis CA, Hill SC & van Hul W 1999 Localization of the gene for sclerosteosis to the Van Buchem disease-gene region on chromosome 17q12-q21. *Am.J.Hum.Genet.* **64** 1661-1669.
- Ballarin M, Fredholm BB, Ambrosio S & Mahy N 1991 Extracellular levels of adenosine and its metabolites in the striatum of awake rats: inhibition of uptake and metabolism. *Acta.Physiol.Scand.* **142** 97-103.
- Bandyopadhyay A, Tsuji K, Cox K, Harfe BD, Rosen V & Tabin CJ 2006 Genetic analysis of the roles of BMP2, BMP4, and BMP7 in limb patterning and skeletogenesis. *PLoS.Genet.* **2** e216.
- Banerjee C, McCabe LR, Choi JY, Hiebert SW, Stein JL, Stein GS & Lian JB 1997 Runt homology domain proteins in osteoblast differentiation: AML3/CBFA1 is a major component of a bone-specific complex. *J.Cell Biochem.* **66** 1-8.
- Barzel US 1995 The skeleton as an ion exchange system: implications for the role of acid-base imbalance in the genesis of osteoporosis. *J.Bone Miner.Res.* **10** 1431-1436.

- Bassett JH, Gogakos A, White JK, Evans H, Jacques RM, van der Spek AH, Sanger Mouse Genetics Project, Ramirez-Solis R, Ryder E, Sunter D, Boyde A, Campbell MJ, Croucher PI & Williams GR 2012 Rapid-throughput skeletal phenotyping of 100 knockout mice identifies 9 new genes that determine bone strength. *Plos Genet.* **8** e1002858
- Basta G, Corciu AI, Vianello A, Del TS, Foffa I, Navarra T, Chiappino D, Berti S & Mazzone A 2010 Circulating soluble receptor for advanced glycation end-product levels are decreased in patients with calcific aortic valve stenosis. *Atherosclerosis* **210** 614-618.
- Baylink DJ & Wergedal JE 1971 Bone formation by osteocytes. *Am.J.Physiol* **221** 669-678.
- Beighton P 1988 Sclerosteosis. *J.Med.Genet* **25** 200-203.
- Beighton P, Cremin BJ & Hamersma H 1976 The radiology of sclerosteosis. *Br.J.Radiol.* **49** 934-939.
- Belanger LF, Belanger C & Semba T 1967 Technical approaches leading to the concept of osteocytic osteolysis. *Clin.Orthop.Relat Res.* **54** 187-196.
- Bellido T, Ali AA, Gubrij I, Plotkin LI, Fu Q, O'Brien CA, Manolagas SC & Jilka RL 2005 Chronic elevation of parathyroid hormone in mice reduces expression of sclerostin by osteocytes: a novel mechanism for hormonal control of osteoblastogenesis. *Endocrinology* **146** 4577-4583.
- Bender E, Buist A, Jurzak M, Langlois X, Baggerman G, Verhasselt P, Ercken M, Guo HQ, Wintmolders C, Van den Wyngaert I, Van Oers I, Schoofs L & Luyten W 2002 Characterization of an orphan G protein-coupled receptor localized in the dorsal root ganglia reveals adenine as a signaling molecule. *Proc.Natl.Acad.Sci.U.S.A* **99** 8573-8578.
- Berg JM, Tymoczko JL & Stryer L 2002 Chapter 25. Purines can be synthesised *de novo* or recycled by salvage pathways. In *Biochemistry 5th edition*, WH Freeman.
- Berne R 1963 Cardiac nucleotides in hypoxia: possible role in regulation of coronary blood flow. *Am.J.Physiol* **204** 317-322.
- Bhatia A, Albazzaz M, Espinoza Orias AA, Inoue N, Miller LM, Acerbo A, George A & Sumner DR 2012 Overexpression of DMP1 accelerates mineralization and alters cortical bone biomechanical properties *in vivo*. *J.Mech.Behav.Biomed.Mater.* **5** 1-8.
- Bhattoa HP, Wamwaki J, Kalina E, Foldesi R, Balogh A & Antal-Szalmás P 2013 Serum sclerostin levels in healthy men over 50 years of age. *J.Bone Miner.Metab* **31** 579-584.
- Bikle DD 2012 Vitamin D and bone. *Curr.Osteoporos.Rep.* **10** 151-159.

- Bilic J, Huang YL, Davidson G, Zimmermann T, Cruciat CM, Bienz M & Niehrs C 2007 Wnt induces LRP6 signalosomes and promotes dishevelled-dependent LRP6 phosphorylation. *Science* **316** 1619-1622.
- Biskobing DM & Fan D 2000 Acid pH increases carbonic anhydrase II and calcitonin receptor mRNA expression in mature osteoclasts. *Calcif.Tissue Int.* **67** 178-183.
- Biswas P & Zanello LP 2009 1alpha,25(OH)(2) vitamin D(3) induction of ATP secretion in osteoblasts. *J.Bone Miner.Res.* **24** 1450-1460.
- Biver G, Wang N, Gartland A, Orriss I, Arnett TR, Boeynaems JM & Robaye B 2013 Role of the P2Y receptor in the differentiation of bone marrow stromal cells into osteoblasts and adipocytes. *Stem Cells*.
- Bivi N, Bereszczak JZ, Romanello M, Zeef LA, Delneri D, Quadrifoglio F, Moro L, Brancia FL & Tell G 2009 Transcriptome and proteome analysis of osteocytes treated with nitrogen-containing bisphosphonates. *J.Proteome.Res.* **8** 1131-1142.
- Blair HC, Schlesinger PH, Ross FP & Teitelbaum SL 1993 Recent advances toward understanding osteoclast physiology. *Clin.Orthop.Relat Res.* 7-22.
- Blair HC, Teitelbaum SL, Ghiselli R & Gluck S 1989 Osteoclastic bone resorption by a polarized vacuolar proton pump. *Science* **245** 855-857.
- Boison D, Scheurer L, Zumsteg V, Rulicke T, Litynski P, Fowler B, Brandner S & Mohler H 2002 Neonatal hepatic steatosis by disruption of the adenosine kinase gene. *Proc.Natl.Acad.Sci.U.S.A* **99** 6985-6990.
- Boland GM, Perkins G, Hall DJ & Tuan RS 2004 Wnt 3a promotes proliferation and suppresses osteogenic differentiation of adult human mesenchymal stem cells. *J.Cell Biochem.* **93** 1210-1230.
- Bonewald LF 2011 The amazing osteocyte. *J.Bone Miner.Res.* **26** 229-238.
- Borrmann T, Abdelrahman A, Volpini R, Lambertucci C, Alksnis E, Gorzalka S, Knosp M, Schiedel AC, Cristalli G & Muller CE 2009 Structure-activity relationships of adenine and deazaadenine derivatives as ligands for adenine receptors, a new purinergic receptor family. *J.Med.Chem.* **52** 5974-5989.
- Boskey AL 2013 Bone composition: relationship to bone fragility and antiosteoporotic drug effects. *Bonekey.Rep.* **2** 447.
- Boskey AL, Spevak L, Paschalis E, Doty SB & McKee MD 2002 Osteopontin deficiency increases mineral content and mineral crystallinity in mouse bone. *Calcif.Tissue Int.* **71** 145-154.
- Bovolenta P, Esteve P, Ruiz JM, Cisneros E & Lopez-Rios J 2008 Beyond Wnt inhibition: new functions of secreted Frizzled-related proteins in development and disease. *J.Cell Sci.* **121** 737-746.

- Bowers PW & Gould DJ 1998 Petrified ears. *Clin.Exp.Dermatol.* **23** 143.
- Bowler WB, Birch MA, Gallagher JA & Bilbe G 1995 Identification and cloning of human P2U purinoceptor present in osteoclastoma, bone, and osteoblasts. *J.Bone Miner.Res.* **10** 1137-1145.
- Boyde A & Jones SJ 1979 Estimation of the size of resorption lacunae in mammalian calcified tissues using SEM stereophotogrammetry. *Scan Electron Microsc.* 393-402.
- Boyden LM, Mao J, Belsky J, Mitzner L, Farhi A, Mitnick MA, Wu D, Insogna K & Lifton RP 2002 High bone density due to a mutation in LDL-receptor-related protein. *N.Engl.J.Med.* **346** 1513-1521.
- Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal.Biochem.* **72** 248-254.
- Brandao-Burch A & Arnett TR 2004 Normal human osteoclasts are activated by acidosis. *Journal of Bone and Mineral Research* **19** 1034.
- Brandao-Burch A, Key ML, Patel JJ, Arnett TR & Orriss IR 2012 The P2X7 Receptor is an Important Regulator of Extracellular ATP Levels. *Front Endocrinol.(Lausanne)* **3** 41.
- Brandao-Burch A, Utting JC, Orriss IR & Arnett TR 2005 Acidosis inhibits bone formation by osteoblasts *in vitro* by preventing mineralization. *Calcif.Tissue Int.* **77** 167-174.
- Brandt S & Jentsch TJ 1995 CIC-6 and CIC-7 are two novel broadly expressed members of the CLC chloride channel family. *FEBS Lett.* **377** 15-20.
- Brantjes H, Barker N, van ES J & Clevers H 2002 TCF: Lady Justice casting the final verdict on the outcome of Wnt signalling. *Biol.Chem.* **383** 255-261.
- Bresler D, Bruder J, Mohnike K, Fraser WD & Rowe PS 2004 Serum MEPE-ASARM-peptides are elevated in X-linked rickets (HYP): implications for phosphaturia and rickets. *J.Endocrinol.* **183** R1-R9.
- Broch OJ & Ueland PM 1980 Regional and subcellular distribution of S-adenosylhomocysteine hydrolase in the adult rat brain. *J.Neurochem.* **35** 484-488.
- Brunet LJ, McMahon JA, McMahon AP & Harland RM 1998 Noggin, cartilage morphogenesis, and joint formation in the mammalian skeleton. *Science* **280** 1455-1457.

- Brunkow ME, Gardner JC, Van Ness J, Paeper BW, Kovacevich BR, Proll S, Skonier JE, Zhao L, Sabo PJ, Fu Y, Alisch RS, Gillett L, Colbert T, Tacconi P, Galas D, Hamersma H, Beighton P & Mulligan J 2001 Bone dysplasia sclerosteosis results from loss of the SOST gene product, a novel cystine knot-containing protein. *Am.J.Hum.Genet* **68** 577-589.
- Buckley KA, Golding SL, Rice JM, Dillon JP & Gallagher JA 2003 Release and interconversion of P2 receptor agonists by human osteoblast-like cells. *FASEB J.* **17** 1401-1410.
- Buckley KA, Hipskind RA, Gartland A, Bowler WB & Gallagher JA 2002 Adenosine triphosphate stimulates human osteoclast activity via upregulation of osteoblast-expressed receptor activator of nuclear factor-kappa B ligand. *Bone* **31** 582-590.
- Buckley KA, Wagstaff SC, McKay G, Gaw A, Hipskind RA, Bilbe G, Gallagher JA & Bowler WB 2001 Parathyroid hormone potentiates nucleotide-induced $[Ca^{2+}]_i$ release in rat osteoblasts independently of Gq activation or cyclic monophosphate accumulation. A mechanism for localizing systemic responses in bone. *J.Biol.Chem.* **276** 9565-9571.
- Buckley MF, Loveland KA, McKinstry WJ, Garson OM & Goding JW 1990 Plasma cell membrane glycoprotein PC-1. cDNA cloning of the human molecule, amino acid sequence, and chromosomal location. *J.Biol.Chem.* **265** 17506-17511.
- Burgess TL, Qian Y, Kaufman S, Ring BD, Van G, Capparelli C, Kelley M, Hsu H, Boyle WJ, Dunstan CR, Hu S & Lacey DL 1999 The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts. *J.Cell Biol.* **145** 527-538.
- Burnstock G 2007 Physiology and pathophysiology of purinergic neurotransmission. *Physiol Rev.* **87** 659-797.
- Burr DB, Robling AG & Turner CH 2002 Effects of biomechanical stress on bones in animals. *Bone* **30** 781-786.
- Burrell HE, Wlodarski B, Foster BJ, Buckley KA, Sharpe GR, Quayle JM, Simpson AW & Gallagher JA 2005 Human keratinocytes release ATP and utilize three mechanisms for nucleotide interconversion at the cell surface. *J.Biol.Chem.* **280** 29667-29676.
- Bushinsky DA, Goldring JM & Coe FL 1985 Cellular contribution to pH-mediated calcium flux in neonatal mouse calvariae. *Am.J.Physiol* **248** F785-F789.
- Busse B, Djonc D, Milovanovic P, Hahn M, Puschel K, Ritchie RO, Djuric M & Amling M 2010 Decrease in the osteocyte lacunar density accompanied by hypermineralized lacunar occlusion reveals failure and delay of remodeling in aged human bone. *Aging Cell* **9** 1065-1075.

- Canals M, Burgueno J, Marcellino D, Cabello N, Canela EI, Mallol J, Agnati L, Ferre S, Bouvier M, Fuxe K, Ciruela F, Lluís C & Franco R 2004 Homodimerization of adenosine A2A receptors: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. *J.Neurochem.* **88** 726-734.
- Candeliere GA, Liu F & Aubin JE 2001 Individual osteoblasts in the developing calvaria express different gene repertoires. *Bone* **28** 351-361.
- Carroll SH, Wigner NA, Kulkarni N, Johnston-Cox H, Gerstenfeld LC & Ravid K 2012 A2B adenosine receptor promotes mesenchymal stem cell differentiation to osteoblasts and bone formation *in vivo*. *J.Biol.Chem.* **287** 15718-15727.
- Carter Y, Thomas CD, Clement JG & Cooper DM 2013 Femoral osteocyte lacunar density, volume and morphology in women across the lifespan. *J.Struct.Biol.* **183** 519-526.
- Carvalho DR & Speck-Martins CE 2011 Additional features of unique Primrose syndrome phenotype. *Am.J.Med.Genet A* **155A** 1379-1383.
- Castrop H, Huang Y, Hashimoto S, Mizel D, Hansen P, Theilig F, Bachmann S, Deng C, Briggs J & Schnermann J 2004 Impairment of tubuloglomerular feedback regulation of GFR in ecto-5'-nucleotidase/CD73-deficient mice. *J.Clin.Invest* **114** 634-642.
- Cecil DL & Terkeltaub RA 2011 Arterial calcification is driven by RAGE in Enpp1^{-/-} mice. *J.Vasc.Res.* **48** 227-235.
- Cederbaum SD, Kaitila I, Rimoin DL & Stiehm ER 1976 The chondro-osseous dysplasia of adenosine deaminase deficiency with severe combined immunodeficiency. *J.Pediatr.* **89** 737-742.
- Cerniway RJ, Morrison RR, Byford AM, Lankford AR, Headrick JP, Van Wylen DG & Matherne GP 2002 A1 adenosine receptor overexpression decreases stunning from anoxia-reoxygenation: role of the mitochondrial K(ATP) channel. *Basic Res.Cardiol.* **97** 232-238.
- Chambers TJ & Moore A 1983 The sensitivity of isolated osteoclasts to morphological transformation by calcitonin. *J.Clin.Endocrinol.Metab* **57** 819-824.
- Chan BY, Fuller ES, Russell AK, Smith SM, Smith MM, Jackson MT, Cake MA, Read RA, Bateman JF, Sambrook PN & Little CB 2011 Increased chondrocyte sclerostin may protect against cartilage degradation in osteoarthritis. *Osteoarthritis Cartilage* **19** 874-885.
- Chandalia M, Davila H, Pan W, Szuszkiewicz M, Tuvdendorj D, Livingston EH & Abate N 2012 Adipose tissue dysfunction in humans: a potential role for the transmembrane protein ENPP1. *J.Clin.Endocrinol.Metab* **97** 4663-4672.

- Chang EJ, Ha J, Oerlemans F, Lee YJ, Lee SW, Ryu J, Kim HJ, Lee Y, Kim HM, Choi JY, Kim JY, Shin CS, Pak YK, Tanaka S, Wieringa B, Lee ZH & Kim HH 2008 Brain-type creatine kinase has a crucial role in osteoclast-mediated bone resorption. *Nature Med.* **14** 966-972.
- Chenu C, Colucci S, Grano M, Zigrino P, Barattolo R, Zambonin G, Baldini N, Vergnaud P, Delmas PD & Zallone AZ 1994 Osteocalcin induces chemotaxis, secretion of matrix proteins, and calcium-mediated intracellular signaling in human osteoclast-like cells. *J.Cell Biol.* **127** 1149-1158.
- Chi SL & Pizzo SV 2006 Angiostatin is directly cytotoxic to tumor cells at low extracellular pH: a mechanism dependent on cell surface-associated ATP synthase. *Cancer Res.* **66** 875-882.
- Chiozzi P, Sanz JM, Ferrari D, Falzoni S, Aleotti A, Buell GN, Collo G & di VF 1997 Spontaneous cell fusion in macrophage cultures expressing high levels of the P2Z/P2X7 receptor. *J.Cell Biol.* **138** 697-706.
- Choi J, Choi SY, Lee SY, Lee JY, Kim HS, Lee SY & Lee NK 2013 Caffeine enhances osteoclast differentiation and maturation through p38 MAP kinase/Mitf and DC-STAMP/CtsK and TRAP pathway. *Cell Signal.* **25** 1222-1227.
- Chopra R, Chaudhary N & Kay J 2013 Relapsing polychondritis. *Rheum.Dis.Clin.North Am.* **39** 263-276.
- Choudhery MS, Badowski M, Muise A, Pierce J & Harris DT 2014 Donor age negatively impacts adipose tissue-derived mesenchymal stem cell expansion and differentiation. *J.Transl.Med.* **12** 8.
- Ciancaglini P, Yadav MC, Simao AM, Narisawa S, Pizauro JM, Farquharson C, Hoylaerts MF & Millan JL 2010 Kinetic analysis of substrate utilization by native and TNAP-, NPP1-, or PHOSPHO1-deficient matrix vesicles. *J.Bone Miner.Res.* **25** 716-723.
- Ciruela F, Casado V, Mallol J, Canela EI, Lluís C & Franco R 1995 Immunological identification of A1 adenosine receptors in brain cortex. *J.Neurosci.Res.* **42** 818-828.
- Ciruela F, Casado V, Rodrigues RJ, Lujan R, Burgueno J, Canals M, Borycz J, Rebola N, Goldberg SR, Mallol J, Cortes A, Canela EI, Lopez-Gimenez JF, Milligan G, Lluís C, Cunha RA, Ferre S & Franco R 2006 Presynaptic control of striatal glutamatergic neurotransmission by adenosine A1-A2A receptor heteromers. *J.Neurosci.* **26** 2080-2087.
- Clair T, Aoki J, Koh E, Bandle RW, Nam SW, Ptaszynska MM, Mills GB, Schiffmann E, Liotta LA & Stracke ML 2003 Autotaxin hydrolyzes sphingosylphosphorylcholine to produce the regulator of migration, sphingosine-1-phosphate. *Cancer Res.* **63** 5446-5453.

- Clair T, Lee HY, Liotta LA & Stracke ML 1997 Autotaxin is an exoenzyme possessing 5'-nucleotide phosphodiesterase/ATP pyrophosphatase and ATPase activities. *J.Biol.Chem.* **272** 996-1001.
- Cohen AM, Talmi YP, Floru S, Tsigelman R, Kalmanovitz M, Zohar Y & Djaldetti M 1991 X-ray microanalysis of ossified auricles in Addison's disease. *Calcif.Tissue Int.* **48** 88-92.
- Collip JB 1925 The internal secretion of the parathyroid glands. *Proc.Natl.Acad.Sci.U.S.A* **11** 484-485.
- Compton SJ & Jones CG 1985 Mechanism of dye response and interference in the Bradford protein assay. *Anal.Biochem.* **151** 369-374.
- Costa AG, Cusano NE, Silva BC, Cremers S & Bilezikian JP 2011a Cathepsin K: its skeletal actions and role as a therapeutic target in osteoporosis. *Nature Rev.Rheumatol.* **7** 447-456.
- Costa MA, Barbosa A, Neto E, Sa-e-Sousa, Freitas R, Neves JM, Magalhaes-Cardoso T, Ferreira F & Correia-de-Sa P 2011b On the role of subtype selective adenosine receptor agonists during proliferation and osteogenic differentiation of human primary bone marrow stromal cells. *J.Cell Physiol* **226** 1353-1366.
- Cronstein BN, Levin RI, Belanoff J, Weissmann G & Hirschhorn R 1986 Adenosine: an endogenous inhibitor of neutrophil-mediated injury to endothelial cells. *J.Clin.Invest* **78** 760-770.
- Dalal P, Leslie ND, Lindor NM, Gilbert DL & Espay AJ 2010 Motor tics, stereotypies, and self-flagellation in Primrose syndrome. *Neurology* **75** 284-286.
- Daly JW, Padgett WL, Secunda SI, Thompson RD & Olsson RA 1993 Structure-activity relationships for 2-substituted adenosines at A1 and A2 adenosine receptors. *Pharmacology* **46** 91-100.
- Dare E, Schulte G, Karovic O, Hammarberg C & Fredholm BB 2007 Modulation of glial cell functions by adenosine receptors. *Physiol Behav.* **92** 15-20.
- Das B, Mondragon MO, Sadeghian M, Hatcher VB & Norin AJ 1994 A novel ligand in lymphocyte-mediated cytotoxicity: expression of the beta subunit of H⁺ transporting ATP synthase on the surface of tumor cell lines. *J.Exp.Med.* **180** 273-281.
- David V, Martin A, Hedge AM & Rowe PS 2009 Matrix extracellular phosphoglycoprotein (MEPE) is a new bone renal hormone and vascularization modulator. *Endocrinology* **150** 4012-4023.
- Davies J, Warwick J, Totty N, Philp R, Helfrich M & Horton M 1989 The osteoclast functional antigen, implicated in the regulation of bone resorption, is biochemically related to the vitronectin receptor. *J.Cell Biol.* **109** 1817-1826.

de la Pompa JL, Timmerman LA, Takimoto H, Yoshida H, Elia AJ, Samper E, Potter J, Wakeham A, Marengere L, Langille BL, Crabtree GR & Mak TW 1998 Role of the NF-ATc transcription factor in morphogenesis of cardiac valves and septum. *Nature* **392** 182-186.

De Corte V, Gettemans J & Vandekerckhove J 1997 Phosphatidylinositol 4,5-bisphosphate specifically stimulates PP60(c-src) catalyzed phosphorylation of gelsolin and related actin-binding proteins. *FEBS Lett.* **401** 191-196.

de Crombrughe B, Lefebvre V, Behringer RR, Bi W, Murakami S & Huang W 2000 Transcriptional mechanisms of chondrocyte differentiation. *Matrix Biol.* **19** 389-394.

De Luca F, Barnes KM, Uyeda JA, De-Levi S, Abad V, Palese T, Mericq V & Baron J 2001 Regulation of growth plate chondrogenesis by bone morphogenetic protein-2. *Endocrinology* **142** 430-436.

Degubareff T & Sleator W 1965 Effects of ceffeine on mamalian atrial muscle, and its interaction with adenosine and calcium. *J.Pharmacol.Exp.Ther.* **148** 202-214.

DeKoter RP, Walsh JC & Singh H 1998 PU.1 regulates both cytokine-dependent proliferation and differentiation of granulocyte/macrophage progenitors. *EMBO J.* **17** 4456-4468.

Delaisse JM, Andersen TL, Engsig MT, Henriksen K, Troen T & Blavier L 2003 Matrix metalloproteinases (MMP) and cathepsin K contribute differently to osteoclastic activities. *Microsc.Res.Tech.* **61** 504-513.

Demenis MA & Leone FA 2000 Kinetic characteristics of ATP hydrolysis by a detergent-solubilized alkaline phosphatase from rat osseous plate. *IUBMB.Life* **49** 113-119.

Dempster DW, Hughes-Begos CE, Plavetic-Chee K, Brandao-Burch A, Cosman F, Nieves J, Neubort S, Lu SS, Iida-Klein A, Arnett T & Lindsay R 2005 Normal human osteoclasts formed from peripheral blood monocytes express PTH type 1 receptors and are stimulated by PTH in the absence of osteoblasts. *J.Cell Biochem.* **95** 139-148.

Dodd JS, Raleigh JA & Gross TS 1999 Osteocyte hypoxia: a novel mechanotransduction pathway. *Am.J.Physiol* **277** C598-C602.

Donaldson SH, Picher M & Boucher RC 2002 Secreted and cell-associated adenylate kinase and nucleoside diphosphokinase contribute to extracellular nucleotide metabolism on human airway surfaces. *Am.J.Respir.Cell Mol.Biol.* **26** 209-215.

Dougall WC, Glaccum M, Charrier K, Rohrbach K, Brasel K, De ST, Daro E, Smith J, Tometsko ME, Maliszewski CR, Armstrong A, Shen V, Bain S, Cosman D, Anderson D, Morrissey PJ, Peschon JJ & Schuh J 1999 RANK is essential for osteoclast and lymph node development. *Genes Dev.* **13** 2412-2424.

- Drury AN & Szent-Gyorgyi A 1929 The physiological activity of adenine compounds with especial reference to their action upon the mammalian heart. *J.Physiol* **68** 213-237.
- Duan RD, Bergman T, Xu N, Wu J, Cheng Y, Duan J, Nelander S, Palmberg C & Nilsson A 2003 Identification of human intestinal alkaline sphingomyelinase as a novel ecto-enzyme related to the nucleotide phosphodiesterase family. *J.Biol.Chem.* **278** 38528-38536.
- Ducy P, Zhang R, Geoffroy V, Ridall AL & Karsenty G 1997 Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell* **89** 747-754.
- Dudley HR & Spiro D 1961 THE FINE STRUCTURE OF BONE CELLS. *J.Biophys.Biochem.Cytol.* **11** 627-649.
- Dzhandzhugazyan K & Bock E 1993 Demonstration of (Ca(2+)-Mg2+)-ATPase activity of the neural cell adhesion molecule. *FEBS Lett.* **336** 279-283.
- Eghbali-Fatourehchi GZ, Lamsam J, Fraser D, Nagel D, Riggs BL & Khosla S 2005 Circulating osteoblast-lineage cells in humans. *N.Engl.J.Med.* **352** 1959-1966.
- Elmenhorst D, Meyer PT, Winz OH, Matusch A, Ermert J, Coenen HH, Basheer R, Haas HL, Zilles K & Bauer A 2007 Sleep deprivation increases A1 adenosine receptor binding in the human brain: a positron emission tomography study. *J.Neurosci.* **27** 2410-2415.
- Emerson CP 1990 Myogenesis and developmental control genes. *Curr.Opin.Cell Biol.* **2** 1065-1075.
- Enyoji K, Sevigny J, Lin Y, Frenette PS, Christie PD, Esch JS, Imai M, Edelberg JM, Rayburn H, Lech M, Beeler DL, Csizmadia E, Wagner DD, Robson SC & Rosenberg RD 1999 Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. *Nature Med.* **5** 1010-1017.
- Ernens I, Leonard F, Vausort M, Rolland-Turner M, Devaux Y & Wagner DR 2010 Adenosine up-regulates vascular endothelial growth factor in human macrophages. *Biochem.Biophys.Res.Commun.* **392** 351-356.
- Fabre AC, Vantourout P, Champagne E, Terce F, Rolland C, Perret B, Collet X, Barbaras R & Martinez LO 2006 Cell surface adenylate kinase activity regulates the F(1)-ATPase/P2Y (13)-mediated HDL endocytosis pathway on human hepatocytes. *Cell Mol.Life Sci.* **63** 2829-2837.
- Fan CM & Tessier-Lavigne M 1994 Patterning of mammalian somites by surface ectoderm and notochord: evidence for sclerotome induction by a hedgehog homolog. *Cell* **79** 1175-1186.

- Fedde KN, Blair L, Silverstein J, Coburn SP, Ryan LM, Weinstein RS, Waymire K, Narisawa S, Millan JL, MacGregor GR & Whyte MP 1999 Alkaline phosphatase knock-out mice recapitulate the metabolic and skeletal defects of infantile hypophosphatasia. *J.Bone Miner.Res.* **14** 2015-2026.
- Fedde KN, Lane CC & Whyte MP 1988 Alkaline phosphatase is an ectoenzyme that acts on micromolar concentrations of natural substrates at physiologic pH in human osteosarcoma (SAOS-2) cells. *Arch.Biochem.Biophys.* **264** 400-409.
- Felix R, Cecchini MG & Fleisch H 1990 Macrophage colony stimulating factor restores *in vivo* bone resorption in the op/op osteopetrotic mouse. *Endocrinology* **127** 2592-2594.
- Fen JQ, Zhang J, Dallas SL, Lu Y, Chen S, Tan X, Owen M, Harris SE & MacDougall M 2002 Dentin matrix protein 1, a target molecule for Cbfa1 in bone, is a unique bone marker gene. *J.Bone Miner.Res.* **17** 1822-1831.
- Feng JQ, Ward LM, Liu S, Lu Y, Xie Y, Yuan B, Yu X, Rauch F, Davis SI, Zhang S, Rios H, Drezner MK, Quarles LD, Bonewald LF & White KE 2006 Loss of DMP1 causes rickets and osteomalacia and identifies a role for osteocytes in mineral metabolism. *Nature Genet.* **38** 1310-1315.
- Ferron M, Hinoi E, Karsenty G & Ducy P 2008 Osteocalcin differentially regulates beta cell and adipocyte gene expression and affects the development of metabolic diseases in wild-type mice. *Proc.Natl.Acad.Sci.U.S.A* **105** 5266-5270.
- Fleisch H & Bisaz S 1962 Isolation from urine of pyrophosphate, a calcification inhibitor. *Am.J.Physiol* **203** 671-675.
- Fleisch H, Russell RG & Straumann F 1966 Effect of pyrophosphate on hydroxyapatite and its implications in calcium homeostasis. *Nature* **212** 901-903.
- Franz-Odendaal TA, Hall BK & Witten PE 2006 Buried alive: how osteoblasts become osteocytes. *Dev.Dyn.* **235** 176-190.
- Franzoso G, Carlson L, Xing L, Poljak L, Shores EW, Brown KD, Leonardi A, Tran T, Boyce BF & Siebenlist U 1997 Requirement for NF-kappaB in osteoclast and B-cell development. *Genes Dev.* **11** 3482-3496.
- Fraser DR & Kodicek E 1973 Regulation of 25-hydroxycholecalciferol-1-hydroxylase activity in kidney by parathyroid hormone. *Nature New Biol.* **241** 163-166.
- Fredholm BB 1982 Adenosine actions and adenosine receptors after 1 week treatment with caffeine. *Acta Physiol Scand.* **115** 283-286.
- Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN & Linden J 2001 International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol.Rev.* **53** 527-552.

Fredholm BB, IJzerman AP, Jacobson KA, Linden J & Muller CE 2011 International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors--an update. *Pharmacol.Rev.* **63** 1-34.

Fredholm BB & Sollevi A 1981 The release of adenosine and inosine from canine subcutaneous adipose tissue by nerve stimulation and noradrenaline. *J.Physiol* **313** 351-367.

Freissmuth M, Selzer E & Schutz W 1991 Interactions of purified bovine brain A1-adenosine receptors with G-proteins. Reciprocal modulation of agonist and antagonist binding. *Biochem.J.* **275** 651-656.

Frick KK & Bushinsky DA 1998 Chronic metabolic acidosis reversibly inhibits extracellular matrix gene expression in mouse osteoblasts. *Am.J.Physiol* **275** F840-F847.

Friedenstein AJ, Chailakhyan RK & Gerasimov UV 1987 Bone marrow osteogenic stem cells: *in vitro* cultivation and transplantation in diffusion chambers. *Cell Tissue Kinet.* **20** 263-272.

Friedenstein AJ, Gorskaja JF & Kulagina NN 1976 Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp.Hematol.* **4** 267-274.

Friedman PA & Gesek FA 1993 Calcium transport in renal epithelial cells. *Am.J.Physiol.* **264** F181-F198.

Frittitta L, Spampinato D, Solini A, Nosadini R, Goldfine ID, Vigneri R & Trischitta V 1998 Elevated PC-1 content in cultured skin fibroblasts correlates with decreased *in vivo* and *in vitro* insulin action in nondiabetic subjects: evidence that PC-1 may be an intrinsic factor in impaired insulin receptor signaling. *Diabetes* **47** 1095-1100.

Frobert O, Haink G, Simonsen U, Gravholt CH, Levin M & Deussen A 2006 Adenosine concentration in the porcine coronary artery wall and A2A receptor involvement in hypoxia-induced vasodilatation. *J.Physiol* **570** 375-384.

Fujiwara S, Kasagi F, Yamada M & Kodama K 1997 Risk factors for hip fracture in a Japanese cohort. *J.Bone Miner.Res.* **12** 998-1004.

Fulzele K, Riddle RC, DiGirolamo DJ, Cao X, Wan C, Chen D, Faugere MC, Aja S, Hussain MA, Bruning JC & Clemens TL 2010 Insulin receptor signaling in osteoblasts regulates postnatal bone acquisition and body composition. *Cell* **142** 309-319.

Gartland A, Buckley KA, Bowler WB & Gallagher JA 2003a Blockade of the pore-forming P2X7 receptor inhibits formation of multinucleated human osteoclasts *in vitro*. *Calcif.Tissue Int.* **73** 361-369.

Gartland A, Buckley KA, Hipkind RA, Perry MJ, Tobias JH, Buell G, Chessell I, Bowler WB & Gallagher JA 2003b Multinucleated osteoclast formation *in vivo* and *in vitro* by P2X7 receptor-deficient mice. *Crit Rev.Eukaryot.Gene Expr.* **13** 243-253.

- Gartland A, Hipskind RA, Gallagher JA & Bowler WB 2001 Expression of a P2X7 receptor by a subpopulation of human osteoblasts. *J.Bone Miner.Res.* **16** 846-856.
- Gelb BD, Shi GP, Chapman HA & Desnick RJ 1996 Pycnodysostosis, a lysosomal disease caused by cathepsin K deficiency. *Science* **273** 1236-1238.
- Genetos DC, Geist DJ, Liu D, Donahue HJ & Duncan RL 2005 Fluid shear-induced ATP secretion mediates prostaglandin release in MC3T3-E1 osteoblasts. *J.Bone Miner.Res.* **20** 41-49.
- Genetos DC, Kephart CJ, Zhang Y, Yellowley CE & Donahue HJ 2007 Oscillating fluid flow activation of gap junction hemichannels induces ATP release from MLO-Y4 osteocytes. *J.Cell Physiol* **212** 207-214.
- George A, Sabsay B, Simonian PA & Veis A 1993 Characterization of a novel dentin matrix acidic phosphoprotein. Implications for induction of biomineralization. *J.Biol.Chem.* **268** 12624-12630.
- Gerlach E & Deuticke B 1966 [Comparative studies on the formation of adenosine in the myocardium of different animal species in oxygen deficiency]. *Klin.Wochenschr.* **44** 1307-1310.
- Gessi S, Fogli E, Sacchetto V, Merighi S, Varani K, Preti D, Leung E, MacLennan S & Borea PA 2010 Adenosine modulates HIF-1{alpha}, VEGF, IL-8, and foam cell formation in a human model of hypoxic foam cells. *Arterioscler.Thromb.Vasc.Biol.* **30** 90-97.
- Gharibi B, Abraham AA, Ham J & Evans BA 2011 Adenosine receptor subtype expression and activation influence the differentiation of mesenchymal stem cells to osteoblasts and adipocytes. *J.Bone Miner.Res.* **26** 2112-2124.
- Gijsbers R, Aoki J, Arai H & Bollen M 2003 The hydrolysis of lysophospholipids and nucleotides by autotaxin (NPP2) involves a single catalytic site. *FEBS Lett.* **538** 60-64.
- Ginsborg BL & Hirst GD 1971 Cyclic AMP, transmitter release and the effect of adenosine on neuromuscular transmission. *Nature New Biol.* **232** 63-64.
- Gitelman SE, Kirk M, Ye JQ, Filvaroff EH, Kahn AJ & Derynck R 1995 Vgr-1/BMP-6 induces osteoblastic differentiation of pluripotential mesenchymal cells. *Cell Growth Differ.* **6** 827-836.
- Gluhak-Heinrich J, Ye L, Bonewald LF, Feng JQ, MacDougall M, Harris SE & Pavlin D 2003 Mechanical loading stimulates dentin matrix protein 1 (DMP1) expression in osteocytes *in vivo*. *J.Bone Miner.Res.* **18** 807-817.
- Goding JW & Shen FW 1982 Structure of the murine plasma cell alloantigen PC-1: comparison with the receptor for transferrin. *J.Immunol.* **129** 2636-2640.

- Goepfert C, Sundberg C, Sevigny J, Enjoji K, Hoshi T, Csizmadia E & Robson S 2001 Disordered cellular migration and angiogenesis in cd39-null mice. *Circulation* **104** 3109-3115.
- Gogate Y, Gangadhar P, Walia RR & Bhansali A 2012 "Petrified ears" with idiopathic adult-onset pituitary insufficiency. *Indian J.Endocrinol.Metab* **16** 830-832.
- Goldring MB & Goldring SR 2010 Articular cartilage and subchondral bone in the pathogenesis of osteoarthritis. *Ann.N.Y.Acad.Sci.* **1192** 230-237.
- Goltzman D 2011 LRP5, serotonin, and bone: complexity, contradictions, and conundrums. *J.Bone Miner.Res.* **26** 1997-2001.
- Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM, Wang H, Cundy T, Glorieux FH, Lev D, Zacharin M, Oexle K, Marcelino J, Suwairi W, Heeger S, Sabatakos G, Apte S, Adkins WN, Allgrove J, Arslan-Kirchner M, Batch JA, Beighton P, Black GC, Boles RG, Boon LM, Borrone C, Brunner HG, Carle GF, Dallapiccola B, De PA, Floege B, Halfhide ML, Hall B, Hennekam RC, Hirose T, Jans A, Juppner H, Kim CA, Keppler-Noreuil K, Kohlschuetter A, LaCombe D, Lambert M, Lemyre E, Letteboer T, Peltonen L, Ramesar RS, Romanengo M, Somer H, Steichen-Gersdorf E, Steinmann B, Sullivan B, Superti-Furga A, Swoboda W, van den Boogaard MJ, Van Hul W, Vikkula M, Votruba M, Zabel B, Garcia T, Baron R, Olsen BR & Warman ML 2001 LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. *Cell* **107** 513-523.
- Gordon DL 1964 Calcification of auricular cartilage. *Arch.Intern.Med.* **113** 23-27.
- Gordon MK & Hahn RA 2010 Collagens. *Cell Tissue Res.* **339** 247-257.
- Gorzalka S, Vittori S, Volpini R, Cristalli G, von K, I & Muller CE 2005 Evidence for the functional expression and pharmacological characterization of adenine receptors in native cells and tissues. *Mol.Pharmacol.* **67** 955-964.
- Goto K 1918 Mineral metabolism in experimental acidosis. *Journal of Biological Chemistry* **36** 355-376.
- Gowen LC, Petersen DN, Mansolf AL, Qi H, Stock JL, Tkalcevic GT, Simmons HA, Crawford DT, Chidsey-Frink KL, Ke HZ, McNeish JD & Brown TA 2003 Targeted disruption of the osteoblast/osteocyte factor 45 gene (OF45) results in increased bone formation and bone mass. *J.Biol.Chem.* **278** 1998-2007.
- Greig AV, Linge C & Burnstock G 2008 Purinergic receptors are part of a signalling system for proliferation and differentiation in distinct cell lineages in human anagen hair follicles. *Purinergic.Signal.* **4** 331-338.
- Grimaud E, Soubigou L, Couillaud S, Coipeau P, Moreau A, Passuti N, Gouin F, Redini F & Heymann D 2003 Receptor activator of nuclear factor kappaB ligand (RANKL)/osteoprotegerin (OPG) ratio is increased in severe osteolysis. *Am.J.Pathol.* **163** 2021-2031.

- Grimsrud CD, Romano PR, D'Souza M, Puzas JE, Schwarz EM, Reynolds PR, Roiser RN & O'Keefe RJ 2001 BMP signaling stimulates chondrocyte maturation and the expression of Indian hedgehog. *J.Orthop.Res.* **19** 18-25.
- Grinthal A & Guidotti G 2006 CD39, NTPDase 1, is attached to the plasma membrane by two transmembrane domains. Why? *Purinergic.Signal.* **2** 391-398.
- Grondal EJ & Zimmermann H 1987 Purification, characterization and cellular localization of 5'-nucleotidase from *Torpedo* electric organ. *Biochem.J.* **245** 805-810.
- Gross TS, Akeno N, Clemens TL, Komarova S, Srinivasan S, Weimer DA & Mayorov S 2001 Selected Contribution: Osteocytes upregulate HIF-1alpha in response to acute disuse and oxygen deprivation. *J.Appl.Physiol (1985.)* **90** 2514-2519.
- Grupe A, Alleman J, Goldfine ID, Sadick M & Stewart TA 1995 Inhibition of insulin receptor phosphorylation by PC-1 is not mediated by the hydrolysis of adenosine triphosphate or the generation of adenosine. *J.Biol.Chem.* **270** 22085-22088.
- Gubb D & Garcia-Bellido A 1982 A genetic analysis of the determination of cuticular polarity during development in *Drosophila melanogaster*. *J.Embryol.Exp.Morphol.* **68** 37-57.
- Guerrini MM, Sobacchi C, Cassani B, Abinun M, Kilic SS, Pangrazio A, Moratto D, Mazzolari E, Clayton-Smith J, Orchard P, Coxon FP, Helfrich MH, Crockett JC, Mellis D, Vellodi A, Tezcan I, Notarangelo LD, Rogers MJ, Vezzoni P, Villa A & Frattini A 2008 Human osteoclast-poor osteopetrosis with hypogammaglobulinemia due to TNFRSF11A (RANK) mutations. *Am.J.Hum.Genet* **83** 64-76.
- Gundberg CM, Lian JB & Booth SL 2012 Vitamin K-dependent carboxylation of osteocalcin: friend or foe?. *Adv.Nutr.* **3** 149-157.
- Guo RT, Chong YE, Guo M & Yang XL 2009 Crystal structures and biochemical analyses suggest a unique mechanism and role for human glycyl-tRNA synthetase in Ap4A homeostasis. *J.Biol.Chem.* **284** 28968-28976.
- Halling LC, Narisawa S, Millan JL & Magnusson P 2009 Glycosylation differences contribute to distinct catalytic properties among bone alkaline phosphatase isoforms. *Bone* **45** 987-993.
- Hallstrom H, Wolk A, Glynn A & Michaelsson K 2006 Coffee, tea and caffeine consumption in relation to osteoporotic fracture risk in a cohort of Swedish women. *Osteoporos.Int.* **17** 1055-1064.
- Ham J & Evans BA 2012 An emerging role for adenosine and its receptors in bone homeostasis. *Front Endocrinol.(Lausanne)* **3** 113.
- Hamdy RC & Daley DN 2012 Oral calcitonin. *Int.J.Womens Health* **4** 471-479.

- Hamersma H, Gardner J & Beighton P 2003 The natural history of sclerosteosis. *Clin.Genet* **63** 192-197.
- Hansen SA, Folsom AR, Kushi LH & Sellers TA 2000 Association of fractures with caffeine and alcohol in postmenopausal women: the Iowa Women's Health Study. *Public Health Nutr.* **3** 253-261.
- Hardouin P, Pansini V & Cortet B 2014 Bone marrow fat. *Joint Bone Spine* in press doi: 10.1016/j.jbspin.2014.02.013
- Harmey D, Hessle L, Narisawa S, Johnson KA, Terkeltaub R & Millan JL 2004 Concerted regulation of inorganic pyrophosphate and osteopontin by *akp2*, *enpp1*, and *ank*: an integrated model of the pathogenesis of mineralization disorders. *Am.J.Pathol.* **164** 1199-1209.
- Harrison JS, Rameshwar P, Chang V & Bandari P 2002 Oxygen saturation in the bone marrow of healthy volunteers. *Blood* **99** 394.
- Hasko G, Szabo C, Nemeth ZH, Kvetan V, Pastores SM & Vizi ES 1996 Adenosine receptor agonists differentially regulate IL-10, TNF-alpha, and nitric oxide production in RAW 264.7 macrophages and in endotoxemic mice. *J.Immunol.* **157** 4634-4640.
- Haussler MR, Jurutka PW, Mizwicki M & Norman AW 2011 Vitamin D receptor (VDR)-mediated actions of 1alpha,25(OH)(2)vitamin D(3): genomic and non-genomic mechanisms. *Best.Pract.Res.Clin.Endocrinol.Metab* **25** 543-559.
- Hayman AR & Cox TM 2003 Tartrate-resistant acid phosphatase knockout mice. *J.Bone Miner.Res.* **18** 1905-1907.
- Haynesworth SE, Baber MA & Caplan AI 1992 Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies. *Bone* **13** 69-80.
- He G & George A 2004 Dentin matrix protein 1 immobilized on type I collagen fibrils facilitates apatite deposition *in vitro*. *J.Biol.Chem.* **279** 11649-11656.
- He Q, Wan C & Li G 2007 Concise review: multipotent mesenchymal stromal cells in blood. *Stem Cells* **25** 69-77.
- He W & Cronstein BN 2012 Adenosine A1 receptor regulates osteoclast formation by altering TRAF6/TAK1 signaling. *Purinergic Signal.* **8** 327-337.
- He W, Mazumder A, Wilder T & Cronstein BN 2013a Adenosine regulates bone metabolism via A1, A2A, and A2B receptors in bone marrow cells from normal humans and patients with multiple myeloma. *FASEB J.* **27** 3446-3454.

- He W, Wilder T & Cronstein BN 2013b Rolofylline, an adenosine a receptor antagonist, inhibits osteoclast differentiation as an inverse agonist. *Br.J.Pharmacol.* **170** 1167-1176.
- Helms JA, Cordero D & Tapadia MD 2005 New insights into craniofacial morphogenesis. *Development* **132** 851-861.
- Hernandez-Avila M, Colditz GA, Stampfer MJ, Rosner B, Speizer FE & Willett WC 1991 Caffeine, moderate alcohol intake, and risk of fractures of the hip and forearm in middle-aged women. *Am.J.Clin.Nutr.* **54** 157-163.
- Hershfield MS 1979 Apparent suicide inactivation of human lymphoblast S-adenosylhomocysteine hydrolase by 2'-deoxyadenosine and adenine arabinoside. A basis for direct toxic effects of analogs of adenosine. *J.Biol.Chem.* **254** 22-25.
- Hessle L, Johnson KA, Anderson HC, Narisawa S, Sali A, Goding JW, Terkeltaub R & Millan JL 2002 Tissue-nonspecific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization. *Proc.Natl.Acad.Sci.U.S.A* **99** 9445-9449.
- Hiken JF & Steinberg TH 2004 ATP downregulates P2X7 and inhibits osteoclast formation in RAW cells. *Am.J.Physiol Cell Physiol* **287** C403-C412.
- Hinton DJ, McGee-Lawrence ME, Lee MR, Kwong HK, Westendorf JJ & Choi DS 2014 Aberrant bone density in ageing mice lacking the adenosine transporter ENT1. *PLoS One* **9** e88818.
- Ho AM, Johnson MD & Kingsley DM 2000 Role of the mouse ank gene in control of tissue calcification and arthritis. *Science* **289** 265-270.
- Hoebertz A, Mahendran S, Burnstock G & Arnett TR 2002 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** 413-419.
- Hoebertz A, Meghji S, Burnstock G & Arnett TR 2001 Extracellular ADP is a powerful osteolytic agent: evidence for signaling through the P2Y(1) receptor on bone cells. *FASEB J.* **15** 1139-1148.
- Hoebertz A, Townsend-Nicholson A, Glass R, Burnstock G & Arnett TR 2000 Expression of P2 receptors in bone and cultured bone cells. *Bone* **27** 503-510.
- Holbrook TL, Barrett-Connor E & Wingard DL 1988 Dietary calcium and risk of hip fracture: 14-year prospective population study. *Lancet* **2** 1046-1049.
- Holick MF, MacLaughlin JA, Clark MB, Holick SA, Potts JT, Jr., Anderson RR, Blank IH, Parrish JA & Elias P 1980 Photosynthesis of previtamin D3 in human skin and the physiologic consequences. *Science* **210** 203-205.

Holmbeck K, Bianco P, Pidoux I, Inoue S, Billingham RC, Wu W, Chrysovergis K, Yamada S, Birkedal-Hansen H & Poole AR 2005 The metalloproteinase MT1-MMP is required for normal development and maintenance of osteocyte processes in bone. *J.Cell Sci.* **118** 147-156.

Hooper NM 1997 Glycosyl-phosphatidylinositol anchored membrane enzymes. *Clin.Chim.Acta* **266** 3-12.

Horton MA, Taylor ML, Arnett TR & Helfrich MH 1991 Arg-Gly-Asp (RGD) peptides and the anti-vitronectin receptor antibody 23C6 inhibit dentine resorption and cell spreading by osteoclasts. *Exp.Cell Res.* **195** 368-375.

Hosoda Y, Yoshimura Y & Higaki S 1981 A new breed of mouse showing multiple osteochondral lesions--twy mouse. *Ryumachi* **21 Suppl** 157-164.

Houston B, Seawright E, Jefferies D, Hoogland E, Lester D, Whitehead C & Farquharson C 1999 Identification and cloning of a novel phosphatase expressed at high levels in differentiating growth plate chondrocytes. *Biochim.Biophys.Acta* **1448** 500-506.

Hsu DR, Economides AN, Wang X, Eimon PM & Harland RM 1998 The *Xenopus* dorsalizing factor Gremlin identifies a novel family of secreted proteins that antagonize BMP activities. *Mol.Cell* **1** 673-683.

Huesa C, Yadav MC, Finnila MA, Goodyear SR, Robins SP, Tanner KE, Aspden RM, Millan JL & Farquharson C 2011 PHOSPHO1 is essential for mechanically competent mineralization and the avoidance of spontaneous fractures. *Bone* **48** 1066-1074.

Hukins DW, Harries JE & Hasnain SS 1986 Extended X-ray absorption fine structure studies of calcification. *Biochem.Soc.Trans.* **14** 545-549.

Huopio J, Kroger H, Honkanen R, Saarikoski S & Alhava E 2000 Risk factors for perimenopausal fractures: a prospective study. *Osteoporos.Int.* **11** 219-227.

Huq NL, Cross KJ, Ung M & Reynolds EC 2005 A review of protein structure and gene organisation for proteins associated with mineralised tissue and calcium phosphate stabilisation encoded on human chromosome 4. *Arch.Oral Biol.* **50** 599-609.

Ihara H, Denhardt DT, Furuya K, Yamashita T, Muguruma Y, Tsuji K, Hruska KA, Higashio K, Enomoto S, Nifuji A, Rittling SR & Noda M 2001 Parathyroid hormone-induced bone resorption does not occur in the absence of osteopontin. *J.Biol.Chem.* **276** 13065-13071.

Ihara H, Hirukawa K, Goto S & Togari A 2005 ATP-stimulated interleukin-6 synthesis through P2Y receptors on human osteoblasts. *Biochem.Biophys.Res.Commun.* **326** 329-334.

- Ikegame M, Ejiri S & Ozawa H 2004 Calcitonin-induced change in serum calcium levels and its relationship to osteoclast morphology and number of calcitonin receptors. *Bone* **35** 27-33.
- Imai S, Heino TJ, Hienola A, Kurata K, Buki K, Matsusue Y, Vaananen HK & Rauvala H 2009 Osteocyte-derived HB-GAM (pleiotrophin) is associated with bone formation and mechanical loading. *Bone* **44** 785-794.
- Inada M, Wang Y, Byrne MH, Rahman MU, Miyaura C, Lopez-Otin C & Krane SM 2004 Critical roles for collagenase-3 (Mmp13) in development of growth plate cartilage and in endochondral ossification. *Proc.Natl.Acad.Sci.U.S.A* **101** 17192-17197.
- Iotsova V, Caamano J, Loy J, Yang Y, Lewin A & Bravo R 1997 Osteopetrosis in mice lacking NF-kappaB1 and NF-kappaB2. *Nature Med.* **3** 1285-1289.
- Irie K, Ejiri S, Sakakura Y, Shibui T & Yajima T 2008 Matrix mineralization as a trigger for osteocyte maturation. *J.Histochem.Cytochem.* **56** 561-567.
- Ishii M, Egen JG, Klauschen F, Meier-Schellersheim M, Saeki Y, Vacher J, Proia RL & Germain RN 2009 Sphingosine-1-phosphate mobilizes osteoclast precursors and regulates bone homeostasis. *Nature* **458** 524-528.
- Ishizuya T, Yokose S, Hori M, Noda T, Suda T, Yoshiki S & Yamaguchi A 1997 Parathyroid hormone exerts disparate effects on osteoblast differentiation depending on exposure time in rat osteoblastic cells. *J.Clin.Invest* **99** 2961-2970.
- Itasaki N, Jones CM, Mercurio S, Rowe A, Domingos PM, Smith JC & Krumlauf R 2003 Wise, a context-dependent activator and inhibitor of Wnt signalling. *Development* **130** 4295-4305.
- Iwaniec UT, Wronski TJ, Liu J, Rivera MF, Arzaga RR, Hansen G & Brommage R 2007 PTH stimulates bone formation in mice deficient in Lrp5. *J.Bone Miner.Res.* **22** 394-402.
- Iwaniec UT, Yuan D, Power RA & Wronski TJ 2006 Strain-dependent variations in the response of cancellous bone to ovariectomy in mice. *J.Bone Miner.Res.* **21** 1068-1074.
- Jankowski RJ, Deasy BM & Huard J 2002 Muscle-derived stem cells. *Gene Ther.* **9** 642-647.
- Jansen RS, Kucukosmanoglu A, de HM, Sapthu S, Otero JA, Hegman IE, Bergen AA, Gorgels TG, Borst P & van de Wetering K 2013 ABCC6 prevents ectopic mineralization seen in pseudoxanthoma elasticum by inducing cellular nucleotide release. *Proc.Natl.Acad.Sci.U.S.A* **110** 20206-20211.

- Jansen S, Stefan C, Creemers JW, Waelkens E, Van Eynde A, Stalmans W & Bollen M 2005 Proteolytic maturation and activation of autotaxin (NPP2), a secreted metastasis-enhancing lysophospholipase D. *J. Cell Sci.* **118** 3081-3089.
- Janssen E, Dzeja PP, Oerlemans F, Simonetti AW, Heerschap A, de HA, Rush PS, Terjung RR, Wieringa B & Terzic A 2000 Adenylate kinase 1 gene deletion disrupts muscle energetic economy despite metabolic rearrangement. *EMBO J.* **19** 6371-6381.
- Jia D, O'Brien CA, Stewart SA, Manolagas SC & Weinstein RS 2006 Glucocorticoids act directly on osteoclasts to increase their life span and reduce bone density. *Endocrinology* **147** 5592-5599.
- Jilka RL, Weinstein RS, Bellido T, Roberson P, Parfitt AM & Manolagas SC 1999 Increased bone formation by prevention of osteoblast apoptosis with parathyroid hormone. *J. Clin. Invest* **104** 439-446.
- Johnson K, Goding J, van Etten D, Sali A, Hu SI, Farley D, Krug H, Hessle L, Millan JL & Terkeltaub R 2003 Linked deficiencies in extracellular PP(i) and osteopontin mediate pathologic calcification associated with defective PC-1 and ANK expression. *J. Bone Miner. Res.* **18** 994-1004.
- Johnson K, Polewski M, van Etten D & Terkeltaub R 2005 Chondrogenesis mediated by PPI depletion promotes spontaneous aortic calcification in NPP1^{-/-} mice. *Arterioscler. Thromb. Vasc. Biol.* **25** 686-691.
- Johnson K & Terkeltaub R 2005 Inorganic pyrophosphate (PPI) in pathologic calcification of articular cartilage. *Front Biosci.* **10** 988-997.
- Jones SJ, Gray C, Boyde A & Burnstock G 1997 Purinergic transmitters inhibit bone formation by cultured osteoblasts. *Bone* **21** 393-399.
- Jorgensen NR, Geist ST, Civitelli R & Steinberg TH 1997 ATP- and gap junction-dependent intercellular calcium signaling in osteoblastic cells. *J. Cell Biol.* **139** 497-506.
- Jurdic P, Saltel F, Chabadel A & Destaing O 2006 Podosome and sealing zone: specificity of the osteoclast model. *Eur. J. Cell Biol.* **85** 195-202.
- Kaczmarek-Hajek K, Lorinczi E, Hausmann R & Nicke A 2012 Molecular and functional properties of P2X receptors--recent progress and persisting challenges. *Purinergic Signal.* **8** 375-417.
- Kara FM, Chitu V, Sloane J, Axelrod M, Fredholm BB, Stanley ER & Cronstein BN 2010a Adenosine A1 receptors (A1Rs) play a critical role in osteoclast formation and function. *FASEB J.* **24** 2325-2333.

- Kara FM, Doty SB, Boskey A, Goldring S, Zaidi M, Fredholm BB & Cronstein BN 2010b Adenosine A(1) receptors regulate bone resorption in mice: adenosine A(1) receptor blockade or deletion increases bone density and prevents ovariectomy-induced bone loss in adenosine A(1) receptor-knockout mice. *Arthritis Rheum.* **62** 534-541.
- Karsdal MA, Henriksen K, Arnold M & Christiansen C 2008 Calcitonin: a drug of the past or for the future? Physiologic inhibition of bone resorption while sustaining osteoclast numbers improves bone quality. *BioDrugs.* **22** 137-144.
- Kassis I, Zangi L, Rivkin R, Levdansky L, Samuel S, Marx G & Gorodetsky R 2006 Isolation of mesenchymal stem cells from G-CSF-mobilized human peripheral blood using fibrin microbeads. *Bone Marrow Transplant.* **37** 967-976.
- Kato K, Nishimasu H, Okudaira S, Mihara E, Ishitani R, Takagi J, Aoki J & Nureki O 2012 Crystal structure of Enpp1, an extracellular glycoprotein involved in bone mineralization and insulin signaling. *Proc.Natl.Acad.Sci.U.S.A* **109** 16876-16881.
- Kato M, Patel MS, Levasseur R, Lobov I, Chang BH, Glass DA, Hartmann C, Li L, Hwang TH, Brayton CF, Lang RA, Karsenty G & Chan L 2002 Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. *J.Cell Biol.* **157** 303-314.
- Katsara O, Mahaira LG, Iliopoulou EG, Moustaki A, Antsaklis A, Loutradis D, Stefanidis K, Baxevanis CN, Papamichail M & Perez SA 2011 Effects of donor age, gender, and *in vitro* cellular ageing on the phenotypic, functional, and molecular characteristics of mouse bone marrow-derived mesenchymal stem cells. *Stem Cells Dev.* **20** 1549-1561.
- Katz S, Boland R & Santillan G 2006 Modulation of ERK 1/2 and p38 MAPK signaling pathways by ATP in osteoblasts: involvement of mechanical stress-activated calcium influx, PKC and Src activation. *Int.J.Biochem.Cell Biol.* **38** 2082-2091.
- Katz S, Boland R & Santillan G 2008 Purinergic (ATP) signaling stimulates JNK1 but not JNK2 MAPK in osteoblast-like cells: contribution of intracellular Ca²⁺ release, stress activated and L-voltage-dependent calcium influx, PKC and Src kinases. *Arch.Biochem.Biophys.* **477** 244-252.
- Ke HZ, Qi H, Weidema AF, Zhang Q, Panupinthu N, Crawford DT, Grasser WA, Paralkar VM, Li M, Audoly LP, Gabel CA, Jee WS, Dixon SJ, Sims SM & Thompson DD 2003 Deletion of the P2X7 nucleotide receptor reveals its regulatory roles in bone formation and resorption. *Mol.Endocrinol.* **17** 1356-1367.
- Keller H & Kneissel M 2005 SOST is a target gene for PTH in bone. *Bone* **37** 148-158.
- Kempson SA, Lotscher M, Kaissling B, Biber J, Murer H & Levi M 1995 Parathyroid hormone action on phosphate transporter mRNA and protein in rat renal proximal tubules. *Am.J.Physiol* **268** F784-F791.

- Kennedy C & Burnstock G 1985 Evidence for two types of P2-purinoceptor in longitudinal muscle of the rabbit portal vein. *Eur.J.Pharmacol.* **111** 49-56.
- Keusch I, Traebert M, Lotscher M, Kaissling B, Murer H & Biber J 1998 Parathyroid hormone and dietary phosphate provoke a lysosomal routing of the proximal tubular Na/Pi-cotransporter type II. *Kidney Int.* **54** 1224-1232.
- Khan AN, Al-Jahdali HH, Allen CM, Irion KL, Al GS & Koteyar SS 2010 The calcified lung nodule: What does it mean? *Ann.Thorac.Med.* **5** 67-79.
- Kiel DP, Felson DT, Hannan MT, Anderson JJ & Wilson PW 1990 Caffeine and the risk of hip fracture: the Framingham Study. *Am.J.Epidemiol.* **132** 675-684.
- Kim BW, Choo HJ, Lee JW, Kim JH & Ko YG 2004 Extracellular ATP is generated by ATP synthase complex in adipocyte lipid rafts. *Exp.Mol.Med.* **36** 476-485.
- Kim HJ, Minashima T, McCarthy EF, Winkles JA & Kirsch T 2010 Progressive ankylosis protein (ANK) in osteoblasts and osteoclasts controls bone formation and bone remodeling. *J.Bone Miner.Res.* **25** 1771-1783.
- King BF, Wildman SS, Townsend-Nicholson A & Burnstock G 1998 Antagonism of an adenosine/ATP receptor in follicular *Xenopus* oocytes. *J.Pharmacol.Exp.Ther.* **285** 1005-1011.
- Kirby BS, Crecelius AR, Voyles WF & Dinunno FA 2012 Impaired skeletal muscle blood flow control with advancing age in humans: attenuated ATP release and local vasodilation during erythrocyte deoxygenation. *Circ.Res.* **111** 220-230.
- Klein-Nulend J, Bakker AD, Bacabac RG, Vatsa A & Weinbaum S 2013 Mechanosensation and transduction in osteocytes. *Bone* **54** 182-190.
- Klement JF, Matsuzaki Y, Jiang QJ, Terlizzi J, Choi HY, Fujimoto N, Li K, Pulkkinen L, Birk DE, Sundberg JP & Uitto J 2005 Targeted ablation of the *abcc6* gene results in ectopic mineralization of connective tissues. *Mol.Cell Biol.* **25** 8299-8310.
- Knospe M, Muller CE, Rosa P, Abdelrahman A, von K, I, Thimm D & Schiedel AC 2013 The rat adenine receptor: pharmacological characterization and mutagenesis studies to investigate its putative ligand binding site. *Purinergic.Signal.* **9** 367-381.
- Knowles AF 2011 The GDA1_CD39 superfamily: NTPDases with diverse functions. *Purinergic Signal.* **7** 21-45.
- Knowles HJ & Athanasou NA 2009 Acute hypoxia and osteoclast activity: a balance between enhanced resorption and increased apoptosis. *J.Pathol.* **218** 256-264.
- Kode A, Mosialou I, Silva BC, Joshi S, Ferron M, Rached MT & Kousteni S 2012 FoxO1 protein cooperates with ATF4 protein in osteoblasts to control glucose homeostasis. *J.Biol.Chem.* **287** 8757-8768.

- Koga T, Matsui Y, Asagiri M, Kodama T, de CB, Nakashima K & Takayanagi H 2005 NFAT and Osterix cooperatively regulate bone formation. *Nature Med.* **11** 880-885.
- Kogawa M, Wijenayaka AR, Ormsby RT, Thomas GP, Anderson PH, Bonewald LF, Findlay DM & Atkins GJ 2013 Sclerostin regulates release of bone mineral by osteocytes by induction of carbonic anhydrase 2. *J.Bone Miner.Res.* **28** 2436-2448.
- Kokabu S, Gamer L, Cox K, Lowery J, Tsuji K, Raz R, Economides A, Katagiri T & Rosen V 2012 BMP3 suppresses osteoblast differentiation of bone marrow stromal cells via interaction with Acvr2b. *Mol.Endocrinol.* **26** 87-94.
- Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S & Kishimoto T 1997 Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* **89** 755-764.
- Kong YY, Boyle WJ & Penninger JM 1999a Osteoprotegerin ligand: a common link between osteoclastogenesis, lymph node formation and lymphocyte development. *Immunol.Cell Biol.* **77** 188-193.
- Kong YY, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, Capparelli C, Li J, Elliott R, McCabe S, Wong T, Campagnuolo G, Moran E, Bogoch ER, Van G, Nguyen LT, Ohashi PS, Lacey DL, Fish E, Boyle WJ & Penninger JM 1999b Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* **402** 304-309.
- Korcok J, Raimundo LN, Du X, Sims SM & Dixon SJ 2005 P2Y6 nucleotide receptors activate NF-kappaB and increase survival of osteoclasts. *J.Biol.Chem.* **280** 16909-16915.
- Kornak U, Kasper D, Bosl MR, Kaiser E, Schweizer M, Schulz A, Friedrich W, Delling G & Jentsch TJ 2001 Loss of the ClC-7 chloride channel leads to osteopetrosis in mice and man. *Cell* **104** 205-215.
- Koszalka P, Ozuyaman B, Huo Y, Zerneck A, Flogel U, Braun N, Buchheiser A, Decking UK, Smith ML, Sevigny J, Gear A, Weber AA, Molojavyi A, Ding Z, Weber C, Ley K, Zimmermann H, Godecke A & Schrader J 2004 Targeted disruption of cd73/ecto-5'-nucleotidase alters thromboregulation and augments vascular inflammatory response. *Circ.Res.* **95** 814-821.
- Kozawa O, Niwa M, Matsuno H, Tokuda H, Miwa M, Ito H, Kato K & Uematsu T 1999 Sphingosine 1-phosphate induces heat shock protein 27 via p38 mitogen-activated protein kinase activation in osteoblasts. *J.Bone Miner.Res.* **14** 1761-1767.
- Kramer I, Loots GG, Studer A, Keller H & Kneissel M 2010 Parathyroid hormone (PTH)-induced bone gain is blunted in SOST overexpressing and deficient mice. *J.Bone Miner.Res.* **25** 178-189.

- Kremer R & Goltzman D 1982 Parathyroid hormone stimulates mammalian renal 25-hydroxyvitamin D₃-1 alpha-hydroxylase *in vitro*. *Endocrinology* **110** 294-296.
- Kretlow JD, Jin YQ, Liu W, Zhang WJ, Hong TH, Zhou G, Baggett LS, Mikos AG & Cao Y 2008 Donor age and cell passage affects differentiation potential of murine bone marrow-derived stem cells. *BMC Cell Biol.* **9** 60.
- Kringelbach TM, Aslan D, Novak I, Schwarz P & Jorgensen NR 2013 UTP-induced ATP release is a fine-tuned signalling pathway in osteocytes. *Purinergic Signal.* **10** 337-47
- Kruger T, Oelenberg S, Kaesler N, Schurgers LJ, van de Sandt AM, Boor P, Schlieper G, Brandenburg VM, Fekete BC, Veulemans V, Ketteler M, Vermeer C, Jahnen-Dechent W, Floege J & Westenfeld R 2013 Warfarin induces cardiovascular damage in mice. *Arterioscler.Thromb.Vasc.Biol.* **33** 2618-2624.
- Kulkarni NH, Halladay DL, Miles RR, Gilbert LM, Frolik CA, Galvin RJ, Martin TJ, Gillespie MT & Onyia JE 2005 Effects of parathyroid hormone on Wnt signaling pathway in bone. *J.Cell Biochem.* **95** 1178-1190.
- Kumagai H, Sakamoto H, Guggino S, Filburn CR & Sacktor B 1989 Neurotransmitter regulation of cytosolic calcium in osteoblast-like bone cells. *Calcif.Tissue Int.* **45** 251-254.
- Kusafuka K, Yamaguchi A, Kayano T & Takemura T 2001 Ossification of tracheal cartilage in aged humans: a histological and immunohistochemical analysis. *J.Bone Miner.Metab.* **19** 168-174.
- Lacey DL, Tan HL, Lu J, Kaufman S, Van G, Qiu W, Rattan A, Scully S, Fletcher F, Juan T, Kelley M, Burgess TL, Boyle WJ & Polverino AJ 2000 Osteoprotegerin ligand modulates murine osteoclast survival *in vitro* and *in vivo*. *Am.J.Pathol.* **157** 435-448.
- Landolt HP, Werth E, Borbely AA & Dijk DJ 1995 Caffeine intake (200 mg) in the morning affects human sleep and EEG power spectra at night. *Brain Res.* **675** 67-74.
- Laurikkala J, Kassai Y, Pakkasjarvi L, Thesleff I & Itoh N 2003 Identification of a secreted BMP antagonist, ectodin, integrating BMP, FGF, and SHH signals from the tooth enamel knot. *Dev.Biol.* **264** 91-105.
- Lautenschlager S, Itin PH & Rufli T 1994 The petrified ear. *Dermatology* **189** 435-436.
- Lazarowski ER 2012 Vesicular and conductive mechanisms of nucleotide release. *Purinergic Signal.* **8** 359-373.
- Lazarowski ER, Homolya L, Boucher RC & Harden TK 1997 Identification of an ecto-nucleoside diphosphokinase and its contribution to interconversion of P₂ receptor agonists. *J.Biol.Chem.* **272** 20402-20407.

- Le BK & Mougiakakos D 2012 Multipotent mesenchymal stromal cells and the innate immune system. *Nature Rev.Immunol.* **12** 383-396.
- Le SO, Urban Z, Tschuch C, Csiszar K, Bacchelli B, Quaglino D, Pasquali-Ronchetti I, Pope FM, Richards A, Terry S, Bercovitch L, De PA & Boyd CD 2000 Mutations in a gene encoding an ABC transporter cause pseudoxanthoma elasticum. *Nature Genet.* **25** 223-227.
- Lee NK, Sowa H, Hinoi E, Ferron M, Ahn JD, Confavreux C, Dacquin R, Mee PJ, McKee MD, Jung DY, Zhang Z, Kim JK, Mauvais-Jarvis F, Ducy P & Karsenty G 2007 Endocrine regulation of energy metabolism by the skeleton. *Cell* **130** 456-469.
- Lee OK, Kuo TK, Chen WM, Lee KD, Hsieh SL & Chen TH 2004 Isolation of multipotent mesenchymal stem cells from umbilical cord blood. *Blood* **103** 1669-1675.
- Lerner UH, Sahlberg K & Fredholm BB 1987 Characterization of adenosine receptors in bone. Studies on the effect of adenosine analogues on cyclic AMP formation and bone resorption in cultured mouse calvaria. *Acta Physiol Scand.* **131** 287-296.
- Leupin O, Kramer I, Collette NM, Loots GG, Natt F, Kneissel M & Keller H 2007 Control of the SOST bone enhancer by PTH using MEF2 transcription factors. *J.Bone Miner.Res.* **22** 1957-1967.
- Li CY, Jepsen KJ, Majeska RJ, Zhang J, Ni R, Gelb BD & Schaffler MB 2006 Mice lacking cathepsin K maintain bone remodeling but develop bone fragility despite high bone mass. *J.Bone Miner.Res.* **21** 865-875.
- Li J, Liu D, Ke HZ, Duncan RL & Turner CH 2005a The P2X7 nucleotide receptor mediates skeletal mechanotransduction. *J.Biol.Chem.* **280** 42952-42959.
- Li Q, Schumacher W, Jablonski D, Siegel D & Uitto J 2012 Cutaneous features of pseudoxanthoma elasticum in a patient with generalized arterial calcification of infancy due to a homozygous missense mutation in the ENPP1 gene. *Br.J.Dermatol.* **166** 1107-1111.
- Li X, Ominsky MS, Niu QT, Sun N, Daugherty B, D'Agostin D, Kurahara C, Gao Y, Cao J, Gong J, Asuncion F, Barrero M, Warmington K, Dwyer D, Stolina M, Morony S, Sarosi I, Kostenuik PJ, Lacey DL, Simonet WS, Ke HZ & Paszty C 2008 Targeted deletion of the sclerostin gene in mice results in increased bone formation and bone strength. *J.Bone Miner.Res.* **23** 860-869.
- Li X, Ominsky MS, Warmington KS, Morony S, Gong J, Cao J, Gao Y, Shalhoub V, Tipton B, Haldankar R, Chen Q, Winters A, Boone T, Geng Z, Niu QT, Ke HZ, Kostenuik PJ, Simonet WS, Lacey DL & Paszty C 2009 Sclerostin antibody treatment increases bone formation, bone mass, and bone strength in a rat model of postmenopausal osteoporosis. *J.Bone Miner.Res.* **24** 578-588.

Li X, Zhang Y, Kang H, Liu W, Liu P, Zhang J, Harris SE & Wu D 2005b Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling. *J.Biol.Chem.* **280** 19883-19887.

Liang J, Fu M, Ciociola E, Chandalia M & Abate N 2007 Role of ENPP1 on adipocyte maturation. *PLoS One* **2** e882.

Lin C, Jiang X, Dai Z, Guo X, Weng T, Wang J, Li Y, Feng G, Gao X & He L 2009 Sclerostin mediates bone response to mechanical unloading through antagonizing Wnt/beta-catenin signaling. *J.Bone Miner.Res.* **24** 1651-1661.

Little RD, Carulli JP, Del Mastro RG, Dupuis J, Osborne M, Folz C, Manning SP, Swain PM, Zhao SC, Eustace B, Lappe MM, Spitzer L, Zweier S, Braunschweiger K, Benchekroun Y, Hu X, Adair R, Chee L, FitzGerald MG, Tulig C, Caruso A, Tzellas N, Bawa A, Franklin B, McGuire S, Nogues X, Gong G, Allen KM, Anisowicz A, Morales AJ, Lomedico PT, Recker SM, Van Eerdewegh P, Recker RR & Johnson ML 2002 A mutation in the LDL receptor-related protein 5 gene results in the autosomal dominant high-bone-mass trait. *Am.J.Hum.Genet.* **70** 11-19.

Liu SH, Chen C, Yang RS, Yen YP, Yang YT & Tsai C 2011 Caffeine enhances osteoclast differentiation from bone marrow hematopoietic cells and reduces bone mineral density in growing rats. *J.Orthop.Res.* **29** 954-960.

Lloyd HG & Fredholm BB 1995 Involvement of adenosine deaminase and adenosine kinase in regulating extracellular adenosine concentration in rat hippocampal slices. *Neurochem.Int.* **26** 387-395.

Lorenz-Depiereux B, Schnabel D, Tiosano D, Hausler G & Strom TM 2010 Loss-of-function ENPP1 mutations cause both generalized arterial calcification of infancy and autosomal-recessive hypophosphatemic rickets. *Am.J.Hum.Genet* **86** 267-272.

Lowry B, Miller JR & Fraser FC 1971 A new dominant gene mental retardation syndrome. Association with small stature, tapering fingers, characteristic facies, and possible hydrocephalus. *Am.J.Dis.Child* **121** 496-500.

Lu Y, Yuan B, Qin C, Cao Z, Xie Y, Dallas SL, McKee MD, Drezner MK, Bonewald LF & Feng JQ 2011 The biological function of DMP-1 in osteocyte maturation is mediated by its 57-kDa C-terminal fragment. *J.Bone Miner.Res.* **26** 331-340.

Luxenburg C, Geblinger D, Klein E, Anderson K, Hanein D, Geiger B & Addadi L 2007 The architecture of the adhesive apparatus of cultured osteoclasts: from podosome formation to sealing zone assembly. *PLoS One* **2** e179.

Ma YL, Cain RL, Halladay DL, Yang X, Zeng Q, Miles RR, Chandrasekhar S, Martin TJ & Onyia JE 2001 Catabolic effects of continuous human PTH (1--38) *in vivo* is associated with sustained stimulation of RANKL and inhibition of osteoprotegerin and gene-associated bone formation. *Endocrinology* **142** 4047-4054.

- MacDermot KD, Winter RM, Wigglesworth JS & Strobel S 1991 Short stature/short limb skeletal dysplasia with severe combined immunodeficiency and bowing of the femora: report of two patients and review. *J.Med.Genet.* **28** 10-17.
- MacDonald BR, Gallagher JA & Russell RG 1986 Parathyroid hormone stimulates the proliferation of cells derived from human bone. *Endocrinology* **118** 2445-2449.
- Macias BR, Aspenberg P & Agholme F 2013 Paradoxical Sost gene expression response to mechanical unloading in metaphyseal bone. *Bone* **53** 515-519.
- Macias BR, Swift JM, Nilsson MI, Hogan HA, Bouse SD & Bloomfield SA 2012 Simulated resistance training, but not alendronate, increases cortical bone formation and suppresses sclerostin during disuse. *J.Appl.Physiol (1985.)* **112** 918-925.
- Mackenzie NC, Huesa C, Rutsch F & MacRae VE 2012a New insights into NPP1 function: lessons from clinical and animal studies. *Bone* **51** 961-968.
- Mackenzie NC, Zhu D, Milne EM, van 't Hoff R, Martin A, Darryl QL, Millan JL, Farquharson C & MacRae VE 2012b Altered bone development and an increase in FGF-23 expression in *Enpp1(-/-)* mice. *PLoS One* **7** e32177.
- Maddux BA, Chang YN, Accili D, McGuinness OP, Youngren JF & Goldfine ID 2006 Overexpression of the insulin receptor inhibitor PC-1/ENPP1 induces insulin resistance and hyperglycemia. *Am.J.Physiol Endocrinol.Metab* **290** E746-E749.
- Maddux BA & Goldfine ID 2000 Membrane glycoprotein PC-1 inhibition of insulin receptor function occurs via direct interaction with the receptor alpha-subunit. *Diabetes* **49** 13-19.
- Maddux BA, Sbraccia P, Kumakura S, Sasson S, Youngren J, Fisher A, Spencer S, Grupe A, Henzel W, Stewart TA, Reaven GM & Goldfine ID. 1995 Membrane glycoprotein PC-1 and insulin resistance in non-insulin-dependent diabetes mellitus. *Nature* **373** 448-451.
- Magnusson P & Farley JR 2002 Differences in sialic acid residues among bone alkaline phosphatase isoforms: a physical, biochemical, and immunological characterization. *Calcif.Tissue Int.* **71** 508-518.
- Maier R, Glatz A, Mosbacher J & Bilbe G 1997 Cloning of P2Y6 cDNAs and identification of a pseudogene: comparison of P2Y receptor subtype expression in bone and brain tissues. *Biochem.Biophys.Res.Commun.* **240** 298-302.
- Malinauskas T, Aricescu AR, Lu W, Siebold C & Jones EY 2011 Modular mechanism of Wnt signaling inhibition by Wnt inhibitory factor 1. *Nature Struct.Mol.Biol.* **18** 886-893.

- Maliszewski CR, Delespesse GJ, Schoenborn MA, Armitage RJ, Fanslow WC, Nakajima T, Baker E, Sutherland GR, Poindexter K, Birks C & . 1994 The CD39 lymphoid cell activation antigen. Molecular cloning and structural characterization. *J.Immunol.* **153** 3574-3583.
- Mao B & Niehrs C 2003 Kremen2 modulates Dickkopf2 activity during Wnt/LRP6 signaling. *Gene* **302** 179-183.
- Mardon HJ, Bee J, von der MK & Owen ME 1987 Development of osteogenic tissue in diffusion chambers from early precursor cells in bone marrow of adult rats. *Cell Tissue Res.* **250** 157-165.
- Marini JC, Forlino A, Cabral WA, Barnes AM, San Antonio JD, Milgrom S, Hyland JC, Korkko J, Prockop DJ, De PA, Coucke P, Symoens S, Glorieux FH, Roughley PJ, Lund AM, Kuurila-Svahn K, Hartikka H, Cohn DH, Krakow D, Mottes M, Schwarze U, Chen D, Yang K, Kuslich C, Troendle J, Dagleish R & Byers PH 2007 Consortium for osteogenesis imperfecta mutations in the helical domain of type I collagen: regions rich in lethal mutations align with collagen binding sites for integrins and proteoglycans. *Hum.Mutat.* **28** 209-221.
- Martin A, David V, Laurence JS, Schwarz PM, Lafer EM, Hedge AM & Rowe PS 2008 Degradation of MEPE, DMP1, and release of SIBLING ASARM-peptides (minhibins): ASARM-peptide(s) are directly responsible for defective mineralization in HYP. *Endocrinology* **149** 1757-1772.
- Martin A, David V & Quarles LD 2012 Regulation and function of the FGF23/klotho endocrine pathways. *Physiol Rev.* **92** 131-155.
- Martini F 1998 Osseous tissue and skeletal structure. In *Fundamentals of anatomy and physiology* 4th edition, Prentice Hall International, New Jersey, US; pp 172-251.
- Matherne GP, Linden J, Byford AM, Gauthier NS & Headrick JP 1997 Transgenic A1 adenosine receptor overexpression increases myocardial resistance to ischemia. *Proc.Natl.Acad.Sci.U.S.A* **94** 6541-6546.
- McClung MR, Grauer A, Boonen S, Bolognese MA, Brown JP, Diez-Perez A, Langdahl BL, Reginster JY, Zanchetta JR, Wasserman SM, Katz L, Maddox J, Yang YC, Libanati C & Bone HG 2014 Romosozumab in postmenopausal women with low bone mineral density. *N.Engl.J.Med.* **370** 412-420.
- McConnell D, Frajola WJ & Deamer DW 1961 Relation between the inorganic chemistry and biochemistry of bone mineralization. *Science* **133** 281-282.
- McHugh KP, Hodivala-Dilke K, Zheng MH, Namba N, Lam J, Novack D, Feng X, Ross FP, Hynes RO & Teitelbaum SL 2000 Mice lacking beta3 integrins are osteosclerotic because of dysfunctional osteoclasts. *J.Clin.Invest* **105** 433-440.
- McLennan AG 2000 Dinucleoside polyphosphates-friend or foe? *Pharmacol.Ther.* **87** 73-89.

- Mediero A, Kara FM, Wilder T & Cronstein BN 2012 Adenosine A_{2A} receptor ligation inhibits osteoclast formation. *Am.J.Pathol.* **180** 775-786.
- Merrill JT, Shen C, Schreiber D, Coffey D, Zakharenko O, Fisher R, Lahita RG, Salmon J & Cronstein BN 1997 Adenosine A₁ receptor promotion of multinucleated giant cell formation by human monocytes: a mechanism for methotrexate-induced nodulosis in rheumatoid arthritis. *Arthritis Rheum.* **40** 1308-1315.
- Meyer JL 1984 Can biological calcification occur in the presence of pyrophosphate? *Arch.Biochem.Biophys.* **231** 1-8.
- Meyer RA, Jr., Meyer MH & Gray RW 1989 Parabiosis suggests a humoral factor is involved in X-linked hypophosphatemia in mice. *J.Bone Miner.Res.* **4** 493-500.
- Migchielsen AA, Breuer ML, van Roon MA, te RH, Zurcher C, Ossendorp F, Toutain S, Hershfield MS, Berns A & Valerio D 1995 Adenosine-deaminase-deficient mice die perinatally and exhibit liver-cell degeneration, atelectasis and small intestinal cell death. *Nature Genet.* **10** 279-287.
- Mikels AJ & Nusse R 2006 Purified Wnt5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context. *PLoS Biol.* **4** e115.
- Millan JL 2006 Alkaline Phosphatases : Structure, substrate specificity and functional relatedness to other members of a large superfamily of enzymes. *Purinergic Signal.* **2** 335-341.
- Millan JL 2013 The role of phosphatases in the initiation of skeletal mineralization. *Calcif.Tissue Int.* **93** 299-306.
- Miller SC & Jee WS 1987 The bone lining cell: a distinct phenotype? *Calcif.Tissue Int.* **41** 1-5.
- Mitic N, Valizadeh M, Leung EW, de JJ, Hamilton S, Hume DA, Cassady AI & Schenk G 2005 Human tartrate-resistant acid phosphatase becomes an effective ATPase upon proteolytic activation. *Arch.Biochem.Biophys.* **439** 154-164.
- Mitsuyama H, Healey RM, Terkeltaub RA, Coutts RD & Amiel D 2007 Calcification of human articular knee cartilage is primarily an effect of ageing rather than osteoarthritis. *Osteoarthritis.Cartilage.* **15** 559-565.
- Miyamoto H, Suzuki T, Miyauchi Y, Iwasaki R, Kobayashi T, Sato Y, Miyamoto K, Hoshi H, Hashimoto K, Yoshida S, Hao W, Mori T, Kanagawa H, Katsuyama E, Fujie A, Morioka H, Matsumoto M, Chiba K, Takeya M, Toyama Y & Miyamoto T 2012 Osteoclast stimulatory transmembrane protein and dendritic cell-specific transmembrane protein cooperatively modulate cell-cell fusion to form osteoclasts and foreign body giant cells. *J.Bone Miner.Res.* **27** 1289-1297.

- Modder UI, Hoey KA, Amin S, McCready LK, Achenbach SJ, Riggs BL, Melton LJ, III & Khosla S 2011 Relation of age, gender, and bone mass to circulating sclerostin levels in women and men. *J.Bone Miner.Res.* **26** 373-379.
- Moncada RM, Venta LA, Venta ER, Fareed J, Walenga JM & Messmore HL 1992 Tracheal and bronchial cartilaginous rings: warfarin sodium-induced calcification. *Radiology* **184** 437-439.
- Monroe DG, McGee-Lawrence ME, Oursler MJ & Westendorf JJ 2012 Update on Wnt signaling in bone cell biology and bone disease. *Gene* **492** 1-18.
- Moochhala SH, Sayer JA, Carr G & Simmons NL 2008 Renal calcium stones: insights from the control of mineralisation. *Exp.Physiol* **93** 43-49
- Mori S, Harruff R, Ambrosius W & Burr DB 1997 Trabecular bone volume and microdamage accumulation in the femoral heads of women with and without femoral neck fractures. *Bone* **21** 521-526.
- Morikawa T, Tanaka N, Kubota Y, Mizuno H, Nakamura K, Kunitomo M & Shinozuka K 2007 ATP modulates the release of noradrenaline through two different prejunctional receptors on the adrenergic nerves of rat prostate. *Clin.Exp.Pharmacol.Physiol* **34** 601-605.
- Mornet E, Taillandier A, Peyramaure S, Kaper F, Muller F, Brenner R, Bussiere P, Freisinger P, Godard J, Le MM, Oury JF, Plauchu H, Puddu R, Rival JM, Superti-Furga A, Touraine RL, Serre JL & Simon-Bouy B 1998 Identification of fifteen novel mutations in the tissue-nonspecific alkaline phosphatase (TNSALP) gene in European patients with severe hypophosphatasia. *Eur.J.Hum.Genet* **6** 308-314.
- Morrison MS, Turin L, King BF, Burnstock G & Arnett TR 1998 ATP is a potent stimulator of the activation and formation of rodent osteoclasts. *J.Physiol* **511** 495-500.
- Morrison RR, Talukder MA, Ledent C & Mustafa SJ 2002 Cardiac effects of adenosine in A(2A) receptor knockout hearts: uncovering A(2B) receptors. *Am.J.Physiol Heart Circ.Physiol* **282** H437-H444.
- Mueller E, Drori S, Aiyer A, Yie J, Sarraf P, Chen H, Hauser S, Rosen ED, Ge K, Roeder RG & Spiegelman BM 2002 Genetic analysis of adipogenesis through peroxisome proliferator-activated receptor gamma isoforms. *J.Biol.Chem.* **277** 41925-41930.
- Mullender MG, van der Meer DD, Huiskes R & Lips P 1996 Osteocyte density changes in ageing and osteoporosis. *Bone* **18** 109-113.
- Murrills RJ, Stein LS & Dempster DW 1993 Stimulation of bone resorption and osteoclast clear zone formation by low pH: a time-course study. *J.Cell Physiol* **154** 511-518.

- Murshed M, Harmey D, Millan JL, McKee MD & Karsenty G 2005 Unique coexpression in osteoblasts of broadly expressed genes accounts for the spatial restriction of ECM mineralization to bone. *Genes Dev.* **19** 1093-1104.
- Muzylak M, Arnett TR, Price JS & Horton MA 2007 The *in vitro* effect of pH on osteoclasts and bone resorption in the cat: implications for the pathogenesis of FORL. *J.Cell Physiol* **213** 144-150.
- Nakamura E, Uezono Y, Narusawa K, Shibuya I, Oishi Y, Tanaka M, Yanagihara N, Nakamura T & Izumi F 2000 ATP activates DNA synthesis by acting on P2X receptors in human osteoblast-like MG-63 cells. *Am.J.Physiol Cell Physiol* **279** C510-C519.
- Nakamura I, Pilkington MF, Lakkakorpi PT, Lipfert L, Sims SM, Dixon SJ, Rodan GA & Duong LT 1999 Role of alpha(v)beta(3) integrin in osteoclast migration and formation of the sealing zone. *J.Cell Sci.* **112 (Pt 22)** 3985-3993.
- Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR & de CB 2002 The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* **108** 17-29.
- Nakashima T, Hayashi M, Fukunaga T, Kurata K, Oh-Hora M, Feng JQ, Bonewald LF, Kodama T, Wutz A, Wagner EF, Penninger JM & Takayanagi H 2011 Evidence for osteocyte regulation of bone homeostasis through RANKL expression. *Nature Med.* **17** 1231-1234.
- Nam HK, Liu J, Li Y, Kragor A & Hatch NE 2011 Ectonucleotide pyrophosphatase/phosphodiesterase-1 (ENPP1) protein regulates osteoblast differentiation. *J.Biol.Chem.* **286** 39059-39071.
- Narisawa S, Frohlander N & Millan JL 1997 Inactivation of two mouse alkaline phosphatase genes and establishment of a model of infantile hypophosphatasia. *Dev.Dyn.* **208** 432-446.
- Nemeth ZH, Lutz CS, Csoka B, Deitch EA, Leibovich SJ, Gause WC, Tone M, Pacher P, Vizi ES & Hasko G 2005 Adenosine augments IL-10 production by macrophages through an A2B receptor-mediated posttranscriptional mechanism. *J.Immunol.* **175** 8260-8270.
- Neubauer S 2007 The failing heart--an engine out of fuel. *N.Engl.J.Med.* **356** 1140-1151.
- Nishimasu H, Ishitani R, Aoki J & Nureki O 2012 A 3D view of autotaxin. *Trends Pharmacol.Sci.* **33** 138-45.
- Nishio Y, Dong Y, Paris M, O'Keefe RJ, Schwarz EM & Drissi H 2006 Runx2-mediated regulation of the zinc finger Osterix/Sp7 gene. *Gene* **372** 62-70.

- Nitschke Y, Baujat G, Botschen U, Wittkamp T, du MM, Stella J, Le MM, Guest G, Lambot K, Tazarourte-Pinturier MF, Chassaing N, Roche O, Feenstra I, Loechner K, Deshpande C, Garber SJ, Chikarmane R, Steinmann B, Shahinyan T, Martorell L, Davies J, Smith WE, Kahler SG, McCulloch M, Wraige E, Loidi L, Hohne W, Martin L, Hadj-Rabia S, Terkeltaub R & Rutsch F 2012 Generalized arterial calcification of infancy and pseudoxanthoma elasticum can be caused by mutations in either ENPP1 or ABCC6. *Am.J.Hum.Genet* **90** 25-39.
- Nitschke Y, Hartmann S, Torsello G, Horstmann R, Seifarth H, Weissen-Plenz G & Rutsch F 2011 Expression of NPP1 is regulated during atheromatous plaque calcification. *J.Cell Mol.Med.* **15** 220-231.
- Noguchi K, Herr D, Mutoh T & Chun J 2009 Lysophosphatidic acid (LPA) and its receptors. *Curr.Opin.Pharmacol.* **9** 15-23.
- Norton ED, Jackson EK, Turner MB, Virmani R & Forman MB 1992 The effects of intravenous infusions of selective adenosine A1-receptor and A2-receptor agonists on myocardial reperfusion injury. *Am.Heart J.* **123** 332-338.
- Nusse R, Brown A, Papkoff J, Scambler P, Shackleford G, McMahon A, Moon R & Varmus H 1991 A new nomenclature for int-1 and related genes: the Wnt gene family. *Cell* **64** 231.
- Nyirenda MJ, Tang JI, Padfield PL & Seckl JR 2009 Hyperkalaemia. *BMJ* **339** b4114.
- O'Brien CA, Plotkin LI, Galli C, Goellner JJ, Gortazar AR, Allen MR, Robling AG, Bouxsein M, Schipani E, Turner CH, Jilka RL, Weinstein RS, Manolagas SC & Bellido T 2008 Control of bone mass and remodeling by PTH receptor signaling in osteocytes. *PLoS.One.* **3** e2942.
- Ohkubo S, Kimura J & Matsuoka I 2000 Ecto-alkaline phosphatase in NG108-15 cells : a key enzyme mediating P1 antagonist-sensitive ATP response. *Br.J.Pharmacol.* **131** 1667-1672.
- Ohnaka K, Taniguchi H, Kawate H, Nawata H & Takayanagi R 2004 Glucocorticoid enhances the expression of dickkopf-1 in human osteoblasts: novel mechanism of glucocorticoid-induced osteoporosis. *Biochem.Biophys.Res.Commun.* **318** 259-264.
- Okabe-Kado J & Kasukabe T 2003 Physiological and pathological relevance of extracellular NM23/NDP kinases. *J.Bioenerg.Biomembr.* **35** 89-93.
- Okawa A, Goto S & Moriya H 1999 Calcitonin simultaneously regulates both periosteal hyperostosis and trabecular osteopenia in the spinal hyperostotic mouse (twy/twy) *in vivo*. *Calcif.Tissue Int.* **64** 239-247.
- Okawa A, Nakamura I, Goto S, Moriya H, Nakamura Y & Ikegawa S 1998 Mutation in Npps in a mouse model of ossification of the posterior longitudinal ligament of the spine. *Nature Genet* **19** 271-273.

- Olah ME 1997 Identification of A2a adenosine receptor domains involved in selective coupling to Gs. Analysis of chimeric A1/A2a adenosine receptors. *J.Biol.Chem.* **272** 337-344.
- Oldknow K, Huesa C, Yadav M, Macrae V, Millan JL & Farquharson C 2012 Does Phospho1 Regulate Insulin Signalling in Osteoblasts? *Osteoporosis International* **23** S579-S580.
- Orimo H 2010 The mechanism of mineralization and the role of alkaline phosphatase in health and disease. *J.Nippon Med.Sch* **77** 4-12.
- Orriss IR & Arnett TR 2012 Rodent osteoclast cultures. *Methods Mol.Biol.* **816** 103-117.
- Orriss IR, Burnstock G & Arnett TR 2010 Purinergic signalling and bone remodelling. *Curr.Opin.Pharmacol.* **10** 322-330.
- Orriss IR, Key ML, Brandao-Burch A, Patel JJ, Burnstock G & Arnett TR 2012a The regulation of osteoblast function and bone mineralisation by extracellular nucleotides: The role of P2X receptors. *Bone* **51** 389-400.
- Orriss IR, Key ML, Hajjawi MO & Arnett TR 2013 Extracellular ATP released by osteoblasts is a key local inhibitor of bone mineralisation. *PLoS One* **8** e69057.
- Orriss IR, Knight GE, Ranasinghe S, Burnstock G & Arnett TR 2006 Osteoblast responses to nucleotides increase during differentiation. *Bone* **39** 300-309.
- Orriss IR, Knight GE, Utting JC, Taylor SE, Burnstock G & Arnett TR 2009 Hypoxia stimulates vesicular ATP release from rat osteoblasts. *J.Cell Physiol* **220** 155-162.
- Orriss IR, Taylor SE & Arnett TR 2012b Rat osteoblast cultures. *Methods Mol.Biol.* **816** 31-41.
- Orriss IR, Utting JC, Brandao-Burch A, Colston K, Grubb BR, Burnstock G & Arnett TR 2007 Extracellular nucleotides block bone mineralization *in vitro*: evidence for dual inhibitory mechanisms involving both P2Y2 receptors and pyrophosphate. *Endocrinology* **148** 4208-4216.
- Ortega N, Behonick DJ & Werb Z 2004 Matrix remodeling during endochondral ossification. *Trends Cell Biol.* **14** 86-93.
- Otero AS 2000 NM23/nucleoside diphosphate kinase and signal transduction. *J.Bioenerg.Biomembr.* **32** 269-275.
- Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, Selby PB & Owen MJ 1997 Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell* **89** 765-771.

- Padhi D, Jang G, Stouch B, Fang L & Posvar E 2011 Single-dose, placebo-controlled, randomized study of AMG 785, a sclerostin monoclonal antibody. *J. Bone Miner. Res.* **26** 19-26.
- Palmer TM, Gettys TW & Stiles GL 1995 Differential interaction with and regulation of multiple G-proteins by the rat A3 adenosine receptor. *J. Biol. Chem.* **270** 16895-16902.
- Palumbo C 1986 A three-dimensional ultrastructural study of osteoid-osteocytes in the tibia of chick embryos. *Cell Tissue Res.* **246** 125-131.
- Parfitt AM 1977 The cellular basis of bone turnover and bone loss: a rebuttal of the osteocytic resorption--bone flow theory. *Clin. Orthop. Relat Res.* 236-247.
- Paszty C, Turner CH & Robinson MK 2010 Sclerostin: a gem from the genome leads to bone-building antibodies. *J. Bone Miner. Res.* **25** 1897-1904.
- Pearce EI, Cousins FB & Smillie AC 1972 The mineralization of hair follicle tissue. I. An *in vivo* study. *Calcif. Tissue Res.* **8** 228-236.
- Pearce EI & Smillie AC 1973 The mineralization of hair follicle tissue. II. An *in vitro* study. *Calcif. Tissue Res.* **11** 23-38.
- Peart JN & Headrick JP 2007 Adenosinergic cardioprotection: multiple receptors, multiple pathways. *Pharmacol. Ther.* **114** 208-221.
- Pellegatti P, Falzoni S, Donvito G, Lemaire I & di Virgilio F. 2011 P2X7 receptor drives osteoclast fusion by increasing the extracellular adenosine concentration. *FASEB J.* **25** 1264-1274.
- Pellegatti P, Falzoni S, Pinton P, Rizzuto R & di Virgilio F 2005 A novel recombinant plasma membrane-targeted luciferase reveals a new pathway for ATP secretion. *Mol. Biol. Cell* **16** 3659-3665.
- Penolazzi L, Bianchini E, Lambertini E, Baraldi PG, Romagnoli R, Piva R & Gambari R 2005 N-Arylpiperazine modified analogues of the P2X7 receptor KN-62 antagonist are potent inducers of apoptosis of human primary osteoclasts. *J. Biomed. Sci.* **12** 1013-1020.
- Piccolo S, Sasai Y, Lu B & De Robertis EM 1996 Dorsoventral patterning in *Xenopus*: inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell* **86** 589-598.
- Picher M & Boucher RC 2003 Human airway ecto-adenylate kinase. A mechanism to propagate ATP signaling on airway surfaces. *J. Biol. Chem.* **278** 11256-11264.
- Picher M, Burch LH, Hirsh AJ, Spychala J & Boucher RC 2003 Ecto 5'-nucleotidase and nonspecific alkaline phosphatase. Two AMP-hydrolyzing ectoenzymes with distinct roles in human airways. *J. Biol. Chem.* **278** 13468-13479.

- Pierce KD, Furlong TJ, Selbie LA & Shine J 1992 Molecular cloning and expression of an adenosine A2b receptor from human brain. *Biochem.Biophys.Res.Commun.* **187** 86-93.
- Pinsky DJ, Broekman MJ, Peschon JJ, Stocking KL, Fujita T, Ramasamy R, Connolly ES, Jr., Huang J, Kiss S, Zhang Y, Choudhri TF, McTaggart RA, Liao H, Drosopoulos JH, Price VL, Marcus AJ & Maliszewski CR 2002 Elucidation of the thromboregulatory role of CD39/ectoapyrase in the ischemic brain. *J.Clin.Invest.* **109** 1031-1040.
- Pinson KI, Brennan J, Monkley S, Avery BJ & Skarnes WC 2000 An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* **407** 535-538.
- Pintor J, Diaz-Hernandez M, Gualix J, Gomez-Villafuertes R, Hernando F & Miras-Portugal MT 2000 Diadenosine polyphosphate receptors: from rat and guinea-pig brain to human nervous system. *Pharmacol.Ther.* **87** 103-115.
- Pintor J & Miras-Portugal MT 1995 A novel receptor for diadenosine polyphosphates coupled to calcium increase in rat midbrain synaptosomes. *Br.J.Pharmacol.* **115** 895-902.
- Pitsillides AA, Rawlinson SC, Suswillo RF, Bourrin S, Zaman G & Lanyon LE 1995 Mechanical strain-induced NO production by bone cells: a possible role in adaptive bone (re)modeling? *FASEB J.* **9** 1614-1622.
- Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S & Marshak DR 1999 Multilineage potential of adult human mesenchymal stem cells. *Science* **284** 143-147.
- Pizette S & Niswander L 2000 BMPs are required at two steps of limb chondrogenesis: formation of prechondrogenic condensations and their differentiation into chondrocytes. *Dev.Biol.* **219** 237-249.
- Poole KE, van Bezooijen RL, Loveridge N, Hamersma H, Papapoulos SE, Lowik CW & Reeve J 2005 Sclerostin is a delayed secreted product of osteocytes that inhibits bone formation. *FASEB J.* **19** 1842-1844.
- Porkka-Heiskanen T, Strecker RE, Thakkar M, Bjorkum AA, Greene RW & McCarley RW 1997 Adenosine: a mediator of the sleep-inducing effects of prolonged wakefulness. *Science* **276** 1265-1268.
- Potts JT 2005 Parathyroid hormone: past and present. *J.Endocrinol.* **187** 311-325.
- Power J, Loveridge N, Rushton N, Parker M & Reeve J 2002 Osteocyte density in ageing subjects is enhanced in bone adjacent to remodeling haversian systems. *Bone* **30** 859-865.
- Pradeep AR, Agarwal E, Raju PA, Rao MSN & Faizuddin M 2011 Study of the orthophosphate, pyrophosphate and pyrophosphatases in saliva with reference to calculus formation and inhibition. *J.Periodontol* **82** 445-451

Price C, Zhou X, Li W & Wang L 2011 Real-time measurement of solute transport within the lacunar-canalicular system of mechanically loaded bone: direct evidence for load-induced fluid flow. *J.Bone Miner.Res.* **26** 277-285.

Prideaux M, Loveridge N, Pitsillides AA & Farquharson C 2012 Extracellular matrix mineralization promotes E11/gp38 glycoprotein expression and drives osteocytic differentiation. *PLoS One* **7** e36786.

Qing H, Ardeshirpour L, Pajevic PD, Dusevich V, Jahn K, Kato S, Wysolmerski J & Bonewald LF 2012 Demonstration of osteocytic perilacunar/canalicular remodeling in mice during lactation. *J.Bone Miner.Res.* **27** 1018-1029.

Qing H & Bonewald LF 2009 Osteocyte remodeling of the perilacunar and pericanalicular matrix. *Int.J.Oral Sci.* **1** 59-65.

Qiu S, Rao DS, Palnitkar S & Parfitt AM 2002 Age and distance from the surface but not menopause reduce osteocyte density in human cancellous bone. *Bone* **31** 313-318.

Ramanathan M, Pinhal-Enfield G, Hao I & Leibovich SJ 2007 Synergistic up-regulation of vascular endothelial growth factor (VEGF) expression in macrophages by adenosine A2A receptor agonists and endotoxin involves transcriptional regulation via the hypoxia response element in the VEGF promoter. *Mol.Biol.Cell* **18** 14-23.

Ramasamy R, Yan SF, Herold K, Clynes R & Schmidt AM 2008 Receptor for advanced glycation end products: fundamental roles in the inflammatory response: winding the way to the pathogenesis of endothelial dysfunction and atherosclerosis. *Ann.N.Y.Acad.Sci.* **1126** 7-13.

Ramirez MI, Millien G, Hinds A, Cao Y, Seldin DC & Williams MC 2003 T1alpha, a lung type I cell differentiation gene, is required for normal lung cell proliferation and alveolus formation at birth. *Dev.Biol.* **256** 61-72.

Ranger AM, Grusby MJ, Hodge MR, Gravallesse EM, de la Brousse FC, Hoey T, Mickanin C, Baldwin HS & Glimcher LH 1998 The transcription factor NF-ATc is essential for cardiac valve formation. *Nature* **392** 186-190.

Rapaport E & Zamecnik PC 1976 Presence of diadenosine 5',5''' -P1, P4-tetraphosphate (Ap4A) in mammalian cells in levels varying widely with proliferative activity of the tissue: a possible positive "pleiotypic activator". *Proc.Natl.Acad.Sci.U.S.A* **73** 3984-3988.

Ratech H, Greco MA, Gallo G, Rimoin DL, Kamino H & Hirschhorn R 1985 Pathologic findings in adenosine deaminase-deficient severe combined immunodeficiency. I. Kidney, adrenal, and chondro-osseous tissue alterations. *Am.J.Pathol.* **120** 157-169.

- Rawlinson SC, Mohan S, Baylink DJ & Lanyon LE 1993 Exogenous prostacyclin, but not prostaglandin E2, produces similar responses in both G6PD activity and RNA production as mechanical loading, and increases IGF-II release, in adult cancellous bone in culture. *Calcif.Tissue Int.* **53** 324-329.
- Razzell WE & Khorana HG HG 1959 Studies on polynucleotides. III. Enzymic degradation; substrate specificity and properties of snake venom phosphodiesterase. *J.Biol.Chem.* **234** 2105-2113.
- Reimold AM, Grusby MJ, Kosaras B, Fries JW, Mori R, Maniwa S, Clauss IM, Collins T, Sidman RL, Glimcher MJ & Glimcher LH 1996 Chondrodysplasia and neurological abnormalities in ATF-2-deficient mice. *Nature* **379** 262-265.
- Retey JV, Adam M, Khatami R, Luhmann UF, Jung HH, Berger W & Landolt HP 2007 A genetic variation in the adenosine A2A receptor gene (ADORA2A) contributes to individual sensitivity to caffeine effects on sleep. *Clin.Pharmacol.Ther.* **81** 692-698.
- Rey JP & Ellies DL 2010 Wnt modulators in the biotech pipeline. *Dev.Dyn.* **239** 102-114.
- Ringpfeil F, Lebwohl MG, Christiano AM & Uitto J 2000 Pseudoxanthoma elasticum: mutations in the MRP6 gene encoding a transmembrane ATP-binding cassette (ABC) transporter. *Proc.Natl.Acad.Sci.U.S.A* **97** 6001-6006.
- Roach HI & Clarke NM 2000 Physiological cell death of chondrocytes *in vivo* is not confined to apoptosis. New observations on the mammalian growth plate. *J.Bone Joint Surg.Br.* **82** 601-613.
- Roberts SJ, Stewart AJ, Sadler PJ & Farquharson C 2004 Human PHOSPHO1 exhibits high specific phosphoethanolamine and phosphocholine phosphatase activities. *Biochem.J.* **382** 59-65.
- Robey P & Boskey A 2009 The composition of bone. In *Primer on metabolic bone diseases and disorders of mineral metabolism*, pp 32-38. Ed C Rosen. ASBMR.
- Robinson MK, Caminis J & Brunkow ME 2013 Sclerostin: how human mutations have helped reveal a new target for the treatment of osteoporosis. *Drug Discov.Today* **18** 637-643.
- Robison R 1923 The possible significance of hexosephosphoric esters in ossification. *Biochem.J.* **17** 286-293.
- Robling AG, Bellido T & Turner CH 2006 Mechanical stimulation *in vivo* reduces osteocyte expression of sclerostin. *J.Musculoskelet.Neuronal Interact.* **6** 354.
- Robling AG, Niziolek PJ, Baldrige LA, Condon KW, Allen MR, Alam I, Mantila SM, Gluhak-Heinrich J, Bellido TM, Harris SE & Turner CH 2008 Mechanical stimulation of bone *in vivo* reduces osteocyte expression of Sost/sclerostin. *J.Biol.Chem.* **283** 5866-5875.

- Robson SC, Seigny J & Zimmermann H 2006 The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. *Purinergic Signal.* **2** 409-430.
- Roforth MM, Fujita K, McGregor UI, Kirmani S, McCready LK, Peterson JM, Drake MT, Monroe DG & Khosla S 2014 Effects of age on bone mRNA levels of sclerostin and other genes relevant to bone metabolism in humans. *Bone* **59** 1-6.
- Romanello M, Pani B, Bicego M & D'Andrea P 2001 Mechanically induced ATP release from human osteoblastic cells. *Biochem.Biophys.Res.Commun.* **289** 1275-1281.
- Ronquist G 1968 Formation of extracellular adenosine triphosphate by human erythrocytes. *Acta Physiol Scand.* **74** 594-605.
- Rosen V 2006 BMP and BMP inhibitors in bone. *Ann.N.Y.Acad.Sci.* **1068** 19-25.
- Roszek K, Blaszczyk A, Wujak M & Komoszynski M 2013 Nucleotides metabolizing ectoenzymes as possible markers of mesenchymal stem cell osteogenic differentiation. *Biochem.Cell Biol.* **91** 176-181.
- Rowe PS, Garrett IR, Schwarz PM, Carnes DL, Lafer EM, Mundy GR & Gutierrez GE 2005 Surface plasmon resonance (SPR) confirms that MEPE binds to PHEX via the MEPE-ASARM motif: a model for impaired mineralization in X-linked rickets (HYP). *Bone* **36** 33-46.
- Rowe PS, Kumagai Y, Gutierrez G, Garrett IR, Blacher R, Rosen D, Cundy J, Navvab S, Chen D, Drezner MK, Quarles LD & Mundy GR 2004 MEPE has the properties of an osteoblastic phosphatonin and minihibin. *Bone* **34** 303-319.
- Ruf N, Uhlenberg B, Terkeltaub R, Nurnberg P & Rutsch F 2005 The mutational spectrum of ENPP1 as arising after the analysis of 23 unrelated patients with generalized arterial calcification of infancy (GACI). *Hum.Mutat.* **25** 98.
- Rumney RM, Wang N, Agrawal A & Gartland A 2012 Purinergic signalling in bone. *Front Endocrinol.(Lausanne)* **3** 116.
- Russell RG 2011 Bisphosphonates: the first 40 years. *Bone* **49** 2-19.
- Rutsch F, Nitschke Y & Terkeltaub R 2011 Genetics in arterial calcification: pieces of a puzzle and cogs in a wheel. *Circ.Res.* **109** 578-592.
- Rutsch F, Ruf N, Vaingankar S, Toliat MR, Suk A, Hohne W, Schauer G, Lehmann M, Roscioli T, Schnabel D, Epplen JT, Knisely A, Superti-Furga A, McGill J, Filippone M, Sinaiko AR, Vallance H, Hinrichs B, Smith W, Ferre M, Terkeltaub R & Nurnberg P 2003 Mutations in ENPP1 are associated with 'idiopathic' infantile arterial calcification. *Nature Genet* **34** 379-381.

- Rutsch F, Schauerte P, Kalhoff H, Petrarulo M, August C & Diekmann L 2000 Low levels of urinary inorganic pyrophosphate indicating systemic pyrophosphate deficiency in a boy with idiopathic infantile arterial calcification. *Acta Paediatr.* **89** 1265-1269.
- Ryzhov S, Zaynagetdinov R, Goldstein AE, Novitskiy SV, Blackburn MR, Biaggioni I & Feoktistov I 2008 Effect of A2B adenosine receptor gene ablation on adenosine-dependent regulation of proinflammatory cytokines. *J.Pharmacol.Exp.Ther.* **324** 694-700.
- Saetia K, Cho D, Lee S, Kim DH & Kim SD 2011 Ossification of the posterior longitudinal ligament: a review. *Neurosurg.Focus.* **30** E1 doi: 10.3171/2010.11.FOCUS10276.
- Saftig P, Hunziker E, Wehmeyer O, Jones S, Boyde A, Rommerskirch W, Moritz JD, Schu P & von FK 1998 Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin-K-deficient mice. *Proc.Natl.Acad.Sci.U.S.A* **95** 13453-13458.
- Sakagami H, Aoki J, Natori Y, Nishikawa K, Kakehi Y, Natori Y & Arai H 2005 Biochemical and molecular characterization of a novel choline-specific glycerophosphodiester phosphodiesterase belonging to the nucleotide pyrophosphatase/phosphodiesterase family. *J.Biol.Chem.* **280** 23084-23093.
- Sakamoto M, Hosoda Y, Kojimahara K, Yamazaki T & Yoshimura Y 1994 Arthritis and ankylosis in twy mice with hereditary multiple osteochondral lesions: with special reference to calcium deposition. *Pathol.Int.* **44** 420-427.
- Sali A, Favalaro JM, Terkeltaub R & Goding JW 1999 Germline deletion of the nucleoside triphosphate pyrophosphohydrolase (NTPPPH) plasma cell membrane glycoprotein (PC-1) produces abnormal calcification of the periarticular tissues. In: Vanduffe L, Lemmens R, editors. Ecto-ATPases and related ectonucleotides, Shaker publishing BV, Maastricht, the Netherlands; pp 267-82.
- Salvatore CA, Tilley SL, Latour AM, Fletcher DS, Koller BH & Jacobson MA 2000 Disruption of the A(3) adenosine receptor gene in mice and its effect on stimulated inflammatory cells. *J.Biol.Chem.* **275** 4429-4434.
- Sandona D, Gastaldello S, Martinello T & Betto R 2004 Characterization of the ATP-hydrolysing activity of alpha-sarcoglycan. *Biochem.J.* **381** 105-112.
- Santagati F & Rijli FM 2003 Cranial neural crest and the building of the vertebrate head. *Nature Rev.Neurosci.* **4** 806-818.
- Santos A, Bakker AD, Zandieh-Doulabi B, Semeins CM & Klein-Nulend J 2009 Pulsating fluid flow modulates gene expression of proteins involved in WNT signaling pathways in osteocytes. *J.Orthop.Res.* **27** 1280-1287.
- Sauer AV, Brigida I, Carriglio N & Aiuti A 2012 Autoimmune dysregulation and purine metabolism in adenosine deaminase deficiency. *Front Immunol.* **3** 265.

Sauer AV, Mrak E, Hernandez RJ, Zacchi E, Cavani F, Casiraghi M, Grunebaum E, Roifman CM, Cervi MC, Ambrosi A, Carlucci F, Roncarolo MG, Villa A, Rubinacci A & Aiuti A 2009 ADA-deficient SCID is associated with a specific microenvironment and bone phenotype characterized by RANKL/OPG imbalance and osteoblast insufficiency. *Blood* **114** 3216-3226.

Sawakami K, Robling AG, Ai M, Pitner ND, Liu D, Warden SJ, Li J, Maye P, Rowe DW, Duncan RL, Warman ML & Turner CH 2006 The Wnt co-receptor LRP5 is essential for skeletal mechanotransduction but not for the anabolic bone response to parathyroid hormone treatment. *J.Biol.Chem.* **281** 23698-23711.

Schiller PC, D'Ippolito G, Roos BA & Howard GA 1999 Anabolic or catabolic responses of MC3T3-E1 osteoblastic cells to parathyroid hormone depend on time and duration of treatment. *J.Bone Miner.Res.* **14** 1504-1512.

Schofl C, Cuthbertson KS, Walsh CA, Mayne C, Cobbold P, von zur MA, Hesch RD & Gallagher JA 1992 Evidence for P2-purinoceptors on human osteoblast-like cells. *J.Bone Miner.Res.* **7** 485-491.

Schrauwen I & Van Camp G 2010 The etiology of otosclerosis: a combination of genes and environment. *Laryngoscope* **120** 1195-1202.

Schreiber WE & Whitta L 1986 Alkaline phosphatase isoenzymes resolved by electrophoresis on lectin-containing agarose gel. *Clin.Chem.* **32** 1570-1573.

Schubert P, Komp W & Kreutzberg GW 1979 Correlation of 5'-nucleotidase activity and selective transneuronal transfer of adenosine in the hippocampus. *Brain Res.* **168** 419-424.

Schulte G 2010 International Union of Basic and Clinical Pharmacology. LXXX. The class Frizzled receptors. *Pharmacol.Rev.* **62** 632-667.

Schulze E, Witt M, Kasper M, Lowik CW & Funk RH 1999 Immunohistochemical investigations on the differentiation marker protein E11 in rat calvaria, calvaria cell culture and the osteoblastic cell line ROS 17/2.8. *Histochem.Cell Biol.* **111** 61-69.

Schwartz RP & Heath AL 1947 The definition of human locomotion on the basis of measurement; with description of oscillographic method. *J.Bone Joint Surg.Am.* **29** 203-214.

Scott EW, Simon MC, Anastasi J & Singh H 1994 Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science* **265** 1573-1577.

Semenov M, Tamai K & He X 2005 SOST is a ligand for LRP5/LRP6 and a Wnt signaling inhibitor. *J.Biol.Chem.* **280** 26770-26775.

Semenov MV, Zhang X & He X 2008 DKK1 antagonizes Wnt signaling without promotion of LRP6 internalization and degradation. *J.Biol.Chem.* **283** 21427-21432.

- Sessarego N, Parodi A, Podesta M, Benvenuto F, Moggi M, Raviolo V, Lituania M, Kunkl A, Ferlazzo G, Bricarelli FD, Uccelli A & Frasson F 2008 Multipotent mesenchymal stromal cells from amniotic fluid: solid perspectives for clinical application. *Haematologica* **93** 339-346.
- Shimada T, Muto T, Urakawa I, Yoneya T, Yamazaki Y, Okawa K, Takeuchi Y, Fujita T, Fukumoto S & Yamashita T 2002 Mutant FGF-23 responsible for autosomal dominant hypophosphatemic rickets is resistant to proteolytic cleavage and causes hypophosphatemia *in vivo*. *Endocrinology* **143** 3179-3182.
- Shimada T, Yamazaki Y, Takahashi M, Hasegawa H, Urakawa I, Oshima T, Ono K, Kakitani M, Tomizuka K, Fujita T, Fukumoto S & Yamashita T 2005 Vitamin D receptor-independent FGF23 actions in regulating phosphate and vitamin D metabolism. *Am.J.Physiol Renal Physiol* **289** F1088-F1095.
- Shyu JF, Shih C, Tseng CY, Lin CH, Sun DT, Liu HT, Tsung HC, Chen TH & Lu RB 2007 Calcitonin induces podosome disassembly and detachment of osteoclasts by modulating Pyk2 and Src activities. *Bone* **40** 1329-1342.
- Sieber C, Kopf J, Hiepen C & Knaus P 2009 Recent advances in BMP receptor signaling. *Cytokine Growth Factor Rev.* **20** 343-355.
- Simao AM, Yadav MC, Narisawa S, Bolean M, Pizauro JM, Hoylaerts MF, Ciancaglini P & Millan JL 2010 Proteoliposomes harboring alkaline phosphatase and nucleotide pyrophosphatase as matrix vesicle biomimetics. *J.Biol.Chem.* **285** 7598-7609.
- Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Sander S, Van G, Tarpley J, Derby P, Lee R & Boyle WJ 1997 Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* **89** 309-319.
- Singer SJ, Maher PA & Yaffe MP 1987 On the translocation of proteins across membranes. *Proc.Natl.Acad.Sci.U.S.A* **84** 1015-1019.
- Skerry TM, Bitensky L, Chayen J & Lanyon LE 1989 Early strain-related changes in enzyme activity in osteocytes following bone loading *in vivo*. *J.Bone Miner.Res.* **4** 783-788.
- Skillington J, Choy L & Derynck R 2002 Bone morphogenetic protein and retinoic acid signaling cooperate to induce osteoblast differentiation of preadipocytes. *J.Cell Biol.* **159** 135-146.
- Slominska EM, Szolkiewicz M, Smolenski RT, Rutkowski B & Swierczynski J 2002 High plasma adenine concentration in chronic renal failure and its relation to erythrocyte ATP. *Nephron* **91** 286-291.

- Smith AD, Cheek DJ, Buxton IL & Westfall DP 1997 Competition of adenine nucleotides for a 1,3-[3H]-dipropyl-8-cyclopentylxanthine binding site in rat vas deferens. *Clin.Exp.Pharmacol.Physiol* **24** 492-497.
- Smith TM & Kirley TL 2006 The calcium activated nucleotidases: A diverse family of soluble and membrane associated nucleotide hydrolyzing enzymes. *Purinergic Signal.* **2** 327-333.
- Sobacchi C, Frattini A, Guerrini MM, Abinun M, Pangrazio A, Susani L, Bredius R, Mancini G, Cant A, Bishop N, Grabowski P, Del FA, Messina C, Errigo G, Coxon FP, Scott DI, Teti A, Rogers MJ, Vezzoni P, Villa A & Helfrich MH 2007 Osteoclast-poor human osteopetrosis due to mutations in the gene encoding RANKL. *Nature Genet.* **39** 960-962.
- Soriano P, Montgomery C, Geske R & Bradley A 1991 Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. *Cell* **64** 693-702.
- Soro-Paavonen A, Watson AM, Li J, Paavonen K, Koitka A, Calkin AC, Barit D, Coughlan MT, Drew BG, Lancaster GI, Thomas M, Forbes JM, Nawroth PP, Bierhaus A, Cooper ME & Jandeleit-Dahm KA 2008 Receptor for advanced glycation end products (RAGE) deficiency attenuates the development of atherosclerosis in diabetes. *Diabetes* **57** 2461-2469.
- Spatz JM, Ellman R, Cloutier AM, Louis L, van Vliet M, Suva LJ, Dwyer D, Stolina M, Ke HZ & Bouxsein ML 2013 Sclerostin antibody inhibits skeletal deterioration due to reduced mechanical loading. *J.Bone Miner.Res.* **28** 865-874.
- Spencer GJ, Utting JC, Etheridge SL, Arnett TR & Genever PG 2006 Wnt signalling in osteoblasts regulates expression of the receptor activator of NFkappaB ligand and inhibits osteoclastogenesis *in vitro*. *J.Cell Sci.* **119** 1283-1296.
- Sprague RS, Bowles EA, Achilleus D & Ellsworth ML 2011 Erythrocytes as controllers of perfusion distribution in the microvasculature of skeletal muscle. *Acta Physiol (Oxf)* **202** 285-292.
- Spychala J, Datta NS, Takabayashi K, Datta M, Fox IH, Gribbin T & Mitchell BS 1996 Cloning of human adenosine kinase cDNA: sequence similarity to microbial ribokinases and fructokinases. *Proc.Natl.Acad.Sci.U.S.A* **93** 1232-1237.
- Spychala J & Kitajewski J 2004 Wnt and beta-catenin signaling target the expression of ecto-5'-nucleotidase and increase extracellular adenosine generation. *Exp.Cell Res.* **296** 99-108.
- St Hilaire C, Ziegler SG, Markello TC, Brusco A, Groden C, Gill F, Carlson-Donohoe H, Lederman RJ, Chen MY, Yang D, Siegenthaler MP, Arduino C, Mancini C, Freudenthal B, Stanescu HC, Zdebik AA, Chaganti RK, Nussbaum RL, Kleta R, Gahl WA & Boehm M 2011 NT5E mutations and arterial calcifications. *N.Engl.J.Med.* **364** 432-442.

- Staebling-Hampton K, Proll S, Paepfer BW, Zhao L, Charmley P, Brown A, Gardner JC, Galas D, Schatzman RC, Beighton P, Papapoulos S, Hamersma H & Brunkow ME 2002 A 52-kb deletion in the SOST-MEOX1 intergenic region on 17q12-q21 is associated with van Buchem disease in the Dutch population. *Am.J.Med.Genet.* **110** 144-152.
- Stavrou BM 2003 Diadenosine polyphosphates: postulated mechanisms mediating the cardiac effects. *Curr.Med.Chem.Cardiovasc.Hematol.Agents* **1** 151-169.
- Stearne PA, Van Driel IR, Grego B, Simpson RJ & Goding JW 1985 The murine plasma cell antigen PC-1: purification and partial amino acid sequence. *J.Immunol.* **134** 443-448.
- Stefan C, Jansen S & Bollen M 2005 NPP-type ectophosphodiesterases: unity in diversity. *Trends Biochem.Sci.* **30** 542-550.
- Stern AR, Stern MM, Van Dyke ME, Jahn K, Prideaux M & Bonewald LF 2012 Isolation and culture of primary osteocytes from the long bones of skeletally mature and aged mice. *Biotechniques* **52** 361-373.
- Stewart AJ, Roberts SJ, Seawright E, Davey MG, Fleming RH & Farquharson C 2006 The presence of PHOSPHO1 in matrix vesicles and its developmental expression prior to skeletal mineralization. *Bone* **39** 1000-1007.
- Stewart AJ, Schmid R, Blindauer CA, Paisey SJ & Farquharson C 2003 Comparative modelling of human PHOSPHO1 reveals a new group of phosphatases within the haloacid dehalogenase superfamily. *Protein Eng.* **16** 889-895.
- Stites PC, Boyd AS & Zic J 2003 Auricular ossificans (ectopic ossification of the auricle). *J.Am.Acad.Dermatol.* **49** 142-144.
- Strumia R, Lombardi AR & Altieri E 1997 The petrified ear--a manifestation of dystrophic calcification. *Dermatology* **194** 371-373.
- Su SJ, Chang KL, Su SH, Yeh YT, Shyu HW & Chen KM 2013 Caffeine regulates osteogenic differentiation and mineralization of primary adipose-derived stem cells and a bone marrow stromal cell line. *Int.J.Food Sci.Nutr.* **64** 429-436.
- Suadicani SO, Brosnan CF & Scemes E 2006 P2X7 receptors mediate ATP release and amplification of astrocytic intercellular Ca²⁺ signaling. *J.Neurosci.* **26** 1378-1385.
- Suzuki T, Namba K, Tsuga H & Nakata H 2006 Regulation of pharmacology by hetero-oligomerization between A1 adenosine receptor and P2Y2 receptor. *Biochem.Biophys.Res.Commun.* **351** 559-565.
- Syberg S, Brandao-Burch A, Patel JJ, Hajjawi M, Arnett TR, Schwarz P, Jorgensen NR & Orriss IR 2012 Clopidogrel (Plavix), a P2Y12 receptor antagonist, inhibits bone cell function *in vitro* and decreases trabecular bone *in vivo*. *J.Bone Miner.Res.* **27** 2373-2386.

- Takada I, Mihara M, Suzawa M, Ohtake F, Kobayashi S, Igarashi M, Youn MY, Takeyama K, Nakamura T, Mezaki Y, Takezawa S, Yogiashi Y, Kitagawa H, Yamada G, Takada S, Minami Y, Shibuya H, Matsumoto K & Kato S 2007 A histone lysine methyltransferase activated by non-canonical Wnt signalling suppresses PPAR-gamma transactivation. *Nature Cell Biol.* **9** 1273-1285.
- Takahashi T, Old LJ & Boyse EA 1970 Surface alloantigens of plasma cells. *J.Exp.Med.* **131** 1325-1341.
- Takayanagi H, Kim S, Koga T, Nishina H, Isshiki M, Yoshida H, Saiura A, Isobe M, Yokochi T, Inoue J, Wagner EF, Mak TW, Kodama T & Taniguchi T 2002 Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev.Cell* **3** 889-901.
- Takeda S, Yoshizawa T, Nagai Y, Yamato H, Fukumoto S, Sekine K, Kato S, Matsumoto T & Fujita T 1999 Stimulation of osteoclast formation by 1,25-dihydroxyvitamin D requires its binding to vitamin D receptor (VDR) in osteoblastic cells: studies using VDR knockout mice. *Endocrinology* **140** 1005-1008.
- Takedachi M, Oohara H, Smith BJ, Iyama M, Kobashi M, Maeda K, Long CL, Humphrey MB, Stoecker BJ, Toyosawa S, Thompson LF & Murakami S 2012 CD73-generated adenosine promotes osteoblast differentiation. *J.Cell Physiol.* **227** 2622-2631.
- Tanaka M, Okudaira S, Kishi Y, Ohkawa R, Iseki S, Ota M, Noji S, Yatomi Y, Aoki J & Arai H 2006 Autotaxin stabilizes blood vessels and is required for embryonic vasculature by producing lysophosphatidic acid. *J.Biol.Chem.* **281** 25822-25830.
- Tang QQ, Otto TC & Lane MD 2003 CCAAT/enhancer-binding protein beta is required for mitotic clonal expansion during adipogenesis. *Proc.Natl.Acad.Sci.U.S.A* **100** 850-855.
- Tatsumi S, Ishii K, Amizuka N, Li M, Kobayashi T, Kohno K, Ito M, Takeshita S & Ikeda K 2007 Targeted ablation of osteocytes induces osteoporosis with defective mechanotransduction. *Cell Metab.* **5** 464-475.
- Tautenhahn M, Leichsenring A, Servettini I, Pesic M, Sperlagh B, Norenberg W & Illies P 2012 Purinergic modulation of the excitatory synaptic input onto rat striatal neurons. *Neuropharmacology* **62** 1756-1766.
- Taylor PR, Martinez-Pomares L, Stacey M, Lin HH, Brown GD & Gordon S 2005 Macrophage receptors and immune recognition. *Annu.Rev.Immunol.* **23** 901-944.
- Tazawa K, Hoshi K, Kawamoto S, Tanaka M, Ejiri S & Ozawa H 2004 Osteocytic osteolysis observed in rats to which parathyroid hormone was continuously administered. *J.Bone Miner.Metab.* **22** 524-529.
- Teale C, Romaniuk C & Mulley G 1989 Calcification on chest radiographs: the association with age. *Age Ageing* **18** 333-336.

- Tehrani S, Tomasevic N, Weed S, Sakowicz R & Cooper JA 2007 Src phosphorylation of cortactin enhances actin assembly. *Proc.Natl.Acad.Sci.U.S.A* **104** 11933-11938.
- Terkeltaub R, Rosenbach M, Fong F & Goding J 1994 Causal link between nucleotide pyrophosphohydrolase overactivity and increased intracellular inorganic pyrophosphate generation demonstrated by transfection of cultured fibroblasts and osteoblasts with plasma cell membrane glycoprotein-1. Relevance to calcium pyrophosphate dihydrate deposition disease. *Arthritis Rheum.* **37** 934-941.
- Terkeltaub RA 2001 Inorganic pyrophosphate generation and disposition in pathophysiology. *Am.J.Physiol.Cell Physiol.* **281** C1-C11.
- Teti A, Blair HC, Schlesinger P, Grano M, Zamboni-Zallone A, Kahn AJ, Teitelbaum SL & Hruska KA 1989 Extracellular protons acidify osteoclasts, reduce cytosolic calcium, and promote expression of cell-matrix attachment structures. *J.Clin.Invest.* **84** 773-780.
- Thimm D, Knosp M, Abdelrahman A, Moutinho M, Alsdorf BB, von K, I, Schiedel AC & Muller CE 2013 Characterization of new G protein-coupled adenine receptors in mouse and hamster. *Purinergic Signal.* **9** 415-426.
- Thompson LF, Eltzschig HK, Ibla JC, Van De Wiele CJ, Resta R, Morote-Garcia JC & Colgan SP 2004 Crucial role for ecto-5'-nucleotidase (CD73) in vascular leakage during hypoxia. *J.Exp.Med.* **200** 1395-1405.
- Thoongsuwan N & Stern EJ 2003 Warfarin-induced tracheobronchial calcification. *J.Thorac.Imaging* **18** 110-112.
- Thorsen K, Kristoffersson AO, Lerner UH & Lorentzon RP 1996 *In situ* microdialysis in bone tissue. Stimulation of prostaglandin E2 release by weight-bearing mechanical loading. *J.Clin.Invest.* **98** 2446-2449.
- Tondravi MM, McKercher SR, Anderson K, Erdmann JM, Quiroz M, Maki R & Teitelbaum SL 1997 Osteopetrosis in mice lacking haematopoietic transcription factor PU.1. *Nature* **386** 81-84.
- Torres RJ & Puig JG 2007 Hypoxanthine-guanine phosphoribosyltransferase (HPRT) deficiency: Lesch-Nyhan syndrome. *Orphanet.J.Rare.Dis.* **2** 48.
- Torres-Lagares D, Tulasne JF, Pouget C, Llorens A, Saffar JL & Lesclous P 2010 Structure and remodelling of the human parietal bone: an age and gender histomorphometric study. *J.Craniomaxillofac.Surg.* **38** 325-330.
- Toyosawa S, Shintani S, Fujiwara T, Ooshima T, Sato A, Ijuhin N & Komori T 2001 Dentin matrix protein 1 is predominantly expressed in chicken and rat osteocytes but not in osteoblasts. *J.Bone Miner.Res.* **16** 2017-2026.

- Traebert M, Roth J, Biber J, Murer H & Kaissling B 2000 Internalization of proximal tubular type II Na-P(i) cotransporter by PTH: immunogold electron microscopy. *Am.J.Physiol.Renal Physiol.* **278** F148-F154.
- Traub W, Arad T & Weiner S 1992 Origin of mineral crystal growth in collagen fibrils. *Matrix* **12** 251-255.
- Tsuang YH, Sun JS, Chen LT, Sun SC & Chen SC 2006 Direct effects of caffeine on osteoblastic cells metabolism: the possible causal effect of caffeine on the formation of osteoporosis. *J.Orthop.Surg.Res.* **1** 7.
- Tsuji K, Bandyopadhyay A, Harfe BD, Cox K, Kakar S, Gerstenfeld L, Einhorn T, Tabin CJ & Rosen V 2006 BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing. *Nature Genet.* **38** 1424-1429.
- Tu X, Joeng KS, Nakayama KI, Nakayama K, Rajagopal J, Carroll TJ, McMahon AP & Long F 2007 Noncanonical Wnt signaling through G protein-linked PKCdelta activation promotes bone formation. *Dev.Cell* **12** 113-127.
- Ubaidus S, Li M, Sultana S, de Freitas PH, Oda K, Maeda T, Takagi R & Amizuka N 2009 FGF23 is mainly synthesized by osteocytes in the regularly distributed osteocytic lacunar canalicular system established after physiological bone remodeling. *J.Electron Microsc.(Tokyo)* **58** 381-392.
- Umezū-Goto M, Kishi Y, Taira A, Hama K, Dohmae N, Takio K, Yamori T, Mills GB, Inoue K, Aoki J & Arai H 2002 Autotaxin has lysophospholipase D activity leading to tumor cell growth and motility by lysophosphatidic acid production. *J.Cell Biol.* **158** 227-233.
- Urist MR 1965 Bone: formation by autoinduction. *Science* **150** 893-899.
- Utting JC, Flanagan AM, Brandao-Burch A, Orriss IR & Arnett TR 2010 Hypoxia stimulates osteoclast formation from human peripheral blood. *Cell Biochem.Funct.* **28** 374-380.
- Utting JC, Robins SP, Brandao-Burch A, Orriss IR, Behar J & Arnett TR 2006 Hypoxia inhibits the growth, differentiation and bone-forming capacity of rat osteoblasts. *Exp.Cell Res.* **312** 1693-1702.
- van Bezooijen RL, Bronckers AL, Gortzak RA, Hogendoorn PC, Wee-Pals L, Balemans W, Oostenbroek HJ, Van Hul W, Hamersma H, Dijkers FG, Hamdy NA, Papapoulos SE & Lowik CW 2009 Sclerostin in mineralized matrices and van Buchem disease. *J.Dent.Res.* **88** 569-574.
- van Bezooijen RL, Roelen BA, Visser A, Wee-Pals L, de WE, Karperien M, Hamersma H, Papapoulos SE, ten DP & Lowik CW 2004 Sclerostin is an osteocyte-expressed negative regulator of bone formation, but not a classical BMP antagonist. *J.Exp.Med.* **199** 805-814.

- Van Der Plas A & Nijweide PJ 1992 Isolation and purification of osteocytes. *J.Bone Miner.Res.* **7** 389-396.
- Van Driel IR & Goding JW 1987 Plasma cell membrane glycoprotein PC-1. Primary structure deduced from cDNA clones. *J.Biol.Chem.* **262** 4882-4887.
- van Galen PJ, van Bergen AH, Gallo-Rodriguez C, Melman N, Olah ME, IJzerman AP, Stiles GL & Jacobson KA 1994 A binding site model and structure-activity relationships for the rat A3 adenosine receptor. *Mol.Pharmacol.* **45** 1101-1111.
- van Driel M, Koedam M, Buurman CJ, Roelse M, Weyts F, Chiba H, Uitterlinden AG, Pols HA & van Leeuwen JP 2006 Evidence that both 1 α ,25-dihydroxyvitamin D3 and 24-hydroxylated D3 enhance human osteoblast differentiation and mineralization. *J.Cell Biochem.* **99** 922-935.
- Van Hul W, Balemans W, Van Hul E, Dijkers FG, Obee H, Stokroos RJ, Hildering P, Vanhoenacker F, Van Camp G & Willems PJ 1998 Van Buchem disease (hyperostosis corticalis generalisata) maps to chromosome 17q12-q21. *Am.J.Hum.Genet.* **62** 391-399.
- Vashishth D 2007 The role of the collagen matrix in skeletal fragility. *Curr.Osteoporos.Rep.* **5** 62-66.
- Villa-Bellosta R, Wang X, Millan JL, Dubyak GR & O'Neill WC 2011 Extracellular pyrophosphate metabolism and calcification in vascular smooth muscle. *Am.J.Physiol Heart Circ.Physiol.* **301** H61-H68.
- Vincent C, Findlay DM, Welldon KJ, Wijenayaka AR, Zheng TS, Haynes DR, Fazzalari NL, Evdokiou A & Atkins GJ 2009 Pro-inflammatory cytokines TNF-related weak inducer of apoptosis (TWEAK) and TNF α induce the mitogen-activated protein kinase (MAPK)-dependent expression of sclerostin in human osteoblasts. *J.Bone Miner.Res.* **24** 1434-1449.
- Vincenzi F, Targa M, Corciulo C, Gessi S, Merighi S, Setti S, Cadossi R, Goldring MB, Borea PA & Varani K 2013 Pulsed electromagnetic fields increased the anti-inflammatory effect of A(2)A and A(3) adenosine receptors in human T/C-28a2 chondrocytes and hFOB 1.19 osteoblasts. *PLoS One* **8** e65561.
- von Kugelgen I, Schiedel AC, Hoffmann K, Alsdorf BB, Abdelrahman A & Muller CE 2008 Cloning and functional expression of a novel Gi protein-coupled receptor for adenine from mouse brain. *Mol.Pharmacol.* **73** 469-477.
- Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, Shapiro SD, Senior RM & Werb Z 1998 MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell* **93** 411-422.
- Wada T, McKee MD, Steitz S & Giachelli CM 1999 Calcification of vascular smooth muscle cell cultures: inhibition by osteopontin. *Circ.Res.* **84** 166-178.

- Wakamiya M, Blackburn MR, Jurecic R, McArthur MJ, Geske RS, Cartwright J, Jr., Mitani K, Vaishnav S, Belmont JW, Kellems RE & . 1995 Disruption of the adenosine deaminase gene causes hepatocellular impairment and perinatal lethality in mice. *Proc.Natl.Acad.Sci.U.S.A* **92** 3673-3677.
- Wakitani S, Saito T & Caplan AI 1995 Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. *Muscle Nerve* **18** 1417-1426.
- Walker DG 1973 Osteopetrosis cured by temporary parabiosis. *Science* **180** 875.
- Walker DG 1975a Bone resorption restored in osteopetrotic mice by transplants of normal bone marrow and spleen cells. *Science* **190** 784-785.
- Walker DG 1975b Spleen cells transmit osteopetrosis in mice. *Science* **190** 785-787.
- Wan M, Yang C, Li J, Wu X, Yuan H, Ma H, He X, Nie S, Chang C & Cao X 2008 Parathyroid hormone signaling through low-density lipoprotein-related protein 6. *Genes Dev.* **22** 2968-2979.
- Wang N, Robaye B, Agrawal A, Skerry TM, Boeynaems JM & Gartland A 2012 Reduced bone turnover in mice lacking the P2Y(13) receptor of ADP. *Mol.Endocrinol.* **26** 142-152.
- Wang YH, Liu Y & Rowe DW 2007 Effects of transient PTH on early proliferation, apoptosis, and subsequent differentiation of osteoblast in primary osteoblast cultures. *Am.J.Physiol Endocrinol.Metab* **292** E594-E603.
- Weinstein RS 2012 Glucocorticoid-induced osteoporosis and osteonecrosis. *Endocrinol.Metab Clin.North Am.* **41** 595-611.
- Weinstein RS, Wan C, Liu Q, Wang Y, Almeida M, O'Brien CA, Thostenson J, Roberson PK, Boskey AL, Clemens TL & Manolagas SC 2010 Endogenous glucocorticoids decrease skeletal angiogenesis, vascularity, hydration, and strength in aged mice. *Aging Cell* **9** 147-161.
- Wennberg C, Hessel L, Lundberg P, Mauro S, Narisawa S, Lerner UH & Millan JL 2000 Functional characterization of osteoblasts and osteoclasts from alkaline phosphatase knockout mice. *J.Bone Miner.Res.* **15** 1879-1888.
- White K, Evans W, O'Riordan J, Speer M, Lorenz-Depiereux B, Grabowski M, Meitinger T & Strom T 2000 Autosomal dominant hypophosphataemic rickets is associated with mutations in FGF23. *Nature Genet.* **26** 345-348.
- Whyte MP 2010 Physiological role of alkaline phosphatase explored in hypophosphatasia. *Ann.N.Y.Acad.Sci.* **1192** 190-200.
- Whyte MP, Obrecht SE, Finnegan PM, Jones JL, Podgornik MN, McAlister WH & Mumm S 2002 Osteoprotegerin deficiency and juvenile Paget's disease. *N.Engl.J.Med.* **347** 175-184.

- Wijenayaka AR, Kogawa M, Lim HP, Bonewald LF, Findlay DM & Atkins GJ 2011 Sclerostin stimulates osteocyte support of osteoclast activity by a RANKL-dependent pathway. *PLoS One* **6** e25900.
- Williams BO & Insogna KL 2009 Where Wnts went: the exploding field of Lrp5 and Lrp6 signaling in bone. *J.Bone Miner.Res.* **24** 171-178.
- Winkler DG, Sutherland MK, Geoghegan JC, Yu C, Hayes T, Skonier JE, Shpektor D, Jonas M, Kovacevich BR, Staehling-Hampton K, Appleby M, Brunkow ME & Latham JA 2003 Osteocyte control of bone formation via sclerostin, a novel BMP antagonist. *EMBO J.* **22** 6267-6276.
- Winslow MM, Pan M, Starbuck M, Gallo EM, Deng L, Karsenty G & Crabtree GR 2006 Calcineurin/NFAT signaling in osteoblasts regulates bone mass. *Dev.Cell* **10** 771-782.
- Wu J, Nilsson A, Jonsson BA, Stenstad H, Agace W, Cheng Y & Duan RD 2006 Intestinal alkaline sphingomyelinase hydrolyses and inactivates platelet-activating factor by a phospholipase C activity. *Biochem.J.* **394** 299-308.
- Xiong J, Onal M, Jilka RL, Weinstein RS, Manolagas SC & O'Brien CA 2011 Matrix-embedded cells control osteoclast formation. *Nature Med.* **17** 1235-1241.
- Yadav MC, Simao AM, Narisawa S, Huesa C, McKee MD, Farquharson C & Millan JL 2011 Loss of skeletal mineralization by the simultaneous ablation of PHOSPHO1 and alkaline phosphatase function: a unified model of the mechanisms of initiation of skeletal calcification. *J.Bone Miner.Res.* **26** 286-297.
- Yadav VK, Ryu JH, Suda N, Tanaka KF, Gingrich JA, Schutz G, Glorieux FH, Chiang CY, Zajac JD, Insogna KL, Mann JJ, Hen R, Ducy P & Karsenty G 2008 Lrp5 controls bone formation by inhibiting serotonin synthesis in the duodenum. *Cell* **135** 825-837.
- Yamaguchi A, Ishizuya T, Kintou N, Wada Y, Katagiri T, Wozney JM, Rosen V & Yoshiki S 1996 Effects of BMP-2, BMP-4, and BMP-6 on osteoblastic differentiation of bone marrow-derived stromal cell lines, ST2 and MC3T3-G2/PA6. *Biochem.Biophys.Res.Comm.* **220** 366-371.
- Yamamoto K, Shimizu N, Obi S, Kumagaya S, Taketani Y, Kamiya A & Ando J 2007 Involvement of cell surface ATP synthase in flow-induced ATP release by vascular endothelial cells. *Am.J.Physiol Heart Circ.Physiol.* **293** H1646-H1653.
- Yan L, Burbiel JC, Maass A & Muller CE 2003 Adenosine receptor agonists: from basic medicinal chemistry to clinical development. *Expert.Opin.Emerg.Drugs* **8** 537-576.
- Yan SF, Ramasamy R & Schmidt AM 2008 Mechanisms of disease: advanced glycation end-products and their receptor in inflammation and diabetes complications. *Nature Clin.Pract.Endocrinol.Metab* **4** 285-293.

- Yanagita M, Oka M, Watabe T, Iguchi H, Niida A, Takahashi S, Akiyama T, Miyazono K, Yanagisawa M & Sakurai T 2004 USAG-1: a bone morphogenetic protein antagonist abundantly expressed in the kidney. *Biochem.Biophys.Res.Commun.* **316** 490-500.
- Yang W, Lu Y, Kalajzic I, Guo D, Harris MA, Gluhak-Heinrich J, Kotha S, Bonewald LF, Feng JQ, Rowe DW, Turner CH, Robling AG & Harris SE 2005a Dentin matrix protein 1 gene cis-regulation: use in osteocytes to characterize local responses to mechanical loading *in vitro* and *in vivo*. *J.Biol.Chem.* **280** 20680-20690.
- Yang X, Matsuda K, Bialek P, Jacquot S, Masuoka HC, Schinke T, Li L, Brancorsini S, Sassone-Corsi P, Townes TM, Hanauer A & Karsenty G 2004 ATF4 is a substrate of RSK2 and an essential regulator of osteoblast biology; implication for Coffin-Lowry Syndrome. *Cell* **117** 387-398.
- Yang Z, Day YJ, Toufektsian MC, Ramos SI, Marshall M, Wang XQ, French BA & Linden J 2005b Infarct-sparing effect of A2A-adenosine receptor activation is due primarily to its action on lymphocytes. *Circulation* **111** 2190-2197.
- Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N & Suda T 1998 Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc.Natl.Acad.Sci.U.S.A* **95** 3597-3602.
- Yegutkin GG 2008 Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. *Biochim.Biophys.Acta* **1783** 673-694.
- Yegutkin GG, Henttinen T & Jalkanen S 2001 Extracellular ATP formation on vascular endothelial cells is mediated by ecto-nucleotide kinase activities via phosphotransfer reactions. *FASEB J.* **15** 251-260.
- Yegutkin GG, Henttinen T, Samburski SS, Spychala J & Jalkanen S 2002 The evidence for two opposite, ATP-generating and ATP-consuming, extracellular pathways on endothelial and lymphoid cells. *Biochem.J.* **367** 121-128.
- Yoshida H, Hayashi S, Kunisada T, Ogawa M, Nishikawa S, Okamura H, Sudo T, Shultz LD & Nishikawa S 1990 The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. *Nature* **345** 442-444.
- Yoshida M, Muneyuki E & Hisabori T 2001 ATP synthase--a marvellous rotary engine of the cell. *Nature Rev.Mol.Cell Biol.* **2** 669-677.
- Yoshioka K, Saitoh O & Nakata H 2001 Heteromeric association creates a P2Y-like adenosine receptor. *Proc.Natl.Acad.Sci.U.S.A* **98** 7617-7622.

- Yoshitake H, Rittling SR, Denhardt DT & Noda M 1999 Osteopontin-deficient mice are resistant to ovariectomy-induced bone resorption. *Proc.Natl.Acad.Sci.U.S.A* **96** 8156-8160.
- Young JD, Yao SY, Baldwin JM, Cass CE & Baldwin SA 2013 The human concentrative and equilibrative nucleoside transporter families, SLC28 and SLC29. *Mol.Aspects Med.* **34** 529-547.
- Yu VW, Ambartsoumian G, Verlinden L, Moir JM, Prud'homme J, Gauthier C, Roughley PJ & St-Arnaud R 2005 FIAT represses ATF4-mediated transcription to regulate bone mass in transgenic mice. *J.Cell Biol.* **169** 591-601.
- Zambonin ZA, Teti A, Primavera MV & Pace G 1983 Mature osteocytes behaviour in a repletion period: the occurrence of osteoplastic activity. *Basic Appl.Histochem.* **27** 191-204.
- Zanini D, Schmatz R, Pimentel VC, Gutierrez JM, Maldonado PA, Thome GR, Cardoso AM, Stefanello N, Oliveira L, Chiesa J, Leal DB, Morsch VM & Schetinger MR 2012 Lung cancer alters the hydrolysis of nucleotides and nucleosides in platelets. *Biomed.Pharmacother.* **66** 40-45.
- Zeng X, Tamai K, Doble B, Li S, Huang H, Habas R, Okamura H, Woodgett J & He X 2005 A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. *Nature* **438** 873-877.
- Zetterstrom T, Vernet L, Ungerstedt U, Tossman U, Jonzon B & Fredholm BB 1982 Purine levels in the intact rat brain. Studies with an implanted perfused hollow fibre. *Neurosci.Lett.* **29** 111-115.
- Zhang K, Barragan-Adjemian C, Ye L, Kotha S, Dallas M, Lu Y, Zhao S, Harris M, Harris SE, Feng JQ & Bonewald LF 2006 E11/gp38 selective expression in osteocytes: regulation by mechanical strain and role in dendrite elongation. *Mol.Cell Biol.* **26** 4539-4552.
- Zhou HH, Chin CN, Wu M, Ni W, Quan S, Liu F, Dallas-Yang Q, Ellsworth K, Ho T, Zhang A, Natasha T, Li J, Chapman K, Strohl W, Li C, Wang IM, Berger J, An Z, Zhang BB & Jiang G 2009 Suppression of PC-1/ENPP-1 expression improves insulin sensitivity *in vitro* and *in vivo*. *Eur.J.Pharmacol.* **616** 346-352.
- Zhou Z, Immel D, Xi CX, Bierhaus A, Feng X, Mei L, Nawroth P, Stern DM & Xiong WC 2006 Regulation of osteoclast function and bone mass by RAGE. *J.Exp.Med.* **203** 1067-1080.
- Zhu D, Mackenzie NC, Millan JL, Farquharson C & MacRae VE 2011 The appearance and modulation of osteocyte marker expression during calcification of vascular smooth muscle cells. *PLoS One* **6** e19595.

- Zimring MB, Caldarella FA, White KE & Econs MJ 2005 Persistent tumor-induced osteomalacia confirmed by elevated postoperative levels of serum fibroblast growth factor-23 and 5-year follow-up of bone density changes. *Endocr.Pract.* **11** 108-114.
- Zimmermann H 2000 Extracellular metabolism of ATP and other nucleotides. *Naunyn Schmiedebergs Arch.Pharmacol.* **362** 299-309.
- Zimmermann H, Zebisch M & Strater N 2012 Cellular function and molecular structure of ecto-nucleotidases. *Purinergic Signal.* **8** 437-502.
- Zorn AM 2001 Wnt signalling: antagonistic Dickkopfs. *Curr.Biol.* **11** R592-R595.
- Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P & Hedrick MH 2002 Human adipose tissue is a source of multipotent stem cells. *Mol.Biol.Cell* **13** 4279-4295.
- Zylka MJ, Sowa NA, Taylor-Blake B, Twomey MA, Herrala A, Voikar V & Vihko P 2008 Prostatic acid phosphatase is an ectonucleotidase and suppresses pain by generating adenosine. *Neuron* **60** 111-122.

Appendix 1 – PCR primer sequences

| Rattus norvegicus gene | Sense 5' – 3' | Anti-sense 5' – 3' |
|--------------------------------|-----------------------|-------------------------|
| <i>A₁ receptor</i> | CTCCATTCTGGCTCTGCTCG | CTCCATTCTGGCTCTGCTCG |
| <i>A_{2A} receptor</i> | CCATGCTGGGCTGGAACA | GAAGGGGCAGTAACACGAACG |
| <i>A_{2B} receptor</i> | TGGCGCTGGAGCTGGTTA | GCAAAGGGGATGGCGAAG |
| <i>A₃ receptor</i> | AGAGCTAGGTCCACTGGC | GCACATGACAACCAGGGGGATGA |
| <i>β-actin</i> | GTTCCGCATGGATGACGAT | TCTGGGTCATCTTTTCACGG |
| <i>Sost</i> | CTCCTGAGAACAACCAGAC | TGGAGAACGCCTATAGAG |
| <i>DMP1</i> | AAGTCAAGCTAGCCGAGA | CGATGAGGACAATGATCTAG |
| <i>ADA</i> | TCCTGGCCAAGTTCGATTCA | AGCGAACTTCCACGTACACC |
| <i>Enpp1</i> | GTCAGTATGCGTGCTAAC | TGGCACACTGAACTGTAG |
| <i>ALP (TNAP)</i> | CTCATTGTGCCAGAGAA | GTTGTACGTCTTGGAGAG |
| <i>ANK</i> | AAGGCAGCCAGATACAGGAA | CATCACCAACATAGCCATGC |
| <i>NTPdase1</i> | AGATGAACAGCCCTGTGA | GGGTTCAATTTCTGGGTCT |
| <i>Ecto-5-nucleotidase</i> | CAGGAAATCCACCTTCCAAA | AACCTTCAGGTAGCCCAGGT |
| <i>OPG</i> | GCAACACATGACAATGTATG | CAAGCTCTCCATCAAGATGC |
| <i>RANKL</i> | CGAGCGCAGATCGATCCTAAC | GACTTTATGGGAACCCGATGG |

Table 2. The primer sequences used for RT-PCR analysis of rat mRNAs expression

| Mus musculus gene | Sense 5' – 3' | Anti-sense 5' – 3' |
|--------------------------------|----------------------|------------------------|
| <i>A₁ receptor</i> | CTACCTTCTGCTTCATCGTA | ACAAGACAGTGGTGACTCAG |
| <i>A_{2A} receptor</i> | CTATTGCCATCGACAGATAC | GAACAACACTGCAGTCAGAAAG |
| <i>A_{2B} receptor</i> | CCACCAACTACTTTCTGGTA | AACAGTAAAGACAGTGCCAC |
| <i>A₃ receptor</i> | TCATTGTCTCCCTAGCACT | GACARCRRRCACARCACCCG |
| <i>GAPDH</i> | CTCACTCAAGATTGTCAGCA | GTCATCATACTTGGCAGGTT |
| <i>Enpp1</i> | ACAGCTTAATCTGACCACAG | GATCCTGGTACAGACAGTTG |
| <i>Enpp2</i> | GTATGACCCTGTCTTTGATG | GAAAGCCACTGAAGGATAGT |
| <i>Enpp3</i> | CTGCTGACTGTGGTTTTACT | CTGTGGTAAAGGAGACAGTG |
| <i>NTPdase1</i> | CTTTGGCGCTTTGGATCTCG | TCTGGTGGCACTGTTCGTAG |
| <i>NTPdase2</i> | CTGGAGGCAGTGACACAGAC | TGGGTGGAGTAGCCCTTTGG |
| <i>NTPdase3</i> | GTGAGCATTGTGGTACTTGT | TGACCACTCCTGTGTTATTC |
| <i>ANK</i> | CAGTTTCCTGGTGGGATGTG | TTGATGTGGGCTGAGGTG |
| <i>ADA</i> | AAGCATTGGCATCAAGGTC | CATAGCCACCACGGTCTTCT |

Table 3. The primer sequences used for RT-PCR analysis of mouse mRNAs expression

GAPDH=glyceraldehyde-3-phosphate dehydrogenase.

Appendix 2 – Abbreviations

| | |
|--------------------------------|---|
| 2-Cado | 2-Chloroadenosine |
| A₁ receptor | Adenosine receptor A ₁ |
| A_{2A} receptor | Adenosine receptor A _{2B} |
| A_{2B} receptor | Adenosine receptor A _{2B} |
| A₃ receptor | Adenosine receptor A ₃ |
| Ab | Antibody |
| ABAM | Antibiotic – antimycotic |
| ABCC6 | ATP-binding cassette subfamily-C member 6 gene |
| ADA | Adenosine deaminase |
| ADA-SCID | ADA - severe combined immunodeficiency |
| ADHR | Autosomal dominant hypophosphatemic rickets |
| Ado | Adenosine |
| ADP | Adenosine diphosphate |
| Akp2 | Mouse tissue non-specific alkaline phosphatase gene |
| ATF4 | Activating transcription factor 4 |
| ALP | Alkaline phosphatase |
| α-MEM | α-modified essential medium supplemented with 10% foetal calf serum, 70 µg/ml gentamicin, 50 U/ml penicillin, 50 µg/ml streptomycin, 0.125 µg/ml amphotericin |

| | |
|--------------------|--|
| AMP | Adenosine monophosphate |
| Ank | Progressive ankylosis gene |
| ANOVA | Analysis of variance |
| Ap4A | Diadenosine 5',5'''P ¹ ,P ⁴ -tetrphosphate |
| APC | Adenomatous polyposis coli |
| APRT | Adenine phosphoribosyltransferase |
| Arg-Gly-Asp | Arginine – glycine – aspartic acid |
| ASARM | Acid serine and aspartic acid-rich |
| ATP | Adenosine triphosphate |
| BGP | β - glycerophosphate |
| BMP | Bone morphogenetic protein |
| BSA | Bovine serum albumin |
| BSP | Bone sialoprotein |
| Bz-ATP | 2'(3')-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate |
| CAN | Calcium activated nucleotidase |
| CCAAT | Cytidine-cytidine-adenosine-adenosine-thymidine |
| CD39 | Ecto-nucleoside triphosphate diphosphohydrolase (NTPdase) |
| CD73 | Ecto-5' nucleotidase (eN) |
| cAMP | Cyclic adenosine monophosphate |
| cDNA | Complimentary deoxyribonucleic acid |
| C/EBPα | CCAAT-enhancer-binding proteins |

| | |
|-----------------------|--|
| CK | Creatine kinase |
| CK1 | Casein kinase 1 |
| CK-BB | Creatine kinase brain type |
| CK-MB | Creatine kinase cardiac type |
| CK-MM | Creatine kinase muscle type |
| CO₂ | Carbon dioxide |
| DC-STAMP | Dendritic cell stimulatory transmembrane protein |
| DKK | Dickkopfs |
| DMEM | Dulbecco's modified essential medium supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin |
| DMP1 | Dentine matrix protein-1 |
| DNase | Deoxyribonuclease |
| DPCPX | P1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine |
| Dpi | Dots per inch |
| Dvl | Dishevelled |
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme linked immunosorbent assay |
| eN | Ecto-5'nucleotidase |
| ENT | Equilibrative nucleoside transporter |
| FCS | Foetal calf serum |

| | |
|----------------|---|
| FGF23 | Fibroblast growth factor 23 |
| FGFR | Fibroblast growth factor receptor |
| FIAT | Factor inhibiting activating transcription factor 4 |
| FoxO | Forkhead box O |
| FZD | Frizzled |
| GACI | Generalised arterial calcification of infancy |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GDP | Guanosine diphosphate |
| GMP | Guanosine monophosphate |
| GPI | Glycosylphosphatidylinositol |
| GSK3 | Glycogen synthase kinase 3 |
| GTP | Guanosine-5'-triphosphate |
| HB-GAM | Heparin binding growth associated molecule |
| HBSS | Hank's buffered salt solution |
| HGPRT | Hypoxanthine-guanine phosphoribosyltransferase |
| HIFs | Hypoxia inducible factors |
| IMP | Inosine monophosphate |
| I-Smads | Inhibitory Smads |
| JNK | C-Jun N-terminal kinases |
| LDH | Lactate dehydrogenase |
| LPA1-6 | Lysophosphatidic acid receptors 1 - 6 |

| | |
|------------------------|--|
| LRP5/6 | Low-density-lipoprotein receptor 5 / 6 |
| MC3T3 | Mouse osteoblast cell line |
| M-CSF | Macrophage colony-stimulating factor |
| MEM | Modified essential medium |
| MEPE | Matrix extracellular phosphoglycoprotein |
| MicroCT | Micro – computed tomography |
| MLO-Y4 | Mouse long bone osteocytes cell line Y4 |
| MMPs | matrix metalloproteinases |
| MrgA | Mas-related gene receptor A (adenine receptor) |
| mRNA | Messenger ribonucleic acid |
| MRP6 | Multi-drug resistant protein 6 |
| MSCs | Mesenchymal stem cells |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NAD⁺ | Nicotinamide adenine dinucleotide |
| NBF | Neutral buffered formalin |
| NCAM | Neural cell adhesion molecule |
| NDP | Nucleoside diphosphates |
| NDPK | Nucleoside diphosphate kinase |
| NECA | 5' -N- Ethylcarboxamidoadenosine |
| NFAT2 | Nuclear factor for activated T-cells 2 |
| NFκβ | Transcription factor nuclear factor κβ |

| | |
|--------------------------------|---|
| NMP | Nucleoside monophosphates |
| Np_{n-1} | Nucleoside 5'(n-1) polyphosphate (n = number of phosphates) |
| NPP | Ecto-nucleotide pyrophosphatase / phosphodiesterase |
| NPP1 | Ecto-nucleotide pyrophosphatase / phosphodiesterase - 1 |
| NTP | Nucleoside triphosphates |
| NTPdase | Ecto-nucleoside triphosphate diphosphohydrolase |
| OCN | Osteocalcin |
| OC-STAMP | Osteoclast stimulatory transmembrane protein |
| OPG | Osteoprotegerin |
| OPLL | Ossification of the posterior longitudinal ligament |
| OPN | Osteopontin |
| ORF-1 | Osteoblast stimulating facto-1 |
| Pi | Inorganic phosphate |
| PC-1 | Ecto-nucleotide pyrophosphatase / phosphodiesterase - 1 |
| PAP | Prostatic acid phosphatase |
| PBS | Phosphate buffered saline |
| PCP | Planar cell polarity pathway |
| PHOSPHO1 | Phosphatase orphan 1 |
| PPARγ | Peroxisome proliferator-activated receptors γ |
| PPi | Pyrophosphate |
| PPi/Pi ratio | Pyrophosphate / phosphate ratio |

| | |
|-------------------------------|--|
| PTH | Parathyroid hormone |
| QMUL | Queen Mary, University of London |
| RAGE | Receptor for advanced glycation end products |
| RANK | Receptor activator of nuclear factor $\kappa\beta$ |
| RANKL | Receptor activator of nuclear factor $\kappa\beta$ ligand |
| RGD | Arginine – glycine – aspartic acid |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| SEM | Scanning electron microscopy |
| SIBLING | Small integrin-binding ligand N-linked glycoprotein |
| <i>Sost</i> | Sclerostin gene |
| SOST | Sclerostin |
| Sox9 | SRY sex determining region Y box 9 |
| sRAGE | Receptor for advanced glycation end products inhibitor |
| sFZP | Secreted frizzled related proteins |
| Tcf/Lef | T-cell specific transcription factor / lymphoid enhancer-binding factor transcription factor |
| TGF-β | Transforming growth factor- β |
| TNAP | Tissue non-specific alkaline phosphatase |
| TNAP | Tissue non-specific alkaline phosphatase (human gene) |
| TNF | Tumour necrosis factor |
| TNFRSF11B | Tumour necrosis factor receptor super-family member 11b (OPG) |

| | |
|----------------|--|
| TNFSF11 | Tumour necrosis factor ligand super-family member 11 (RANKL) |
| TRAF | TNF receptor associated proteins |
| TRAP | tartrate resistant acid phosphatase |
| TRPV5 | Transient receptor potential, vanilloid, members 5 |
| TRPV6 | Transient receptor potential, vanilloid, members 6 |
| Ttw | Tiptoe walking mouse |
| UDP | Uridine diphosphate |
| UTP | Uridine triphosphate |
| VDREs | Vitamin D response elements |
| VEGF | Vascular endothelial growth factor |
| Vit D2 | Ergocalciferol |
| Vit D3 | Cholecalciferol |
| Wif-1 | WNT inhibitory factor-1 |
| WNT | <i>Wingless</i> type and <i>int-1</i> |
| w/v | Weight per volume |
| XLH | X-linked hypophosphatemic rickets |

Appendix 3 – Publications

The following is a list of publications produced during the course of this thesis.

Peer Reviewed Journal Articles

Hajjawi MOR, Boyde A, Huesa C, MacRae V, Millán JL, Arnett TR, Orriss IR (2014) Hyper mineralisation of collagen rich soft tissues and osteocyte lacunae in *Enpp1*^{-/-} mice. Submitted to *Bone* June 2014

Hajjawi MOR, Orriss IR, Arnett TR (2014) Lack of effect of adenosine on the function of rodent osteoblasts and osteoclasts *in vitro*. Submitted to *J. Endocrinol.* June 2014

Wornham DP, **Hajjawi MO**, Orriss IR, Arnett TR (2012) Strontium potently inhibits mineralisation in bone-forming primary rat osteoblast cultures and reduces osteoclastogenesis in mouse marrow. Accepted to *Osteoporos. Int.* June 2014

Davey T, Lanham-New SA, Shaw AM, Cobley R, Allsopp AJ, **Hajjawi MOR**, Arnett TR, Taylor P, Cooper C, Fallowfield JL (2014) Fundamental differences in axial and appendicular bone density and markers of bone resorption in stress fractures and uninjured royal marine recruits – a matched case control study. Submitted to *J. Bone Miner. Res.* April 2014

Orriss IR, Key ML, **Hajjawi MOR**, Arnett TR (2013) Extracellular ATP released by osteoblasts is a key local inhibitor of mineralisation. *PLoS One* 8 e69057

Syberg S, Brandao-Burch A, Patel JJ, **Hajjawi M**, Arnett TR, Schwarz P, Jorgensen NR, Orriss IR (2012) Clopidogrel (Plavix®), a P2Y₁₂ receptor antagonist, inhibits bone cell function *in vitro* and decreases trabecular bone *in vivo* *J. Bone Miner. Res.* 27 2373 - 2386

Xirouchakis E, Marelli L, Cholongitas E, Manousou P, Calvaruso V, Pleguezuelo M, Guerrini GP, Maimone S, Kerry A, **Hajjawi M**, Nair D, Thomas M, Patch D, Burroughs AK (2011) Comparison of cystatin C and creatinine-based glomerular filtration rate formulas with ⁵¹Cr-EDTA clearance in patients with cirrhosis. *Clin. J. Am. Soc. Nephrol.* 6: 84-92

Conference abstracts and other articles

Orriss I, **Hajjawi M**, Arnett T (2014) Activation of the P2Y₂ receptor enhances osteoclast function by stimulating the release of ATP, a pro-resorptive extracellular nucleotide. *Bone Abstracts* Vol 1, OC3.2

Hajjawi MOR, Huesa C, MacRae VE, Millan JL, Boyde A, Arnett TR, Orriss IR (2013) μ CT as tool to study cortical porosity and soft tissue calcification in *Enpp1* knockout mice. *Skyscan user meeting Hasselt Belgium* ISSN:2033-8031. 160 - 166

Orriss IR, **Hajjawi MOR**, Millan JL, Poulet B, Arnett TR (2013) μ CT as a tool for investigating structural changes in the knees of *Enpp1* knockout mice. *Skyscan user meeting Hasselt Belgium* ISSN:2033-8031. 228-231

Hajjawi MOR, MacRae VE, Huesa C, Millan JL, Poulet B, Boyde A, Arnett TR, Orriss IR (2013) Regulation of skeletal and soft tissue mineralisation by NPP1 (ecto-nucleotide pyrophosphatase / phosphodiesterase) *Bone Res. Soc. Abstracts* ISBN: 978-2-88919-174-1

Orriss IR, **Hajjawi MOR**, Millan JL, Arnett TR (2013) The inhibitory actions of ATP and UTP on bone mineralisation are partially mediated by the activity of NPP1. *Bone Res. Soc. Abstracts* ISBN: 978-2-88919-174-1

Orriss IR, Zhu D, Mackenzie NCW, **Hajjawi MOR**, Millan JL, Arnett TR, MacRae VE (2013) ATP and UTP are potent inhibitors of vascular calcification. *Bone Res. Soc. Abstracts* ISBN: 978-2-88919-174-1

Hajjawi MOR, MacRae VE, Huesa C, Millán JL, Arnett TR, Orriss IR (2013) Do ecto-nucleotidases play a role in the regulation of osteoclast function? *Bone abstracts Vol1* ISSN 2052 - 1219

Hajjawi MOR, MacRae VE, Huesa C, Millán JL, Poulet B, Arnett TR, Orriss IR (2013) Npp1 is a key regulator of skeletal and soft tissue mineralisation. *Bone abstracts Vol1* ISSN 2052 - 1219

Orriss IR, **Hajjawi MOR**, Huesa C, MacRae VE, Arnett TR (2013) Bone-forming cultures of rat and mouse calvarial osteoblasts: key differences in protocols *Bone abstracts Vol1* ISSN 2052 – 1219

Wornham DP, **Hajjawi MO**, Orriss IR, Arnett TR (2013) Strontium potently inhibits mineralisation in bone-forming osteoblast cultures while osteoclast formation from marrow mononuclear cells is moderately reduced. *Bone abstracts Vol1* ISSN 2052 - 1219

Orriss IR, **Hajjawi MOR**, Arnett TR (2013) Endogenous extracellular nucleotides are important autocrine/paracrine regulators of bone cell function. *Purinergic Signal.* 9 (4) 697-706

Hajjawi MOR, Boyde A, Millán JL, Arnett TR and IR Orriss (2013) Ecto-nucleotide pyrophosphate / phosphodiesterase-1 (*Enpp1*) gene deletion affects osteoclast gene expression and cortical bone porosity *Purinergic Signal.* 9 (4) 697-706

Davey T, Lanham-New SA, Allsopp AJ, **Hajjawi M**, Arnett T, Fallowfield JL (2012) Differences in bone resorption during Royal Marine training and in relation to stress fracture. *J. Bone Miner. Res.* (suppl.1)

Hajjawi MOR, Arnett TR, Orriss IR (2012) Ecto-nucleotide pyrophosphate / phosphodiesterase-1 is expressed by osteoclasts but does not affect osteoclast function. *Osteoporos. Int.* 23 (5) s535

Strontium directly inhibits mineral deposition in bone-forming primary osteoblast cultures (2012) Wornham DP, **Hajjawi MO**, Arnett TR. *Osteoporos. Int.* 23 (5) s537

Syberg S, Brandao-Burch A, Patel JJ, **Hajjawi M**, Arnett TR, Schwarz P, Jorgensen NR, Orriss IR (2012) Clopidogrel (Plavix®), a P2Y₁₂ receptor antagonist, inhibits bone cell function *in vitro* and decreases trabecular bone *in vivo*. *Bone* 50 s45

Hajjawi MOR, Orriss IR, Arnett TR (2011) Lack of effect of adenosine on rodent osteoblast and osteoclasts *Front. Endocrinol.* doi. 10.3389/conf.fendo.2011.02.00026

Hajjawi M, Arnett TR, Orriss IR (2011) Can μ CT be used as a tool to study *in vitro* bone formation? Skyscan user meeting Leuven Belgium ISSN:2033-8031. 227-229