Acidosis, a fundamental regulator of bone cell function

Michelle Laura Key

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Department of Cell and Developmental Biology

University College London

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Declaration of Authorship

I, Michelle Laura Key, declare that this thesis titled ‘Acidosis, a fundamental regulator of bone cell function’ and the work presented in it are my own. I confirm that:

- This work was done wholly while in candidature for a research degree at this university.
- No part of this thesis has previously been submitted for a degree or any other qualification at this university or any other institution.
- Where I have consulted the published work of others, this is always clearly attributed.
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- With the exception of such quotations, this thesis is entirely my own work.
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Abstract

Previous work has demonstrated that bone cells are highly sensitive to extracellular acidification. Low pH is a key osteoclast activation factor, and reduces bone formation by inhibiting mineralisation of the matrix. The aim of this thesis was to investigate further the roles played by pH in modulating bone cell function. Transient receptor potential (TRP) channels detect protons within the pathophysiological range, so are potential candidates for mediating the observed effects of acid on bone cells. I found that TRPV1 agonists/antagonists had no effect on osteoclast formation and activation or bone mineralisation by osteoblasts. Additionally, *TRPV1*$^{-/-}$ osteoclasts demonstrated no functional differences from wildtypes. Furthermore, agonists/antagonists for TRPM8, TRPV4 and TRPV3 were without effect. Pyrophosphate (PPi) a fundamental inhibitor of mineralisation in the bone microenvironment is generated from extracellular nucleotides by ecto-nucleotidases such as E-NPP1. I showed that E-NPP1 mRNA and protein were up-regulated in osteoblasts cultured at pH 6.9; total E-NPP activity was also increased. To determine the role of acidification in tumour-induced osteolysis, I devised an *in vitro* model to investigate interactions of osteoclasts with breast cancer cells, which metastasise to bone, sometimes causing osteolytic disease. Surprisingly, the overall effect of breast cancer cells on osteoclasts was inhibitory; however, resorption was significantly stimulated relative to buffered controls when breast cancer cells were allowed to acidify the culture medium. These results suggest that local acidification in tumour settings *in vivo* could be an important factor in determining the degree of osteolysis that occurs. Finally, I investigated whether two key cytokines implicated in the vicious cycle model of tumour-mediated osteolysis, B-cell activating factor (BAFF) and activin A influence osteoclast function. In all experiments only acidosis stimulated osteoclast resorption. These results provide further evidence for the fundamental role of acid as a regulator of bone cell function.

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Chapter 1

Introduction

The skeletal system

The skeletal system is a dynamic living tissue comprising bone and cartilage. The skeleton has numerous important roles in the body, including: (i) it provides a rigid framework of support for the body; (ii) it protects the major internal organs from damage; and (iii) it acts as a large reservoir of ions to support both calcium and phosphate homeostasis (Dempster 2006). Bone is one of the most highly vascularised tissues in the human body (Ortega *et al.* 2004).

Anatomy

The bones that comprise the skeletal system can be classified into two groups: long bones (i.e., femur, tibia and radius); and flat bones such as the skull and scapula. Long bones contain a hollow central shaft known as the diaphysis, which contains the bone marrow and is the site of haematopoiesis. There is a membranous sheath covering the inner surface of the bone called the endosteum. It contains blood vessels, osteoblasts and osteoclasts and is in direct contact with the bone marrow. On the outer surface of the long bones is the periosteum, comprising fibrous connective tissue. It contains blood vessels, nerve endings and both osteoblasts and osteoclasts. The periosteum is attached to the bone by collagenous fibres known as Sharpey's fibres. The periosteum covers all bone surfaces except at joints where articular cartilage is found. The ends of the long bones are formed of the metaphyses below the growth plate and the epiphysis above the growth plate. The metaphyses and epiphysis are mainly cancellous bone covered in cortical bone, whereas the long bones are primarily cortical bone. Approximately 80% (by weight) of the adult skeleton is cortical bone and the remaining 20% is cancellous. Cortical bone is dense and solid, whereas cancellous bone has a structure akin to

honeycomb. Cancellous bone is the more metabolically active of the two types (Dempster 2006).

Development of bone

Bone is formed in two different ways, either by intramembranous ossification or by endochondral ossification.

Intramembranous ossification

Intramembranous ossification is the process by which mesenchymal tissue is converted directly into bone, and is how flat bones and the jaw is generated. Neural-crest-derived mesenchymal cells proliferate and condense; some of these cells become osteoblasts, the bone forming cells. The bone morphogenetic proteins BMP2, BMP4 and BMP7 are thought to instruct these mesenchymal cells to express runt-related transcription factor 2 (RUNX2) (also known as core-binding factor alpha 1 (*Cbfa1*), osteoblast specific factor 2 (*Osf2*) and acute-myeloid-leukaemia protein 3 (*Aml3*)) which results in osteoblastic differentiation as outlined below (Ducy *et al.* 1997).

Endochondral ossification

Most of the skeleton is formed by endochondral ossification. Initially cartilage is generated from mesenchymal cells. The avascular cartilage framework is then gradually replaced by bone (Ortega *et al.* 2004). Initially mesenchymal cells commit to becoming cartilage cells as a result of mesodermal cells expressing *Pax1* and *Scleraxis* transcription factors which activate cartilage-specific genes. These cells then condense and differentiate into chondrocytes, the cartilage forming cells, which coincides with expression of the DNA-binding protein SOX9, a known chondrogenic transcription factor (Cserjesi *et al.* 1995; Wright *et al.* 1995). The chondrocytes then proliferate into a model for bone; at this stage, the chondrocytes stop dividing but increase their volume. These are known as hypertrophic chondrocytes. The conversion to bone is also dependent on expression of vascular endothelial growth factor (VEGF). Increased

expression of VEGF also coincides with up-regulation of collagen type X and matrix metalloproteinase 13 (MMP 13). Endochondral ossification happens at two distinct sites within the long bones, the primary site of which is diaphyseal (shaft of the long bone), and the secondary site is epiphyseal (end of a long bone). The secondary site is under independent control and is ossified after the primary site. Ossification at the secondary site is dependent upon signalling from Indian hedgehog (IHH), bone morphogenetic proteins (BMPs) and fibroblast growth factor 18 (FGF 18). This results in 'resting' chondrocytes moving into an area where chondrocytes are proliferating. These chondrocytes then undergo hypertrophy and apoptosis. The extracellular matrix (ECM) that surrounds the terminally differentiated chondrocytes, comprising type X collagen, is calcified and partially degraded by MMP13 expressed by the chondrocytes themselves as well as by invading osteoclasts (Behonick *et al.* 2007). This allows for invasion of blood vessels. Following this, osteogenic progenitors are recruited to the area and deposit trabecular bone. The area between the hypertrophic chondrocytes and bone marrow where the bone is initially formed is known as the ossification front (Ortega *et al.* 2004). MMPs are key molecules that have been implicated in the degradation of extracellular matrix molecules. MMPs are structurally related zinc-dependent proteases; during endochondral ossification, MMP 10 and MMP 13 (a collagenase) are particularly important. MMP 13 expression is controlled by RUNX2 (Ortega *et al.* 2004; Mak *et al.* 2009).

Osteoblasts

There are three main cell types in bone, of which osteoblasts are responsible for bone formation. They are derived from mesenchymal cell precursors. This is the lineage that also produces adipocytes, chondrocytes, myoblasts, fibroblasts and tendon cells. Activation of different transcription factors gives rise to the different cell types listed. Both *Runx2* and osterix (*Osx*) have been identified as important transcription factors in the control of differentiation of osteoblasts. RUNX2 is expressed throughout bone formation (Byers *et al.* 2002). *Runx2*

mRNA first appears at embryonic day 9.5 in mice, coinciding with formation of mesenchymal condensations that precede branching of chondrogenic and osteogenic lineages (Franceschi *et al.* 2009). *Runx2*-null mice have a cartilaginous skeleton, although the cartilage is not hypertrophic and additionally there are neither osteoblasts present in the skeleton, nor any evidence of alkaline phosphatase activity as a marker of bone formation (Otto *et al.* 1997). Furthermore, there is no bony collar development, no vascular invasion and no marrow formation, all of which provide evidence for the importance of RUNX2 in skeletal development and osteoblast differentiation (Harada & Rodan 2003; Franceschi *et al.* 2009). In *Runx2*$^{-/-}$ mice, chondrocyte maturation is delayed, an observation which is consistent with RUNX2 being expressed in hypertrophic chondrocytes. Mice heterozygous for *Runx2* have a defect in intramembranous ossification, a phenotype that closely resembles the human condition cleidocranial dysplasia, which is a condition in which the clavicles are underdeveloped or missing, there is a wide pubic symphysis and sufferers are short in stature (Otto *et al.* 1997). Transgenic expression of *Runx2* in chondrocytes resulted in ectopic chondrocyte hypertrophy and endochondral ossification (Harada & Rodan 2003). Together, these observations have demonstrated that RUNX2 has a role in the control of differentiation of osteoblasts and chondrocytes and that there must be other factors downstream of *Runx2* that act upon osteoblasts alone. Expression of *Runx2* is controlled by the down-regulation of expression of the helix-loop-helix protein TWIST, a negative regulator of osteoblast maturation. *Twist* haploinsufficiency causes premature cranial suture fusion, the result of premature osteoblast maturation (Marie 2008).

Additionally, the zinc finger transcription factor osterix (OSX) was discovered; its expression has been induced in myoblasts in response to bone morphogenetic protein (BMP) (Harada & Rodan 2003; Marie 2008). *Osx*-null mice have a normally patterned skeleton of cartilage but no bone formation occurs. Mesenchymal cells, osteoclasts and blood vessels invade the cartilage matrix but there are no osteoblasts present to lay down mineralised bone

matrix. Osterix has been shown to direct pre-osteoblasts to become immature osteoblasts and, therefore, without it, cells are unable to differentiate into osteoblasts (Marie 2008). *Osx*$^{-}$/$^{-}$ cells do express *Runx2,* but *Runx2*$^{-}$/$^{-}$ mice do not express *Osx*, demonstrating that OSX functions downstream of RUNX2 (Nakashima *et al.* 2002).

Terminal osteoblast differentiation is demonstrated by depositions of an organised collagenous matrix which is subsequently mineralised and requires activating transcription factor 4 (ATF4), also known as cAMP-response element-binding protein (CREB2) (Matsuguchi *et al.* 2009). *Atf4*-null mice at embryonic day 13 (E13) show no differences in skeletal elements, with wildtype animals indicating that *Atf4* is not important in the earlier stages of skeletal development. However, by E14 and E16 *Atf4* knockouts have less mineralised tissue compared to wildtype. Specifically, the delay in differentiation of osteoblasts resulted in absent trabeculae in the frontal bones of the skull in the *Atf*-null mice. By E16, trabeculae were present only rarely in the skulls of *Atf4*-null mice; when present they were short and thin in contrast to wildtype animals where trabeculae were long, thick and numerous. Many *Atf4*$^{-}$/$^{-}$ die perinatally, possibly because the skull is much thinner in these animals and thus the brain is poorly protected during birth. The same delay in bone formation was observed in the rest of the skeleton (Yang *et al.* 2004). Bone sialoprotein (*Bsp*) is a molecular marker of differentiated osteoblasts and it was absent in *Atf4* knockouts at E15 and E16 when normally present. Osteocalcin (*Ocn*) is the last marker of differentiated osteoblasts to appear; it was decreased in both Atf4-null mice at E16 and shortly after birth when compared to wildtypes. Transcription factors expressed earlier in osteoblast differentiation (i.e. *Runx2* and *Osx*) were unaffected by the absence of *Atf4*, which provides evidence that *Atf4* acts downstream of both *Runx2* and *Osx* (Yang *et al.* 2004). ATF4 acts with Runx2 to control transcriptional activity of osteocalcin (Marie 2008). ATF4 has also been shown to be important for bone mass acquisition post-natally. Osteocalcin expression was decreased 2-fold in *Atf*-deficient mice, demonstrating lack of terminal differentiation of osteoblasts. As a consequence mice never gain a

normal bone mass. This is the result of decreased amino acid import by ATF4 which results in deficient synthesis of type I collagen (Yang *et al.* 2004).

Two other transcription factors, distal-less homeobox 5 (*Dlx5*) and MSH homeobox homologue 2 (*Msx2*), are important in the early stages of osteoblast differentiation. Functional analysis of the pair *in vitro* has revealed a reciprocal relationship between the two whereby *Dlx5* functions as an activator of transcription and *Msx2* a repressor. Mutations of these genes in both humans and mice have demonstrated that they are essential for normal intramembranous ossification (Samee *et al.* 2008).

Distal-less homeobox 5 (DLX5) is a homeobox protein and an activator of *Runx2* that has been shown to also function as a regulator of bone formation. Analysis of *Dlx5*-deficient osteoblasts *in vitro* has demonstrated a decrease in both osteoblast proliferation and differentiation. Furthermore, decreases in expression of the following were observed: *Runx2, Osx, Ocn* and *Bsp*, demonstrating that DLX5 functions upstream of these. Additionally, in co-cultures containing osteoclast precursor cells and *Dlx5*-null osteoblasts, there was a significant increase in osteoclast number and resorption. The RANKL/OPG ratio was also increased. These results indicate that as well as inducing expression of Runx2 directly, DLX5 has a role in coupling osteoblast–osteoclast activity and therefore functions as a regulator of bone turnover (Samee *et al.* 2008).

Another member of this family of transcription factors, *Dlx3*, is expressed at all stages of osteoblast differentiation, and is highest in mature osteoblasts. *Dlx3* has both positive and negative effects on *Ocn* expression. *Msx1* and *Msx2* are also homeodomain proteins that function as transcription factors. In both *Msx1*$^{-/-}$ and *Msx2*$^{-/-}$ mice, there is a delay in formation of the cranial bones. Double knockouts of both of these genes result in severely inhibited cranial bone formation. *Msx2* is expressed most highly in osteoprogenitor cells and then down-regulated as differentiation progresses, inactivation of its expression causes delay in skull ossification associated with decreased *Runx2* expression

(Marie 2008). The exact role *Msx2* in osteoblast differentiation is unknown, with some studies showing both suppression of differentiation of preosteoblasts and others showing promotion of differentiation and/or proliferation of osteoblasts (Komori 2006). *In vitro*, *Msx2* promotes osteoblastic differentiation and proliferation from mesenchymal stem cells but inhibits *Runx2* activity and osteoblastic gene expression in mature osteoblasts. *Msx2* also controls osteoblast apoptosis *in vitro*, in contrast however, *in vivo Msx2* is a positive regulator of osteoblasts differentiation (Marie 2008). More work is needed to elucidate the precise roles of the genes *in vivo*.

Wnt signalling

Wnt (Wingless and Int-1) signalling is important in many processes during both development and throughout life. Canonical Wnt signalling involves binding of one of the Wnt molecules to a frizzled (Fz) receptor on the cell surface along with a co-receptor low density lipoprotein-5 (LRP5) or LRP6 (Piters *et al.* 2008). Binding of Wnts to the Fz/LRP5/6 complex activates the cytoplasmic protein Dishevelled (Dvl) which subsequently phosphorylates glycogen synthase kinase 3b (GSK3b) and renders it inactive. This allows for stabilisation of β-catenin followed by translocation to the nucleus where it activates target genes (Johnson *et al.* 2004). If Wnt signalling is prevented, β-catenin is phosphorylated by GSK3b following its association with the β-catenin destruction complex comprising GSK3b, axin and adenomatous polyposis coli (APC). β-catenin is therefore marked for degradation, and ultimately not translocated to the nucleus and does not activate gene transcription (Johnson *et al.* 2004; Yavropoulou & Yovos 2007). Wnt signalling can be regulated by binding of the secreted regulator Dickkopf (Dkk) to LRP5/6 which then binds to the transmembrane protein Kremen. The tertiary complex is internalised, making LRP5/6 unavailable at the cell surface and so Wnt signalling is prevented (Yavropoulou & Yovos 2007; Piters *et al.* 2008).

Wnt signalling is important during bone development, where Wnts enhance expression of *Runx2* (Komori 2006). Later on in life, Wnt signalling

plays an important role in the regulation of bone mass via the action of sclerostin (Moester *et al.* 2010) and this is discussed later in this chapter.

Bone matrix

Once osteoblasts are terminally differentiated they begin to produce and secrete proteins that comprise the extracellular matrix (ECM), the organic portion of the bone (Xiao *et al.* 2007). This organic scaffold provides bone with its strength, while also providing elasticity and flexibility. The proportions of mineral, organic material and water are critical to ensure the correct balance between stiffness and flexibility; in adult human cortical bone, the proportions are 60% mineral, 20% organic material and 20% water (Crockett *et al.* 2011). The major constituent of the ECM is collagen, comprising approximately 80% of the organic content of bone. Non-collagenous protein is also found in the ECM. These proteins comprise between 10 and 15% of the total protein found in bone.

Collagen

Of the collagen found in bone, around 95% is type 1. Other collagens present at much lower levels are types III and V; these types are thought to have a role in collagen fibril diameter regulation (Tavassoli & Yoffey 1983; Robey & Boskey 2006). Type 1 collagen is a fibrillar collagen comprising three α-polypeptide chains arranged into a triple helix. Two of the chains are genetically and structurally identical and are encoded by the gene COL1A1. These are designated α1(I) chains. The third chain is genetically different COL1A2, but structurally similar, designated the α2(I) chain (Prockop & Kivirikko 1995; Robey & Boskey 2006). The polypeptide chain is made of a repetitive sequence of amino acids, glycine-X-Y, where X can be any amino acid, although it is usually proline, and Y is usually hydroxyproline. The polypeptide chains are also subject to the post-translational modifications: hydroxylation of lysyl residues and glycosylation of lysyl or hydroxlysyl residues. This composition allows the three chains to interweave tightly together and enables the formation of both

intra- and inter-molecular covalent cross links (Robey & Boskey 2006). These helices are then arranged into fibrils, which are positioned according to their role. In type I collagen, fibrils have a diameter of between 50 and 200nm, and adjacent collagen molecules are 67nm apart from one another, displaced by approximately a quarter of their length. In bone, collagen is arranged in concentric weaves (Prockop 1995; Lodish 2000; Viguet-Carrin 2006). Prolyl hydroxylases require vitamin C as a co-factor, without it hydroxylation does not occur. This results in failure of helix formation at normal body temperature and, consequently, fibrils are also not formed. Non-hydroxylated procollagen chains are degraded, which results in the disease scurvy (Lodish 2000).

Non-collagenous proteins

There are several different classes of non-collagenous proteins made and secreted by bone cells, each of which performs a different role. Some of the specific roles of some of these individual proteins are still undefined. Many are thought to be involved in the control of hydroxyapatite crystal propagation. A list of non-collagenous proteins and their specific roles can be found in Table 1.1.

Protein	Role
Albumin	inhibits hydroxyapatite growth
α2-HS glycoprotein	promotes endocytosis; opsonic properties; chemo attractant for monocytic cells; bovine analogue (fetuin) a growth factor
Aggrecan	matrix organisation; retention of water and ions; resilience to mechanical forces
Versican	‘capture’ space destined to become bone
Decorin	binds to collagen; regulates fibril diameter; binds to TGF-β; may modulate activity; inhibits cell attachment to fibronectin
Biglycan	binds to collagen; may bind to TGF-β; pericellular environment; genetic determinant of peak bone mass
Asporin / Periodontal ligament associated	involved in chondrogensis; negatively regulates mineralisation of periodontal ligament cells by down-

protein-1 (PLAP-1)	regulating BMP-2
Fibromodulin	binds to collagen and regulates fibril formation; binds to TGF-β
Osteoadherin/osteomodulin	may mediate cell attachment
Lumican	binds to collagen; may regulate fibril formation
Osteoglycin/mimecan	Binds to TGF-β
Hyaluronan	May work with versican molecule to 'capture' space destined to become bone
Alkaline phosphatase	hydrolyses inhibitors of mineral deposition; potential Ca^{2+} carrier
Osteonectin/SPARC	regulates collagen fibrillogenesis; binds to growth factors; may influence cell cycle; positive regulator of bone formation
Tetranectin	binds to plasminogen; may regulate matrix mineralisation
Tenascin-C	interferes with cell-FN interactions
Tenascin-W	may be involved in regulating osteogenesis
Osteopontin	binds to cells; inhibits mineralisation in bone and soft tissues; inhibits remodelling; involved in tumourogenesis; inhibits nitric oxide synthase; may regulate resistance to viral infection
Bone sialoprotein	binds to cells; initiates mineralisation *in vitro*
DMP-1	*in vitro* regulator of mineralisation
MEPE	possible regulator of phosphate metabolism
Dentin sialophosphoprotein (DSPP)	possible role in osteogenesis
Thrombospondins	cell attachment; binds to: heparin, platelets, collagen (types 1 and 5), thrombin, fibrinogen, laminin, plasminogen, plasminogen activator inhibitor and histidine rich glycoprotein
Fibronectin	binds to: cells, fibrin heparin, gelatine and collagen
Vitronectin	cell attachment protein; binds to: collagen, plasminogen, plasminogen activator inhibitor and heparin
Fibrillin 1 and 2	regulates elastic fibre formation

Matrix Gla Protein (MGP)	negative regulator of mineralisation
Osteocalcin (OCN)	regulates activity of osteoclasts and precursors; may mark turning point between bone formation and resorption
Protein S	primarily a liver product; may be made by osteogenic cells

Table 1.1 *Non-collagenous proteins found in the matrix and their roles*

Adapted from (Robey & Boskey 2006). Additional information from (Roach 1994; Qin *et al.* 2002; Yamada *et al.* 2007)

Mineralisation of the extracellular matrix

Matrix mineralisation is a tightly controlled process, which is regulated *in vivo* by the ratio of phosphate (Pi) to pyrophosphate (PPi). Phosphate is required for mineralisation because it is a component of hydroxyapatite and pyrophosphate is a potent inhibitor of the process. Tissue-nonspecific alkaline phosphatase (TNAP) is the primary enzyme within the ECM that hydrolyses PPi; PPi itself is generated from extracellular nucleotides by the action of ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (E-NPP1). PPi is also channelled into the extracellular space from within cells via progressive ankylosis (ANK) protein (Yadav *et al.* 2011). During endochondral bone formation, mineralisation begins in membrane bound matrix vesicles; once they have budded from osteoblasts and are located within the matrix; formation of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) is promoted within the lumen of the vesicles (Anderson 1995; Anderson 2003; Thouverey *et al.* 2009). Matrix vesicles were initially identified in the calcifying zone of the mouse bone epiphyseal plate where the vesicles were closely associated with needle-like structures identified to be hydroxyapatite and were deduced to be required to initiate hydroxyapatite nucleation (Anderson 1969). The matrix vesicles are full of phosphatases to provide Pi for hydroxyapatite and to remove pyrophosphate, which inhibits mineralisation (Fleisch *et al.* 1966; Ali *et al.* 1970). Matrix vesicles provide a site for Ca^{2+} and PO_4^{3-} accumulation which are both required to initiate mineralisation and are key components of hydroxyapatite (i.e. the mineral component of bone). Once crystal formation has

started, crystals will eventually propagate so much as to perforate the matrix vesicle membrane, which causes the hydroxyapatite to be exposed to the extracellular fluid; mineralisation of the organic component of bone is then governed by levels of Ca^{2+} and/or PO_4^{3-} in the extracellular fluid, the pH and presence of non-collagenous proteins (see Table 1.1) which control the rate of crystal propagation. Mineral will continue to grow until the matrix is full (Anderson 1995; Thouverey *et al.* 2009; Yadav *et al.* 2011).

Matrix mineralisation in mature bone differs, however, insofar that grooves within the collagen provide nucleation sites. Crystals begin to form with a specific orientation at these sites, where large amounts of calcium and phosphate ions have been electrostatically attracted by phosphoproteins within the collagen matrix (Mann 1988).

Osteocytes

Osteocytes account for approximately 90% of the cells in bone (Noble 2008; Dallas & Bonewald 2010). Osteocytes start life as osteoblasts, but those that eventually become embedded within the bone matrix are osteocytes. As the osteoblast becomes embedded, changes to the cell occur that result in its classification as an osteocyte. Osteocytes form a network of cytoplasmic dendritic processes known as canaliculi (Noble 2008).

Once osteoblasts have completed the bone formation phase of their lives, there are several stages through which they can proceed: 1) they become embedded in bone as osteocytes, this is thought to account for between 10 and 20% of osteoblasts; 2) they become bone lining cells (inactive osteoblasts); or 3) they undergo apoptosis (Noble 2008; Dallas & Bonewald 2010).

Current opinion states that ostocytogenesis is an active rather than a simply passive process in which osteoblasts are ‘buried alive’. The change from osteoblast to osteocyte involves substantial changes to the cell, specifically the formation of dendrites. This process involves the cell becoming polarised and dendrites first extend towards the mineralising front of bone and then toward the

vascular space or the bone surface. Once the cell is embedded, this polarity is maintained. Formation of dendrites is thought to require collagen cleavage and that of other matrix proteins; mice that are homozygous for metalloproteinase 14 (*Mmp 14*), a collagenase, have less and shorter dendritic processes than wildtype (Dallas & Bonewald 2010). MMP 2 is thought to have a role in formation of the canalicular system. *Mmp 2* null animals have reduced numbers of canaliculi (Noble 2008).

As expected, owing to the significant changes in cell morphology that result from the transition from osteoblast to osteocyte, the actin-associated proteins fimbrin, filamin and α-actinin translocate to within the dendritic processes. It is proposed that these proteins are required to maintain the unique shape of the osteocyte (Kamioka *et al.* 2004). More recent work has demonstrated that osteocytes are enriched with proteins associated with cytoskeletal function (Dallas & Bonewald 2010).

A key marker of osteocytes is dentine matrix protein 1 (DMP-1), which in bone is predominantly expressed by osteocytes but also at much lower levels by osteoblasts. Cells null for DMP-1 do not differentiate into mature osteocytes. Furthermore, *Dmp-1* knock-out mice demonstrate pathological changes in their osteocyte lacunocanalicular system. DMP-1 controls maturation by down-regulating the osteoblast specific genes osterix and type I collagen and by up-regulating expression of the osteocyte specific gene sclerostin (*Sost)* (Lu *et al.* 2011). Osteocytes inhibit bone formation via the action of SOST. SOST binds to low-density lipoprotein (LDL) receptor-related protein 5 (LRP5) and LRP6 which inhibit the Wnt signalling pathway, thus β-catenin does not promote expression of *Runx2* to stimulate osteoblastogenesis (Marie 2008; Piters *et al.* 2008; Krause *et al.* 2008).

Osteoclasts

Osteoclasts are derived from haematopoietic stem cells in the bone marrow. This is the same lineage that gives rise to all the cells of blood. Osteoclasts are

multinucleated cells that form from the fusion of mononuclear progenitors of, more specifically, the monocyte/macrophage lineage (Teitelbaum 2000). The first transcription factor necessary for osteoclast formation is PU.1 which is essential for macrophage maturation. Nuclear factor of activated T cells, cytoplasmic 1 (NFATc1) is also required to induce osteoclast-specific genes (Crockett *et al.* 2011). NFATc1 has been described as the master regulator of osteoclast differentiation (Nakashima & Takayanagi 2008). Subsequently, M-CSF is required for the proliferation and survival of these macrophages. In order for osteoclasts to form *in vivo*, osteoblasts or their progenitor cells or monocytes, granulocytes, endothelial cells, fibroblasts or dendritic cells are required, as they produce two factors that are essential for osteoclast formation; these are macrophage colony-stimulating factor (M-CSF) and receptor for activation of nuclear factor kappa B (NF-κB) (RANK) ligand (RANKL) (Teitelbaum 2000). M-CSF is required initially for differentiation of cells, and RANKL is required subsequently. (RANKL is also known as osteoprotegerin ligand (OPGL), tumour necrosis factor (TNF) ligand super family member 11 (TNFSF11), TNF-related activation-induced cytokine (TRANCE) and osteoclast differentiation factor). Mice lacking either of these two factors do not form osteoclasts (Teitelbaum 2000). Differentiation *in vivo* then depends upon expression of RANKL in addition to C-FOS, encoded for by the gene *Fos*; this commits the precursors to the osteoclastic lineage (Teitelbaum 2000). In haematopoietic precursors deficient in *Fos*, osteoclasts are not produced and these knockout animals develop osteopetrosis (Grigoriadis *et al.* 1994; Matsuo *et al.* 2000). The NF-κB transcription complex is needed for differentiation into osteoclasts (Teitelbaum 2000).

A number of other factors are thought to have a role in monocyte fusion to produce multinucleated osteoclasts (Oursler 2010) these include: E-cadherin, the V-ATPase V0 subunit d2 and, the protein: a disintegrin and metalloproteinase (ADAM) 8 and 12. ADAMS cleave extracellular parts of transmembrane proteins that are involved in cell–cell and cell–matrix interactions (Oursler 2010).

More recently, is has been proposed that osteoclast fusion is driven by a specific protein, namely dendritic cell specific transmembrane protein (DC-STAMP) (Iwasaki *et al.* 2008), which was identified by microarray on RAW 264.7 cells (Oursler 2010), DC-STAMP has also been demonstrated on primary human monocytes that give rise to osteoclasts. Blocking DC-STAMP with an antibody blocked formation of osteoclasts (Chiu *et al.* 2011). Expression of DC-STAMP by osteoclast precursors is rapid following RANKL application. siRNA knockdown of DC-STAMP resulted in suppression of osteoclast fusion. DC-STAMP also directly induces expression of tartrate resistant acid phosphatase (TRAP) (Kukita *et al.* 2004). A separate protein that has a c-terminal structure to DC-STAMP, osteoclastic stimulatory transmembrane protein (OC-STAMP), has been shown to be up-regulated following administration of RANKL (Verron *et al.* 2010), and this may have a role in driving osteoclast fusion.

A number of P2 receptors, have been implicated in osteoclast formation, ATP has been shown to cause increased osteoclast formation as well as activity (Morrison *et al.* 1998), additionally ADP signalling via the $P2Y_1$ receptor, causes significant increases in osteoclast activation (Hoebertz *et al.* 2001). A role for the purinergic receptor $P2X_7$ has been proposed. When primary osteoclasts were cultured with an antibody blocking the $P2X_7$ receptor, multinucleated osteoclasts were prevented from forming; and conversely, over expression of the $P2X_7$ receptor resulted in spontaneous fusion of osteoclast precursors. It has been proposed that one means by which ATP exits cells is via $P2X_7$ which forms a pore in the cell membrane. Once released into the extracellular environment, ATP is hydrolysed to adenosine, AMP or ADP. Adenosine has been proposed to facilitate osteoclast fusion (Pellegatti *et al.* 2011) Therefore, blocking the $P2X_7$ receptor prevents ATP release from cells, adenosine is thus not generated by hydrolysis of ATP and fusion is inhibited; if $P2X_7$ is over-expressed, more ATP leaves the cell and more adenosine is generated, thereby driving an increase in fusion, so that more osteoclasts are formed (Pellegatti *et al.* 2011). Also, removal of extracellular adenosine prevented fusion and addition of exogenous adenosine resulted in increased fusion (Pellegatti *et al.*

2011) however, these data could not be confirmed in our laboratory (Morrison *et al.* 1998; Hajjawi *et al.* 2011). $P2X_7$ antagonists have been shown to inhibit human osteoclast precursor fusion, and the authors note that mononuclear cells appear to aggregate and are thus following a normal path to fusion but are then unable to fuse. (Agrawal *et al.* 2010)

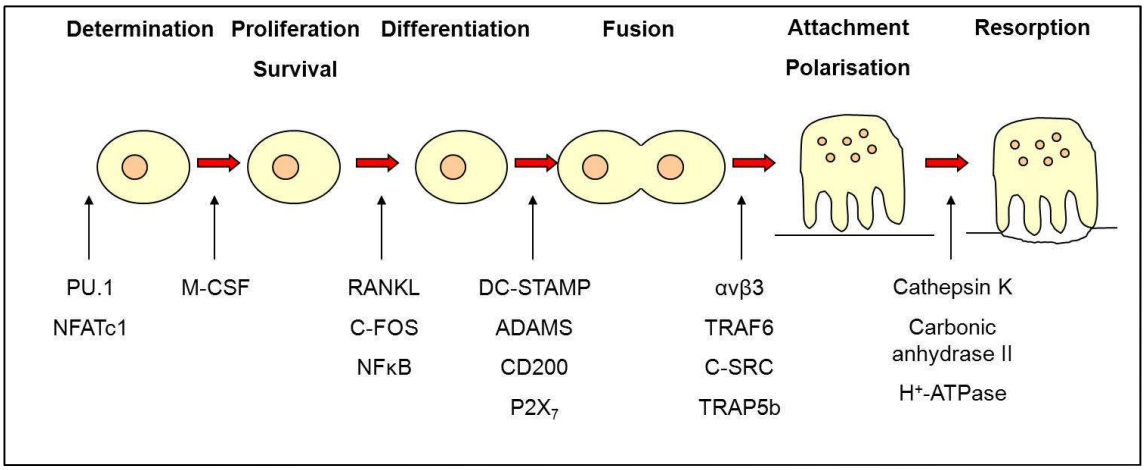


Figure 1.1 *Proteins important in osteoclast development and function*

Overview of proteins necessary for each stage of osteoclast development and/or activity are indicated below the black arrows. For details of the abbreviations see Appendix II.

Osteoclastic resorption and bone remodelling

Bone remodelling is the process whereby mature bone is removed by osteoclasts in a process called resorption. This area is then filled with newly made bone by the osteoblasts (Crockett *et al.* 2011). Osteoblasts and osteoclasts work in coordination with each other at discrete locations in the (Andersen *et al.* 2009).

Osteoclasts seal tightly to the bone surface, where they become polarised. Recognition of the bone surface by the osteoclast involves integrins, in particular integrin β1, which recognises type I collagen and integrin αvβ3 which is the major attachment molecule; it recognises the Arg-Gly-Asp amino acid motif which is present in osteopontin and bone sialoprotein. Without αvβ3 actin rings will not form and the ruffled border is abnormal (Teitelbaum 2000;

Udagawa 2003). The αvβ3 integrin comprises part of the podosomes, which are the equivalent of focal adhesions in other cells. Their role is to form a stable structure between the extracellular matrix, cytoskeletal and signalling molecules of the osteoclast. Podosomes are found associated with the actin ring and comprise an actin core surrounded by αvβ3 and other cytoskeletal proteins (Teti *et al.* 1991).

Once sealed to the bone surface, osteoclasts become polarised, which requires expression of TNF receptor activating factor 6 (TRAF-6) (Zaidi *et al.* 2003). The rearrangement of the osteoclast's cytoskelton is dependent on the actions of the Rho family of GTPases. Following attachment to the cell surface, Rho and Rac bind GTP and translocate to the cytoskelton. Rho signalling mediates formation of the actin ring the role of which occurs primarily in cell attachment. Rac, however, is responsible for cytoskeletal changes necessary to allow osteoclast migration (Faccio *et al.* 2005). TRAP5b, which is commonly used as a marker of osteoclasts, is thought to modulate osteoclast attachment to the bone surface by dephosphorylating the matrix proteins bone sialoprotein and osteopontin (Ek-Rylander *et al.* 1994). Osteoclasts form a 'ruffled border' which involves formation of infolds of the plasma membrane, a process which is dependent on cellular-src (C-SRC) (Edwards *et al.* 2006). TRAP is targeted to the ruffled border but how this initial targeting occurs here is not known (Crockett *et al.* 2011). Surrounding the ruffled border is an area known as the sealing zone which is contained within an actin ring. Appearance of the ring indicates that the osteoclast is resorbing the bone at this point and is not motile (Teitelbaum 2000). The sealing zone allows the osteoclasts to create a micro environment between it and the bone matrix beneath it, while protecting the surrounding area from exposure to the drop in pH. This area is acidified to approximately pH 4.5 via the actions of an H^+-ATPase. This ATPase locates to the ruffled border upon polarisation and associates with the actin ring via its N-terminal domain (Zaidi *et al.* 2003). The chloride-proton antiporter ClC7 works in conjunction with the ATPase, to transport Cl^- into the resorption lacuna, necessary to maintain electoneutraility (Kornak *et al.* 2001; Crockett *et al.*

2011). The intra-osteoclastic pH is maintained via an energy-independent Cl^- /HCO_3^- exchanger at the anti-resorptive surface of the osteoclast (Teitelbaum 2000). The protons that are required to decrease the pH are generated by the actions of carbonic anhydrase II which hydrates CO_2 and yields H_2CO_3 which then spontaneously dissociates, generating protons (Vaananen & Laitala-Leinonen 2008). The drop in pH within the sealing zone results in dissolution of the mineral component of bone and leaves the organic phase exposed to the lysosomal enzyme cathepsin K and matrix metalloproteinases which are exocytosed into the sealing zone from the osteoclast (Teitelbaum & Ross 2003) Products of the bone degradation, namely collagen fragments, calcium and phosphate ions, are then endocytosed by the osteoclast and transported to the anti-resorptive surface where they are released and eventually reach the blood stream (Teitelbaum 2000; Crockett *et al.* 2011).

Transcription of cathepsin K occurs in response to stimulation of the osteoclast by RANKL. Cathepsin K is a protease which is synthesised as a pre-proenzyme and is secreted by osteoclasts into the resorption lacuna once the osteoclast has sealed onto the bone surface. The low pH results in activation of cathepsin K. Cathepsin K is able to cleave triple helix collagens as well as osteopontin, thereby degrading the organic portion of the extracellular bone matrix (Zhao *et al.* 2009). It is thought that TRAP may also have a role in further proteolytic degradation of the collagen fragments (Crockett *et al.* 2011).

Regulation of bone remodelling

Bone remodelling is a process that is tightly controlled in healthy individuals to ensure that appropriate amounts of bone are removed and replaced with new bone. One of the most important regulators of osteoclast activity is the osteoblast, in a process known as coupling (Andersen *et al.* 2009). The process must be regulated, as osteoclasts are able to remove 2–4 times their own volume in one day (Vignery & Baron 1980).

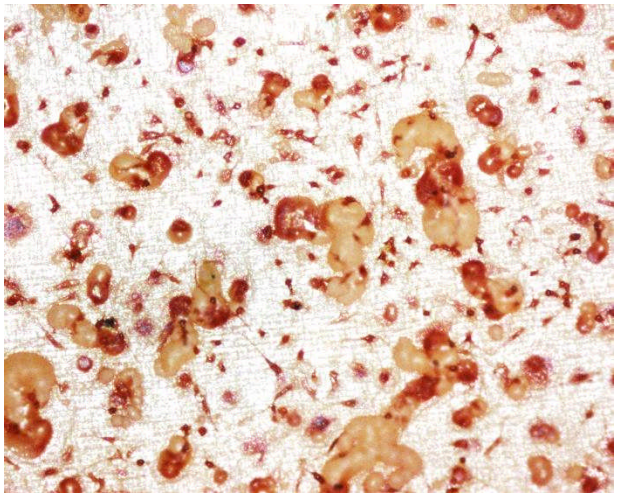


Figure 1.2 *Resorption pits created by acid-activated human peripheral blood-derived osteoclasts over a 48 hour period (in the presence of RANKL)*

(osteoclasts – red, resorption pits – orange)

Osteoprotegerin (OPG)

One of the most important endogenous factors that prevent osteoclast formation and activity is OPG. OPG is produced by both osteoblasts and stromal cells. It is a soluble decoy receptor to RANKL and prevents binding of RANKL to the RANK receptor on osteoclast precursor cells and osteoclasts by out competing it. This prevents new osteoclasts from forming and as RANKL is required for osteoclast survival it negatively affects osteoclasts that are already formed, therefore down-regulating bone resorption (Crockett *et al.* 2011; Kohli & Kohli 2011).

There are numerous other factors important in the regulation of bone remodelling; some of the key factors are discussed briefly below.

Mechanical loading

One of the most fundamental regulators of bone turnover is mechanical strain. Increasing strain results in increased bone formation by osteoblasts and a decrease in osteoclastic resorption and therefore increased bone mass. Unloading has the opposite effect, a decrease in strain requires less mechanical strength and thus less bone mass (Harada & Rodan 2003). How mechanical strain is detected and then transduced to changes in transcription is complex. The osteocyte is now widely regarded as the mechano-sensor in bone. When load is applied to any cell it will deform the cell membrane, in bone cells this is

detected by the stretch-activated cation channel (SA-CAT). This results in an increase in cytosolic Ca^{2+}, from internal stores and influx via the voltage-sensitive calcium channel (VSCC). Increased calcium causes initiation of various kinase cascades, these ultimately lead to up-regulation of *c-fos* which in turn up-regulates expression of *Runx2* (Ziros *et al.* 2002); Pavalko et al., 2003a). There is increasing evidence that dentin matrix protein 1 (DMP1) expressed by osteocytes is able to act as a mechano-sensor; *Dmp1* mRNA is up-regulated in osteocytes *in vivo* in response to mechanical loading (Gluhak-Heinrich *et al.* 2003; Harris *et al.* 2007). Fluid shear stress also affects bone cells, causing release of prostaglandin E2 (Jiang & Cheng 2001; Pavalko *et al.* 2003), which is anabolic. The overall effect of the detection of mechanical stimulation is the up-regulation of osteoblastic genes and the down-regulation of osteoclastic genes, which therefore results in increased bone formation and decreased bone resorption (Marie 2009).

Oxygen / hypoxia

Bone is one of the most vascular tissues in the body (Ortega *et al.* 2004), with the skeleton receiving approximately 10% of the cardiac output (Vakaet & Boterberg 2004) and as such is well oxygenated. There are certain situations when this oxygen supply to the bone is disrupted and oxygen tension drops. Oxygen tension in arterial blood is approximately 95mmHg (12%) and in venous and capillary blood is around 40mmHg (5%). In normal tissue the interstitial pO_2 is between 24 and 66mmHg (three and 9%) (Vaupel *et al.* 1989; Lewis *et al.* 1999). In rabbits, pO_2 within a haematoma, 4 days post fracture, was measured to be 0.8% (Brighton & Krebs 1972). Hypoxia is not only associated with fractures but also with inflammation, tissue damage and tumours (Thomlinson 1977; Murdoch *et al.* 2005). It has been demonstrated that hypoxia has a strong inhibitory effect on osteoblast function, at levels of extreme hypoxia, (0.2% O_2) bone nodule formation *in vitro* was reduced by more than ten times. Hypoxia down-regulates alkaline phosphatase activity and appearance of osteocalcin was also delayed (Utting *et al.* 2006; Lechler *et al.* 2011). Additionally the three

procollagen lysine, 2-oxoglutarate, 5-dioxygenases (PLOD1-3) were down-regulated in response to hypoxia. These enzymes are needed for formation of hydroxylysine residues (Utting *et al.* 2006), therefore, collagen production is reduced. Hypoxia has also been shown to cause ATP release from osteoblasts (Orriss *et al.* 2009). Discussed later in this chapter, ATP has an inhibitory effect on bone formation and this may therefore contribute to this effect. These effects demonstrate that osteoblast function and bone formation is a process highly dependent on proper oxygenation (Utting *et al.* 2006).

Hypoxia also has profound effects on osteoclast function, hypoxia causes an increase in number of osteoclasts formed, they are additionally of greater size, with increased numbers of nuclei and have greater resorptive activity (Arnett *et al.* 2003; Utting *et al.* 2010). Exposure to hypoxia also results in stabilisation of the hypoxia inducible factors (HIF) 1α and 2α, and up-regulation of vascular endothelial growth factor (VEGF) and interleukin 6 (IL 6). VEGF has been shown to stimulate osteoclast formation and IL 6 has also been reported to be osteoclastogenic. The HIF subunits are proteasomaly degraded under normoxic conditions but hypoxia results in their stabilisation and they allow the transactivation of genes involved in angiogenesis and metabolic adaptation (Knowles *et al.* 2010; Utting *et al.* 2010). This adaptation to hypoxia may be important when bone is damaged because it would stimulate removal of damaged bone before the fracture is repaired by generation of new bone by osteoblasts, for example. This additionally demonstrates the importance of the vasculature supply in normal bone turnover.

Temperature

Core body temperature is known to fall as we age, with young healthy adults typically having a core body temperature of 36.9˚C, falling to 35.5˚C in 10% of people aged 65 (Howell 1948; Fox *et al.* 1973). Temperature is known to have an effect on cell function, typically slowing down enzyme reaction rates, cell proliferation and differentiation by slowing progression through the cell cycle. Osteoblasts are negatively affected by exposure to cooler temperatures.

Exposure to mild hypothermia (35.5ºC) and severe hypothermia (34ºC) reduced bone formation *in vitro* by 75 and 95% respectively. Additionally, *Runx2*, alkaline phosphatase, type 1 collagen and osteocalcin were all down-regulated in osteoblasts cultured in hypothermic conditions (Patel *et al.* 2009). Hypothermic conditions have reciprocal effects on osteoclast formation and activity, whereby formation and resorption by osteoclasts were both increased by between 1.5- and 2-fold respectively (Patel *et al.* 2009). The reciprocal effects of hypothermia seen on osteoclasts are similar to the reciprocal effects caused by hypoxia and acidosis, whereby the overall effect on bone formation is negative. Exactly why decreases in temperature may also have this effect are not currently known but this clearly has significant implications to bone health in the elderly.

Hyperthermia (40ºC) has been shown to decrease osteoblast proliferation and bone nodule formation by 75%; furthermore, ATP release from osteoblasts was also significantly increased (Orriss 2005). As outlined later, ATP has a negative effect on bone formation and this may contribute to the decrease in bone formation. Additionally, it has been reported that procollagen denatures at 37.9ºC (Rosenbloom *et al.* 1973); without stable collagen, normal bone formation will not be able to take place. The effect of severe hyperthermia (40ºC) on human osteoclasts is also striking: osteoclast number was decreased by 4 times and resorption per osteoclast was decreased by 10 times (Utting *et al.* 2005). The effects of hyperthermia are overall bone formation being reduced and osteoclastic bone removal being also decreased (Patel *et al.* 2009). Temperatures of 40ºC may occur in bone as a consequence of infection and/or inflammation (Utting *et al.* 2005).

Oestrogen

Oestrogen is a steroid hormone derived from cholesterol which is initially converted into androgens including testosterone and androstendione and then into the oestrogens oestrone and oestradiol via the action of an aromatase. Oestrogen has profound effects on bone turnover. Increases in levels of

oestrogen in girls at menarche are associated with a large reduction in bone turnover markers. At this stage of life, the effects of oestrogen are biphasic, low doses accelerate growth rates, but at higher doses growth is inhibited and fusion of the epiphyses is stimulated. Oestrogen is required for the closure of the growth plate, therefore higher levels of oestrogen in girls compared to boys of the same age account for women eventually having shorter bones than men as the growth plate is closed more quickly. The effect of oestrogen on closure of the growth plate is also more potent than that of testosterone in men (Eastell 2005). At menopause, the decreases in oestrogen levels result in increased levels of bone turnover (Parfitt 1991). The mechanism by which oestrogen works involve it binding to the oestrogen receptor on osteoblasts, which results in increased production of OPG and decreases in production of M-CSF. Oestrogen also causes decreased secretion of interleukin 1 (IL-1) and TNFα by monocytes; additionally, oestrogen acting via oestrogen receptor α (ERα) results in up-regulation of Fas ligand (FasL), binding of which to its receptor induces apoptosis. Combined, these effects result in decreased osteoclast production, activity and survival (Eastell 2005; Jeong & Choi 2011). Decreases in the level of oestrogen in women at the menopause therefore result in loss of this mechanism of osteoclast regulation and as such, and bone remodelling is altered in favour of bone destruction.

Parathyroid hormone (PTH)

There are four parathyroid glands in humans, located behind the thyroid gland. The majority of each gland comprises chief cells and it is these that detect calcium ion levels. It is also from these that PTH is secreted. Ca^{2+} is detected via a calcium sensing GPCR, decreased Ca^{2+} results in decreased phospholipase C and increased adenylate cyclase activity, cyclic adenosine monophosphate (cAMP) generation results in PTH secretion (Nussey & Whitehead 2001). PTH is synthesised as a preprohormone, the amino terminal sequence is the only region of the polypeptide absolutely required for mineral homeostasis, as such this segment, PTH(1-84), is highly conserved in all

vertebrate species. The principle physiological role of PTH is to generate release of calcium from the bones in order to maintain plasma calcium levels, and a lack of PTH results in hypocalcaemia. Decrease of calcium ion concentration from normal levels results in PTH secretion within minutes, persistent decreases in calcium ion levels can cause hypertrophy of the glands, in some cases up to five fold (Potts 2005).

The actions of PTH are mediated via its receptor, the GPCR PTHR1. PTH acts at the kidney where it promotes formation of $1,25(OH)_2D_3$ in order to promote intestinal calcium absorption as outlined previously. Additionally, PTH regulates calcium and phosphate transport in the kidneys. Dietary lack of phosphate is therefore uncommon; PTH promotes its excretion simply by preventing its reuptake. When calcium levels are lacking, PTH promotes calcium reabsorption in the distal tubules of the kidneys in order to prevent excretion (Agus *et al.* 1973; Potts 2005).

PTH also exerts direct actions on bone cells. PTH, acting on osteoblasts causes increased expression of RANKL and decreased expression of OPG, therefore resulting in increased osteoclast formation (Lee & Lorenzo 1999). Primary human osteoclasts express the PTHR1 and addition of PTH(1-34) to osteoclasts *in vitro*, caused a 2–3-fold increase in resorption, demonstrating that PTH has direct effects on osteoclasts (Dempster *et al.* 2005). This demonstrates that PTH has dual effects on osteoclasts via interacting with them directly and via the action of PTH on osteoblasts; whereby increased resorption ultimately increases calcium release from the bones.

Parathyroid hormone related protein (PTHrp)

PTHrp is derived from a different gene to PTH, although they are thought to be evolutionary related. PTHrp is produced by many tissues in the body and exists in three different isoforms that are generated by alternative splicing. PTHrp functions in a paracrine manner and exerts its effect via PTHR1, the same receptor as PTH. The role of PTHrp is thought to be more important during

development, where, along with Indian hedgehog (*Ihh*) and other morphogenetic proteins, it functions to delay differentiation of chondrocytes in the growth plate of developing long bones (Gardella & Juppner 2001). The role of PTHrp in the adult is not entirely clear, although it may have a role in Ca^{2+} regulation between maternal and foetal circulations and regulate calcium ion concentration in breast milk as (Nussey & Whitehead 2001) PTHrp has been shown in induce osteoclast formation *in vitro* (Akatsu *et al.* 1989).

Prostaglandin E_2 (PGE_2)

Prostaglandins are a class of eicosonoid hormones which are derived from arachidonic acid. There are at least sixteen different prostaglandins split into nine chemical classes designated PGA-PGI. Prostaglandins are continuously made and secreted by many cell types but are often broken down rapidly by enzymes in the extracellular fluid; as a consequence, they mostly function in an autocrine or paracrine manner (Lodish *et al.* 2000).

PGE_2 is the most widely produced of the prostaglandins and is also the most important in terms of skeletal biology. The physiological roles of PGE_2 are difficult to elucidate as *in vivo* PGE_2 has a strongly osteogenic and anabolic effect (Yao *et al.* 1999). *In vitro*, PGE_2 induces osteoclast formation (Collins & Chambers 1992) and stimulates osteoclasts to resorb (Klein & Raisz 1970).

Transforming growth factor beta (TGFβ)

Transforming growth factor beta is a family of related growth factors that account for a substantial portion of intercellular signalling. Members of the family regulate cell proliferation, lineage determination, differentiation, motility, adhesion and cell death. Family members have significant roles in development, homeostasis and repair of almost all tissues. Both activins and bone morphogenetic proteins (BMPs) are members of the TGFβ super family (Kingsley 1994). TGFβ regulate gene expression via activation of a serine/threonine kinase that activates the SMAD signalling pathway (Massague 1998). TGFβ is highly abundant within the ECM of bone, although here it is

present in its latent form; upon bone resorption it is released into the extracellular environment where it becomes active (Juarez & Guise 2011).

Three TGFβ isoforms have been identified in humans, designated TGFβ 1—3. All three isoforms are crucial for bone development; knockout mice for each of the isoforms demonstrated substantial defects in normal bone development. Changes in temporal expression of the isoforms control bone remodelling. TGFβ1 stimulates bone formation by increasing migration of osteoblast progenitors and stimulating proliferation (Janssens *et al.* 2005); later, TGFβ blocks osteoblast differentiation and prevents mineralisation (Alliston *et al.* 2001). It does, however, also prevent apoptosis of osteoblasts in order to allow for some to transdifferentiate into osteocytes (Juarez & Guise 2011). The role of TGFβ in osteoclast formation and function is complex and depends on cellular context. In mouse foetal long bones for example, TGFβ prevents osteoclastic resorption. It has been demonstrated that TGFβ can induce OPG production by osteoblasts which therefore leads to decreased availability of RANKL and so osteoclastogenesis is decreased (Takai *et al.* 1998). In contrast, however, in the presence of M-CSF and RANKL, but absence of stromal cells or osteoblasts, TGFβ increased osteoclast formation. This suggests that TGFβ might mediate its effects via acting on osteoclast precursors. Despite conflicting results from *in vitro* experiments, *in vivo* TGFβ inhibitors consistently show that TGFβ stimulates both osteoclast formation and resorption (Mohammad *et al.* 2009; Juarez & Guise 2011).

Bone morphogenetic proteins (BMPs) are important members of the TGFβ superfamily, initially named as early members of this sub family were able to induce bone formation. However, they have many and diverse roles including regulation of cell division, apoptosis, cell migration and differentiation (Dimitriou & Giannoudis 2005).

Fibroblast growth factors (FGFs)

Fibroblast growth factors are a group of signalling factors that have an important role in skeletal biology. Some members are expressed almost all of the time and others are restricted temporally and spatially. There is a certain amount of redundancy between some of the FGFs, but others have very specific roles; there are upwards of twenty family members so far identified (Ornitz & Marie 2002). There are three FGF receptors (FGFR), designated FGFR1–3, and binding of different ligands to these receptors activate different intracellular signalling pathways to ultimately alter transcription of target genes (Dailey *et al.* 2005).

In bone development, the FGF receptors are particularly important. Mutations in FGFR3 result in dwarfism, which occurs as a result of abnormal endochondral ossification (Rousseau *et al.* 1994; Shiang *et al.* 1994). Mutations in FGFR1 and 2 mostly effect intramembranous ossification and as a consequence result in abnormal craniofacial development, including premature closure of the skull sutures (Dailey *et al.* 2005). FGFs themselves are also important for normal bone development; *Fgf2*$^{-/-}$ mice had inhibited bone formation (Montero *et al.* 2000) and *Fgf18*$^{-/-}$ mice demonstrated delayed suture closure and an expanded growth plate (Liu *et al.* 2002; Ohbayashi *et al.* 2002).

Insulin-like growth factors (IGFs)

Bone is rich in growth factors including IGF-1 and -2, both of which enhance production of type I collagen by osteoblasts. Both mRNA expression and protein production of IGF-1 and -2 are increased in response to PTH (McCarthy *et al.* 1989; Giustina *et al.* 2008). IGF is also important during bone development because it stimulates growth during endochondral ossification (Giustina *et al.* 2008).

Vascular endothelial growth factor (VEGF)

VEGF is essential for bone formation as vascular invasion is needed for endochondral bone formation and fracture healing. VEGF promotes differentiation of osteoblasts and mineralisation of the new bone (Carlevaro *et al.* 2000; Keramaris *et al.* 2008).

Tumour necrosis factor (TNF)

TNFα is a cytokine that mediates a wide variety of cellular functions. Levels of serum TNFα increase with age and have a negative effect on bone formation. TNFα prevents deposition of the ECM (Lam *et al.* 2000). TNFα causes osteoblast apoptosis; this may occur via generation of reactive oxygen species (ROS) which go on to antagonise the anabolic effects of WNT signalling by suppressing of RUNX2, osterix and bone morphogenetic protein (BMP) induced osteoblast differentiation (Almeida *et al.* 2011).

TNFα has been shown to stimulate both osteoclast formation and resorption (Pfeilschifter *et al.* 1989; Yamashita *et al.* 2007). In order to stimulate formation, there must be RANKL present, although this is only required at 1% of the amount usually required to induce osteoclast formation. RANKL is a member of the TNF superfamily (Wong *et al.* 1997). When TNFα and RANKL are added to *in vitro* osteoclast precursors together, it results in a marked increase in translocation of NFκB to the nucleus. Increases in resorption may be a result of TNFα causing increased MMP synthesis (Lam *et al.* 2000).

TNFβ, also known as lymphotoxin, is homologous to TNFα in terms of activities that they carry out and receptors that they bind too, however, they have limited sequence homology and TNFβ acts only in a paracrine manner, as much smaller amounts are produced (Ruddle 1992). TNFβ has been reported to stimulate resorption by osteoclasts (Garrett *et al.* 1987).

Interleukins

Interleukins are a group of cytokines that mediate a wide variety of cellular responses. Although produced by many cell types, interleukins are mainly produced by immune cells and are critical in the proper functioning of the immune system. As osteoclasts are derived from the same lineage as immune cells, this may explain why interleukins have a role in osteoclast function. Several interleukins are of particular significance in bone, Interleukin-1 (IL-1) is able to stimulate osteoclast formation by enhancing expression of RANKL in stromal cells and therefore stimulating osteoclast precursors to differentiate (Wei *et al.* 2005). Interleukin-6 (IL-6) has also been shown to stimulate osteoclast differentiation via a RANKL independent mechanism, and stimulate osteoclastic resorption (Kurihara *et al.* 1990; Tamura *et al.* 1993; de la Mata *et al.* 1995; Kudo *et al.* 2003). Interleukin-8 (IL-8) has been reported to stimulate both osteoclastogenesis and osteoclastic resorption (Bendre *et al.* 2003). Interleukin-11 (IL-11) has a negative effect on bone volume overall as it both inhibits bone formation by down-regulating alkaline phosphatase expression (Hughes & Howells 1993) and it also stimulates osteoclast formation in a RANKL independent mechanism (Kudo *et al.* 2003).

Sclerostin

Sclerostin (SOST) is the product of *Sost* gene, the loss of which results in two rare, closely related disorders characterised by an increase in bone mass, sclerosteosis and van Buchem disease. SOST acts as an *in vivo* endogenous negative regulator of bone mass. SOST is expressed by osteocytes, and only appears late in differentiation, presumably once osteocytes have become buried within the matrix (Yavropoulou & Yovos 2007). SOST inhibits bone formation by inhibiting Wnt signalling via preventing signalling through LRP5/6, therefore, β catenin is degraded and downstream genes that initiate osteoblast differentiation are not transcribed (Piters *et al.* 2008). It has recently been proposed that BMP7 stimulates *Sost* expression, though BMP7 has been used as an anabolic treatment for long bone non-union fracture so the exact

mechanism by which BMP7 elicits its effects is not fully understood (Kamiya *et al.* 2011).

Nucleotides

Nucleotides mediate a wide variety of biological functions, which includes the activity of bone cells. Nucleotides mediate their effects via P2 receptors. These can be further subdivided into P2X receptors, which are ligand-gated ion channels, and P2Y receptors, which are GPCRs. ATP has been shown to stimulate osteoclast formation and increase resorptive activity (Morrison *et al.* 1998; Orriss *et al.* 2010). Like osteoblasts, nucleotides are detected through P2 receptors which have been reported on osteoclasts (Bowler *et al.* 1995; Naemsch *et al.* 1999; Hoebertz *et al.* 2000; Buckley *et al.* 2002; Ke *et al.* 2003; Gartland *et al.* 2003; Korcok *et al.* 2005). It has been suggested that ATP mediates this effect by signalling through the $P2Y_1$ receptor on osteoclasts, however, this may not be the case as a study has shown that ATP causes this effect indirectly by up-regulating RANKL expression by osteoblasts and thus cause increased osteoclast formation (Buckley *et al.* 2002). More recently UDP has been shown to signal through the $P2Y_6$ receptor to stimulate both osteoclast formation and resorption (Orriss *et al.* 2011). The ADP receptor $P2Y_{13}$ has also been shown to signal via in osteoclasts (Wang *et al.* 2012).

The effect of nucleotides on osteocytes is not currently known as their location within the ECM makes them difficult to study (Orriss *et al.* 2010).

Osteoblasts, have been reported to express several P2X and P2Y receptors (Hoebertz *et al.* 2000). Both adenosine triphosphate (ATP) and uridine triphosphate (UTP) inhibit bone nodule formation *in vitro* by preventing mineralisation of the ECM. The effects of ATP and UTP are mediated via a dual inhibitory mechanism; ATP or UTP acting via $P2Y_2$ results in decreased expression of alkaline phosphatase mRNA, thus, there is less alkaline phosphatase enzyme produced and so hydrolysis of the mineralisation inhibitor PPi is decreased and mineralisation is impeded (Orriss *et al*. 2007).

Unlike most of the rest of the body, bone, teeth and mineralised cartilage are the only tissues where deposition of calcium phosphate in the form of hydroxyapatite is intended. The high concentration of calcium within the body and its high affinity for inorganic phosphate leading to calcium phosphate crystal deposition have led to strategies being developed to prevent ectopic mineralisation in most of the body (Terkeltaub 2006; Kornak 2011). The most important organic inhibitors of this deposition are oxalate and citrate, which function by chelating calcium ions. A number of proteins also inhibit calcium phosphate crystal formation, mainly in the extracellular space, these include: fetuin-A, matrix-Gla-protein, osteopontin and osteocalcin (Kornak 2011). ATP, along with other nucleotides, is hydrolysed to generate the corresponding nucleotide monophosphate and PPi via the actions of the ectonucleotide phosphodiesterase/pyrophosphatase 1 (E-NPP1) (Orriss *et al.* 2007)

Pyrophosphate (PPi) is a potent inhibitor of mineralisation, and the ratio of phosphate (Pi) to pyrophosphate in bone microenvironment is a fundamental regulator of bone mineralisation (Felix & Fleisch 1976; Kornak 2011). Both Pi and PPi can be generated from extracellular nucleotide triphosphates (NTPs) by the actions of ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs) (Stefan *et al.* 2005; Orriss *et al.* 2007) and, ecto-nucleoside triphosphate diphosphohydrolases (E-NTPdases), ecto-5'-nucleotidases and alkaline phosphatases (Yegutkin 2008). Within the bone microenvironment, tissue-nonspecific alkaline phosphatase (TNAP) is the most important enzyme responsible for phosphate generation. In addition to PPi preventing mineralisation, *in vitro*, ATP has been shown to be a potent inhibitor of mineralisation (Orriss *et al.* 2007). Figure 1.3 illustrates the decreased mineralisation by primary rat osteoblasts cultured *in vitro* in the presence of PPi or ATP.

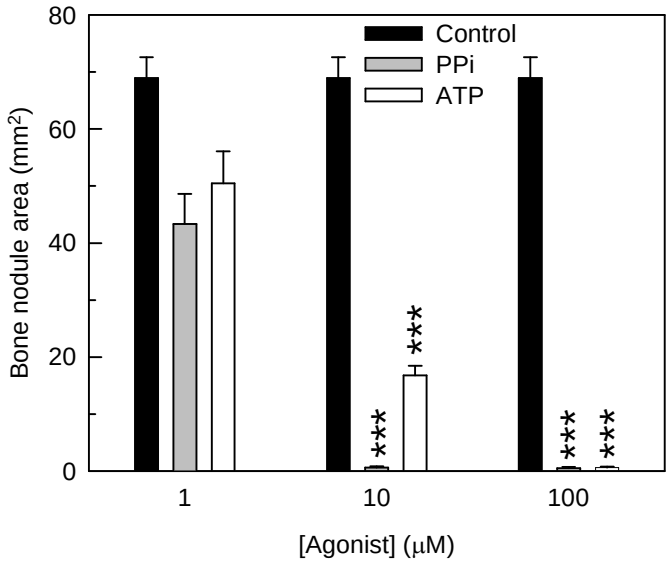


Figure 1.3 *Inhibition of bone nodule mineralisation by osteoblasts cultured with PPi or ATP*

PPi at concentrations of 1μM and above significantly inhibited the amount of mineralisation of matrix nodules. ATP also inhibited mineralisation significantly at concentrations of 10μM and above (n = 6, *** = p <0.001, ** = p<0.001). Data courtesy of Dr Isabel Orriss.

Ecto-nucleotide pyrophosphatase/phosphodiesterases

Ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs) are a family of seven structurally related ecto-enzymes that hydrolyse pyrophosphate or phosphodiester bonds in a number of extracellular compounds, including nucleotides, lysophospholipids and choline phosphate esters. The catalytic domain contains around 400 amino acid residues. The catalytic domains of each enzyme share between 24 and 60% identity at the amino acid level. E-NPP1 and E-NPP3 are both type 2 transmembrane proteins, that is, their c-terminus is on the outside of the cell. E-NPP2 is secreted and E-NPPs 4–7 are type 1 transmembrane proteins (Stefan *et al.* 2005). Table 1.2 gives details of the substrates of each of the E-NPPs.

Gene/protein name	**Alternative protein name(s)**	**Substrate(s)**
E-npp1	major acidic fibroblast-growth-factor stimulated phosphoprotein (MAFP); nucleotide pyrophosphatase phosphodiesterase (NPPase); nucleotide pyrophosphatase phosphodiesterase γ (NPPγ); nucleotide triphosphate pyrophosphohydrolase(NTPPPH); plasma cell differentiation antigen 1 (PC-1)	nucleotides; diadenosine polyphosphates (Ap_3A, Ap_4A, Ap_5A)
E-npp2	Autotaxin; lysophospholipase-D; nucleotide pyrophosphatase phosphodiesterase α (NPPα); phosphodiesterase-Iα (PD- Iα)	nucleotides; diadenosine polyphosphates (Ap_3A, Ap_4A, Ap_5A); lysophospholipids
E-npp3	B10; CD203c (cluster of differentiation203c); $Gp130^{RB13-6}$; phosphodiesterase β (NPPβ); phosphodiesterase-1β (PD-Iβ);	Nucleotides; diadenosine polyphosphates (Ap_3A, Ap_4A, Ap_5A)
E-npp4		unknown
E-npp5		unknown
E-npp6		choline phosphate esters
E-npp7	alkaline sphingomyelinase	choline phosphate esters

Table 1.2 *E-NPP nomenclature and substrates.* (Stefan *et al.* 2005; Yegutkin 2008).

E-NPPs catalyse the following reactions: nucleoside triphosphate (NTP) → nucleoside monophosphate (NMP) + pyrophosphate (PPi) or nucleoside diphosphate (NDP) → nucleoside monophosphate (NMP) + phosphate (Pi). E-NPPs1–3 are expressed by osteoblasts (Vaingankar *et al.* 2004).

E-NNP1

E-NPP1 is thought to be the most important E-NPP in bone mineralisation and is highly expressed in bone and cartilage; additionally, it is expressed by arteries, kidney, liver and B-lymphocytes (Vaingankar *et al.* 2004; Terkeltaub 2006; Yegutkin 2008; Kornak 2011). E-NPP1 is preferentially targeted to the plasma membrane and, in bone, it is mainly associated with matrix vesicles

(Vaingankar *et al.* 2004; Terkeltaub 2006; Yegutkin 2008). E-NPP1 mutations in humans are associated with idiopathic infantile arterial calcification whereby the lack of functional E-NPP1 protein means there is decreased PPi in areas of the body in which calcium build up can lead to life threatening conditions such as arterial and vascular calcification. (Rutsch *et al.* 2003) The hypothesis here is that an increase in E-NPP1 will have the opposite effect where there is an increase in levels of PPi; this will be of concern in areas of the body where PPi is not useful and calcification is necessary (i.e. the bones). There is a naturally occurring mouse E-NPP1 'knockout', known as tip-toe walking (*Ttw/Ttw*) in which a mutation in the gene results in a homozygous stop codon between the catalytic site and the helix-loop-helix EF hand, causing truncation of the gene product and more than a third of the functional protein (Johnson *et al.* 2003). The *Ttw/Ttw* mice exhibit ossification of the spinal ligaments, peripheral joint hyperostosis and calcification of articular cartilage, and this has similarities to the human disease 'Ossification of the posterior longitudinal ligament of the spine' (OPLL) (Okawa *et al.* 1998a; Okawa *et al.* 1998b; Zhang *et al.* 2007). It should be noted that the role of E-NPP1 as an inhibitor of mineralisation is also dependent on the activity of alkaline phosphatase, because the two enzymes together determine the final concentration of PPi.

E-NPP2

E-NPP2 is synthesised as a pre-pro-enzyme. Following N-terminal signal peptide removal and further trimming by furine-type protease, E-NPP2 is secreted. E-NPP2 primarily functions as a lysophospholipase-D, converting lysophosphatidylcholine into lysophosphatidic acid (LPA). LPA binds to many signalling pathways and activates those involved in cell survival, proliferation, contraction and migration. E-NPP2 knockout mice die at embryonic day 9.5 and have profound vascular defects, supporting a role for LPA as a lipid mediator in vascular development (Stefan et al. 2005; Yegutkin 2008).

E-NPP3

E-NPP3 has similar enzymatic activity and substrate specificity as E-NPP1; however, it is not targeted to the matrix vesicles, so its role in bone mineralisation is not thought to be as important as that of E-NPP1 (Vaingankar *et al.* 2004). E-NPP3 is expressed strongly by mature chondrocytes (Johnson *et al.* 2003) and is thought to have a role in allergic responses, as it is present on basophils and mast cells but no other blood cells. Following exposure to an allergen, there is an immunoglobulin E (IgE) response that results in basophils and mast cells releasing histamine and other mediators of an allergic response (Stefan *et al.* 2005) .

E-NPP4 and E-NPP5

The natural substrates for E-NPP4 and E-NPP5 are unknown.

E-NPP6

E-NPP6 is highly expressed in the human brain and kidney. It is shown to hydrolyse the classical phospholipase C substrate p-nitophenyl phosphorylcholine but not the classical nucleotide phosphodiesterase substrate p-nitrophenyl thymidine 5'-monophosphate (Sakagami *et al.* 2005).

E-NPP7

The enzyme intestinal sphingomyelinase was discovered over forty years ago and was recently identified as E-NPP7 by cloning studies (Stefan *et al.* 2005; Duan 2006). E-NPP7 is not thought to have nucleotide pyrophosphatase activity but instead, it has phospholipase C activity against sphingomyelin in the gut. Sphingomyelin is found in animal cell membranes, particularly those that comprise the myelin sheath. Sphingomyelin comprises a ceramide core (the 18 carbon amino alcohol, sphingosine, bonded to a fatty acid) and either a phosphocholine or phosphoethanolamine.

Ecto-nucleoside triphosphate diphosphohydrolases

E-NTPdases hydrolyse NTP → NDP + Pi and NDP → NMP + Pi. They are unable to hydrolyse nucleotide monophosphates (Vaingankar *et al.* 2004; Yegutkin 2008). There are currently eight known family members. E-NTPdases 1, 2, 3 and 8 are located on the cell surface, E-NTPdases 5 and 6 are localised intracellularly and then secreted following heterologous expression and E-NTPdases 4 and 7 are found only intracellularly, facing the lumen of cytoplasmic organelles (Yegutkin 2008).

E-NTPdase 1

Also known as CD39 and is found on vascular endothelial and smooth muscle cells, dendritic cells and lymphocytes (Yegutkin 2008).

E-NTPdase 2

This family member is thought to have an important role in purinergic control of embryonic, post-natal and adult neurogenesis. Expression of the protein is associated with adventitial surfaces of muscularised vessels and astrocytes, non-myelinating Schwann cells and glial cells of the central and peripheral nervous systems (Yegutkin 2008).

E-NTPdases 3–8

E-NTPdase 3 is thought to have a role in pre-synaptic regulation of extracellular ATP levels and coordination of homeostatic systems (Yegutkin 2008). Little is currently known about E-NTPdases 4, 5, 6 and 7. E-NTPdase 8 is most highly expressed in the liver, particularly the bile canaliculi and large blood vessels. It is not clear whether its role is in bile secretion or nucleoside salvage, or possibly both (Yegutkin 2008).

Acid-base balance

Acid-base balance is one of the most tightly regulated processes of homeostasis owing to the effect of pH on numerous biological functions (Orr-

Walker *et al.* 1999). This is a process in constant dynamic equilibrium as the body is constantly producing carbon dioxide and non-volatile acids as by-products of normal metabolic activity.

Regulation of acid-base balance in healthy individuals

The normal [H^+] is maintained between 40–45 nmoles of free hydrogen ions per litre which corresponds to a blood pH of between 7.35 and 7.4. Blood pH below 6.8 or above 7.7 is considered incompatible with life (Pocock & Richards 2009). Any changes in hydrogen ion concentration are combated in a number of ways; by chemical buffering via the body fluids to prevent excessive changes in H^+ concentration, via removal of CO_2 and therefore the acid H_2CO_3 from extracellular fluid, and, finally, via the kidneys which excrete either acid or alkaline urine to adjust the H^+ concentration back to normal (Guyton & Hall 2000).

There are several ways in which the [H^+] is buffered *in vivo*. One is the bicarbonate buffer system. H_2CO_3 is formed in the body by reaction of water and carbon dioxide; carbonic anhydrase catalyses this reaction, which is otherwise very slow. H_2CO_3 is a weak acid and ionises slightly to generate hydrogen ions and HCO_3^-. The second component of this buffer system is sodium bicarbonate which ionises to yield HCO_3^- and Na+. Overall, this gives:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- (+ Na^+)$$

Dissociation of H_2CO_3 is weak and therefore the overall hydrogen ion concentration is low. When buffering metabolic acidosis, the increased hydrogen ions are buffered by the HCO_3^- to give H_2CO_3, which in turn generates CO_2 and H_2O. The increase in CO_2 results in increased respiration and removal of CO_2 from the extracellular fluid and hence its excretion via the lungs. The opposite reaction happens in situations when alkalosis may occur. The bicarbonate buffer system is the most important buffer in the body (Guyton & Hall 2000).

Proteins within the plasma also function as useful buffers as they are plentiful. Free carboxyl and amino groups can dissociate to buffer changes in H^+ concentrations (Ganong 2005). Within the blood, changes in the binding property of haemoglobin makes it an important buffer (Austin & Cullen 1925). The imidazole groups of the histidine residues are much more important than the free carboxyl or amino groups as there are thirty-eight histidine residues in the haemoglobin molecule. In addition, there is a lot of haemoglobin which means it has six times the buffering capacity of plasma proteins (Ganong 2005).

In addition to buffering within the body, acid-base balance is maintained via respiratory regulation which functions to eliminate carbon dioxide from the extracellular fluid. CO_2 is made constantly *in vivo* as a by-product of intracellular metabolic processes. It diffuses out of cells into the interstitial fluid and then into the blood where it is transported to the lungs and is then excreted through the lungs after diffusing into the alveoli. Changes in extracellular fluid pH will alter the alveolar ventilation rate to change increase or decrease CO_2 excretion as required (Guyton & Hall 2000).

The kidneys also have a vital role in regulation of acid-base balance by excreting excess acid or base in the urine. Bicarbonate ions are filtered constantly through the tubules and if excreted into the urine, will remove excess base from the blood. Hydrogen ions are also filtered to remove excess acid. Non-volatile acids (i.e., not H_2CO_3) cannot be excreted via the lungs and are removed by the kidneys. When healthy and under normal conditions, almost all of the bicarbonate is reabsorbed by the kidneys. Hydrogen ions are actually required for this reabsorption. Any excess hydrogen ions are excreted in the urine after combination with ammonia (Guyton & Hall 2000).

Perturbations to acid-base balance

There are a number of situations whereby the pH of the blood drops to below 7.35, and results in acidaemia. Respiratory acidosis is the result of decreased

CO_2 excretion via the lungs. Initially small increases can be compensated for by plasma bicarbonate but certain conditions lead to hypercapnia that cannot be compensated for in this way and therefore acidosis results. These conditions include chronic obstructive or restrictive pulmonary diseases, upper-airway obstruction, and central nervous system (CNS) depression (Adrogue & Madias 1998).

Metabolic acidosis is the term given to all types of acidosis that occur in the body except those caused by an increase in CO_2 in the body fluid. Metabolic acidosis is the result of a number of situations detailed here. Metabolic acidosis can be caused by end stage chronic renal failure, whereby acid excretion is decreased (Kraut 2000). Additionally, acidosis can result from a high protein diet such as that followed on the 'Atkins diet' (Denke 2001; Chen *et al.* 2006a). Despite increases in glomerular filtration rates and increased permeability of renal capillaries, and consequently increased uric acid excretion, the uric acid load is increased to such a degree that not all is excreted and therefore blood pH decreases (Denke 2001).

It is rare that large enough quantities of acid are consumed in food to result in acidosis , however, metabolic acidosis can sometimes arise following ingestion of acetylsalicylic acid (aspirin) or methyl alcohol which is metabolised to formic acid (Guyton & Hall 2000). Ageing also results in worsening low-level metabolic acidosis: at 80 years of age, blood $[H^+]$ is 6-7% higher than at 20 years of age, plasma $[HCO_3^-]$ is 12-16% lower and blood pCO_2 is 7-10% lower. This is due to the progressive decline in renal function with age. The kidney is a major determinant of plasma $[HCO_3^-]$ regulation; renal insufficiency also results in reduced conservation of bicarbonate and decreased excretion of acid as a by-product of normal cellular function. Additionally, acid-excretory ability in response to exogenous acid is reduced (Frassetto & Sebastian 1996). Severe cases of diabetes mellitus can result in metabolic acidosis when fats are split to generate acetoacetic acid which is metabolised by tissues to generate energy in

the place of glucose. Blood levels of the acetoacetic acid can rise to sufficiently high levels to cause metabolic acidosis (Guyton & Hall 2000).

Acidosis also occurs as a consequence of the menopause; loss of progesterone results in respiratory hypoventilation and therefore mild respiratory acidosis (Hodgkinson 1984; Orr-Walker *et al.* 1999; Preston *et al.* 2009).

Acidosis can also occur at a more localised level which can influence bone cells depending on the location itself. Situations in which this may be the case include inflammation, where lactic acid production will result in acidosis at the site (Saadi *et al.* 2002). Also, a reduced vascular supply will result in acidosis as by-products of cellular reactions are not removed at a normal rate and so build up at the location. Circumstances under which reduced vascular supply will occur include: inflammation, infection, tumours, diabetes and aging (Arnett 2010). Consideration of local acidosis in relation to tumours is covered in greater detail in Chapter 6.

Effect of acid base balance on the skeleton

The detrimental effect of acid on the skeleton has been known for many years (Goto 1918). Bone functions as a 'fail-safe' store of alkali if overall bodily pH drops to dangerous levels and requires buffering to maintain the pH within normal but narrow limits (Arnett 2010). Solubility of hydroxyapatite increases as pH decreases; at pH 7.39 calcium ion concentration is 0.16 mmol/l and phosphate ion concentration is 0.21 mmol/l, when pH is reduced to 6.9, solubility has increased to give calcium ion and phosphate ion concentrations of 0.28mmol/l and 0.78mmol/l respectively (Larsen & Jensen 1989). Acidosis does however also have more direct effects on bone cells; culture of osteoblasts at pH 6.9 prevents bone nodule formation *in vitro* by inhibiting mineralisation of the collagenous matrix (Brandao-Burch *et al.* 2005b). The action of TNAP, expressed by osteoblasts, which generates phosphate for incorporation into the hydroxyapatite, is down-regulated as a consequence of acidosis (Brandao-

Burch *et al.* 2005b). As a consequence of this down-regulation there is less Pi for incorporation into the hydroxyapatite but it also alters the ratio of Pi and PPi in the bone microenvironment such that there is more PPi present, which is an important inhibitor of mineralisation (Fleisch *et al.* 1966).

In addition to the direct effects of acid on osteoblasts, acidosis also affects osteoclasts. It was demonstrated over twenty-five years ago that protons are the key stimulator of osteoclast resorption. Small drops in pH from the physiological norm of pH 7.4 to pH 6.8 resulted in dramatic increases in the resorptive activity of osteoclasts (Arnett & Dempster 1986). Once osteoclasts have been activated by acid, cells can be further stimulated with RANKL (Arnett 2010). Acidosis also results in up-regulation of carbonic anhydrase II by osteoclasts (Biskobing & Fan 2000); which is required to generate protons from CO_2 and H_2O which are secreted into the resorption pit in order to generate the acidic environment required for resorption (Vaananen & Laitala-Leinonen 2008). Chronic acidosis causes an increase in activity of the H^+-ATPase at the ruffled border, thus delivering more protons into the resorption pit and ultimately leading to increased resorption as a consequence of metabolic acidosis (Nordstrom *et al.* 1997). The mechanism by which protons are detected by bone cells however, remains elusive; potential acid-sensors include members of the Transient Receptor Potential (TRP) channel super family and these are discussed below.

Acidosis has an overall negative effect on bone whereby the action of bone mineralisation by osteoblasts is decreased and the bone removing activity is increased leading to a net reduction in bone mass; this is likely to be an evolutionary ‘fail-safe’ mechanism to release base into the body to buffer acidosis that may occur as a result of situations previously outlined.

Transient receptor potential (TRP) channels

Transient receptor potential (TRP) channels are a super family of cation channels, made up of seven smaller families of related proteins (Clapham 2003). The different families are listed in Table 1.3 below. Individual members are named according to the different family and then a number (i.e., Transient Receptor Potential Vanilloid type 1 is abbreviated to TRPV1).

All members of the TRP channel family are structurally related and comprise six transmembrane domains; these assemble as homo- or hetero-tetramers to form the active channel, with the pore loop situated between the fifth and sixth transmembrane domains (Abed *et al.* 2009). Both the C- and N-termini are located intracellularly (Holzer 2009). There are varying degrees of sequence homology between the channels and all are cation permeable (Venkatachalam & Montell 2007). The TRP channel family is distinct from other groups of ion channels because of the large range of cellular functions they mediate; their sensory roles include vision, taste, hearing and smell and they also act as acid, mechano-, chemo- and thermo-sensors (Montell 2005; Voets *et al.* 2005). Most of the TRP channels characterised to date are permeable to Ca^{2+} ions, the exceptions being Transient Receptor Potential Melastatin type 4 (TRPM-4) and -5. Different groups of TRP channels also behave in different ways, some open upon activation and result in Ca^{2+} movement into the cell, others are constitutively open (Nilius *et al.* 2007).

Abbreviation	Name	Number of mammalian members
TRPC	-Canonical	7
TRPM	-Melastatin	8
TRPV	-Vanilloid	6
TRPA	-Ankyrin	1
TRPP	-Polycystin	3
TRPML	-Mucolipin	3
TRPN	-No mechanopotential	0

Table 1.3 *Mammalian TRP (transient receptor potential) channel families* (Nilius *et al*, 2007; Wu *et al.* 2010)

Currently TRP channels are the subject of much interest as they appear to be widely expressed throughout the body (Desai & Clapham 2005); furthermore they have been implicated in some human diseases. Mutations in TRPM6 result in hypomagnesmia and hypocalcaemia, mutations in Transient Receptor Potential Canonical type 6 (TRPC6) cause kidney disease, in Transient Receptor Potential Polycystin type 2 (TRPP2) or -1 they cause autosomal dominant polycystic kidney disease, and in Transient Receptor Potential Mucolipin type 1 (TRPML1) they cause mucolipidosis type IV (Venkatachalam & Montell 2007). Furthermore, there is functional evidence from transgenic mouse models that many of the TRP channels could be linked to a whole host of human diseases. There have, however, been few studies that investigate the presence or role of TRP channels on bone cells (Abed *et al.* 2009).

TRPV1

TRPV1 was the first member of the vanilloid family of TRPs to be discovered. It was originally named VR1 (Voets *et al.* 2005), and it is assembled as a homotetramer. TRPV1 is gated open when the pH drops to below pH 6.0,

however, mild acidosis (pH 6.0–7.0) sensitises TRPV1 to other stimuli such as capsaicin (the 'hot ingredient in chilli peppers) and heat. The TRPV1 cation channel is activated and therefore opened in response to temperatures above 43°C, and is additionally stimulated by endovanilloids (Latorre *et al.* 2007; Holzer 2009).

Activation of TRPV1 by protons and merely sensitising TRPV1 to stimulations by other factors depend on two different amino acid residues. Glu-600, which is located on the extracellular side of transmembrane segment 5, is necessary for proton-sensitisation of TRPV1. Several amino acid residues are essential for proton-induced gating of the channel, these residues are: Val-538, located in the intracellular linker between segments three and 4; Thr-633, found within the pore helix; and Glu-648, which is found in the linker between the pore and transmembrane 6 (Holzer 2009).

The TRPV channels are non-selective for cations and moderately permeable to Ca^{2+} and activation of the channel results in cation influx (Nilius *et al.* 2007); (Yang *et al.* 2010). TRPV1 has been shown to be expressed by neurons, the bladder and testis (Venkatachalam & Montell 2007). Preliminary work in our laboratory also indicated that TRPV1 is expressed by osteoclasts (Brandao-Burch 2005a) and that addition of capsaicin to osteoclast cultures resulted in osteoclastic resorption (Brandao-Burch *et al.* 2006). TRPV1 has been implicated in bone cancer pain, where the microenvironment is acidic (Ghilardi *et al.* 2005; Prevarskaya *et al.* 2007). Furthermore, it has been suggested that TRPV1 may have a role in osteoarthritis (Nilius *et al.* 2007).

TRPV4

TRPV4, like TRPV1, responds to multiple stimuli including osmotic cell swelling, warm temperatures (27˚C) and acidosis (Montell 2005; Holzer 2009). Synoviocytes in joints are reported to abundantly express TRPV4, in addition to being expressed by chondrocytes and osteoblasts. TRPV4 has been proposed to act as a mechanical sensor in bone (Nilius *et al.* 2007).

TRPM8

TRPM8 is related to both TRPV1 and TRPV4 because it is also a thermosensor, detecting 'cool' temperatures between 23–28˚C. It is activated by menthol and a synthetic agonist, icilin. Its activation is also pH modulated (Venkatachalam & Montell 2007). An abnormal bone phenotype in *Trpm8*$^{-/-}$ mice has not been reported.

Cancer metastasis to bone

Cancer metastases are a common feature of disease progression and, account for approximately 90% of cancer deaths (Fokas *et al.* 2007; Kingsley *et al.* 2007). Cancers go through similar stepwise processes in order to metastasize to an organ different from the primary tumour site. The simple timeline of cancer metastasis involves a number of key processes: 1) local invasion; 2) intravasation, that is, entry of the tumour cells into the blood stream; 3) survival in the circulation; and 4) extravasation, that is, exit of tumour cells from capillary beds into 'target' organ (Nguyen *et al.* 2009). Different types of cancer preferentially metastasize to different organs; this is known as the 'seed and soil' theory, first described by (Paget 1889). The theory states that certain tumour cells (i.e., the 'seeds') colonise distinct distant organs (i.e., the 'soil') that have conditions most favourable to growth of that particular tumour (Fokas *et al.* 2007).

Both breast and prostate cancer preferentially metastasize to the bone, although the types of disease that typically result are different. Breast cancer most commonly results in incurable osteolytic bone disease caused by aberrant osteoclast stimulation (Guise 2000; Fokas *et al.* 2007; Kingsley *et al.* 2007), whereas prostate cancer more often results in osteoblastic metastases (Roodman 2004). The resulting bone destruction causes hypercalcaemia; pathological fractures and nerve compression, the latter two causing considerable pain which is not attenuated satisfactorily by traditional pain relief regimens such as opiates owing to the intermittent nature of the pain (Mercadante 1997; Guise 2000; Slatkin 2006). Common sites of bone

metastases are the back, pelvis, upper leg, ribs, upper arm and skull (Fokas *et al.* 2007).

The vicious cycle of bone metastases

When tumour cells metastasize to bone, the cancer cells will initially adhere to the endosteal surface of the bone before colonisation. The 'vicious cycle' hypothesis proposes that cancer cells secrete numerous growth factors and cytokines into the micro-environment that stimulate osteoclast activity. Once osteoclasts are stimulated to resorb bone, the growth factors which are trapped in the bone matrix, including transforming growth factor β (TGFβ), of which bone is the richest source (Clezardin 2011; Patil *et al.* 2011), are released into the bone/cancer micro-environment where their presence is postulated to stimulate further cancer cell growth. More tumour cells means there are more growth factors and cytokines in the extracellular environment to stimulate further osteoclast activity and, hence, this is the cycle which results in further tumour growth, more osteoclast stimulation and increased bone destruction (Guise 2000; Yoneda & Hiraga 2005; Kingsley *et al.* 2007). Cytokines produced by the breast cancer cell lines used for this work are detailed here. Some of these have been implicated in the vicious cycle model.

Cytokines produced by breast cancer cells

A number of cytokines known to have a role in bone cell function have been reported to be expressed by both MCF-7 and/or MDA-MB-231 breast cancer cell lines. These are the breast cancer cells most commonly used in research (Lacroix & Leclercq 2004) and are used for the work presented in this chapter. The MDA-MB-231 cell line was isolated from the metastatic pleural effusion of a 51-year-old Caucasian woman. MDA-MB-231 cells are oestrogen receptor negative. The MCF-7 breast cancer cell line was isolated from the metastatic pleural effusion of a 69 year old Caucasian woman. MCF-7 cells are oestrogen receptor positive (Lacroix & Leclercq 2004).

As previously stated, TGFβ induces and regulates many cellular processes; bone provides a rich source of TGFβ (Juarez & Guise 2011; Patil *et al.* 2011). One of the mechanisms TGFβ regulates is cell proliferation; cancer cells, however, often lose this regulatory nature owing to loss of members of the signalling pathway. In the breast cancer microenvironment, TGFβ has been reported to increase tumour cell invasiveness, increase angiogenesis and induce immunosuppression (Buijs *et al.* 2011). TGFβ2 has been reported to both promote osteoclastogenesis, whereby it makes pre-osteoclasts more sensitive to RANKL (Stadelmann *et al.* 2008). TGFβ2 and TGFβ3 have been reported to be produced by MCF-7 cells (Perera *et al.* 2008). TGFβ2 is expressed by MDA-MB-231 cells (Li & Sidell 2005).

Activin A is a multifunctional growth factor and a member of the TGFβ super family (Phillips *et al.* 2005). The diverse roles of activin A include cell proliferation, differentiation, apoptosis, the immune response and wound repair (Sulyok *et al.* 2004; Chen *et al.* 2006b). It has been reported that breast cancer patients with bone metastases have higher serum concentrations of activin A than those patients without cancer metastases to bone; therefore indicating that activin A may play a part in the vicious cycle of cancer metastases (Leto *et al.* 2006).

Interleukin-6 (IL-6) has well-documented effects on osteoclasts, whereby it promotes osteoclast differentiation via a RANKL independent mechanism; additionally, it has been reported to stimulate osteoclastic resorption (Kurihara *et al.* 1990; Tamura *et al.* 1993; de la Mata *et al.* 1995; Kudo *et al.* 2003). IL-6 is expressed by MDA-MB-231 and MCF-7 cells, (Lacroix *et al.* 1998; Li & Sidell 2005; Lee 2006; Joimel *et al.* 2010) . IL-6 has been implicated as a marker of cancer progression in several different cancer types. In patients with metastatic malignant melanoma, higher IL-6 levels are correlated with an increased tumour burden (Mouawad *et al.* 1996). Similarly, patients with metastatic renal cell carcinoma have higher levels of circulating IL-6 compared with healthy adults. Additionally, when these patients were compared with each other those with the

highest levels of IL-6 had a worse prognosis and shorter life expectancy than those with lower IL-6 levels (Blay *et al.* 1992). Equivalently, in patients with prostate cancer, the patients with the highest IL-6 levels were those with metastases, followed by those with only localised cancer who in turn had higher IL-6 levels than normal healthy adults. This study also found that in patients whose cancer recurred, IL-6 levels became elevated when prostate-specific antigen (PSA), (the current standard marker of prostate cancer), became elevated upon recurrence of the disease (Michalaki *et al.* 2004). Furthermore, in breast cancer patients with metastases, higher levels of IL-6 have been correlated with shorter periods of survival (Bachelot *et al.* 2003).

Interleukin(IL)-8 (IL-8) is produced by MCF-7 cells (Lee 2006; Perera *et al.* 2008) and MDA-MB-231 cells (Li & Sidell 2005; Joimel *et al.* 2010) and has been reported to stimulate both osteoclastogenesis and osteoclastic resorption (Bendre *et al.* 2003).

Other cytokines and growth factors produced by MDA-MB-231 breast cancer cells include: granulocyte macrophage-colony stimulating factor (GM-CSF); growth related oncogene (GRO), GRO-α; IL-11; angiogenin; platelet-derived growth factor-B (PDGF-B); insulin-like growth factor-binding protein (IGFBP) -1 and -4; tissue inhibitor of metalloproteinases (TIMP) -1 and -2; and leukaemia inhibitory factor (LIF) (Lacroix *et al.* 1998; Li & Sidell 2005; Joimel *et al.* 2010). MDA-MB-231 cells grown on bone matrix *in vitro* produce the matrix metalloproteinases MMP2 and MMP9 (Yoneda & Hiraga 2005).

MCF-7 cells have also been reported to produce osteoprotegerin (OPG) (Perera *et al.* 2008) which may have a role in disrupting the bone remodelling balance *in vivo*.

Other cytokines and growth factors produced by MCF-7 cells include: VEGF (Lee 2006; Perera *et al.* 2008), TNFβ, macrophage inflammatory protein (MIP)-1β, also known as chemokines (C-C motif) ligand 4 (CCL4); brain derived neurotrophic factor (BDNF); fibroblast growth factor 9 (FGF9); IGFBP-2;

interferon gamma induced protein 10 (IP-10); LIF; neutrophil-activating protein 2 (NAP-2); TIMP-1 and TIMP-2 (Perera *et al.* 2008); TNFα; IL-1β; IL-10; and IL-12p70 (Lee 2006).

Multiple myeloma (MM) is a cancer of mature B lymphoid cells (Skinner 2011) which presents with symptoms including unexplained anaemia, bone pain, renal failure, infection and hypercalcaemia (Scharschmidt *et al.* 2011). Osteolytic bone destruction is a hallmark of multiple myeloma (Scharschmidt *et al.* 2011) and, thus, cytokines implicated in MM may play a part in osteolytic bone disease.

B-cell activating factor (BAFF) is a tumour necrosis factor (TNF) family member expressed by T-cells and dendritic cells (Schneider *et al.* 1999). Members of the TNF super family were initially characterised by their cytotoxicity to tumour cells, causing tumour regression. Since their discovery, however, TNF family members have been implicated in some normal functions and disease states including: the immune response, haematopoiesis and morphogenesis, tumourigenesis, transplant rejection, septic shock, viral replication, rheumatoid arthritis, diabetes and bone resorption (RANKL is a TNF family member) (Aggarwal 2003). Given that multiple myeloma is a B cell neoplasm, it was proposed that inhibiting BAFF, tumour growth may be suppressed and subsequent disease progression thus reduced (Hideshima *et al.* 2007). Preliminary experiments carried out by collaborators at the University of Sheffield using a soluble BAFF receptor-FC construct to block *in vivo* BAFF activity showed that there was no effect on tumour burden but osteolytic bone lesions were reduced compared to control animals. Additionally, in mice that over-express BAFF, bone density and trabecular bone volume were both reduced. These results together suggest that osteoclasts may be affected by BAFF and the decreased number of osteolytic lesions results from decreased osteoclast activity as BAFF is blocked. (Croucher, personal communication; Xu *et al.* 2007)

Despite the fact that much work has been undertaken in an attempt to understand the mechanisms underplaying the vicious cycle of cancer metastasis to bone, the exact mechanisms are still not understood. Additionally, little attention has been paid to the role of acid, which is surprising given that the tumour microenvironment is known to be acidic, and acid is the key factor that results in stimulation of osteoclastic resorption (Arnett 2010).

General aim of the thesis

The overall aims of this PhD are to further elucidate the role that extracellular acidosis has in negatively affecting bone remodelling. More specifically, to determine the mechanisms by which extracellular acidosis prevents mineralisation of the extracellular matrix; to investigate the mechanism by which acid is sensed by osteoblasts and osteoclasts; and to elucidate the role of acidosis in the vicious cycle of cancer metastasis in osteolytic bone disease.

Chapter 2

Materials and methods

All chemicals were purchased from Sigma-Aldrich (Gillingham, UK) unless otherwise stated. Tissue culture reagents were purchased from Gibco (Paisley, UK).

Cell Culture

Osteoblast cell culture

Primary rat osteoblasts were obtained by sequential enzyme digestion of calvarial bones isolated from two-day-old neonatal Sprague-Dawley rats. The process comprised three steps: 1% trypsin in PBS for 10 minutes; 0.2% collagenase type II in Hanks balanced salt solution (HBSS) for 30 minutes; 0.2% collagenase type II in HBSS for 60 minutes. The first two digests were discarded and the cells resuspended in Dulbecco's Modified Essential Medium without phenol red supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin (complete mixture abbreviated to DMEM). Cells were cultured for 3–4 days in a humidified atmosphere of 5% CO_2-95% air at 37°C until confluent. Once confluent, cells were seeded into 6 or 24-well trays (10^5 and 2.5 x 10^4 cells/well, respectively) in DMEM supplemented with β-glycerophosphate (2mM), ascorbic acid (50µg/ml) and dexamethasone (10 nM). Osteoblasts were cultured for 4–15 days with half medium changes every 3–4 days. Throughout the culture, medium pH, pCO_2 and pO_2 were monitored using a blood gas analyser (ABL-700, Radiometer, Copenhagen, Denmark).

To investigate the effects of TRP channel agonists, these were added to the medium at half media changes every 3–4 days.

Quantification of bone nodule formation

After 4–15 days experiments were terminated to determine the extent of bone nodule formation. Cell layers were washed with PBS, fixed with 2.5% glutaraldehyde for 5 minutes, washed again with PBS and washed three times in 70% ethanol. The plates were then left to air dry. Mineralised bone nodules were visualised by staining with alizarin red (1% solution w/v in water) for 5 minutes, rinsed with 50% ethanol to remove excess stain, then air-dried. The plates were imaged at 800 dpi using a high-resolution flat-bed scanner (Epson Perfection Photo 3200). Using 'Adobe Photoshop' (version 6, Adobe Systems Inc.) images of individual wells were first changed to greyscale before conversion to binary images using Scion Image software (Scion Corporation; http://www.scioncorp.com). These images were then subjected to automated analysis, using constant 'threshold' and 'minimum particle' levels, to determine the number and plan surface area of mineralised bone nodules.

Human peripheral blood mononuclear cell culture

Blood was taken from healthy volunteers, and peripheral blood mononuclear cells (PBMNC) were isolated by density gradient separation. PBMNC were washed once in PBS and then sedimented on to 250µM thick, 5mm diameter dentine discs cut transversely from elephant ivory (kindly donated by HMRC, Heathrow, UK) in 96-well trays in Minimum Essential Medium without phenol red, 15% heat inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2mM L-glutamine (complete mixture abbreviated to 'MEM'). After 4 hours, discs were washed in PBS before being placed into 96-well trays in MEM containing 7.5 ng/ml M-CSF (Insight Biotechnology Ltd., Wembley, UK). After 3–4 days incubation, discs were transferred to 6-well trays containing MEM with the pH adjusted to 7.4 by the addition of 7.5 meq/L OH^- (as NaOH), and further supplemented with 7.5 ng/ml M-CSF and 1 ng/ml RANKL (R&D Systems Europe Ltd., Abingdon, UK). Half of the medium was replaced every 3–4 days and medium was acidified to pH 7.0 by the addition of 10 meq/L H^+ (as HCl) 48 hours prior to termination of the culture. Cells were fixed in 2.5% glutaraldehyde

and stained for tartrate resistant acid phosphatase (TRAP) using the leukocyte acid phosphatase kit (387-A, Sigma-Aldrich, Gillingham, UK) according to the manufacturer's instructions. The numbers of TRAP-positive multinucleated osteoclasts were assessed 'blind' using transmitted light microscopy. The area resorbed per disc was quantified by dot-count morphometry under reflected light.

To investigate the effect of TRP channel agonists or cytokines implicated in the vicious cycle of cancer metastases on osteoclast formation, test substances were added at the same time as RANKL, on day 4. To investigate the effect on osteoclast activity they were added upon acidification 48 hours prior to the termination of the culture.

Murine osteoclast culture

Long bones were isolated from two 6–8 week old MF1 mice killed by cervical dislocation. The epiphyses were removed and the marrow was flushed out with PBS using a 25-gauge needle. Marrow was collected and cells pelleted by centrifugation at 1500g, followed by a PBS wash, before being in Minimal Essential Medium without phenol red containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml amphotericin B (mixture abbreviated 'MEM') supplemented with 10^{-7} M prostaglandin E_2 (PGE_2), and 50ng/ml M-CSF (Wyeth, Maidenhead, UK). Cells were placed into a 75 cm^2 tissue culture flask and incubated for 24 hours at 37°C / 5% CO_2 to allow attachment of stromal cells. Non-adherent cells were collected by centrifugation and resuspended in MEM containing 10^{-7} M PGE_2, 150 ng/ml M-CSF and three ng/ml RANKL. Dentine slices in 96-well trays were incubated with 200µl of cell suspension (10^6 cells) overnight at 37°C / 5% CO_2 to allow attachment of osteoclast precursors to dentine discs. Discs were then transferred to 6-well trays containing MEM with the pH adjusted to 7.4 by the addition of 7.5 meq/L OH, and further supplemented with 150 ng/ml M-CSF and three ng/ml RANKL. Half of the medium was replaced every 3–4 days and medium was acidified to pH 7.0 by the addition of 10 meq/L H^+ 48 hours prior to termination of the culture. Cells

were fixed in 2.5% glutaraldehyde and stained for tartrate resistant acid phosphatase (TRAP) using the leukocyte acid phosphatase kit according to the manufacturer's instructions. The numbers of TRAP-positive multinucleate osteoclasts were assessed 'blind' using transmitted light microscopy. The area resorbed per disc was quantified by dot-count morphometry under reflected light.

To investigate the effect of TRP channel agonists or cytokines implicated in the 'vicious cycle' model of cancer metastases on osteoclast formation, test substances were added when RANKL was added. To investigate the effect on osteoclast activity they were added upon acidification 48 hours prior to the termination of the culture.

Maintenance of breast cancer cell lines

Both MCF-7 (passage: 6) and MDA-MB-231 (passage: 48) breast cancer cell lines were a gift from Dr Kay Colston (St. George's Medical School, University of London). Cells were maintained in RPMI medium (Sigma-Aldrich, Poole, UK) with 5% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin (complete mixture abbreviated to RPMI). Cells were passaged every 3–4 days.

Monitoring and adjustment of medium pH

Medium pH, pCO_2 and pO_2 were monitored at every medium change and experiment end using a blood gas analyser (ABL 705, Radiometer, Crawley, UK). The blood gas analyser equilibrates two buffer solutions at a low and high pH with air and CO_2. These equilibrated solutions are then used for electrode calibrations. Accuracy of the measurements is achieved as the blood gas analyser is also internally calibrated with known standards. Approximately 200µl of culture medium is taken into a syringe and injected into the machine. The CO_2 of samples taken subsequently fell therefore causing pH to rise, so these were back-corrected to CO_2 from the first reading which is assumed to

represent the CO_2 in all wells. Calibration curves to allow for back-correction can be found below (**Fig 2.1 A**)

The pH of medium was adjusted using additions of small volumes of concentrated acid or alkali. The graph below (**Fig 2.1 B**) demonstrates the effect of additions of protons or hydroxyl ions on pH of MEM with 10% FCS. To maintain pH of MEM at 7.4, 7.5meq/l OH^- as NaOH was added; to adjust the pH of MEM to 7.2 or 6.9, 5meq/l or 10meq/l H^+ as HCl was added respectively.

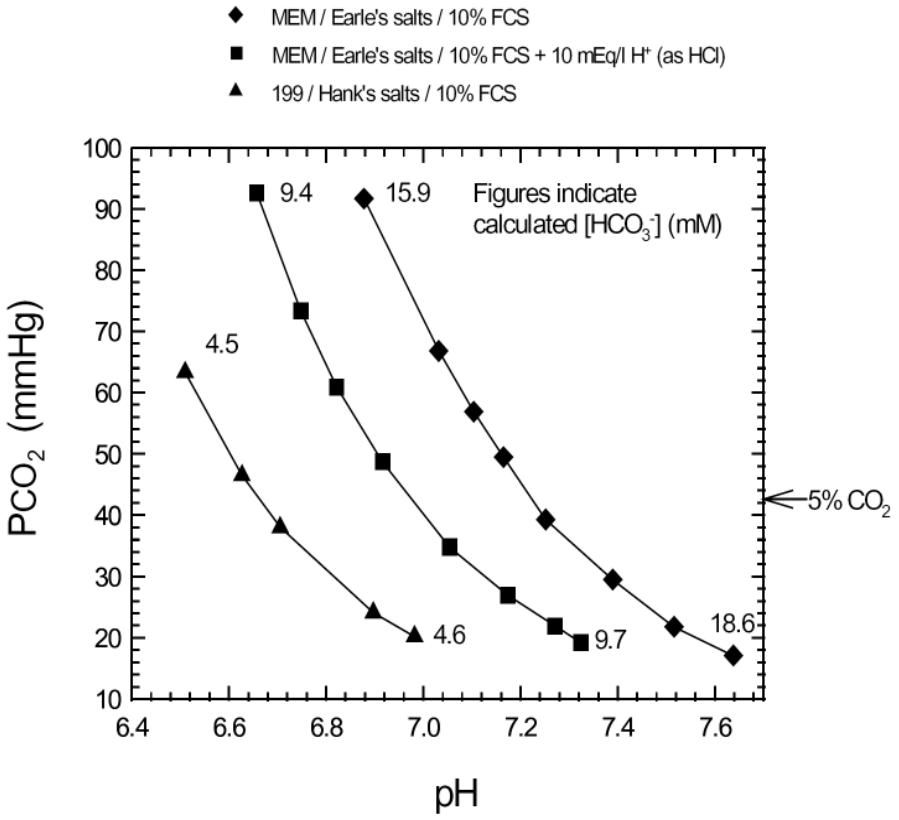


Figure 2.1 A *Relationship between pH, pCO_2 and HCO_3^- in tissue culture medium.*

Data obtained using a blood gas analyser. Courtesy of Prof. Tim Arnett.

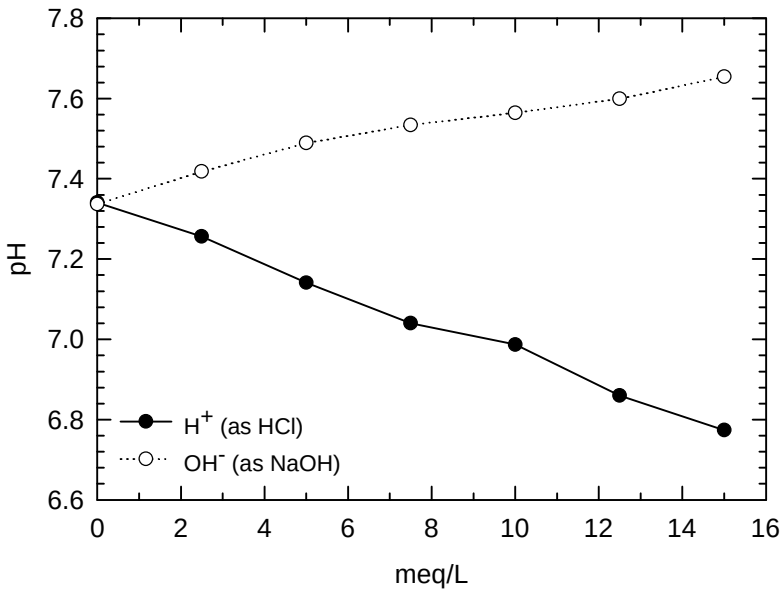


Figure 2.1 B *Adjustment of pH of MEM with 10% FCS by addition of H^+ or OH^- which had been equilibrated with 5% CO_2.*

Assays

Cell proliferation assay

Osteoblast number was measured at 4, 7, 10 and 14 days of culture using the CytoTox 96® non-radioactive cytotoxicity assay (Promega UK, Southampton, UK). This assay quantitatively measures cellular lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. LDH oxidises lactate into pyruvate, generating NADH, which is then used to convert a tetrazolium salt into a red formazan product. The amount of colour formed is proportional to the number of lysed cells. One hour prior to assaying, osteoblasts were switched to serum free DMEM containing lysis buffer (1% (v/v) Triton X-100 in water); following sample collection the assay was performed as per manufacturer's instructions. A standard curve for determination of cell numbers was constructed using cells seeded at 10^2 to 10^6/well. Manual cell counts were performed in parallel for assay validation.

Cell viability assay

Osteoblast viability was determined at days 4, 7, 10 and 14 of culture using the CytoTox 96® non-radioactive cytotoxicity assay (Promega UK, Southampton UK). Cells were cultured with the test substance (TRP channel agonist or antagonist) until the day of the assay. One hour prior to the assay, medium was removed and replaced with serum free DMEM. Samples were collected and the assay performed according to the manufacturer's instructions. Following sample collection, cells were lysed in order to determine cell number (described previously) to calculate percentage viability.

Ecto-nucleotidase activity assay

This assay exploits the fact that ecto-nucleotidases (E-NPPs) are able to hydrolyse p-nitrophenyl thymidine 5'-monophosphate (pNP-TMP) to generate thymidine 5'-monophosphate and p-nitrophenol, which has a yellow colour at alkaline pH and can be measured colorimetrically (RAZZELL 1963). Therefore, enzyme activity can be deduced from the amount of p-nitrophenol generated. Total E-NPP activity was measured on days 4, 7, 10 and 14. Osteoblasts were cultured as described above. Medium was removed from wells and cell monolayers were washed with phosphate-buffered saline (PBS). Lysis buffer (1% Triton X in 0.2M Tris base with 1.6mM $MgCl_2$ pH 8.1) was added to wells and monolayers were collected and kept on ice. Cell debris was pelleted by centrifugation at 500 x g for 5 minutes at 4ºC. 90µl of supernatant was removed to be assayed with 10µl of pNP-TMP (the final concentration of pNP-TMP was 5mM). The assay plate was kept in the dark at 37ºC and measurements taken at regular intervals. Absorbance at 405nM was recorded using a plate reader (EL_x800, Bio-Tek International, Fisher Scientific UK, Loughborough, UK). Amounts were adjusted for total protein content.

Phosphate assay

The amount of phosphate (Pi) released by osteoclasts was determined using the Pi ColorLock™ Gold (Innova Biosciences Ltd., Cambridge, UK) microplate

assay kit. The assay was performed at day 10 of culture. The effect of short and long term acidosis was investigated. For short term effects, osteoblasts were grown in 24-well trays as described previously to day of assay at pH 7.4, they were then transferred to assay buffer at pH 6.9 one hour prior to assay. To investigate long term effects, culture medium was adjusted to pH 6.9 as previously described. Assay buffer was added to cells one hour prior to assay at the same pH as the osteoblasts had been cultured at. Osteoblasts cultured at pH 7.4 were used as a control. One hour prior to assay, DMEM was removed from monolayers and cells were washed with assay buffer (0.01M HEPES, 0.1% NaCl, 1% BSA pH adjusted to be pH 7.4 or pH 6.9 at 37˚C) to remove any phosphate and pyrophosphate. 500µl of the assay buffer was then added to the wells and osteoblasts incubated at 37˚C for one hour. The assay was then performed according to the manufacturer's instructions. The assay is based on the principle that the absorbance of the dye malachite green changes in the presence of phosphomolybdate complexes. When unbound to Pi, the dye is orange, but when bound to phosphate (Pi), it is green. Absorbance was read at 630nm. The amount of Pi was determined using a standard curve produced using known amounts of phosphate.

A cell viability assay using the lactate dehydrogenase assay CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Southampton, UK), described previously, was performed alongside.

Pyrophosphate assay

Osteoblasts were prepared as above to determine pyrophosphate generation. Pyrophosphate was measured using the PiPer™ Pyrophosphate Assay Kit (Invitrogen, Paisley, UK.) The principle is that inorganic phosphatase converts pyrophosphate to two molecules of inorganic phosphate. In the presence of inorganic phosphate, maltose phosphorylase converts maltose to glucose 1-phosphate and glucose. Glucose is converted to gluconolactone and H_2O_2 by glucose oxidase. With hydrogen peroxidise as a catalyst, H_2O_2 reacts with amplex red to generate the product resorufin. The increase in absorption above

the background is proportional to the amount of pyrophosphate in the sample. A standard curve of known amounts of pyrophosphate was prepared alongside samples that were assayed.

ATP release assay

Osteoblasts were grown in 12-well trays, as described previously. ATP release assays were performed at days 4, 7, 10 and 14 of culture. Both the effects of short and long term acidosis were investigated. For long term acidosis, osteoblasts were cultured in medium at pH 6.9 from plating out, for short term acidosis, osteoblasts were transferred to acidic medium immediately before the assay began. Serum free DMEM containing L-glutamine and ABAM and pH adjusted to pH 6.9 or left at the running pH of 7.4 were put into the incubator overnight to equilibrate. On the day of the assay, medium was removed from the wells and replaced with 500µl of the serum free DMEM that had been equilibrating overnight. Samples were taken 60 minutes after addition of serum free DMEM. The cells were returned to the incubator between readings after the 5 minute sample has been taken, taking care not to move the plate more than necessary as this would result in additional ATP release. 25µl per well was removed into an eppendorf that already contained luciferase reagent. An additional 25µl was also removed into a 96-well plate to assay for cell viability. The ATP in the medium was measured using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Southampton, UK). The assay utilises a reaction catalysed by luciferase, whereby in the presence of ATP and O_2 and Mg^{2+}, luciferin is converted to oxyluciferin, AMP, PPi, CO_2 and light. This was detected using the GLOMAX® 20/20 luminometer (Promega, Southampton, UK). A standard curve using known amounts of ATP was generated and, from this, the amount of ATP released from the cells was quantified.

Apoptosis assay

Primary mouse osteoclasts were isolated as described previously. Following collection and cell count, osteoclasts were seeded onto plastic in a 96-well tray,

at 10^6 cells, in 300µl of MEM per well. pH was adjusted to 7.4 or 6.9 with small additions of NaOH or HCl respectively, as outlined previously. Cells were half media changed every other day and pH, pCO_2 and pO_2 monitored throughout as previously described. Cells were fixed on days 4, 6, 8 and 10. Cells were fixed and apoptosis performed as outlined in the manufacturer's instructions. The apoptosis kit used was HT TiterTACS Assay Kit (Trevigen®, Gaithersburg, MD, USA) which allows *in situ* detection of apoptotic cells in either monolayer or suspension formats. The assay utilises one of the final stages, and a hallmark of apoptosis, DNA fragmentation. The DNA breaks generate free 3' hydroxyl groups. The assay exploits this by providing a target for terminal deoxynucleotidyl transferase (TdT) to sequentially add nucleotides to the 3' end of this DNA, the nucleotides are tagged and can be measured colorimetrically. Increases in absorbance levels indicate increased apoptosis. Both positive and negative controls were performed at each time point. This assay has been used to demonstrate apoptosis in mouse osteoclasts by (Orriss *et al.* 2011).

Bradford assay for total protein

Bradford reagent (B6916, Sigma-Aldrich, Gillingham, UK) was used to perform total protein determination in 96-well plate format according to manufacturer's instructions.

Visualisation methods

Western Blotting

Osteoblasts were cultured as outlined previously at pH 7.4 or pH 6.9 for 4, 7, 10 or 14 days. DMEM was removed and cell monolayers washed in PBS; mouse osteoclasts were cultured on dentine for up to 12 days; at days 2, 6, 8 and 12; total protein was extracted from the cells. MEM was removed and dentine discs washed in PBS. The following is the same for both cell types: ice cold radio immunoprecipitation (RIPA) buffer (0.05M Tris HCl pH 7.4, 0.15M NaCl, 1% Triton X 100, 0.1% SDS, 5mM EDTA, 1µl/ml protease inhibitor cocktail (104mM

4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 80µM aprotinin, 4mM bestatin, 1.4mM proteinase inhibitor E-64, 2mM leupeptin,1.5mM pepstatin A), 100µl/ml sodium deoxycholate, 5µl/ml activated sodium vanadate, 10ul/ml phenyl methyl sulfonyl fluoride (PMSF)) was used to lyse the cells and release total cell protein. Following sonication for 5 minutes, cell homogenates were stored at -80ºC until required for blotting. Protein content of the samples was determined using the Bradford assay. 20µg of protein was added per well and before loading these were denatured using reducing buffer (60mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 10% β-mercaptoethanol and 0.1% bromophemol blue) at 100ºC for 5 minutes. Samples were run on a 10% SDS-PAGE resolving gel for one hour at 150V. Samples were then transferred to hydrophobic polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Amersham, UK) using a wet blot tank (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) at 100V for one hour. Following successful protein transfer, the PVDF membrane was blocked with 5% milk in PBS for one hour with gentle agitation then washed in dH_2O. The membrane was then incubated overnight; agitating gently, with the primary antibody diluted in 2% milk solution. The following day, antibody solution was removed and membrane washed three x 15 minutes in 0.05% (v/v) Tween in PBS. The membrane was then incubated with the HRP-conjugated secondary antibody diluted in 1% milk in PBS and incubated for one hour with gentle agitation. Membrane was then washed three x 15 minutes in 0.05% (v/v) Tween in PBS. To visualise the immunoreactivity, Immobilon western chemiluminescent HRP substrate (Millipore (U.K.) Limited, Watford, UK) was used according to the manufacturer's instructions. Following visualisation of chemiluminescence, the membrane was then stained with Ponceau S (0.5% (w/v) Ponceau S, 1% (v/v) acetic acid, 200µM NaOH, 20% (v/v) acetonitrile) for 10 mins at room temperature with shaking, and then rinsed with tap water until clear. The membrane was allowed to air dry.

Anti E-NPP1 antibody was purchased from Abcam (Cambridge, UK) and used at a concentration of 1:500. HRP-conjugated anti-rabbit secondary

antibody diluted to 1:20,000 was purchased from Jackson ImmunoResearch Europe Ltd. (Newmarket, UK).

Anti-TRPV1 primary antibody used at 1:200 (raised in rabbit) was purchased from Alomone Labs Ltd. (Jerusalem, Israel). HRP-conjugated anti-rabbit secondary antibody diluted to 1:30,000 was purchased from Jackson ImmunoResearch Europe Ltd. (Newmarket, UK).

Immunofluorescence

Rat osteoblastic cells were seeded onto 1cm^2 squares of Melinex® clear polyester film (DuPont UK Ltd, Stevenage, UK), in 24-well trays at 2.5 x 10^4 cells/well and cultured in supplemented DMEM for up to 14 days. At required time points, the Melinex® squares were fixed in 3.7% paraformaldehyde in 0.1M phosphate buffer with Triton X 100 for 20 minutes at room temperature washed three x 5minutes in PBS. Each square was incubated with a blocking solution of 10% FCS in PBS containing 0.1% Triton x 100 for one hour. Primary antibody was diluted in 10% FCS in PBS. Melinex® squares were incubated overnight in the primary antibody solution with gentle agitation; negative controls were incubated overnight in 10% FCS in PBS, containing no primary antibody. Following removal of the primary antibody solution, cells were subjected to three further 5-minute washes with PBS before incubation for one hour with the secondary antibody solution and a DAPI counter stain (1:10000), diluted in PBS with 1% FCS. After three further 5-minute PBS washes Melinex® squares were mounted onto microscope slides using Citifluor AF2 solution (Citifluor, London, UK) and viewed under a fluorescence microscope. All images were acquired using identical camera settings to allow comparison of staining intensity.

Murine and human osteoclasts were subject to the same procedure as rat osteoblasts following fixation. Murine osteoclasts were seeded onto Melinex squares at a density of 4 x 10^6 cells/well and cultured in MEM supplemented with 150ng/ml MCSF and three ng/ml RANKL. Murine osteoclasts were fixed on day 10. Human osteoclasts were seeded at 8 x 10^5 cells/well and cultured in

MEM supplemented with 5 ng/ml MCSF and one ng/ml RANKL. Human osteoclasts were fixed on day 10.

Anti E-NPP1 antibody was purchased from Abcam (Cambridge, UK) and used at a concentration of 1:100. Fluorescein isothiocyanate (FITC) labelled rabbit anti-goat secondary antibody (DakoCytomation, Glostrup, Denmark) was used at 1:100.

Anti-TRPV1 primary antibody used at 1:100 (raised in rabbit) was purchased from Alomone Labs Ltd. (Jerusalem, Israel). Donkey anti-rabbit Cy3 labelled secondary antibody used at 1:400 was purchased from Jackson ImmunoResearch Europe Ltd. (Newmarket, UK).

Quinacrine staining

Quinacrine is an acridine derivative and weak base that binds ATP with high affinity. When excited by light at 476nm it fluoresces between 500 and 540nm. Quinacrine has been used to stain ATP in sub-cellular compartments (IRVIN & IRVIN 1954; Olson *et al.* 1976). Osteoblasts were cultured on Melinex polyester film, cut into $1cm^2$ pieces in 24-well trays at 2.5×10^4 cells in supplemented DMEM, pH adjusted to 6.9 with an addition of 10meq/l H^+ as HCl, with a half medium change at day three. DMEM was removed and cells twice washed with PBS. To visualise ATP, 30µM quinacrine in DMEM at pH 7.4 or 6.9 was added for one hour to investigate the effect of acid on ATP release. Melinex pieces were then mounted on a slide and visualised using fluorescence microscopy with a digital camera attachment (AxioCam MRc5, Imaging Associates Ltd., Bicester, UK). This method has been described previously (Orriss *et al.* 2009).

Toluidine blue staining of cancer cells

Toluidine blue stock solution (1% (w/v) toluidine blue in dH_2O, 1% (w/v) sodium borate) was diluted to 2.5% (v/v) in deionised H_2O (dH_2O) with 1% (w/v) sodium borate. Enough of this solution was added to cover the discs completely and

cells allowed staining for between 5 and 10 minutes. Excess dye was washed with water. Discs were allowed to air dry.

Molecular biology

RNA Isolation and RT-PCR

Osteoclasts or osteoblasts were cultured as described previously and were lysed in TRIZOL solution (Invitrogen, Paisley, UK), and total RNA was extracted according to the manufacturer's instructions. RNA was treated with RNase-free DNase I (Promega UK, Southampton, UK) to eliminate any DNA contamination and was quantified by using NanoDrop 1000 spectrophotometer (LabTech International, Ringmer, UK). cDNA was then generated using Superscript III reverse transcriptase (Invitrogen, Paisley, UK) and oligo $(dT)_{15}$ (Promega UK, Southampton, UK) according to the manufacturer's protocol, using 200ng of RNA per reaction. PCR was performed using GoTaq® Flexi DNA polymerase (Promega UK, Southampton, UK) according to the manufacturer's instructions, with cycles of denaturation at 95^{o}C for 30 seconds, annealing at X^{o}C for 30 seconds; extension was performed at 72^{o}C for 40 seconds. Details of primer sequences and annealing temperatures (X) can be found in appendix I.

Quantitative real-time PCR (qRT-PCR)

Cancer cells were seeded at a density of 10^6 cells per well in a 6-well tray in 3ml of RPMI. Cells were grown to confluence and collected into trizol after three days. RNA extraction and cDNA generation were performed as described previously. 300ng/µl cDNA was amplified using SYBR® Green Jumpstart™ Taq ReadyMix™ (Sigma-Aldrich, Poole, UK) on a Chromo 4 PTC-400 real-time detector (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) according to the manufacturer's instructions with initiation of the reaction (94˚C for 2 minutes) followed by forty cycles of denaturation (94˚C for 15 seconds), annealing (60˚C for 1 minute), elongation (72˚C for 1 minute) and detection. Data were analysed using the Pfaffl method whereby the relative quantification of the gene of

interest is compared to a reference gene (Pfaffl 2001). For all genes investigated, the reference gene used was β actin. All reactions were carried out in triplicate. Primer sequences can be found in appendix I.

Statistical Analysis

Statistics

Statistical comparisons were made using one-way analysis of variance (ANOVA) applying the Bonferroni correction, performed using GraphPad InStat (version 3.06, GraphPad software Inc.). Results are one experiment representative of those performed at least in triplicate. All data are presented as means ± SEM, *n* represents the number of replicates for each single experiment, indicated below each graph.

Chapter 3

Characterisation of the effects of acid on osteoclast formation and survival

Introduction

The effects of acid on osteoclast activation are clear; small drops in pH within the physiological range stimulate osteoclasts to resorb (Arnett & Dempster 1986). This short chapter characterises the effects of acid on osteoclast formation and survival in view of the conflicting reports (Murrills *et al.* 1993; Bushinsky 1995; Meghji *et al.* 2001; Pereverzev *et al.* 2008; Kato & Morita 2011; Ahn *et al.* 2012). A substantial amount of work during the course of this PhD has involved careful control and observation of the pH of cell culture medium and, therefore, of the pH environment of both osteoclasts and osteoblasts. It was observed that for osteoclasts cultured at pH 7.4 for the duration of an experiment (i.e. clamped at normal physiological pH, and therefore not resorbing dentine), there were significantly less osteoclasts when compared to those that had been stimulated to resorb for the final 48 hours of culture (**Fig. 3.1**).

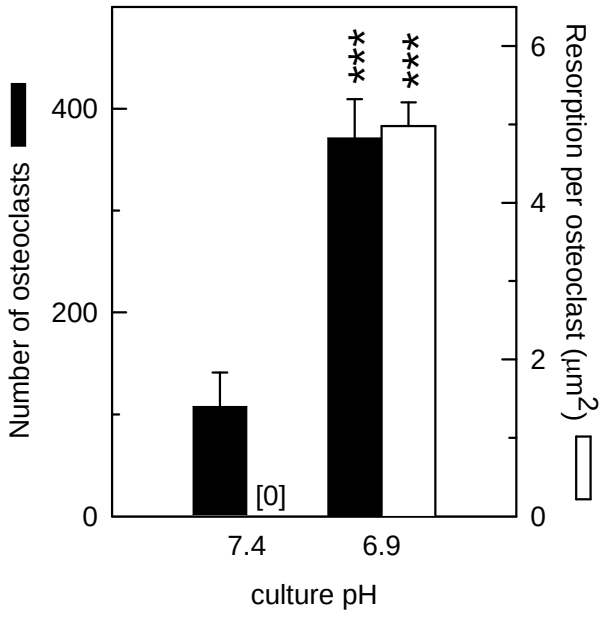


Figure 3.1 *The effect of acid on osteoclast number and resorption*

Murine marrow cells were cultured with M-CSF and RANKL for 7 days at pH 7.4 or for 5 days at pH 7.4 followed by two days at pH 6.9. Acid significantly increased the number of osteoclasts and resorption per osteoclast in comparison to control. (n = 8, *** = p<0.001).

Hypothesis – Exposure to acid will increase osteoclast survival by preventing osteoclast apoptosis.

Results

Number of nuclei per osteoclast is decreased when osteoclasts are cultured at pH 6.9

Osteoclasts were cultured in the presence of M-CSF and RANKL on dentine for 7 days and pH was maintained at either pH 7.4 or pH 6.9 for the duration of the experiment. Osteoclasts cultured in acidic conditions have significantly less nuclei per osteoclast than those cultured at physiological pH (pH 7.4) (**Fig. 3.2**).

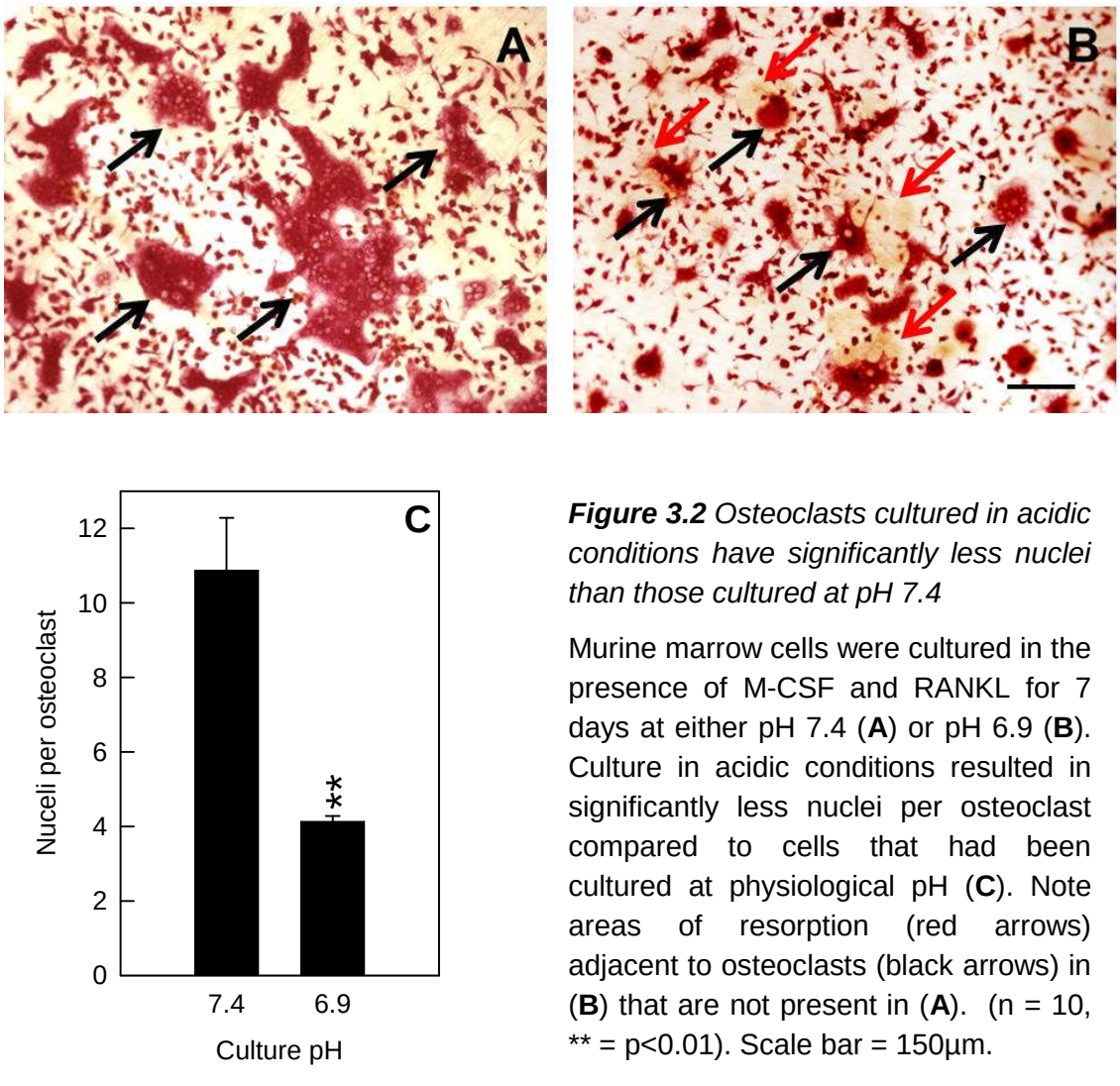


Figure 3.2 *Osteoclasts cultured in acidic conditions have significantly less nuclei than those cultured at pH 7.4*

Murine marrow cells were cultured in the presence of M-CSF and RANKL for 7 days at either pH 7.4 (**A**) or pH 6.9 (**B**). Culture in acidic conditions resulted in significantly less nuclei per osteoclast compared to cells that had been cultured at physiological pH (**C**). Note areas of resorption (red arrows) adjacent to osteoclasts (black arrows) in (**B**) that are not present in (**A**). (n = 10, ** = $p<0.01$). Scale bar = 150µm.

Culture of osteoclasts at acidic pH does not significantly alter the amount of apoptosis which occurs

Osteoclasts were cultured for up to 10 days on plastic and an apoptosis assay performed on these cells at days 4, 6, 8 and 10 of culture. Apoptosis was significantly increased in cells cultured at pH 6.9 compared with pH 7.4 at day 4 only. (**Fig. 3.3**) At all other time points, apoptosis was slightly increased in cells which had been cultured at pH 6.9 throughout or had been transferred from medium at pH 7.4 to pH 6.9 48 hours prior to the assay being performed; these increases, however, were not statistically significant.

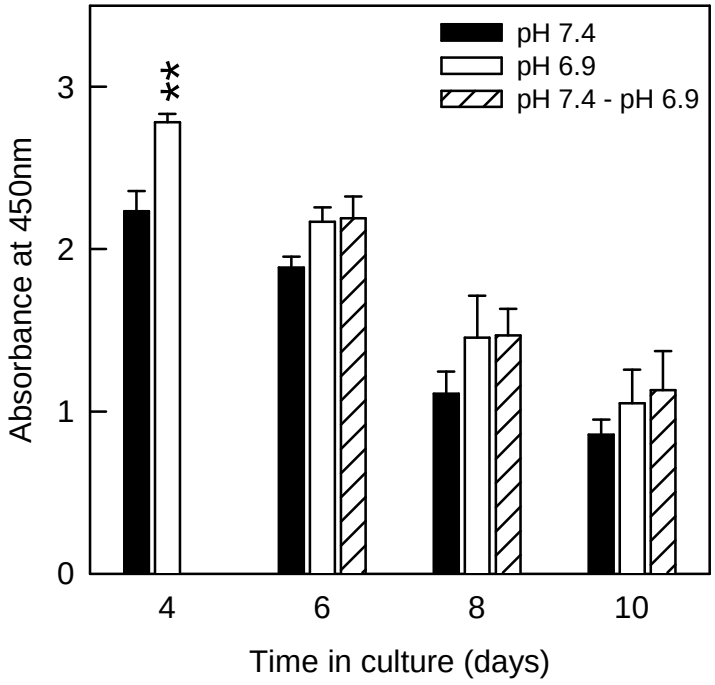


Figure 3.3 *Effect of medium pH on mouse osteoclast apoptosis*

Apoptosis was significantly increased in cells cultured for 4 days at pH 6.9 compared to those at pH 7. Culture of osteoclasts at pH 7.4, pH 6.9 or pH 7.4 then 6.9 for 48 hours did not cause any significant changes in apoptosis at days 6, 8 and 10. Overall apoptosis decreased over time regardless of culture pH (n = 6, ** = $p<0.01$).

Osteoclast numbers are increased when cultured in acidic conditions on dentine, without affecting their viability

Osteoclasts were cultured on either dentine discs or on plastic for up to 9 days in medium adjusted to pH 7.4, pH 6.9 or at pH 7.4 and then adjusted to pH 6.9 48 hours prior to the assay being performed. At all days when cultured on dentine, osteoclast number is increased when osteoclasts were cultured at acidic conditions compared to those at physiological pH whether for the duration of the experiment or for just 48 hours (**Fig. 3.4**). In comparison, osteoclasts cultured on plastic did show the same effect on cell number at day 5 whereby there are significant increases in numbers of osteoclasts when cultured at pH 6.9 for the duration or at pH 7.4 and then pH 6.9 for the 48 hours prior to performing the assay. At days 7 and 9 there were no statistically significant differences observed in numbers of osteoclasts in those cultured on plastic. There were no significant differences in viability at any stage or between the two substrates except at day 5, when cells transferred to pH 6.9 medium for pH 7.4 for 48 hours showed a small but significant decrease in viability. Additionally, there were no differences in viability between the cells when cultured on dentine versus plastic.

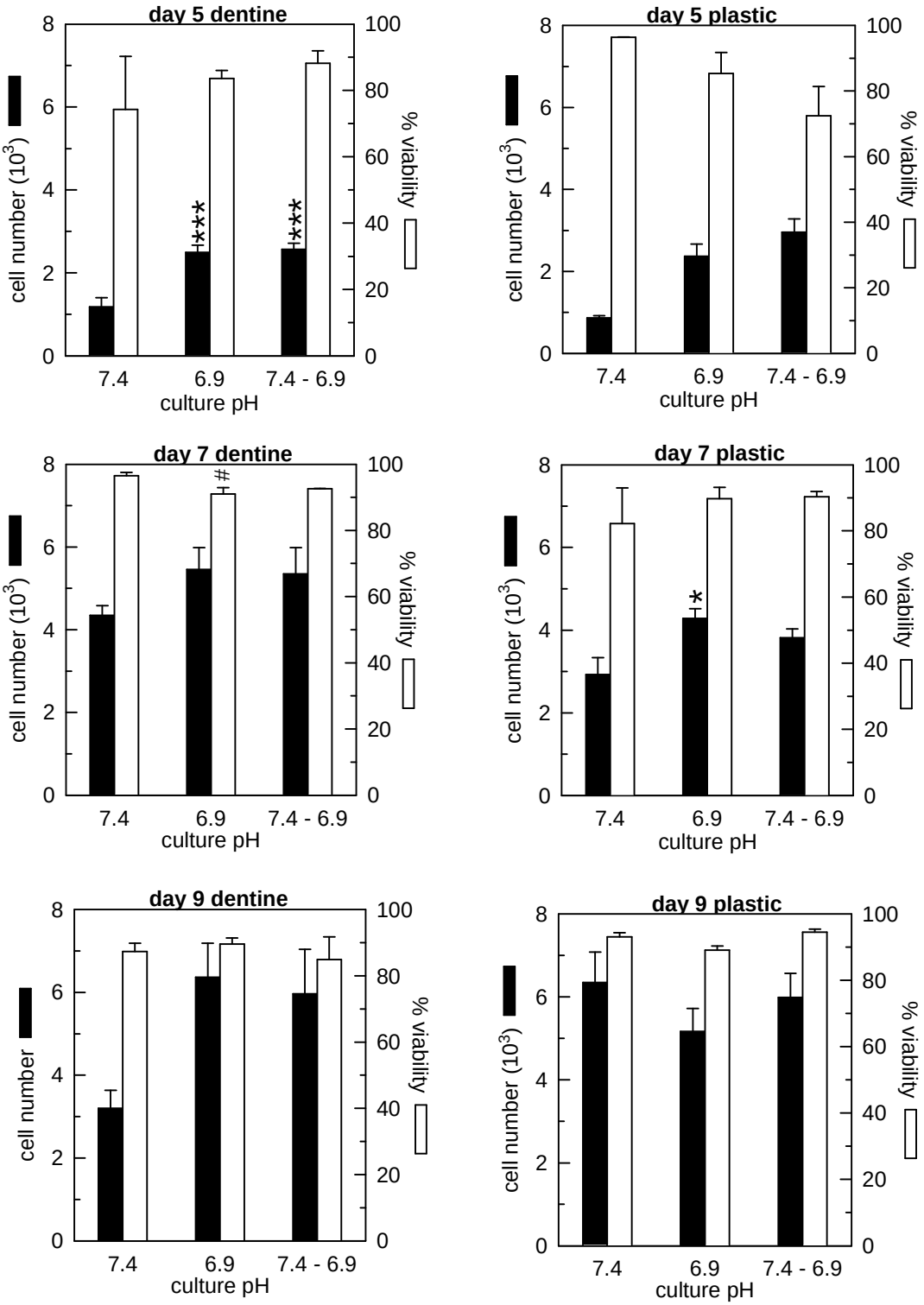


Figure 3.4 *Effect of medium pH on mouse osteoclast viability*

Murine marrow cells were cultured in the presence of M-CSF and RANKL for up to 9 days at pH 7.4, pH 6.9 or pH 7.4 then pH 6.9 for 48 hours prior to assay. Culture for any time period in acidic medium on dentine resulted in increased cell number. On plastic acid resulted in increased cell number at day 5. Viability was only affected in osteoclasts at day 5 when transferred to pH 6.9 for 48 hours prior to the assay being performed. (n = 8, * and # = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$).

Discussion

Over the course of this PhD, it was observed that osteoclasts cultured for the duration of an experiment at pH 7.4 were much larger than those which had been initially clamped at pH 7.4 and then stimulated to resorb by artificially dropping the pH to 6.9 for the final 48 hours of culture. The osteoclasts which had been held at 7.4 did not resorb at all. Additionally, it was observed that there were less osteoclasts at pH 7.4 compared to pH 6.9, both cultures had the same number of osteoclast precursors initially seeded onto dentine discs (**Figs. 3.1, 3.2 A and 3.2 B**).

It has been reported that acidosis promotes osteoclast formation (Kato & Morita 2011), however, the same group reports that these osteoclasts are larger than those cultured at physiological pH which is in contrast to observations made here. I hypothesised that the cause of larger osteoclasts at pH 7.4 was a result of them beginning to undergo apoptosis owing to the cytoplasmic vacuoles that were sometimes present; and are a characteristic of apoptotic cells (Hacker 2000). Apoptosis of osteoclasts at pH 7.4 would also account for the differences in osteoclast number recorded (**Fig. 3.1**). These osteoclasts may have begun to die as the lack of acid resulted in them being inactive as they are not stimulated to resorb and are therefore would not be required in an *in vivo* setting. This is in contrast to those osteoclasts cultured at pH 6.9 or transferred to this medium that were stimulated and 'switched on' to resorb dentine. In order to investigate whether acidosis was preventing osteoclasts from undergoing apoptosis an assay for this was performed.

Owing to the nature of this assay, the osteoclasts had to be grown directly on plastic rather than the dentine substrate, which meant that they were unable to resorb. It was observed that apoptosis was significantly increased in osteoclasts cultured at pH 6.9 compared with those at 7.4 at day 4 of culture. The high levels of apoptosis seen at this stage were likely to be a consequence other cell types dying; this is because these cultures were of primary cells and so are not homogenous. Although the isolation method used allowed for separation of the cell types it is possible for small numbers of other cells to be

present at early stages of culture. As a result these other cells, likely to be stromal cells, and which were not in their optimal growing conditions, died. Also at this stage, pH would have been detrimental to most cell types; hence the greater levels of apoptosis seen at pH 6.9 at the early stages of culture. This is in agreement with data presented by Murrills (1993). It was then observed that apoptosis decreased over the course of the experiment, which was surprising given the observation that when osteoclasts were grown on dentine at pH 7.4, number decreased and sometimes had large vacuoles. It may be that as these osteoclasts were not behaving as they normally would, owing to the fact that they are on plastic rather than dentine. This adds further weight to the argument that osteoclasts should be grown on dentine or an equivalent substrate to allow them to resorb as they would *in vivo*. In order to ascertain the effect of acid on apoptosis in osteoclasts grown on dentine an assay to assess this that allows for osteoclasts to be culture on dentine could be performed in the future.

In order to determine whether growing on plastic rather than dentine was affecting results, an LDH assay to assess cell viability was performed; this assay could be performed on cells grown both on dentine and on plastic. This assay revealed that there were no significant differences in osteoclast viability when grown in acidic conditions compared with normal physiological pH (**Fig. 3.4**), which is broadly in agreement with results from the apoptosis assay (**Fig. 3.3**). Furthermore, no differences in viability were recorded between the two substrates. The substrates did show, however, that there were differences in cell number when osteoclasts were cultured on the more physiologically relevant dentine compared to plastic. This has implications for *in vitro* research as it would indicate that osteoclasts cultured on plastic, in addition to not being to resorb, may not form in the same way as those cultured on dentine. The results presented in this chapter, however, indicate that culture at pH 7.4 does not affect osteoclast viability or induce apoptosis; as a consequence, there must be another process in place to account for the differences in cell number observed.

One likely explanation is that switching osteoclasts to acidic culture medium prevents osteoclast fusion. Osteoclasts cultured at pH 7.4 are not 'switched on' and so do not resorb, therefore they may continue the fusion process. This will generate large cells and therefore decrease the overall cell number, as more precursor cells progressively fuse to become less and less osteoclasts. Osteoclast activation to resorb involves substantial changes to the osteoclast structure and cytoskeleton, observed as polarisation and formation of a ruffled border (Crockett *et al.* 2011) therefore osteoclasts at this point may be unable to fuse to any more cells. This provides an area for further research and regulation of the recently discovered osteoclast fusion molecule DC-STAMP (Miyamoto 2006) may be an opportune place to begin.

Furthermore, this work reinforces the role of acid as the osteoclast activation factor, rather than RANKL as proposed by (Boyle *et al.* 2003). It has already been demonstrated that when osteoclasts are treated with RANKL at physiological pH, they barely resorb, however, treatment with the same amount of RANKL at pH 6.9 results in a 20-fold increase in resorption (Orriss & Arnett 2012). Osteoclasts cultured for the work presented in this chapter were treated with the same amount of RANKL regardless of pH, but osteoclastic resorption occurred when the pH had dropped to approximately 6.9.

Chapter 4

Osteoblasts and the regulatory role of acid

Introduction

As previously outlined, acidosis has an overall detrimental effect on the skeleton. Previous work has shown that acidosis (pH 6.9) prevents bone nodule formation *in vitro* by inhibiting mineralisation of the collagenous matrix; this was partly explained by dissolution of the mineral in a physico-chemical effect (Brandao-Burch *et al.* 2005a). However, in the bone microenvironment most of the phosphate required for incorporation into hydroxyapatite is generated by the actions of alkaline phosphatase; when osteoblasts were cultured at pH 6.9, alkaline phosphatase activity was reduced by approximately 80% compared to those cultured at pH 7.37(Brandao-Burch *et al.* 2005b); therefore acidosis also appears to be causing a cell-mediated negative effect on mineral deposition. The mechanism by which acid prevented mineralisation, however, was unknown and elucidating this mechanism was the focus of the work presented in this chapter.

Hypothesis – Acidosis will cause up-regulation of E-NPP1, therefore resulting in increased pyrophosphate production and so decreased mineralisation of the collagenous matrix.

Results

Acidosis inhibits mineralisation of the collagenous matrix by osteoblasts

Long term culture (14 days) of primary rat osteoblasts in acidotic medium (pH 6.9) prevents mineralisation of the collagenous matrix as demonstrated by lack of alizarin red staining for calcium (**Figs. 4.1C and 4.1D**) compared to osteoblasts cultured in medium at physiological pH (pH 7.4) (**Figs 4.1A and 4.1B**).

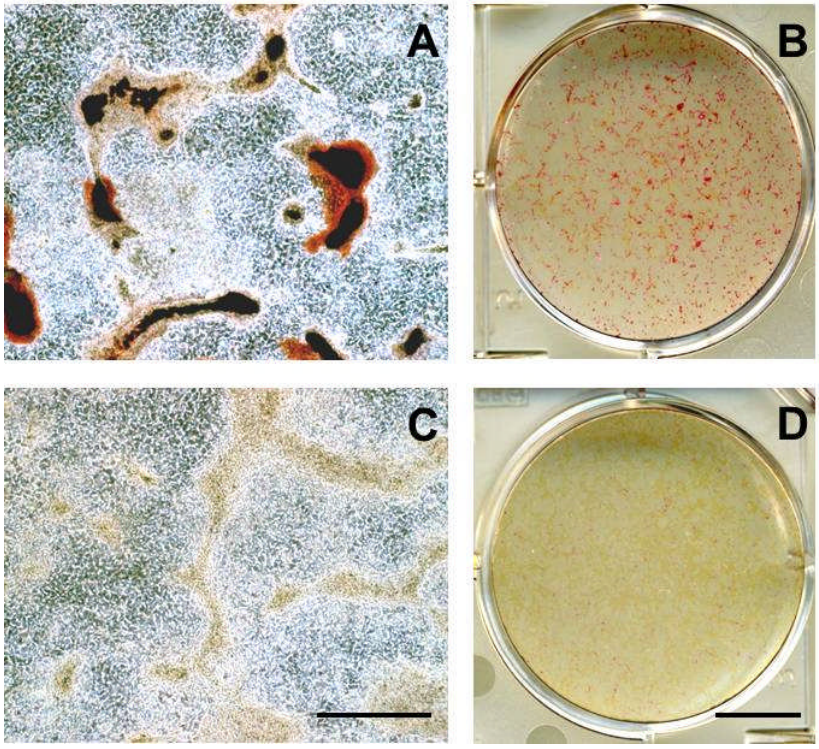


Figure 4.1 *Acid inhibits mineralisation by primary rat osteoblasts*

Osteoblasts were cultured at pH 6.9 or pH 7.4 for 14 days. Low power scans demonstrate a lack of alizarin red stained bone nodules at pH 6.9 (**C**) compared to pH 7.4 (**A**). Phase contrast micrographs (**B**) indicate the presence of collagenous matrix but absence of alizarin red staining at pH 6.9 (**D**) indicating that mineralisation did not occur. Scale bars: tissue culture wells = 8.75mm, phase contrast micrographs = 800μm.

Osteoblasts express numerous E-NPPs and E-NTPdases but only E-NPP1 is up-regulated in osteoblasts cultured at pH 6.9

Osteoblasts were cultured for 14 days at pH 7.4 or pH 6.9. mRNA and protein samples were taken at days 4, 7, 10 and 14 of culture. RT-PCR demonstrated the presence of *E-npp1, E-npp2 and E-npp3* and the *E-ntpdases 2-6*. Of these, *E-npp1* was up-regulated at all stages of differentiation when cells had been cultured in acidosis compared to control (**Fig. 4.2**). Further analysis of E-NPP1 by Western blotting and immunocytochemistry revealed that protein levels of E-NPP1 are also increased when cells had been cultured in acid conditions (**Fig. 4.2**). Multiple bands present are the result of different post-translational modifications.

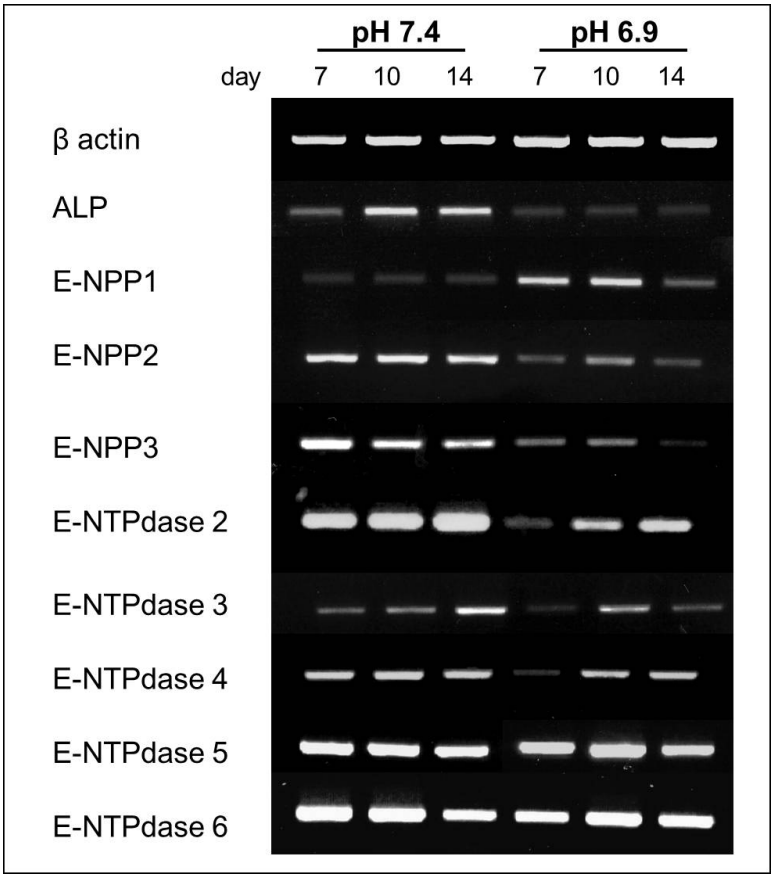


Figure 4.2 *The effect of acid on expression of ecto-nucleotidases*

RT-PCR revealed that expression of mRNA for E-NPP1 is up-regulated when osteoblasts were cultured at pH 6.9. Alkaline phosphatase, E-NPP2, E-NPP3 and E-NTPdase 2 mRNA expression was decreased in acid. Expression of mRNA for E-NTPdase 3–6 showed no difference between the two pH conditions.

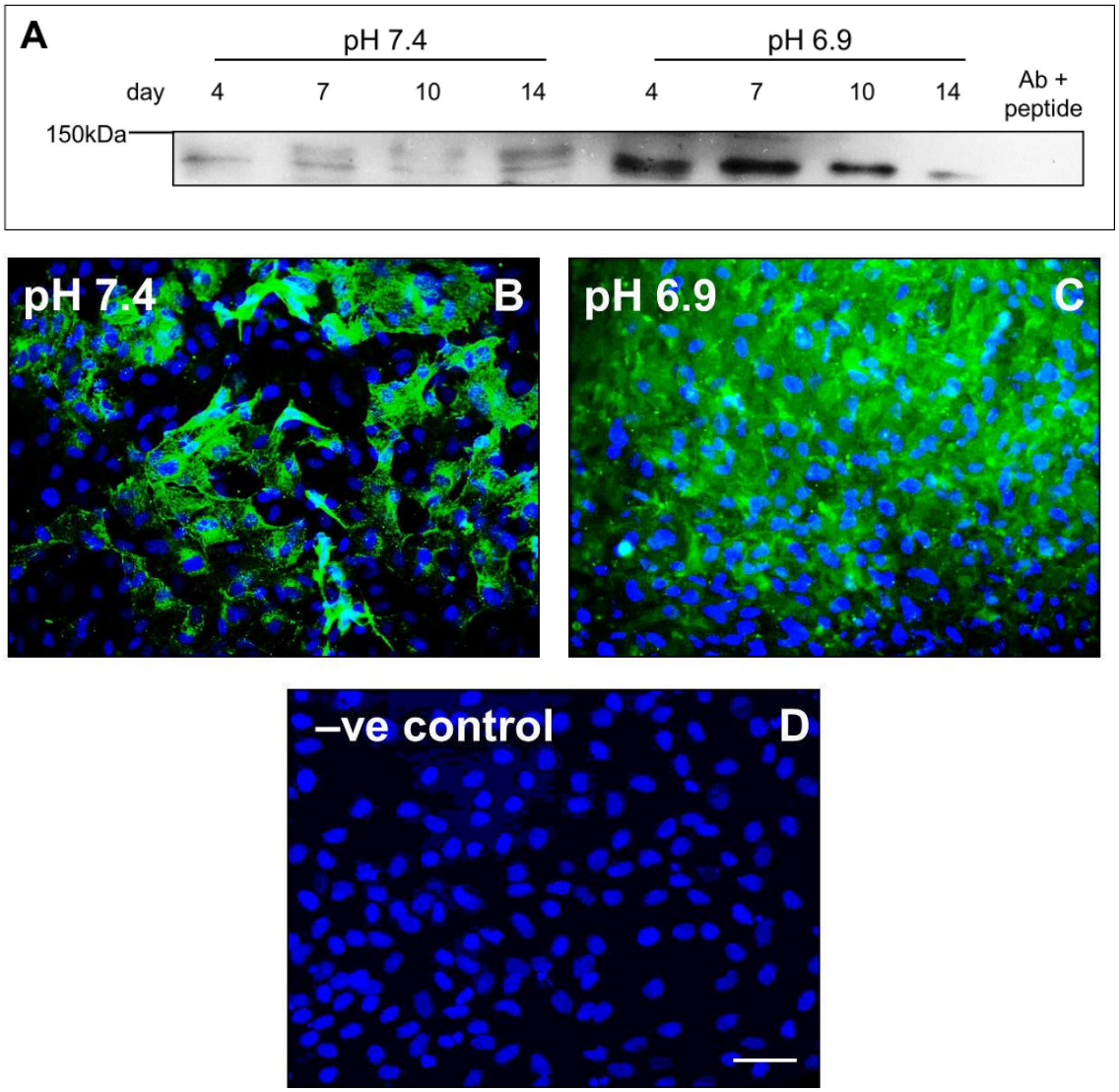


Figure 4.3 *Expression of E-NPP1 protein by osteoblasts cultured at pH 6.9 and pH 7.4*

Western blotting (**A**) demonstrated that E-NPP1 expression was increased at all stages of differentiation when osteoblasts were cultured at pH 6.9 compared with pH 7.4. Immunocytochemistry at day 7 showed increased E-NPP1 expression (green) at pH 6.9 (**C**) compared to pH 7.4 (**B**). DAPI staining of cell nulcei is blue. Negative control = (**D**). Scale bar = 50µm.

Total E-NPP activity is significantly increased at days 7 and 10 of culture in osteoblasts cultured at pH 6.9

An enzyme assay was performed to determine total E-NPP activity in osteoblasts cultured at pH 6.9 compared to pH 7.4 at days 4, 7, 10 and 14 days of culture. Total E-NPP activity was increased at all time points, but significantly at days 7 and 10 (**Fig. 4.4**).

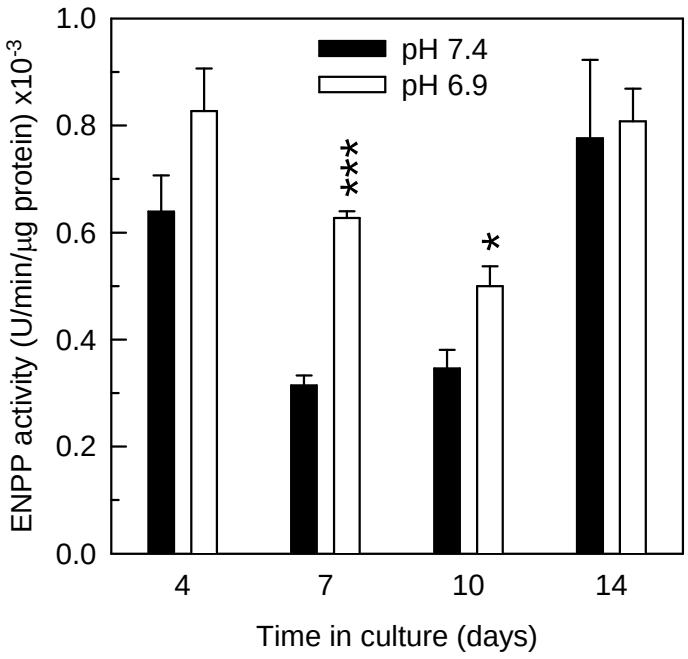


Figure 4.4 *Total E-NPP activity is increased in osteoblasts cultured at pH 6.9*

Osteoblasts show increased E-NPP activity when cultured at pH 6.9 at days 4, 7, 10 and 14. E-NPP activity is increased by 50% at day 7 and 1.4-fold at day 10 at pH 6.9 compared with pH 7.4 (*n* = 6, *** = p <0.001, * = p<0.05).

Exposure to acidotic medium effects phosphate and pyrophosphate generation by osteoblasts

Osteoblasts were either cultured at pH 7.4 for the duration of the experiment and then transferred to pH 6.9 one hour prior to the assays being performed or were cultured for the entirety at pH 6.9. In both cases a control group at pH 7.4 was treated in the same way. Short term exposure resulted in significantly increased generation of phosphate but no significant change in pyrophosphate generation (**Fig. 4.5 A**). Long term culture at pH 6.9 caused significant increases in both phosphate and pyrophosphate generation (**Fig. 4.5 B**).

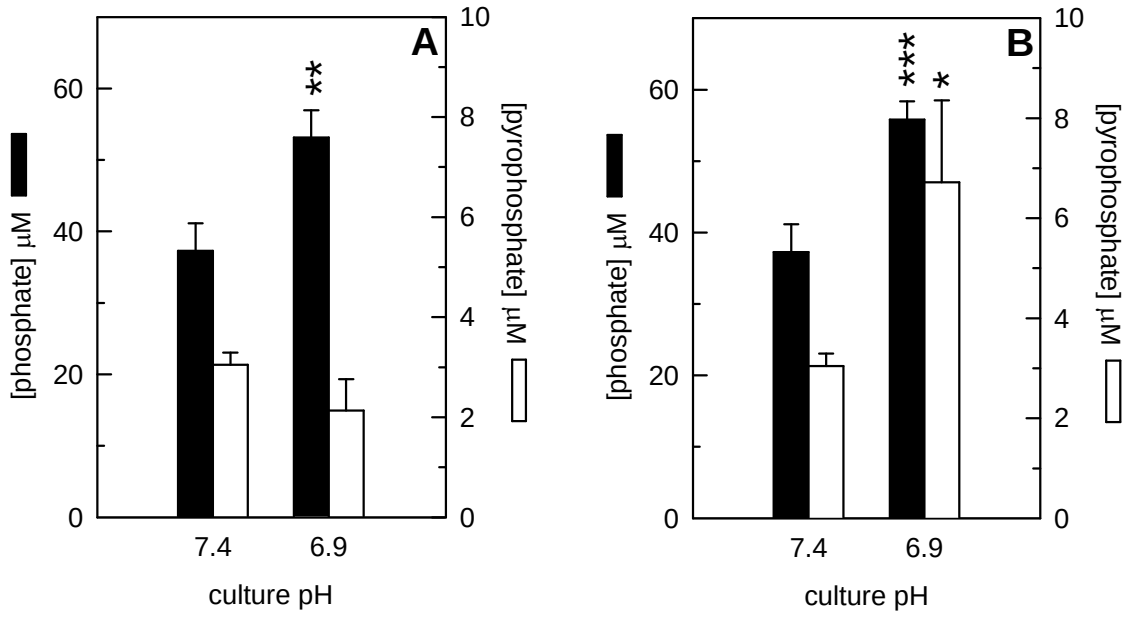


Figure 4.5 *Exposure to short term and long term acidosis affects phosphate and pyrophosphate generation by primary rat osteoblasts*

Osteoblasts were cultured for 10 days at pH 7.4 and then transferred to pH 7.4 or pH 6.9 one hour (**A**). The amount of phosphate generated was significantly increased, but pyrophosphate generation was reduced slightly. When osteoblasts were cultured for 14 days at either pH 7.4 or pH 6.9 (**B**), there were significant increases in amounts of both phosphate and pyrophosphate generated. (*n* = 12, *** = p <0.001, ** = p<0.01 * = p<0.05).

Acidosis does not affect ATP release by osteoblasts in vitro

ATP is a known inhibitor of mineralisation (**Fig 1.3**) (Orriss *et al.* 2007) and is released by osteoblasts following exposure to stress; therefore it was hypothesized that acidosis might stimulate ATP release by osteoblasts which may in turn inhibit mineralisation. Osteoblasts were cultured for up to 14 days at pH 7.4 or pH 6.9, a luminescent assay to detect ATP release was performed at days 4, 7, 10 and 14 of culture. ATP release increased with osteoblast differentiation, however, there was no significant differences in ATP release between the two pH groups (**Fig. 4.6**). Quinacrine staining also showed no differences in ATP containing vesicles in osteoblasts at pH 6.9 compared with pH 7.4.

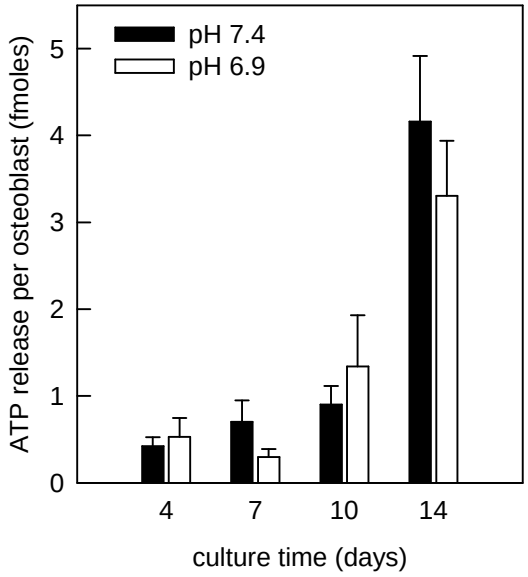


Figure 4.6 *Acidosis does not affect ATP release by osteoblasts*

Osteoblasts were cultured for between 4 and 14 days at either pH 7.4 or pH 6.9. ATP release was measured using a luminometer. Acidosis did not result in any significant changes in ATP release at any stage of culture. Cell viability assays were performed alongside. (*n* = 12).

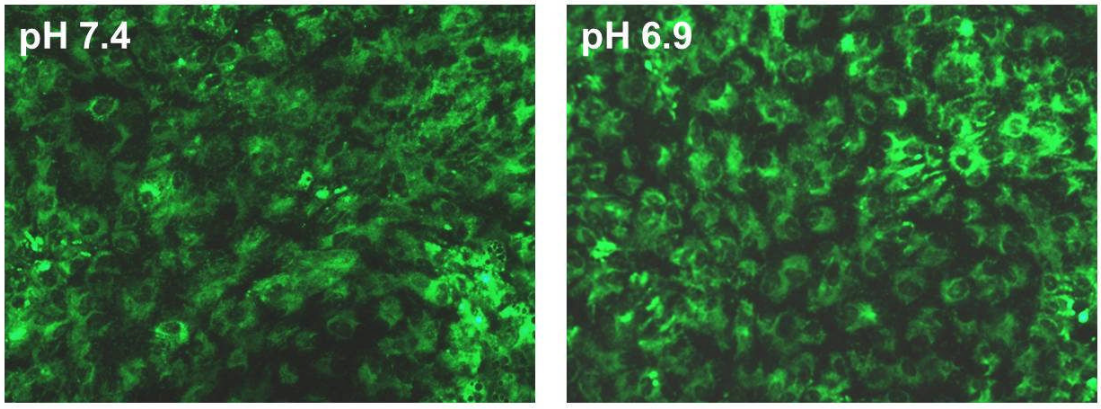


Figure 4.7 *Quinacrine staining of osteoblasts*

Osteoblasts were cultured for 10 days at either pH 7.4 or pH 6.9, they were then transferred to DMEM containing the ATP-binding dye quinacrine, adjusted to the correct pH for one hour before visualising. These images show no differences in quinacrine binding to ATP between the two pH values.

Discussion

The deleterious effect of acid on the skeleton has been known for almost 100 years (Goto 1918). The skeleton provides a large store of base to buffer excess acid that occurs in the body as a consequence of metabolic acidosis. Metabolic acidosis is caused by kidney disease, respiratory acidosis by chronic respiratory disease. Systemic acidosis can result from: a high protein diet, ingestion of inorganic acid, aging and the menopause (Arnett 2010). More localised acidosis occurs owing to reduced vascular supply, which is in turn a consequence of inflammation, infection, tumours, diabetes and aging.

In addition to buffering these body-wide effects, it was observed that acid had a direct effect on osteoblasts whereby acid prevented mineralisation of the deposited collagenous matrix (**Fig. 4.1**). This effect is not simply a physico-chemical instance of acid dissolving mineral (Brandao-Burch 2005a).

Mineralisation of collagenous bone matrix is regulated by the ratio of phosphate to pyrophosphate within the bone microenvironment; inorganic phosphate (Pi) is required as a component of the hydroxyapatite that comprises the mineral matrix. Pyrophosphate (PPi) is a potent inhibitor of mineralisation

(Fleish & Neuman 1961; Thouverey *et al.* 2009). Several enzymes produce either Pi or PPi from nucleotides and these were investigated as potential targets of the action of acid. Of the family of E-NPP and E-NTPdases tested, the only enzyme up-regulated in osteoblasts grown in acidic conditions was E-NPP1; this enzyme has also been reported to be the most highly expressed of this family of enzymes in bone (Vaingankar *et al.* 2004). E-NPP1 has already been shown to have a role in the regulation of mineralisation within the body; E-NPP1 deficiency results in infantile arterial calcification (Rutsch *et al.* 2003)

The disease is characterised by calcification of muscular arteries, and their abnormal narrowing owing to migration of cells from the muscle layer of the arterial wall migrating to a sub-endothelial site and 'organising' deposits on the vessel wall (Rutsch *et al.* 2003). Of twelve individuals identified with this disease, all had mutations that resulted in inactive E-NPP1. Seven of these individuals died within 45 days of birth, one died at 6 months of age, the remaining four were still alive at two, three, 6 and 16 years of age (Rutsch *et al.* 2003). This analysis provides evidence that E-NPP1 is responsible for generating PPi in order to maintain the Pi/PPi at levels to prevent mineralisation of the soft tissue.

E-NPP1 protein was shown to be up-regulated at all stages of osteoblast differentiation. In addition, total E-NPP activity was also up-regulated at all stages of differentiation, though only significantly at days 7 and 10 but, *in vitro*, this would be the stage at which osteoblasts are mineralising. *E-npp1* expression appears to peak and then drop in mature osteoblasts, other *E-npps* and *E-ntpdases* are either unaffected by stage of differentiation or later stages of differentiation result in higher levels of gene expression, thus potentially explaining the lack of significant effect in total E-NPP activity seen at day 14. To date there is not a commercially available assay to determine E-NPP1 activity alone, should one become available, this would provide a useful addition to this work.

As E-NPP1 hydrolyses nucleotide triphosphates to generate a nucleotide monophosphate and PPi, a logical deduction would be that acid would cause an

increase in pyrophosphate generation and a decrease in phosphate generation; however, results presented show something different, when exposed to acidic medium for a period of one hour, there is no significant change in pyrophosphate levels but phosphate generation is significantly increased by approximately 40%. In osteoblasts cultured long term at pH 6.9, phosphate generation is again increased by a similar amount; however, pyrophosphate is also significantly increased, by 54%.

Where osteoblasts have been cultured for an extended period of time at pH 6.9, pyrophosphate is increased as anticipated, this could account for the decreased mineralisation observed. The increase in phosphate generation however, does not appear to fit in with current understanding of the control of mineralisation. It could be that there is a feedback mechanism at work whereby the osteoblasts detect the extracellular acid and consequently generate phosphate to buffer the decrease in pH. However, previous work has shown that in osteoblasts cultured in acidic medium, alkaline phosphatase activity is reduced (Brandao-Burch et al. 2005b), so that would imply the increases in phosphate generation seen here are not due to alkaline phosphatase activity, but rather another enzyme responsible for phosphate generation. RT-PCR presented here has shown that E-NTPdase 5 and E-NTPdase 6 expression (**Fig. 4.1**) is not affected by growing osteoblasts in culture that is slightly acidic. these enzymes, however are currently thought to only be localised intracellularly (Yegutkin 2008) so are unlikely to have a role in Pi generation in the extra cellular environment.

The phosphate generating enzyme PHOSPHO 1 could be a potential candidate responsible for the increased phosphate generation. The enzyme has already been implicated in generating phosphate for matrix mineralisation (Ciancaglini *et al.* 2010); *Phospho1*$^{-}$/$^{-}$ animals have been generated, they are smaller than age matched wildtype controls, show growth retardation and have shorter bones (Yadav *et al.* 2011). These animals eat and drink less than wildtype littermates and show reduced chewing, thought to be due to soft jaw bones and incorrectly developed teeth. Thoracic scoliosis was present in

approximately 30% of animals at day 10 and in 100% when they had reached one month in age. This deformity progressively worsened. Histological analysis demonstrated reduced alizarin red staining, indicative of a reduction in mineralisation of the trabeculae. The femurs of these animals had increased cortical porosity and decreased cortical thickness (Yadav *et al.* 2011).

These observations indicate that PHOSPHO1 has an important role in mineralisation of the bone matrix. The pH optimum for this enzyme is reported to be pH 6.7. (Roberts *et al.* 2004). PHOSPHO1 actually has two types of activity: phosphocholine activity and phosphoethanolamine activity. Each has separate pH dependent activity, however, both peak at pH 6.7. Interestingly, the activity of PHOSPHO1 drops at pH levels above 7.2 (Roberts *et al.* 2004), therefore suggesting that when pH is at normal physiological levels, activity is not as important as at more acidic pHs. As the pH optimum of PHOSPHO 1 is pH 6.7, it may have an important role as a homeostatic regulator of Pi generation.

It is known that at acidic pH, alkaline phosphatase decreases (Brandao-Burch *et al.* 2005b) resulting in decreased production of phosphate; E-NPP1 activity increases, and in theory should result in an increase in pyrophosphate production. Pi and PPi assays, however, have not given the results expected, so potentially, PHOSPHO 1 may function to correct the lack of phosphate as the osteoblasts are able to detect that mineralisation is *not* occurring and this may result in increased phosphate generation via PHOSPHO 1 to provide Pi for incorporation into the hydroxyapatite. This provides a potential area for further investigation.

Another protein that may be worth investigating is progressive ankylosis protein (ANK). ANK spans the cell membrane and transports PPi from within the cell to the extracellular space, it regulates the levels of PPi both intra- and extra-cellularly, where it contributes to the ratio of Pi/PPi (Ho *et al.* 2000). Although E-NPP1 is responsible for the majority of PPi in the extracellular bone microenvironment, ANK does contribute to this store (Johnson *et al.* 2003). The effect of acid on expression of this protein may account for the PPi levels being

lower than expected if acid down-regulates expression. As PPi levels are lower than anticipated I would hypothesise that ANK is down-regulated in acidotic conditions and therefore less PPi is channelled into the extracellular space.

Our laboratory recently acquired *E-npp1*$^{-/-}$ mice and investigating the responses to acid of osteoblasts from these animals may provide a clearer idea of the exact role of E-NPP1 in the striking absence of matrix mineralisation owing to acidosis. Work presented recently demonstrated that *E-npp1* knockout animals have reduced resistance to bending, hypomineralisation of the long bones, reduced trabecular number at both 6 and 22 weeks of age, a decreased percentage bone volume to tissue volume (%BV/TV) ratio, an increased cortical thickness but increased bone mineral density. The researchers propose that this paradox is due to the smaller size of the *E-npp1* deficient animals compared to the wildtype animals. Osteocalcin is decreased when animals are 6 weeks of age but by 22 weeks levels are comparable. The resorption marker C-terminal telopeptide (CTx) is the same at 6 weeks but by 22 weeks is significantly reduced. Alkaline phosphatase is increased, as are levels of the phosphate regulator FGF23 (Mackenzie *et al.* 2011). Preliminary micro X-ray computed tomography (micro-CT) scans from our laboratory of spines from *E-npp1* knockout animals compared with wildtype show ectopic mineralisation of the intervertebral discs (unpublished data).

Adenosine triphosphate (ATP) is well documented as an inhibitor of mineralisation (Boskey *et al.* 1992; Zhang *et al.* 2005; Orriss *et al.* 2007). Cell stress has been reported to cause ATP release for example, hypoxia causes ATP release from primary rat osteoblasts (Orriss *et al.* 2009) and fluid shear stress has been reported to cause ATP release from MC3T3-E1 mouse-derived osteoblast-like cells (Genetos *et al.* 2005). Additionally, mechanical loading of the human osteoblastic cell line Te85 and the human osteosarcoma cell line with osteoblastic properties, SaOS-2, has been reported to increase ATP release from these cells (Rumney *et al.* 2008a; Rumney *et al.* 2008b).

As a consequence, it was investigated whether acidosis could cause ATP release from osteoblasts; this ATP could then act upon the very

osteoblasts it was released from in a feedback loop via P2 receptors to inhibit bone formation (Orriss *et al.* 2007). Results presented here, however, show that osteoblasts exposed to acidosis within the physiological range demonstrate no changes in ATP release and, thus, the decreased mineralisation observed under acidotic condition is not due to increases in ATP release. Certainly more acidic conditions may result in ATP release from osteoblasts but levels of acid needed will be on at least near the borderline of being physiologically relevant and so this has not been investigated further.

There are several animal models of pathological mineralisation states and genes and proteins implicated in these processes may also be affected by changes in pH. The focus of future work could move to these.

The actions of E-NPP1, TNAP, PHOSPHO 1 and ANK are intrinsically linked. In order to understand the exact mechanism by which acid inhibits mineralisation, the actions of both PHOSPHO 1 and ANK under acidic conditions need to be determined in addition to those of E-NPP1 and TNAP.

Chapter 5

TRP channels and bone cell function

Introduction

The mechanisms by which small pH drops within the normal physiological range are detected and transduced to the cells are still unknown. The TRP group of ion channels have been identified as potential sensors of changes in pH by bone cells. The aims of this chapter were to determine the roles of TRPV1 and other TRP channels in mediating the responses of osteoclasts and osteoblasts to the stimuli of extracellular pH.

Hypothesis – TRPV1 will act as the acid-sensor in osteoclasts and osteoblasts, demonstrated by the TRPV1 agonist, capsaicin, (1) stimulating osteoclastic resorption at physiological pH; (2) preventing matrix mineralisation by osteoblasts cultured at pH 7.4. Osteoclasts isolated from *TRPV1*$^{-/-}$ will not resorb when culture medium is acidified.

Results

TRPV1 protein is expressed by mouse and human osteoclasts

TRPV1 expression on osteoclasts was investigated using immunofluorescence. Clear expression of TRPV1 protein was evident on both mouse (**Fig. 5.1 A**) and human (**Fig. 5.1 C**) osteoclasts.

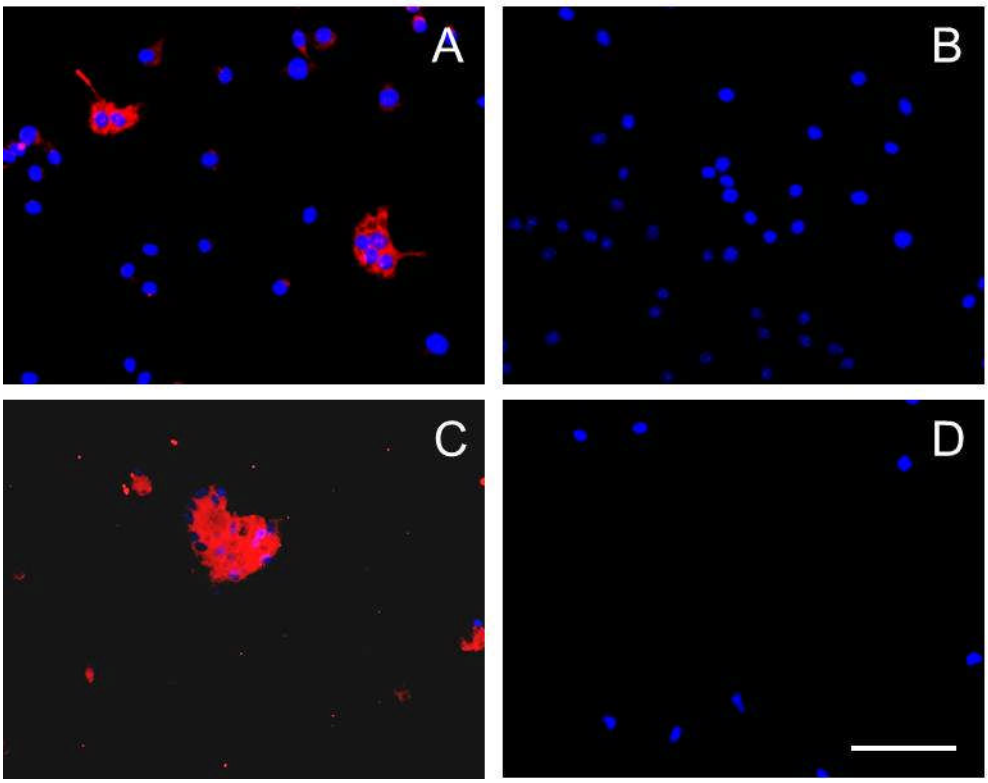


Figure 5.1 *TRPV1 expression by mouse and human osteoclasts*

TRPV1 expression in osteoclasts was studied by immunofluorescence using specific primary polyclonal antibodies, a Cy3-labelled anti-rabbit secondary antibody (red) and DAPI nuclear stain (blue). The image in (**A**) shows TRPV1 expression on a mouse osteoclast at day 10 of culture and (**B**) the negative control. (**C**) Human osteoclasts also express TRPV1 channels. (**D**) Negative control image. Scale bar = 150μm.

TRPV1 protein is up-regulated in mouse osteoclasts cultured at pH 6.9

Mouse osteoclasts were grown at pH 7.4 continuously or pH 7.4 and then pH 6.9 for the 48 hours prior to protein collection. Western blotting demonstrated an increase in amount of TRPV1 detected in osteoclasts that were subject to pH 6.9 medium (**Fig. 5.2**).

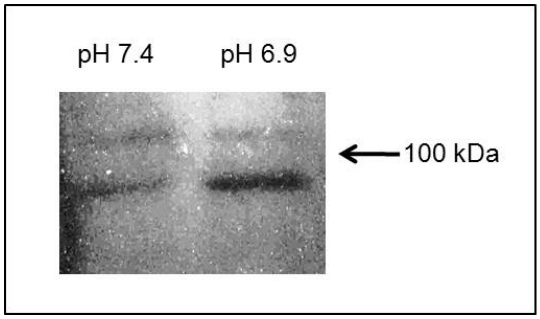


Figure 5.2 *TRPV1 protein expression is increased in mouse osteoclasts cultured at pH 6.9 compared to pH 7.4*

Mouse osteoclasts were cultured for either 12 days at pH 7.4 or 10 days at pH 7.4 followed by two days at pH 6.9. Western blotting detected the TRPV1 protein which, has a size of approximately 100kDa, is up-regulated when osteoclasts were acid activated.

Capsaicin does not affect mouse osteoclast formation or resorption

Osteoclasts were grown for 7 days at pH 7.4 and then 7.4, 7.2 or 6.9 for the final 48 hours. To determine capsaicin's effect on osteoclast formation, capsaicin at concentrations of 5nM–5µM were added at the same time as RANKL. In order to determine effects on osteoclast resorption, capsaicin was added at the same time as acidification. If TRPV1 was the acid sensor, agonists of this channel would be expected to mimic the effects of acid at physiological pH. Capsaicin had no effect on formation of osteoclasts or osteoclastic resorption in contrast to the profound stimulatory effects of acid (**Figs. 5.3 and 5.4**).

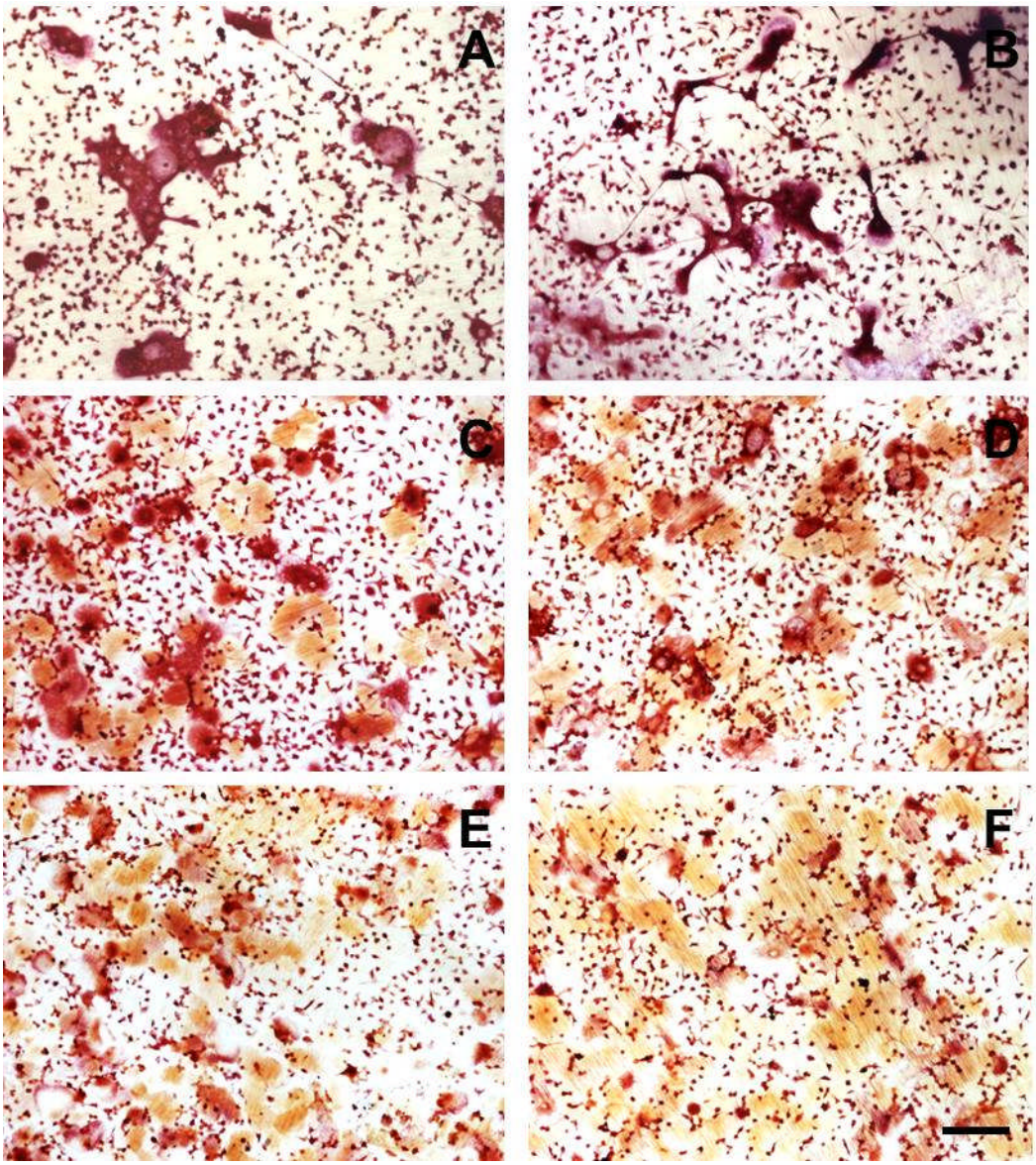


Figure 5.3 *Capsaicin does not affect osteoclast formation or activation*

Osteoclasts were cultured for 8 days at pH 7.4, for the final 48 hours pH was either kept at pH 7.40 or adjusted to 7.23 or 6.96. To investigate the effect of capsaicin on osteoclast activation, capsaicin was added for the final 48 hours of culture at concentrations of 5nM–5μM (ethanol vehicle was added to control groups); capsaicin had no effect on osteoclast activation when compared to control groups. (**A**) pH 7.40 control, (**B**) pH 7.40 5μM capsaicin, (**C**) pH 7.23 control, (**D**) pH 7.23 5μM capsaicin, (**E**) pH 6.96 control, (**F**) pH 6.96 5μM capsaicin. (Red/purple = TRAP stained osteoclasts, orange = resorption pits/trails.) Scale bar = 150μm.

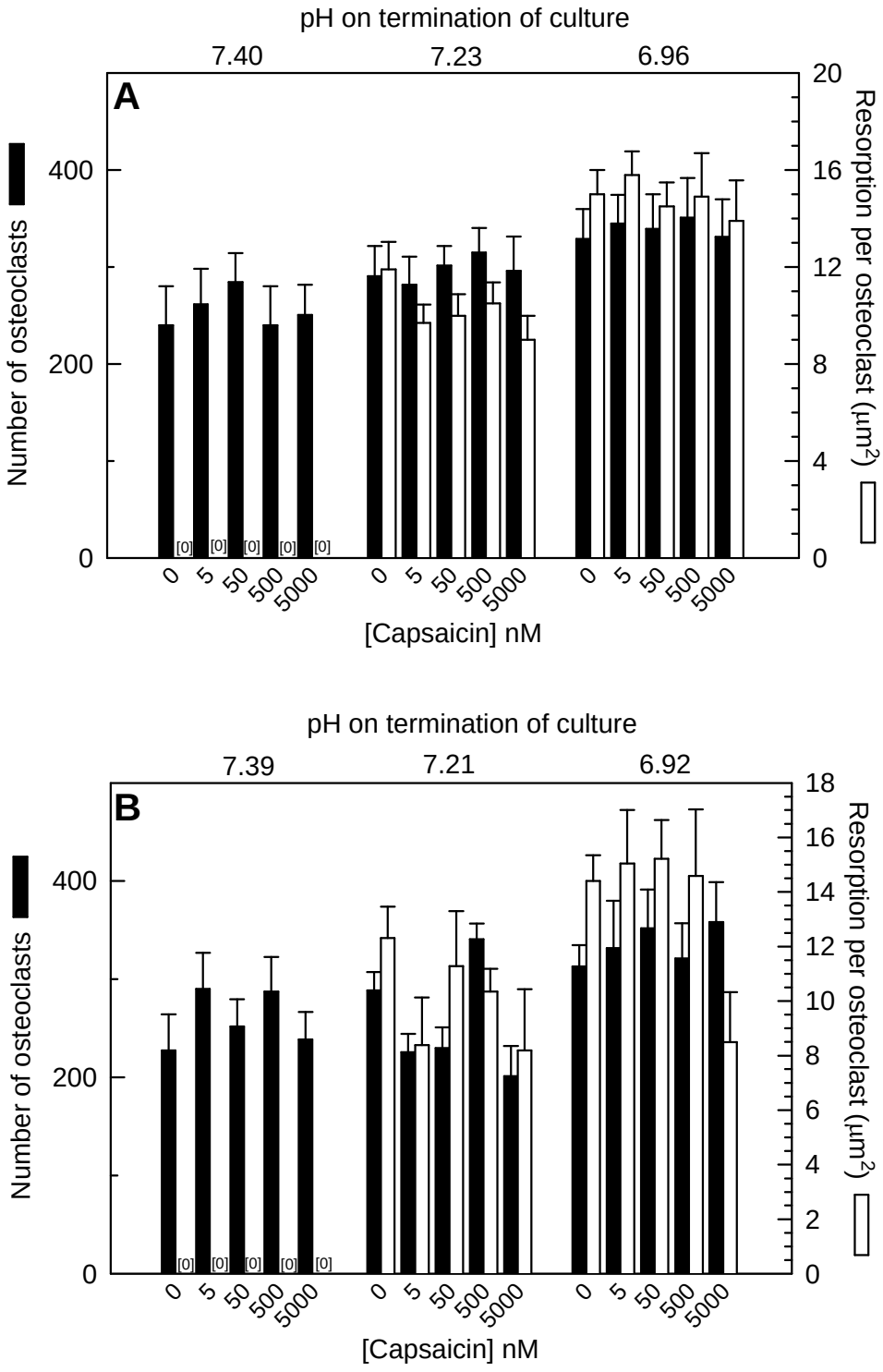


Figure 5.4 *Lack of effect of capsaicin on osteoclast formation or activation*

Osteoclasts were cultured for 8 days at pH 7.4, then at pH 7.4, 7.2 or 6.9 for the final 48 hours. Capsaicin was added at 5nm-5µM, at the same time as RANKL to determine effects on osteoclast formation, or 48 hours prior to termination to determine effects on resorption. Capsaicin had no effect on the number of osteoclasts formed (**A**) or on osteoclast activation (**B**). (*n* = 8).

TRPV1$^{-/-}$ osteoclasts show no differences in their ability to resorb dentine when compared to wildtype osteoclasts

In order to ascertain whether TRPV1 was the 'acid sensor' in bone cells, knockout osteoclasts were cultured to determine responses to acid. *Trpv1$^{-/-}$* mouse marrow was kindly donated by Prof. Bernd Nilius (Catholic University of Leuven, Belgium) and results show that resorption per osteoclast was comparable to wildtype osteoclasts at pH 6.9 (**Fig. 5.5**).

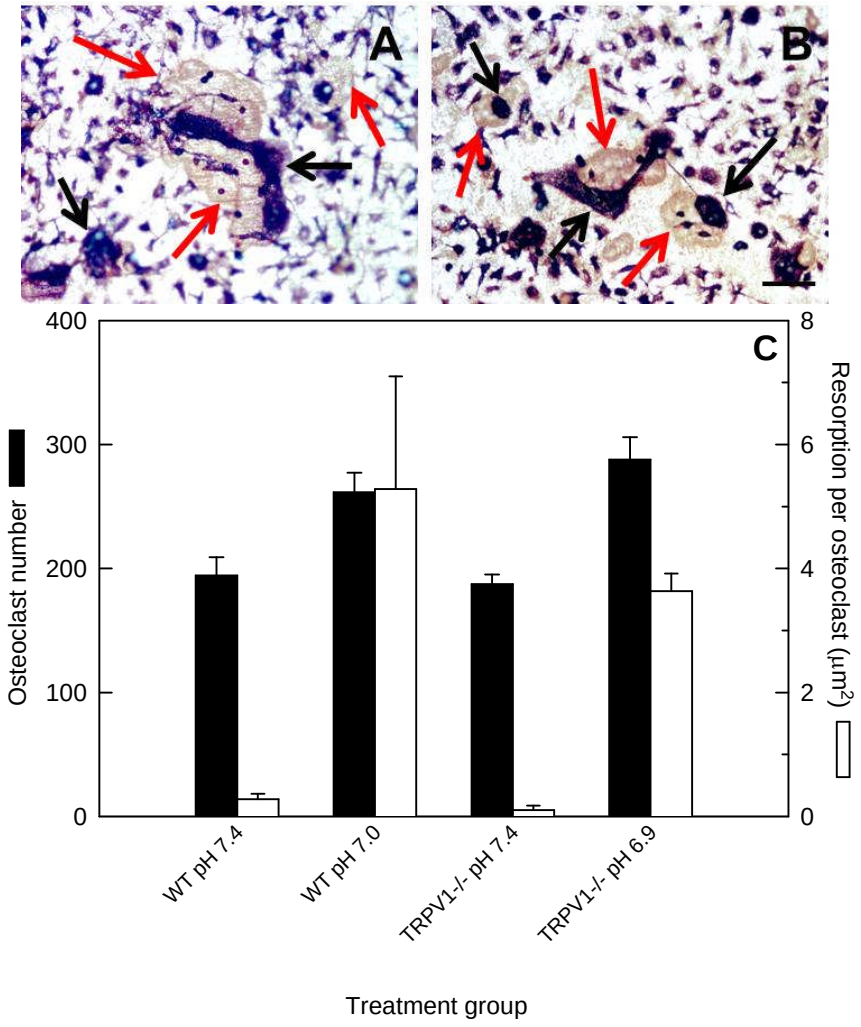


Figure 5.5 *TRPV1$^{-/-}$ osteoclasts are stimulated to resorb by acid*

Wildtype (**A**) and *Trpv1$^{-/-}$* (**B**) osteoclasts (black arrows) show characteristic resorption pits (red arrows) when they have been stimulated to resorb by culture at pH 6.9. Osteoclasts from *Trpv1$^{-/-}$* mice resorb a comparable amount to those isolated from wildtype animals (**C**). Scale bar = 50µm. (*n* = 8).

TRPM8 agonists have no effect on osteoclast formation and activity

Menthol has previously been shown to inhibit osteoclastic bone resorption *in vivo* (Muhlbauer *et al.* 2003). Icilin and menthol were tested to determine whether they influenced osteoclast formation and activity. Both agonists failed to affect either the formation or activation of human osteoclasts from peripheral blood mononuclear cells (**Fig. 5.6**).

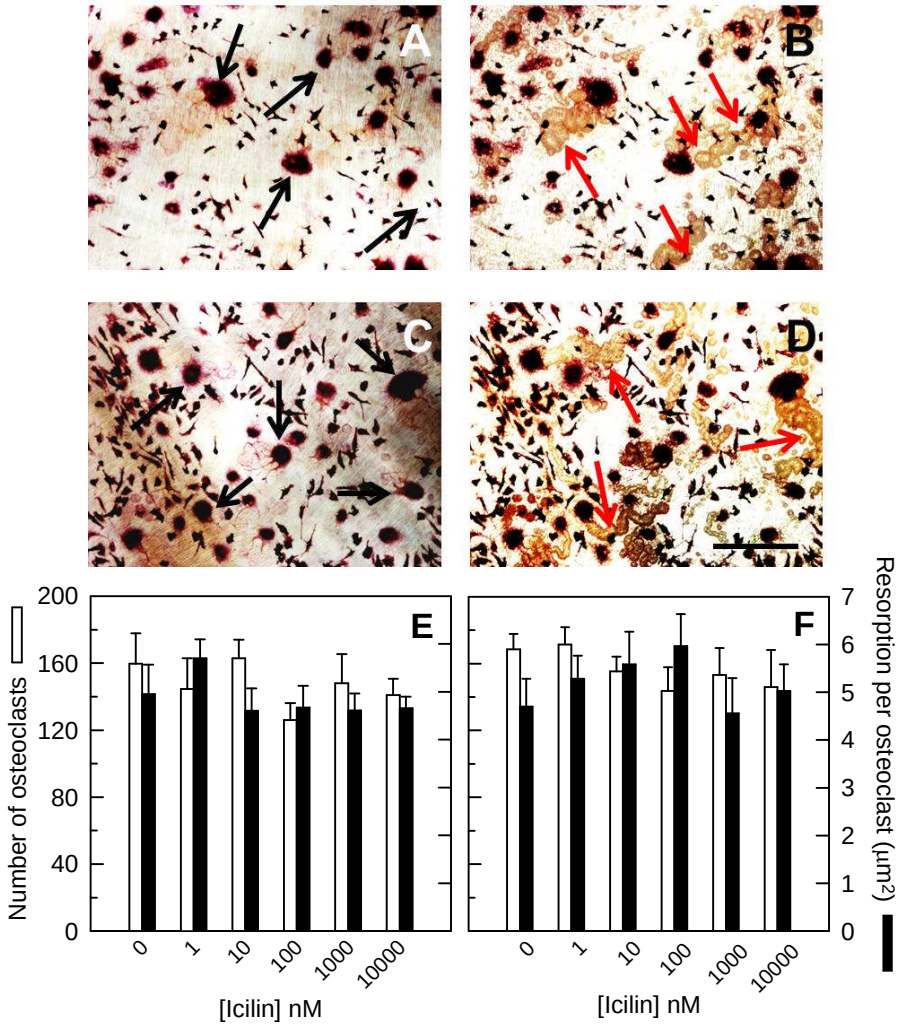


Figure 5.6 *Icilin has no effect on osteoclast formation or activation*

(**A**) Osteoclasts (black arrows) under control conditions and (**B**) associated image under reflected light to visualise resorption (red arrows). (**C**) Osteoclasts exposed to 10µM icilin and (**D**) corresponding reflected light image. Icilin at 1nM–10µM does not affect osteoclast formation (**E**) or activation (**F**). Treatment with menthol at the same concentrations was similarly without effect (data not shown). Scale bar = 150µm. (*n* = 8).

Murine osteoclasts express Trpv4 but not Trpv3 mRNA

RT-PCR was performed on mRNA isolated from mature osteoclasts. Levels of mRNA were normalised using the housekeeping gene, *Gapdh*. *Trpv4* mRNA was clearly expressed by mature osteoclasts, whereas *Trpv3* mRNA was absent (**Fig. 5.7**).

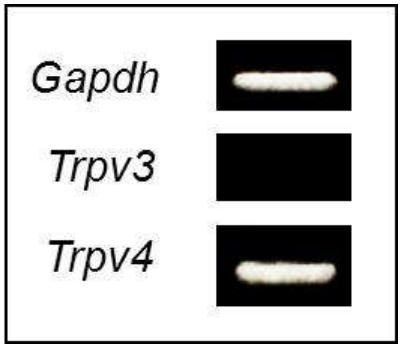


Figure 5.7 *Mouse osteoclasts express Trpv4 but not Trpv3 mRNA*

RNA was extracted from osteoclasts cultured for 10 days at pH 7.4. *Trpv4* mRNA was strongly expressed on mouse osteoclasts; however, *Trpv3* mRNA was not detected. Samples were normalised with mRNA for the housekeeping gene *Gapdh*.

TRPV1 protein is expressed by rat osteoblasts

Immunofluorescence demonstrated TRPV1 expression by mature bone forming osteoblasts (**Fig. 5.8**).

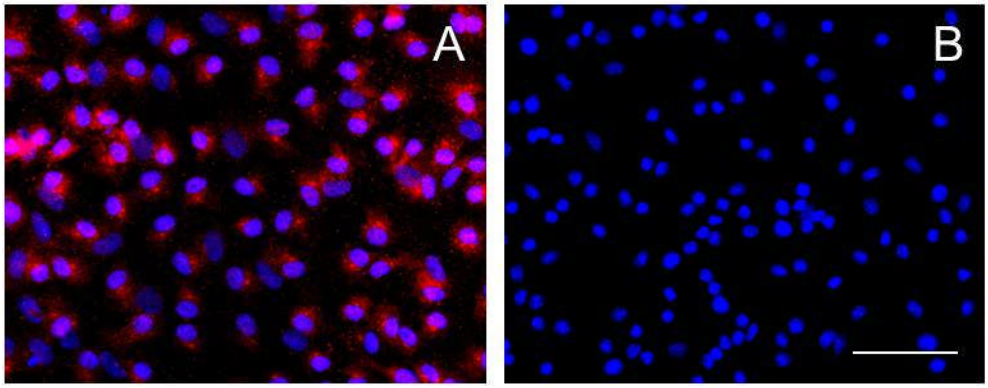


Figure 5.8 *Primary rat osteoblasts express TRPV1*

TRPV1 expression in osteoblasts was studied by immunofluorescence using specific primary polyclonal antibodies, Cy3-labelled anti-rabbit secondary antibody (red) and DAPI nuclear stain (blue). TRPV1 expression was evident in mature osteoblasts and appeared to be localised intracellularly (**A**); corresponding negative staining in (**B**). Scale bar = 150μm.

The TRPV1 agonist capsaicin does not affect bone formation by osteoblasts or their proliferation

Acid inhibits bone mineralisation by osteoblasts (**Fig. 4.2**) and, thus, the TRPV1 agonist capsaicin was added to osteoblast cultures to determine whether it could also mimic the effect of acid and inhibit mineralisation in osteoclasts cultured at physiological pH (pH 7.4). Capsaicin was also added to cells at pH 6.9 to provide an 'acid' reference group. Results obtained indicate that long term culture with capsaicin at concentrations between 2nM-20µM have no effect on bone formation *in vitro*, nor does capsaicin affect the proliferation of osteoblasts at either pH 7.4 or pH 6.9 (**Figs. 5.9, 5.10 and 5.11**).

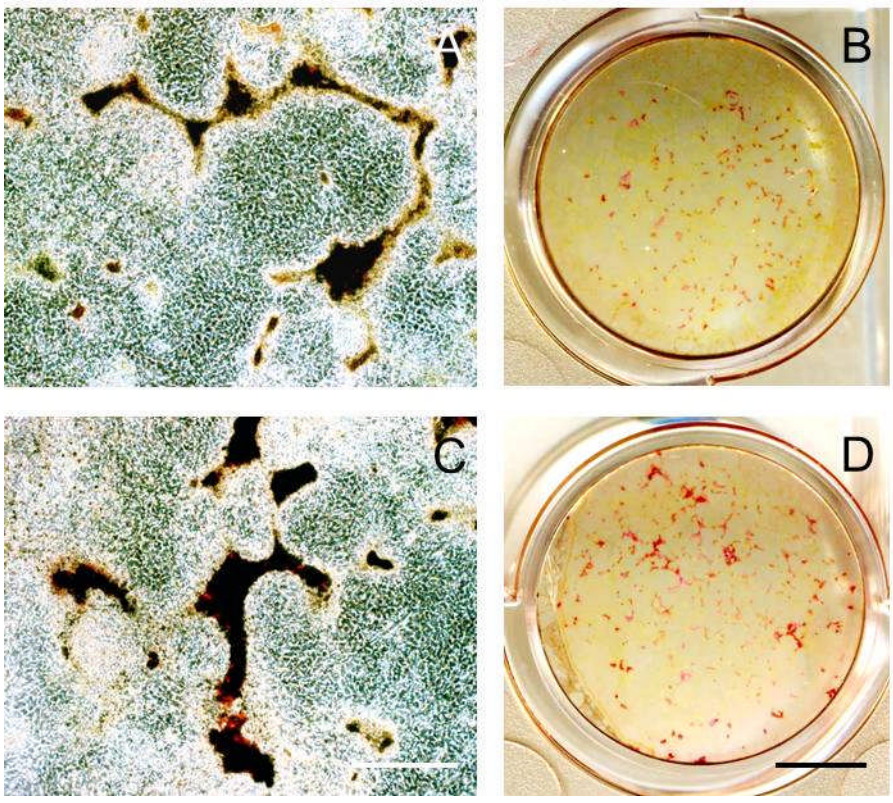


Figure 5.9 *Capsaicin does not affect bone nodule formation by osteoblasts*

Long term culture of primary rat osteoblasts with 20µM capsaicin does affect the number or appearance of alizarin red stained bone nodules formed by primary rat osteoblasts (**C** and **D**) when compared to bone nodules grown under control conditions (**A** and **B**). White scale bar = 800µm, black scale bar = 3.75mm.

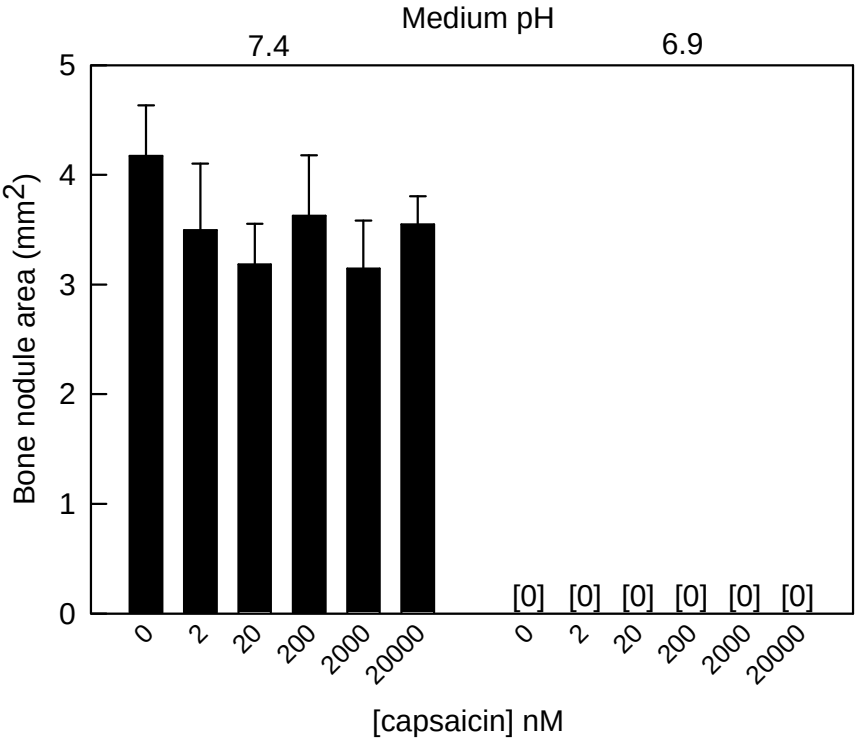


Figure 5.10 *Capsaicin has no effect on the bone forming capacity of osteoblasts.*

Primary rat osteoblasts were cultured for 14 days at pH 7.4 or pH 6.9 with 2nM-20µM capsaicin. Cultures were terminated at day 14. Capsaicin has no reproducible effects on bone formation by osteoblasts (*n*= 6).

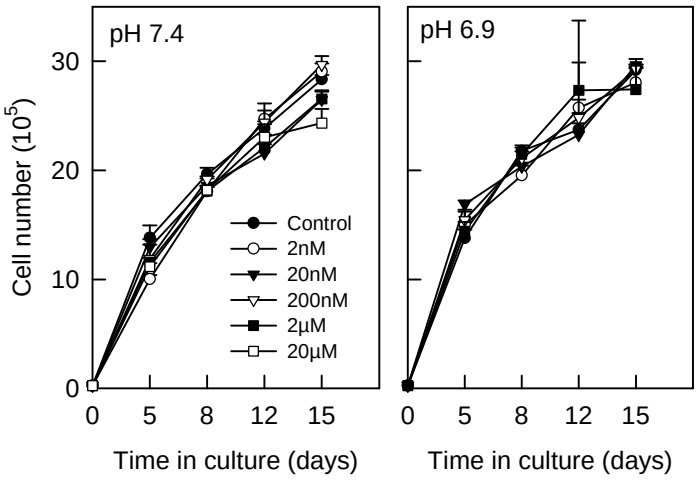


Figure 5.11 *Capsaicin does not affect the proliferation of osteoblasts.*

Capsaicin at 2nM–20µM had no effect on proliferation of rat osteoblasts cultured at pH 7.4 or pH 6.9 for 15 days (*n* = 6).

The TRPV1 antagonists' capsazepine and N-(3-methoxyphenyl)-4-chlorocinnamide (SB 366791) do not affect bone formation by primary rat osteoblasts.

Capsazepine is a synthetic TRPV1 antagonist, generated by altering the side chain region of capsaicin (Walpole *et al.* 1994). Osteoblasts were cultured for 14 days in the presence of capsazepine or SB 366791 at pH 7.4 or 6.9. Both capsazepine and SB 366791 had no effect on primary osteoblasts' ability to form bone nodules *in vitro*. (**Figs. 5.12, 5.13 and 5.14)**. If TRPV1 was the only acid-sensing receptor, adding these antagonists to osteoblasts cultured at pH 6.9 would be expected to block the acid-sensing part of the receptor and so osteoblasts would produce the classic bony nodules made by osteoblasts *in vitro*.

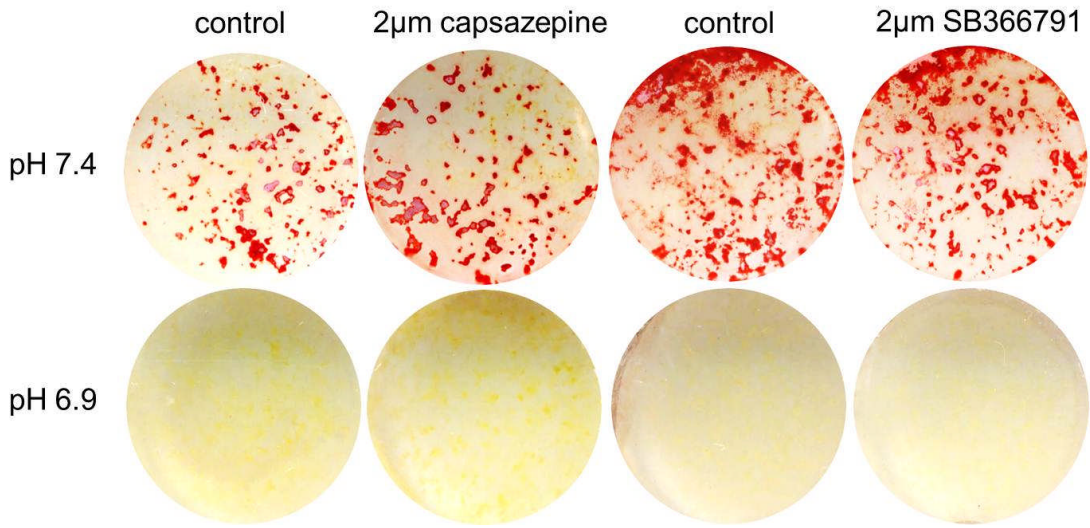


Figure 5.12 *The TRPV1 antagonists capsazepine and SB366791 do not affect bone nodule formation by primary rat osteoblasts in vitro.*

2nM–2µM capsazepine or SB366791 did not affect alizarin red stained bone nodule formation by osteoblasts at either pH 7.4 or pH 6.9. Well width = 1.5mm.

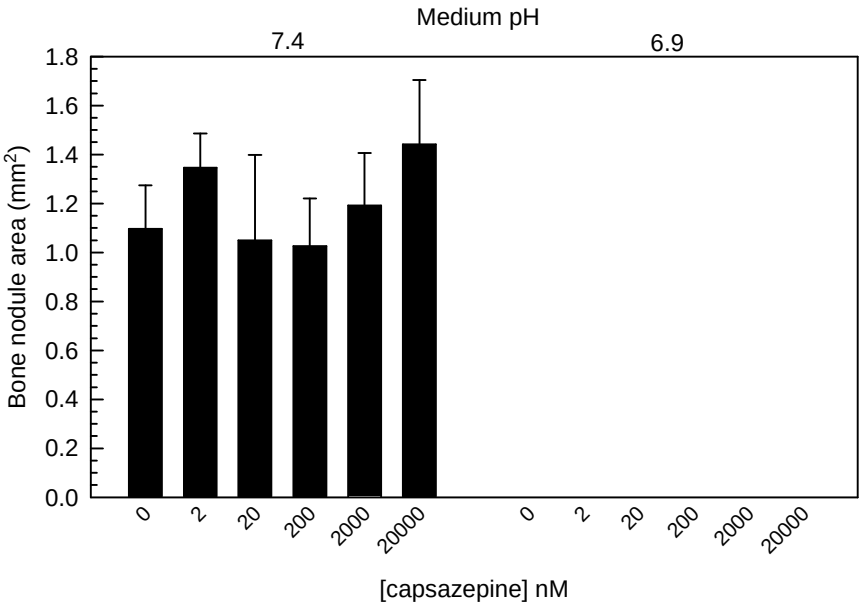


Figure 5.13 C*apsazepine does not affect bone nodule formation*

Capsazepine at concentrations between 2nM–20μM had no effect on bone nodule formation by primary rat osteoblasts at either pH 7.4 or 6.9. (*n* = 6).

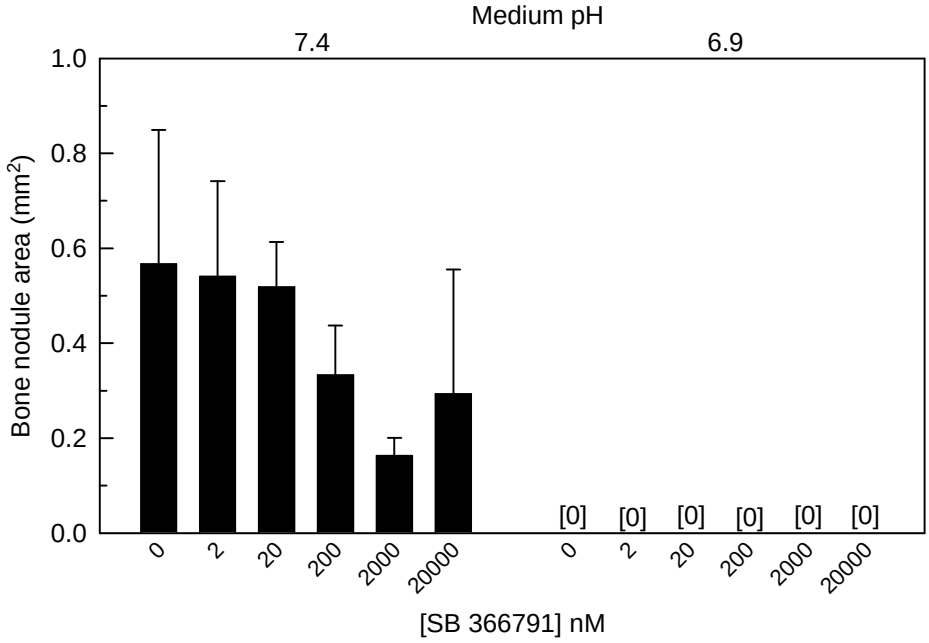


Figure 5.14 *SB366791 does not affect bone nodule formation*

Bone nodule formation by osteoblasts at pH 7.4 is decreased following additions of SB 366791 at concentrations of up 20μM though this is not statistically significant. At pH 6.9, bone nodule formation is unaffected at all concentrations of SB 366791 tested. (*n* = 6).

Rat osteoblasts express Trpv3 and Trpv4 mRNA

In addition to TRPV1, there are a number of other TRP channels which are reported to detect protons, these include TRPV3 and TRPV4 (Bandell *et al.* 2007; Holzer 2009). Both *Trpv3* and *Trpv4* mRNA were expressed on osteoblasts at both 7 and 14 days of culture (**Fig. 5.15**).

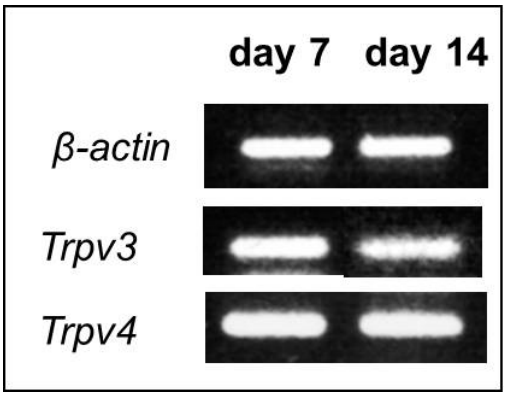


Figure 5.15 *TRPV3 and TRPV4 mRNA is expressed by primary rat osteoblasts*

RNA was extracted from osteoblasts cultured for 7 or 14 days at pH 7.4. *Trpv3* and *Trpv4* mRNA was expressed at both time points Samples were normalised with mRNA for the housekeeping gene, *β-actin*.

The TRPV4 agonist, 4 α-phorbol 12,13-didecanoate (4αPDD) is cytotoxic to osteoblasts at concentrations above 1μM

As TRPV4 has a similar structure and has been reported to work in a similar way to TRPV1, it was investigated as a potential acid-sensor. To determine if 4αPDD was able to mimic acid, it was added to osteoblast cultures at concentrations between 1nM and 10μM at pH 7.4 and also at pH 6.9 to provide an acid control. Bone formation was inhibited at concentrations of 1μM and above (**Fig 5.16**); however, a viability assay performed on osteoblasts showed that at concentrations of above 1μM 4αPDD was cytotoxic to osteoblasts (**Fig. 5.17**).

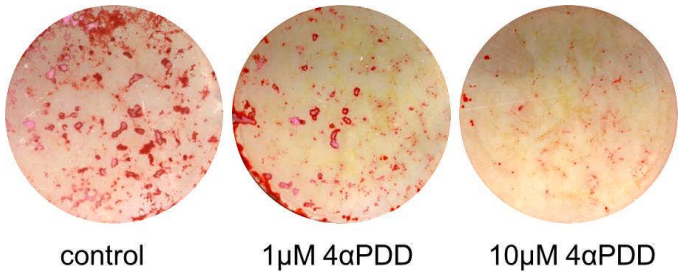


Figure 5.16 TRPV4 *agonist 4αPDD is cytotoxic at concentrations >1μM*

Bony nodule formation at pH 7.4 was inhibited at concentrations of 1μM 4αPDD and above because long term culture results in cytotoxicity.

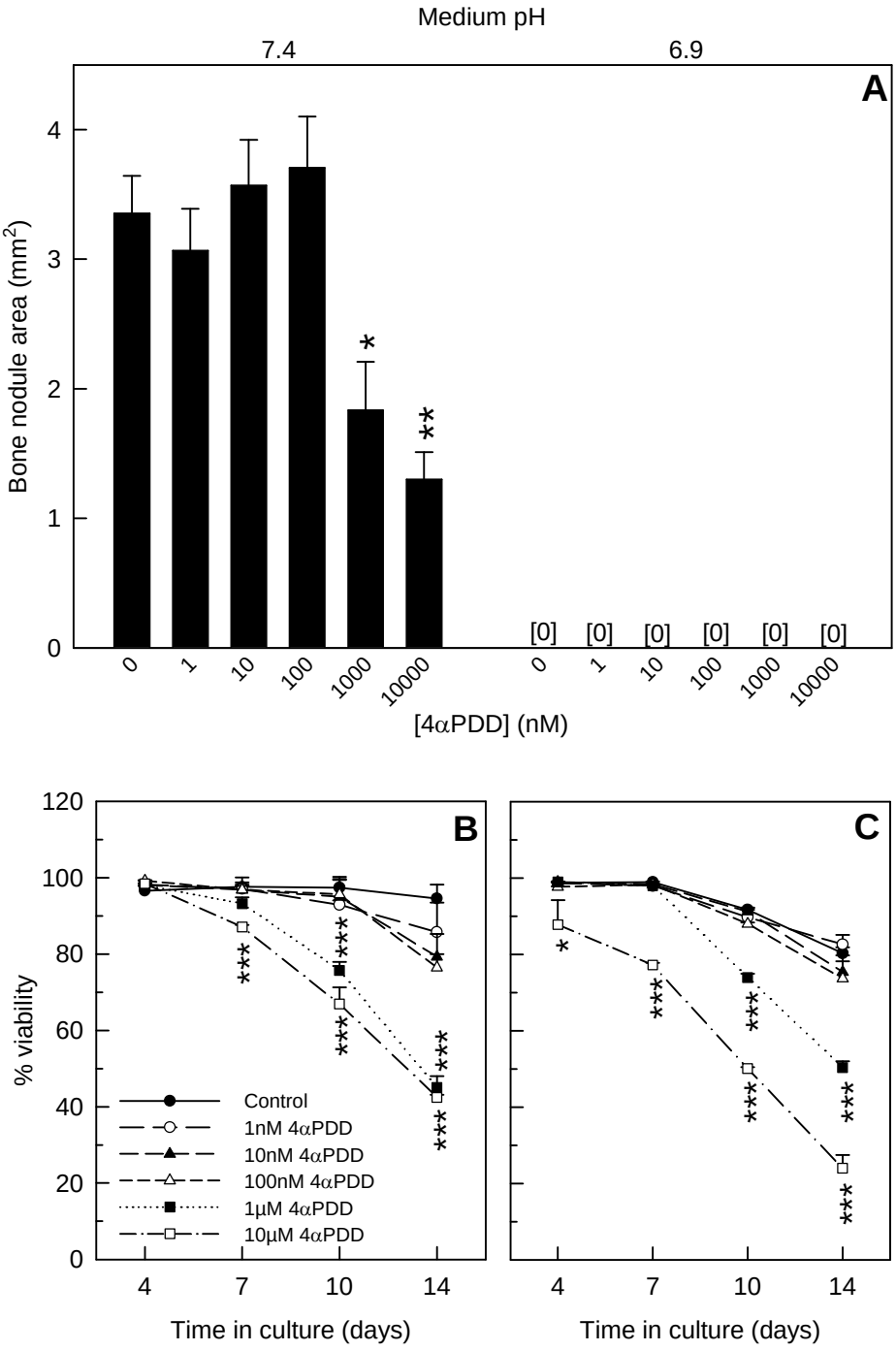


Figure 5.17 *TRPV4 agonist 4αPDD is cytotoxic to osteoblasts at concentrations of 1μM and above*

1nM–100nM 4αPDD did not affect bone formation (**A**); however, at concentrations of 1 and 10μM significant decreases in bone nodule formation were observed. Viability assays demonstrated that 4αPDD (1nM–10μM) is cytotoxic to osteoblasts, at pH 7.4, 10μM was cytotoxic from day 7 and 1μM from day 10 (**B**). At pH 6.9 (**C**), 4αPDD was cytotoxic at 10μM day 4 and 1μM from day 10 of exposure. (*n* = 6, * = p <0.05, ** = p <0.001 *** = p <0.001).

Ruthenium red inhibits bony nodule formation at concentrations greater than 100nM at physiological pH

Ruthenium red (RR) is an antagonist of several TRP channels including TRPV4. Osteoblasts were cultured for 14 days in the presence of ruthenium red at 1nM–10μM at pH 7.4 or pH 6.9. At physiological pH, bone nodule formation was inhibited in osteoblasts cultured at pH 7.4 (**Fig. 5.18, 5.19 and 5.20**). Ruthenium red had no effect on osteoblastic bone formation in cells cultured in acidic medium.

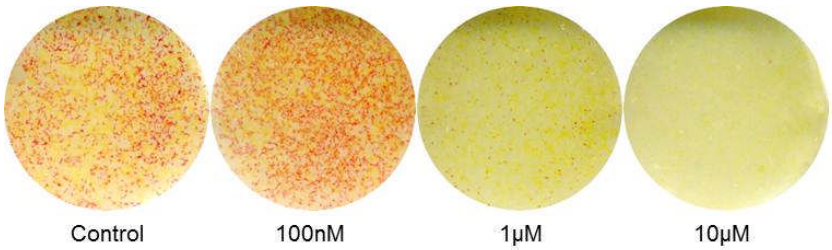
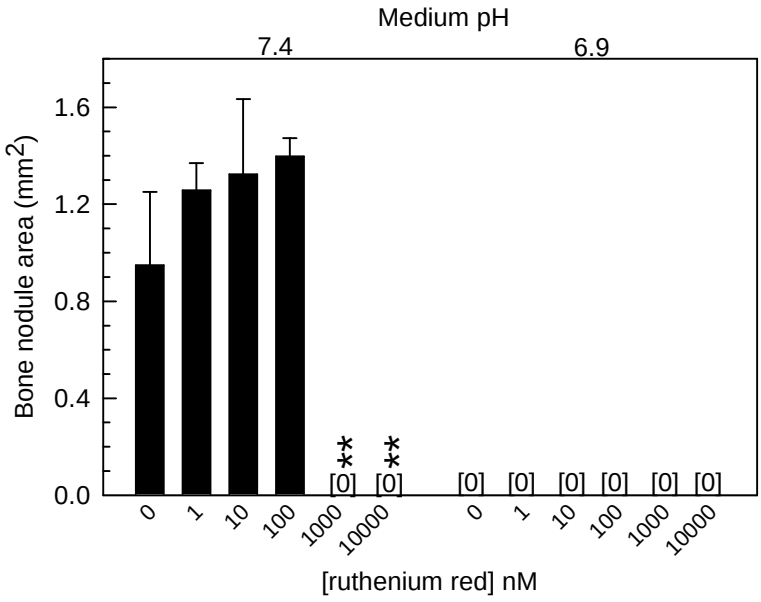


Figure 5.18 *The effect of ruthenium red on bone formation by osteoblasts in vitro*

Ruthenium red at concentrations >1μM inhibited osteoblastic bone nodule formation when cultured for 14 days at pH 7.4. (Well width

Figure 5.19 *The effect of ruthenium red on bone formation by osteoblasts in vitro.*

Ruthenium red significantly inhibited bone nodule formation by primary rat osteoblasts at concentrations above 100nM at physiological pH. At pH 6.9, ruthenium red did not affect bone formation. (*n* = 6, ** = p <0.001).



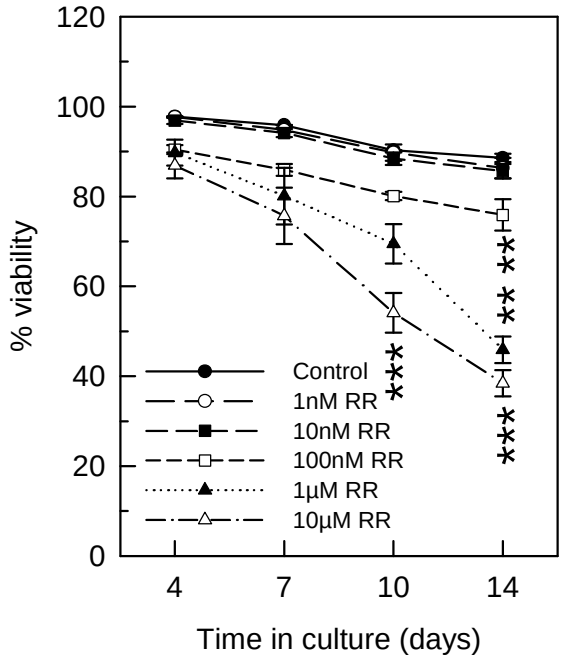


Figure 5.20 *Ruthenium red is cytotoxic to osteoblasts*

Osteoblasts cultured for 14 days at pH 7.4 and with ruthenium red (RR) for 14 days. Concentrations of 100nM and above resulted in cell death. 10μM ruthenium red caused significant decreases in cell viability from day 7. (*n* = 6, * = p <0.05, ** = p <0.001 *** = p <0.001).

Gadolinium chloride, a TRPV4 antagonist, does not affect bone nodule formation by osteoblasts

Primary rat osteoblasts were cultured for 14 days in the presence of the TRPV4 antagonist gadolinium chloride at both pH 7.4 and pH 6.9. There was no effect on bone nodule formation at either pH (**Fig. 5.21**).

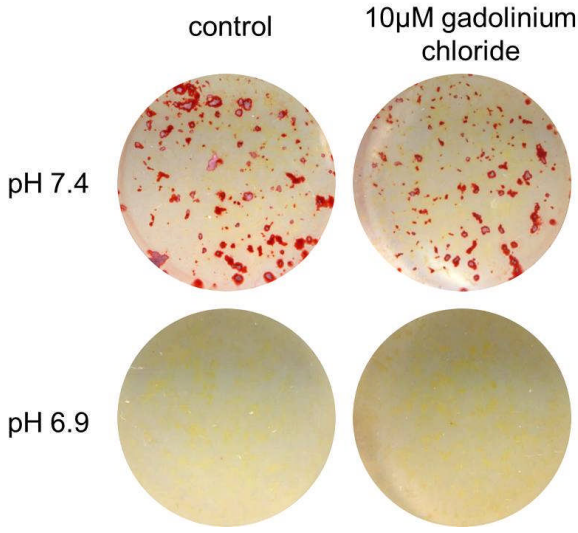


Figure 5.21 *Gadolinium chloride does not affect the bone forming capacity of primary rat osteoblasts*

At pH 7.4 and 6.9, long-term culture of osteoblasts with 1nM–10μM gadolinium chloride does not affect bone nodule formation *in vitro*. Well width = 1.5cm.

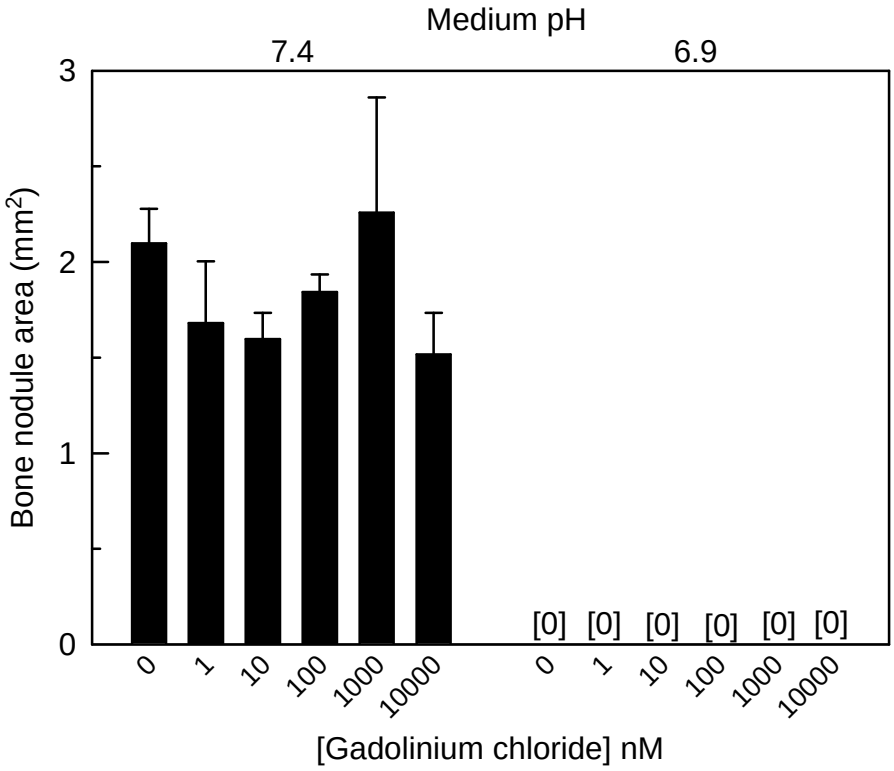


Figure 5.22 *Gadolinium chloride has no effect on bone nodule formation in vitro*

At concentrations of 1nM–10μM gadolinium did not affect the bone forming capacity of primary rat osteoblasts *in vitro* when cultured at pH 7.4 or pH 6.9 (*n* = 6).

TRPM8 mRNA is not expressed by primary rat osteoblasts

RT-PCR was used to investigate the expression of *Trpm8* mRNA by osteoblasts after 7 and 14 days of culture. Levels of mRNA were normalised using the housekeeping gene, *β-actin*. *Trpm8* mRNA was not detected in osteoblasts at either day 7 or 14 (**Fig. 5.23**), in agreement with the lack of effect of both icilin and menthol.

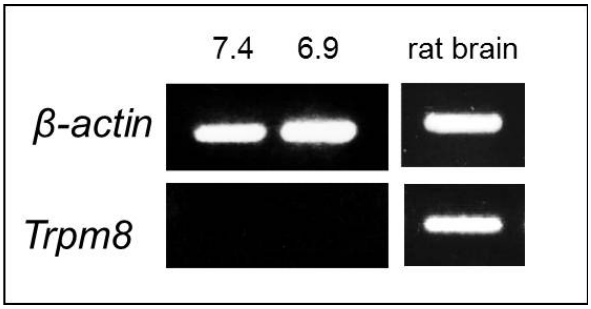


Figure 5.23 *Lack of Trpm8 mRNA expression by primary rat osteoblasts*

Osteoblasts cultured were for 7 or 14 days at pH 7.4 or 6.9; *TRPM8* mRNA expression was not detected at either time point or temperature. Rat brain was used as a positive control.

TRPM8 agonists do not affect osteoblast proliferation and function

The effects of the TRPM8 agonists' menthol and icilin were investigated on both osteoblast and osteoclast formation and activity. Menthol is the natural agonist of TRPM8 and icilin is a higher potency synthetic agonist. Initially, menthol and icilin were investigated at concentrations between 1nM–10μM on bone nodule formation by primary rat osteoblasts. At all concentrations tested, menthol and icilin failed to have any effect on bone formation at either pH 7.4 or pH 6.9. Furthermore, neither agonist affected osteoblast proliferation, assessed using the LDH assay (**Figs. 5.25 B and 5.25 D**). The medium pH was maintained at either physiological (pH 7.4) or acid (pH 6.9) levels throughout the duration of the culture. Bone matrix mineralisation was inhibited by the acid conditions (**Figs. 5.24, 5.25 A and 5.25 C**), in agreement with published results (Brandao-Burch *et al.* 2005b).

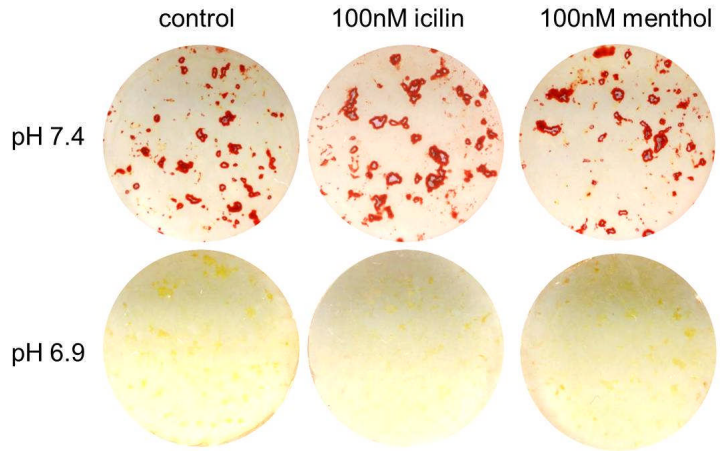


Figure 5.24 *The TRPM8 agonists' **i**cilin and menthol do not affect the bone forming capacity of primary rat osteoblasts*

At pH 7.4 and 6.9, long term culture of osteoblasts with 1–100nM icilin or menthol do not affect bone nodule formation *in vitro*. Well width = 1.5cm.

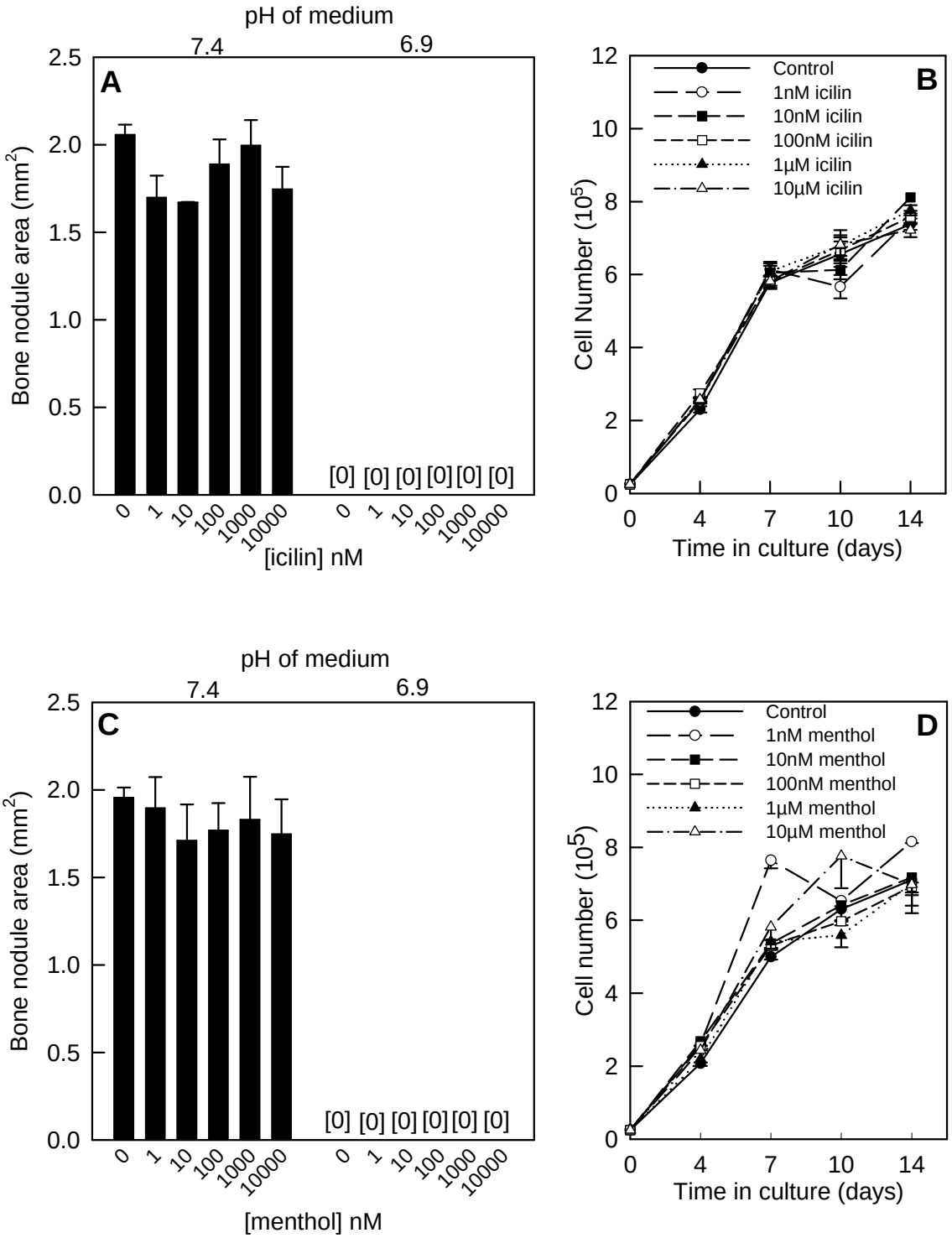


Figure 5.25 *TRPM8 agonists icilin and menthol do not affect the bone forming capacity of osteoblasts or their proliferation*

Icilin at 1nM–10µM had no effect on bone nodule formation by osteoblasts (**A**) or on their proliferation at pH 7.4 (**B**). (**C**) Menthol at 1nM–10µM did not effect on bone nodule formation by osteoblasts (**D**); osteoblast proliferation was unaffected by menthol in the range 1nM–10µM at pH 7.4. (*n* = 6).

Discussion

The negative effect of acid on the skeleton has been known for many years; more recently it was shown that in addition to acting as a fail-safe store of base to buffer low pH during times of acidosis, acid is actually able to elicit direct effects on bone cells themselves (Arnett & Dempster 1986; Brandao-Burch *et al.* 2005b). Currently the mechanism by which osteoclasts and osteoblasts detect low pH is unknown. Experimental work carried out in this chapter set out to determine whether proton-sensing members of the TRP family of ion channels may be responsible for acid-sensing by bone cells.

Work presented in this chapter has demonstrated the presence of several TRP channels on both osteoclasts and osteoblasts. TRPV1 is expressed by both murine and human osteoclasts. TRPV1 protein levels are increased in murine osteoclasts cultured in acidic conditions. TRPV1 has also been shown to be expressed by primary rat osteoblasts. This is in agreement with Idris *et al*, who are the first to have published this (Idris *et al.* 2010). TRPV1 expression however appears to be localised intracellularly within osteoblasts (**Fig. 5.8**) rather than on the cell membrane where it would be expected to be present. Further immunofluorescence could be performed ensuring the cell membrane is not lysed to reveal whether TRPV1 is expressed at the cell membrane of osteoblasts. TRPV4 mRNA was expressed by murine osteoclasts and rat osteoblasts. TRPV3 mRNA was expressed by rat osteoblasts but could not be detected on osteoclasts. TRPM8 could not be detected on osteoblasts.

Capsaicin, a TRPV1 agonist, was added to cultures of pre-osteoclasts and mature osteoclasts to determine if it had an effect on osteoclast formation or activity, respectively. Osteoclasts are known to be stimulated to resorb by drops in pH within the physiological range (Arnett & Dempster 1986). It was hypothesised that if TRPV1 was functioning as the acid-sensor in osteoclasts, then even when cultured at physiological pH, capsaicin may be able to stimulate osteoclasts to resorb. However, the foregoing examples show that this was not the case. TRPV1 activation is complex as it can occur in two distinct ways,

either the channel is stimulated purely by protons which act via several key amino acid residues (Val-538, Thr-633, Glu-648). Alternatively TRPV1 can be initially sensitised by mild acidosis, whereby protons act via the Glu-600 amino, TRPV1 is then activated fully following detection of capsaicin or heat (Ryu *et al.* 2007). Due to the nature of TRPV1 activation, a pH in between physiological (pH 7.4) and acidotic (pH 6.9) was also tested in case the mild acidosis (pH 7.2) was required for capsaicin activation. However, at pH 7.4 no resorption was detected and at pH 7.2 no more resorption was observed in osteoclasts treated with capsaicin compared with those in the control groups. This led to the conclusion that capsaicin could not stimulate osteoclasts to resorb. This is in agreement with results presented by Idris *et al,* who reported that at concentrations of three μM and above, capsaicin has no effect on osteoclasts (Idris *et al.* 2010). In order to confirm this, osteoclasts were obtained from *Trpv1*$^{-/-}$ mice. Osteoclasts from these animals were still able to resorb when the medium was acidified to pH 6.9. The knockout osteoclasts did however resorb less than those from wildtype animals therefore indicating that TRPV1 may contribute to acid-sensing. These results suggest that in osteoclasts, TRPV1 does not act as the sole acid-sensor. Capsaicin also had no effect on the bone forming capacity of primary rat osteoblasts when cultured at either pH 7.4 or pH 6.9.

The TRPV1 antagonists tested in this study, capsazepine and N-(3-methoxyphenyl)-4-chlorocinnamide (SB366791), also had no effect on the bone forming abilities of primary rat osteoblasts cultured *in vitro*. Capsazepine and SB366791 are both selective antagonists for TRPV1, and it is thought that they act competitively against capsaicin for the same binding site. SB366791, although a potent antagonist of TRPV1 when the channel has been stimulated with TRPV1 agonists or noxious heat, is not able to antagonise the channel when it has been stimulated with protons. It is proposed that all TRPV1 antagonists identified to date function by competing with capsaicin for the capsaicin binding site, this causes a conformational change in TRPV1 that keeps the channel closed when it is stimulated by capsaicin. However, when

TRPV1 is stimulated by protons, certain antagonists, including SB366791 are unable to generate the conformational change needed to close the channel. Therefore, Ca^{2+} is still able to pass through TRPV1 and the channel is still active (Gavva *et al.* 2005). The following TRPV1 antagonists, which were not commercially available at the time the work was carried out, inhibit the channel allosterically when it is stimulated by protons and are able to close the channel: AMG 7472; BCTC, AMG 6880 and AMG 9810 (Gavva *et al.* 2005).

Of the other members of the TRPV family, TRPV4 was also investigated. It has been reported to respond to acid in a similar way to TRPV1, although how important its role as an acid-sensing receptor is remains to be determined given that it also functions as both a mechano- and osmo-sensor (Holzer 2009). mRNA for this channel was detected on both osteoblasts and osteoclasts. The specific TRPV4 agonist 4αPDD was added to *in vitro* osteoblast cultures. At concentrations of 1μM and above bone nodule formation was decreased, however, further investigation revealed that at concentrations of 1μM and above, 4αPDD was cytotoxic to osteoblasts *in vitro*. Assessment of viability in this case was important as the results could have easily been misinterpreted as an inhibition of bone formation. The assays used to assess bone formation mimic a chronic system, whereby the cells are exposed to the agonist or antagonist of interest for a sustained period of time thus providing an *in vitro* system akin to the physiological situation. If 4αPDD was able to stimulate TRPV4 and function as an acid-sensor, it would be expected that at physiological pH, bone nodule formation would be inhibited. As this is not the case, it would appear that TRPV4 is not acting as an acid-sensing receptor in osteoblasts.

The effect of two TRPV4 antagonists, gadolinium chloride and ruthenium red, on bone nodule formation was also tested. Given that they are antagonists, it would be anticipated that if osteoblasts were detecting acid via the TRPV4 channel, blocking it would result in a lack of an acid effect and, thus, bone nodules would be made *in vitro* by osteoblasts that were cultured at pH

6.9. For both ruthenium red and gadolinium chloride this was not the case and bone nodule formation was inhibited when osteoblasts were cultured at pH 6.9 at all concentrations tested.

In addition to sensing pH, TRPV4 is more commonly associated with temperature sensing; it detects temperatures between 27 and 34˚C and osmotic cell swelling (Venkatachalam & Montell 2007) and, therefore, it may not have a role in acid sensing in bone cells, as evidence presented in this chapter would suggest. Additionally, the TRPV antagonist, ruthenium red, has been reported to block heat activation of TRPV4 (Watanabe *et al.* 2002) therefore ruthenium red may not inhibit pH sensing. Also, some reports state that TRPV4 is not able to detect acid until pH reaches levels of 6.0 and below (Holzer 2007). As a consequence, TRPV4 may not function as an acid-sensor in bone cells because it is unlikely that pH will drop to such levels in the bone microenvironment in a healthy individual. Furthermore, although I detected mRNA for *Trpv4* on cells, this does not necessarily mean the protein has been translated; therefore it may be that a functional TRPV4 channel is not expressed by bone cells.

Finally, TRPM8 was investigated. TRPM8 was investigated because it is a 'sister' channel of TRPV1; although it has not been reported as an acid sensor, it is stimulated by temperatures in the range 23–28˚C and functions as the 'cool' sensor. It is also stimulated by menthol and icilin. Icilin is a super-agonist of TRPM8 and is 200 times more potent (Montell 2005). Both icilin and menthol at the concentrations tested had no effect on either osteoclasts or osteoblasts. RT-PCR revealed a lack of mRNA for TRPM8 and, therefore, it is likely that the lack of effect seen is because TRPM8 is not expressed by bone cells.

Although numerous TRP channels are expressed by bone cells, evidence presented in this chapter would suggest that they do not act as *the* acid-sensing receptor. Work published recently has shown TRP channels working cooperatively with cannabinoid receptors in bone cells, where this combination of receptors has been implicated as a potential target of

osteoporosis treatments. TRP channels have also been hypothesised to work with purinergic receptors. Both of these provide areas for further investigation.

The question remains, however, as to role TRP channels may play in bone cell function. In addition to the TRP channels detected on bone cells during this study, other TRP channels have also been detected. TRPC1, TRPC3, TRPC4, TRPC6, TRPM4, TRPM6, TRPM7, TRPM8, TRPV2 and TRPV4 mRNA has been detected on human osteoblast-like cells (Abed *et al.* 2009; Suzuki *et al.* 2011), murine osteoblast cell-lines additionally express TRPC7 and TRPM1 but not TRPC3, TRPV2 or TRPV4 (Abed *et al.* 2009). Furthermore the *Trpv5*$^{-/-}$ mouse is reported to have a bone phenotype, whereby bone thickness is increased and bone resorption is defective (Hoenderop *et al.* 2003; van der Eerden *et al.* 2005). TRPV4 has already been shown to play a part in mechanosensation in bone cells (Suzuki *et al.* 2003).

Some members of the TRP channel superfamily are able to act as thermosensors; TRV1–4, TRPM8 and TRPA1 are all activated at varying temperature ranges (Clapham 2003). Work performed at the beginning of this PhD (not presented here) showed that human osteoclast formation was increased 3-fold when osteoclasts were cultured at 34ºC compared to 37ºC; the effect on murine osteoclast formation was similar (Patel *et al.* 2009). Furthermore *in vitro* bone formation was decreased by 95% when cultured for 16 days at 34ºC in comparison to 37ºC (Patel *et al.* 2009). The mechanism by which osteoclasts and osteoblasts detect and respond to changes in temperature is currently unknown, but TRP channels may be involved in this temperature detection.

Previous work in our laboratory has shown that the acid-sensing receptor ovarian cancer G-protein coupled receptor 1 (OGR1) is expressed by primary rat osteoblasts and human osteoclasts and is up-regulated in both when cells are cultured in acidotic conditions. Similar up-regulation was reported for T cell death-associated gene 8 (TDAG8). mRNA for GPR4 could not be detected (Brandao-Burch 2005a). Osteoclasts isolated from *Ogr1*$^{-/-}$ and *Tdag8*$^{-/-}$ mice

were able to respond to acidosis in the same way as wildtype, thus ruling these receptors out as the acid sensor (Gasser *et al.* 2006).

Another set of acid-sensors that may have a role are the acid-sensing ion channels (ASICs); these proteins are encoded by three genes: amiloride-sensitive cation channel (ACCN) -1, - 2 and -3. Alternative splicing gives rise to at least five subunits, these then assemble to generate to the different ASICs, some of which have already been detected on bone cells. Human osteoblasts express mRNA for ASIC1, 2 and 3 and human osteoclasts express mRNA for ASIC1, 2, 3 and 4 (Jahr *et al.* 2005). Previous work in the laboratory has shown that mRNA for ASIC1a was expressed by human osteoclasts, however this was down-regulated when osteoclasts had been acid-activated (Brandao-Burch 2005a). ASIC3 is the most sensitive of the ASICs when pH drops within the pathophysiological range, metabolic acidosis can reach a pH of 7.0 and, ASIC3 begins to open at pH 7.1 and fully activated at pH 6.5 (Birdsong *et al.* 2010). Therefore the role of ASIC3 on bone cells is worth investigating.

TRPC5 detects decreases in pH from 7.4 to 7.0, however, because all other TRP channels studied to date do not affect bone cell function, this may not have a role on these cells either. There are many other families of acid-sensing receptors that may have a role in acid sensing by osteoblasts and osteoclasts (Holzer 2009) including TWIK (tandem of pore domains in weak inward rectifier (K^+) channels, TALK (TWIK-related alkaline pH-activated K^+) channels, TASK (TWIK-related acid-sensitive K^+) and TRESK (TWIK-related spinal cord K+) channels. Of these I have identified the following as the most likely to be physiologically relevant in osteoclasts and osteoblasts because they respond to small decreases in acidosis within the pathophysiological range: TWIK-1, TALK-1 and -2, TASK-1, -2 and -3 (Duprat *et al.* 1997; Holzer 2009).

There is substantial evidence that clearly demonstrates the importance of acid in regulating both osteoblastic bone formation and osteoclastic bone resorption however, it seems likely that that deducing the mechanisms of pH detection and signal transduction is likely to be a multi-factorial problem. Acid-

sensing is one of the most tightly regulated of all the homeostatic mechanisms (Frassetto *et al.* 1996) and it is probable that a degenerate system for acid detection evolved. As a consequence, elucidating the receptor(s) that function as the acid sensor in bone will prove a significant challenge for the future.

Chapter 6

The role of acid in breast cancer-induced osteolytic bone disease

Introduction

The damaging effects that breast cancer metastases have on bone are well documented. Much work has been undertaken to understand the mechanisms involved and to identify growth factors and cytokines that might contribute to the destructive effect of osteolytic bone disease. The cancer microenvironment is known to be acidic, and acid is known to stimulate osteoclastic resorption, however little attention has been paid to the direct effect protons at this location may have on resorption by osteoclasts. The aims of this chapter were to determine the role acid has in stimulating osteoclastic resorption in the vicious cycle model of cancer metastasis to bone. Additionally, cytokines implicated in this model were investigated to determine their ability to stimulate osteoclast formation and activation.

Hypothesis – (1) Activin A and BAFF will only be able to augment osteoclastic resorption when they are first stimulated to resorb by acid; (2) co-culture of breast cancer cells with osteoclast precursors will result in increased osteoclast formation; (3) acid produced by the breast cancer cells will stimulate osteoclast resorption.

Materials and methods

Methods used were as described in chapter 2. A novel system was devised to investigate the effects of acid produced by cancer cells on osteoclast activity. These are outlined below.

Co-culture of breast cancer cells and osteoclasts – without cell-to-cell contact

Osteoclasts were isolated and seeded onto dentine discs (5mm diameter) as described in Chapter 2. Osteoclasts were allowed to adhere to the dentine for 24 hours. On the same day, cancer cells were trypsinised, collected and seeded onto dentine discs, (1cm diameter) at 10^6 cells/disc for long-term culture or $5x10^6$ cells/disc for short term culture in 1ml of MEM (without M-CSF or RANKL) and allowed to adhere for 24 hours. Two dentine discs (5mm diameter) on which osteoclasts had adhered were placed in the bottom of one well of a 24-well plate in 2ml MEM, followed by a $1cm^2$ stainless steel grid on which a large dentine disc (diameter = 1cm) on which cancer cells had been seeded was placed (**Fig. 6.1**).

To investigate either long- or short-term exposure to acid produced by MCF-7 and MDA-MB-231 cells on osteoclasts, cells were cultured for 5 days or 48 hours, respectively. Half of the medium was replaced on day three. Culture medium pH, pCO_2 and pO_2 were monitored daily, as described previously in Chapter 2. Upon termination of the culture, large dentine discs were removed, washed with PBS, trypsinised and a cell count performed. Some from each group were fixed with 2.5% glutaraldehyde and stained with toluidine blue. Small dentine discs were washed with PBS and then fixed in 2.5% glutaraldehyde for 5 minutes and stained for tartrate resistant acid phosphatase (TRAP) using the leukocyte acid phosphatase kit according to the manufacturer's instructions and analysed as outlined in Chapter 2.

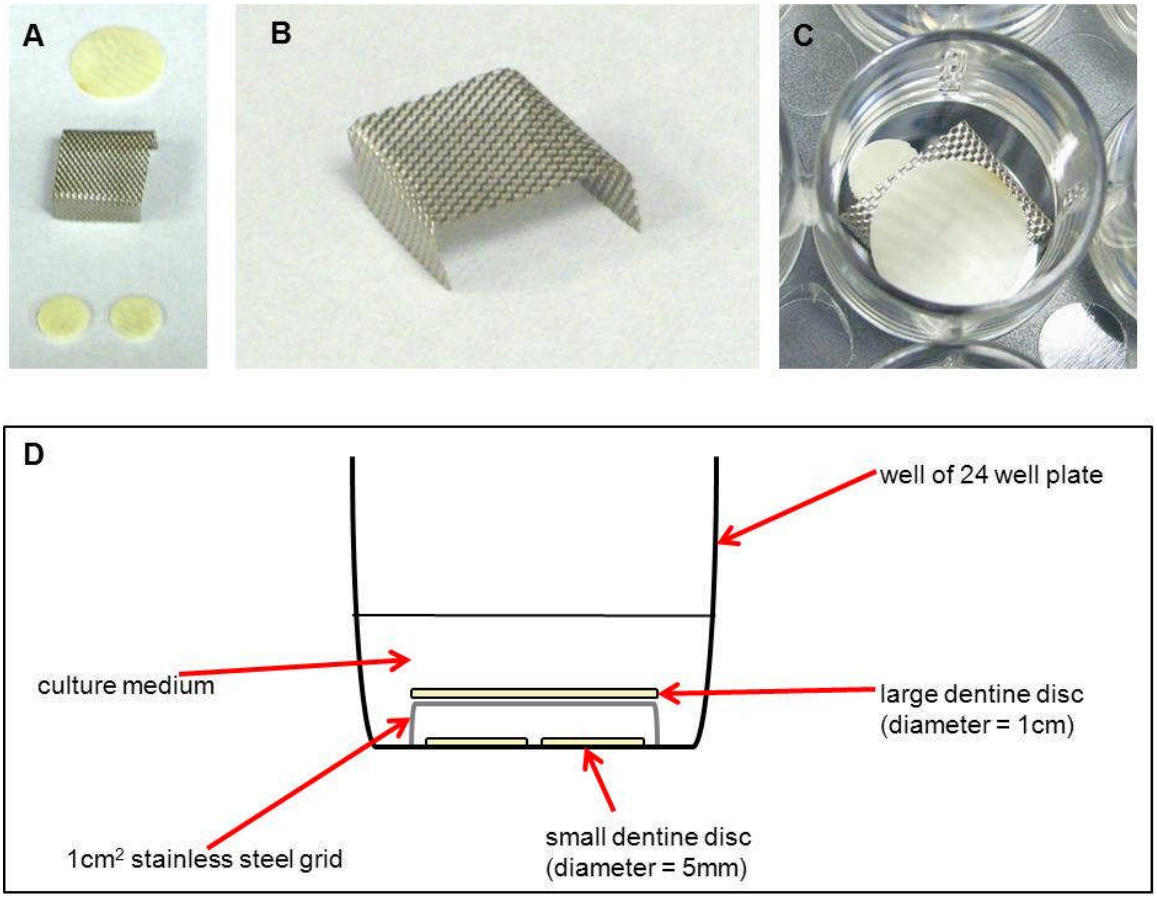


Figure 6.1 *Experimental set up for culture of osteoclasts and breast cancer cells without cell-to-cell contact*

Osteoclasts were seeded onto small dentine discs (5mm diameter), two were placed in the bottom of a well in a 24-well plate, a 1cm^2 stainless steel grid (**B**) was placed above these discs, on top of this a large dentine disc (1cm diameter) on which the breast cancer cells had been seeded was placed in 2ml MEM (**D**). Components of this experimental set up (**A**) were two small dentine discs, a 1cm^2 stainless steel grid, one large dentine disc and *in situ*, without any culture medium (**C**).

Co-culture of breast cancer cells and osteoclasts – allowing cell-to-cell contact

Osteoclasts were isolated as described previously and allowed to adhere to dentine discs (5mm diameter) for 24 hours. Dentine discs were then transferred into 6-well trays in 4 ml MEM/well. In order to determine long-term effects of co-culture with MCF-7 or MDA-MB-231 cells, they were seeded directly into the wells at a 10^6 cells/well. In order to determine short term effects of co-cultures,

cancer cells were added 48 hours prior to the termination of the culture at a density of $5x10^6$/well. Upon termination of the culture, cells were fixed as previously described. Discs were then stained with TRAP to demonstrate osteoclasts; these were counted, and resorption analysed, as previously outlined. Discs were then additionally stained with toluidine blue to identify the cancer cells.

Results

Activin A does not stimulate osteoclasts to resorb at physiological pH

Osteoclasts were cultured for up to 9 days in the presence of activin A at concentrations of 0, 10 and 100 ng/ml. Osteoclasts were maintained at physiological pH (7.4) for 7 days, then half the cells were transferred to medium adjusted to pH 6.9 by addition of 10 meq/L H^+ (as HCl). After 48 hours, pH was recorded using a blood gas analyser, cells were fixed and TRAP stained. Osteoclast cell number and resorption was analysed as outlined in Chapter 2. Figures 6.2 and 6.3 show that activin A did not affect osteoclast formation nor was it able to stimulate osteoclasts to resorb when cultured at physiological pH.

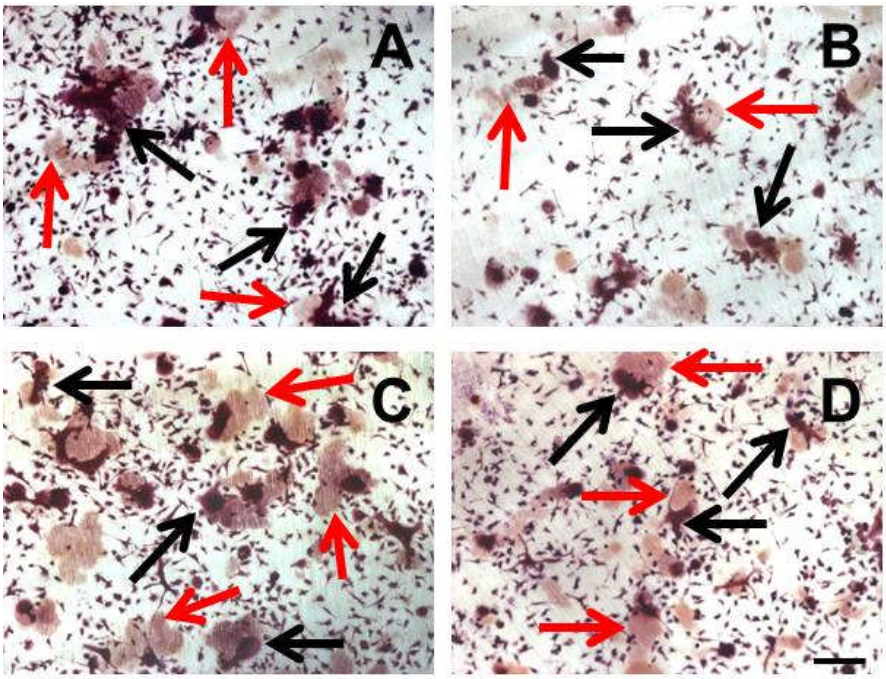


Figure 6.2. *Effect of activin A on osteoclastic resorption*

(**A**) Mouse osteoclasts (black arrows) resorb (red arrows) a small amount when maintained at pH 7.4 for the duration of the experiment; addition of activin A 100ng/ml (**B**) has no effect on osteoclast formation or resorption. (**C**) Resorption by osteoclasts was increased when medium was acidified to pH 6.91 for the final 48 hours in culture. Addition of 100ng/ml activin A to acidified culture medium did not affect resorption by osteoclasts (**D**). Scale bar = 100µM.

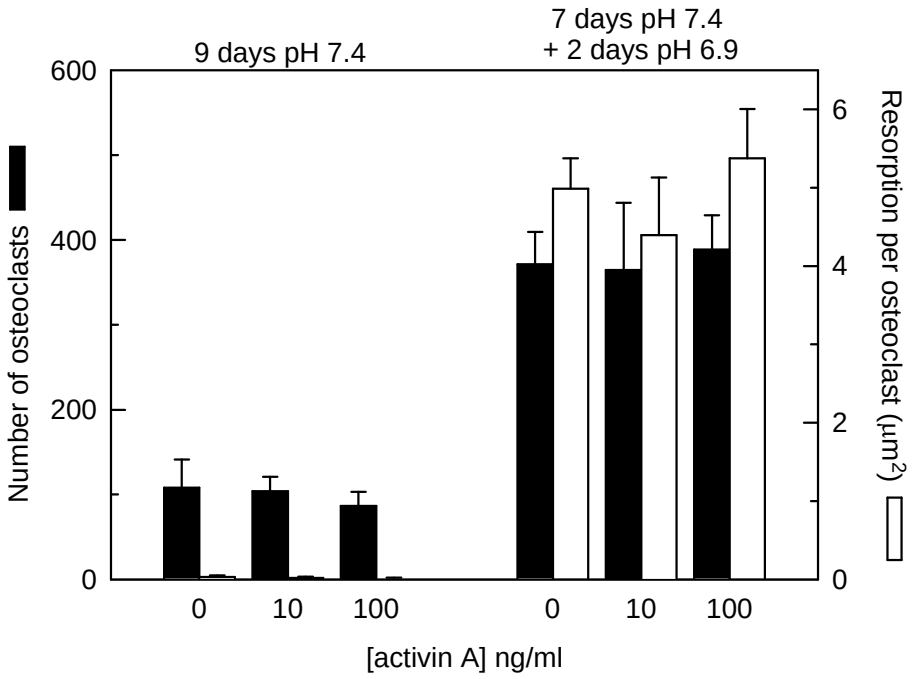


Figure 6.3 *Activin A does not affect osteoclast formation or resorptive activity.*

At concentrations of 10 and 100ng/ml, the cytokine activin A does not affect osteoclast formation. Additionally, activin A does not stimulate osteoclasts to resorb when cultured at physiological pH. Activin A is unable to augment osteoclast resorptive activity when they are already stimulated to resorb by addition of protons. (*n* = 8).

BAFF does not stimulate osteoclasts to resorb at physiological pH.

BAFF was added to osteoclast cultures 48 hours prior to their termination at concentrations of two, 20 and 200 ng/ml in order to determine the effect on osteoclastic resorption. Osteoclasts were maintained at either pH 7.4 for the duration of the experiment or stimulated to resorb by adjusting the pH from 7.41 to 6.93 for the final 48 hours. BAFF was unable to stimulate osteoclasts to resorb when they were maintained at physiological pH. Additionally, when osteoclasts were stimulated by the addition of H^+ to the culture medium, BAFF did not augment resorption (**Figs. 6.4**). Additionally, BAFF had no effect on osteoclast formation.

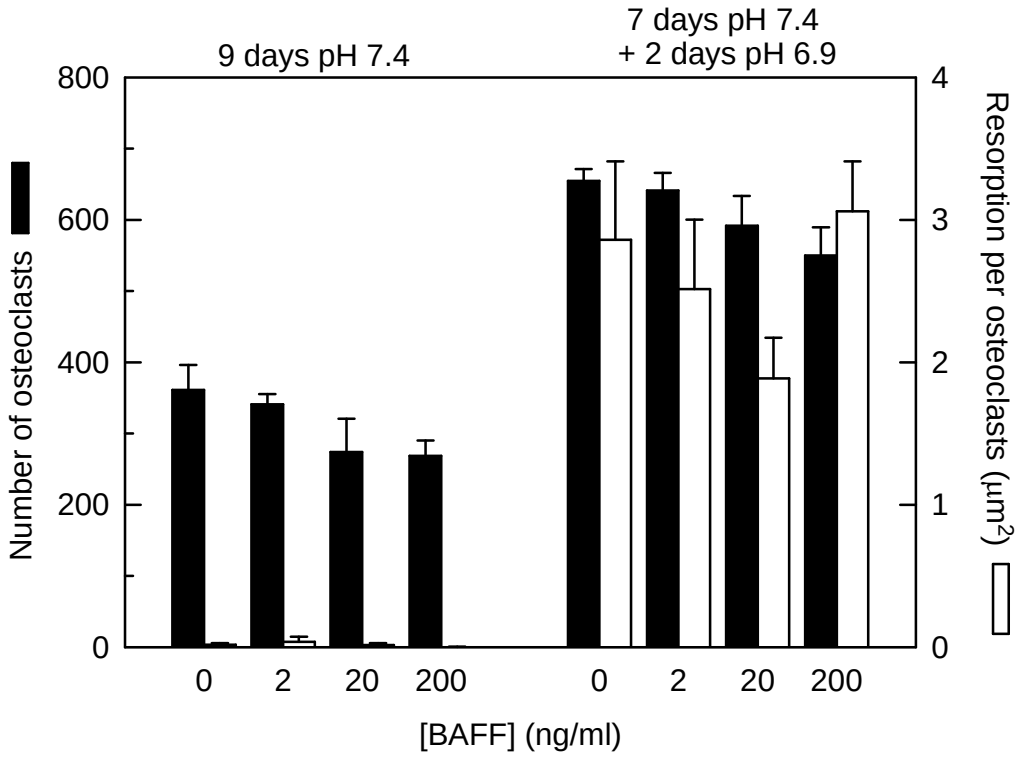


Figure 6.4 *BAFF does not affect osteoclast activation.*

At concentrations of two, 20 and 200ng/ml, BAFF did not stimulate osteoclasts to resorb at physiological pH. When osteoclasts had first been stimulated to resorb by the addition of protons, BAFF is unable to alter the resorptive activity of these osteoclasts. (*n* = 8).

Acidification of culture medium by MDA-MB-231 and MCF-7 human breast cancer cells.

Cancer cells were seeded at different densities onto large dentine discs, (1cm diameter) and placed in 2ml of MEM. Cells were half media changed at day three of culture; medium pH was measured at this point and also at termination on day 5, using a blood gas analyser. MDA-MB-231 and MCF-7, initially seeded at 10^6 cells reduced the pH by 0.18 and 0.21 pH units, respectively (**Figs. 6.5A and 6.5B)**

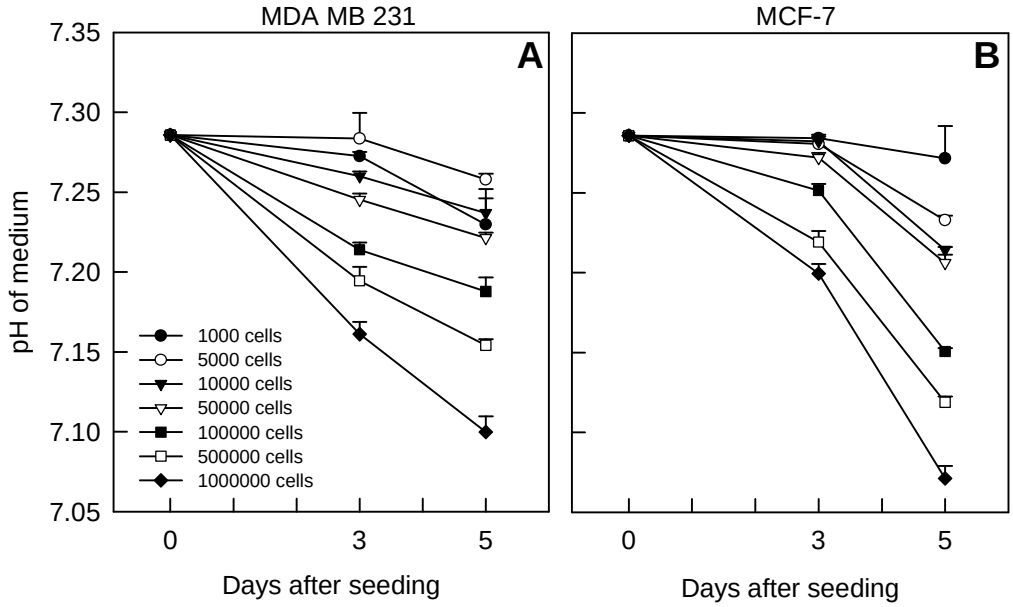


Figure 6.5 *Effect of seeding density of human breast cancer cells on culture medium pH.*

Cells were seeded at 10^6 / 1cm diameter dentine disc. (**A**) MDA-MB-231 cells caused a maximum medium pH decrease of 0.17 units. (**B**) MCF-7 cells caused a maximum pH decrease of 0.21 units.

MDA-MB-231 and MCF-7 human breast cancer cells inhibit osteoclast formation when co-cultured without cell-to-cell contact

Mouse marrow derived osteoclasts were cultured in the presence of MDA-MB-231 or MCF-7 cells for 5 days, medium had either 10meq/l OH^- added in order to maintain pH at ~7.3 for the duration of the experiment, or, 5meq/l OH^- was added to enable cancer cells to self-acidify the medium to approximately pH 7.1 Surprisingly for both cell types, culture with cancer cells resulted in highly significant reductions in numbers of osteoclasts formed (**Fig. 6.6 A**).

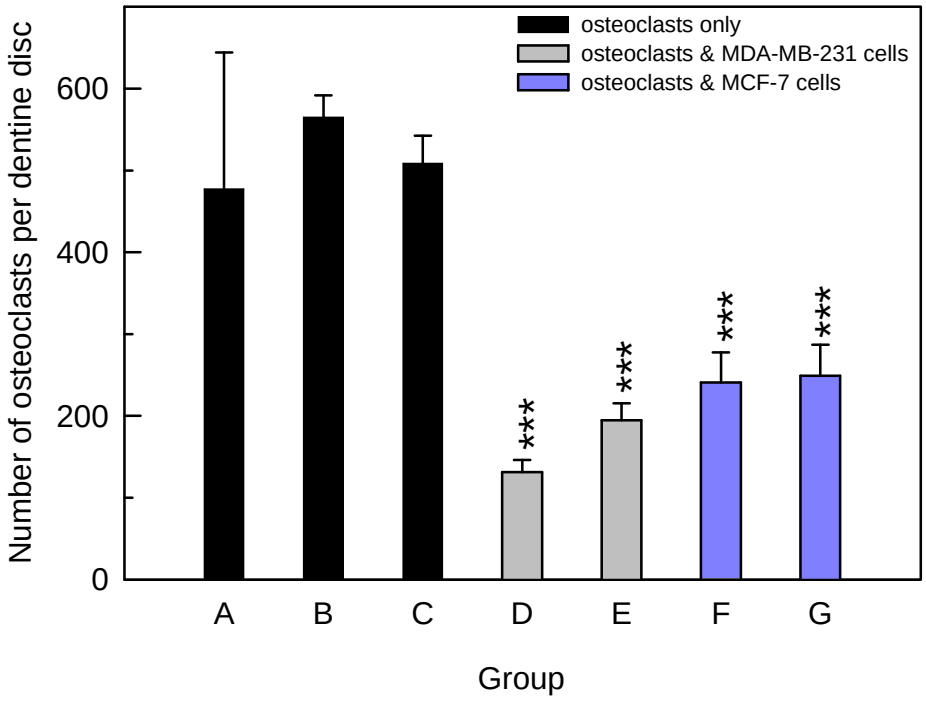


Figure 6.6 A *Effect of pH and co-culture with human breast cancer cells on osteoclast formation in mouse marrow cultures*

Mouse marrow derived osteoclasts were cultured in the presence of M-CSF and RANKL and human breast cancer cells. Co-culture with MDA-MB-231 or MCF-7 resulted in significant decreases in numbers of osteoclasts formed compared to osteoclast only controls regardless of pH.

A, osteoclast formation at pH 7.31 (days 1-7).

B, osteoclast formation at pH 7.31 (days 1-5); resorption stimulated (days 5-7) by acidification with 10meq/L H^+ to pH 7.12.

C, osteoclast formation at pH 7.31 (days 1-5), resorption stimulated (days 5-7) by acidification with 15meq/L H^+ to 6.82.

D, osteoclast formation with MDA-MB-231 cells at pH 7.33 (days 1-7).

E, osteoclast formation with MDA-MB-231 cells at pH 7.33 (1-5 days); self-acidification by MDA-MB-231 cells (days 5-7) to pH 7.14.

F, osteoclast formation with MCF-7 cells pH 7.37 (days 1-7).

G, osteoclasts formation with MCF-7 cells at pH 7.37 (1-5 days); self-acidification by MCF-7 cells (days 5-7) to pH 7.

(*n* = 10, *** = p <0.001).

MDA-MB-231 and MCF-7 human breast cancer cells significantly inhibit osteoclastic resorption

Mouse marrow derived osteoclasts were cultured in the presence of M-CSF and RANKL for 5 days before addition of MDA-MB-231 or MCF-7 cells for two days. Culture medium had either 10meq/l OH- added in order to maintain pH at 7.3 for the duration of the experiment, or 5meq/l OH^- was added to allow cancer cells to self-acidify the medium to approximately pH 7.00. For both cell types, culture with cancer cells resulted in significant reductions in both the amount of resorption carried out per osteoclast and, as osteoclast number was not affected by addition of cancer cells for only two days, total resorption was also significantly reduced. (**Fig. 6.6 B**).

Osteoclastic resorption is significantly increased when cultures are allowed to self-acidify

Mouse marrow derived osteoclasts were cultured in the presence of M-CSF and RANKL for 5 days. Human breast cancer cells without contact with the osteoclasts were added for the final 48 hours of culture to determine the effect on resorption. One group was maintained at approximately physiological pH by addition of OH^-, the other system was allowed to self-acidify via the metabolic activity of the cancer cells. This self-acidification resulted in a decrease in pH large enough to significantly increase the resorption carried out by osteoclasts, as expected, without affecting their number (**Fig. 6.6 C**).

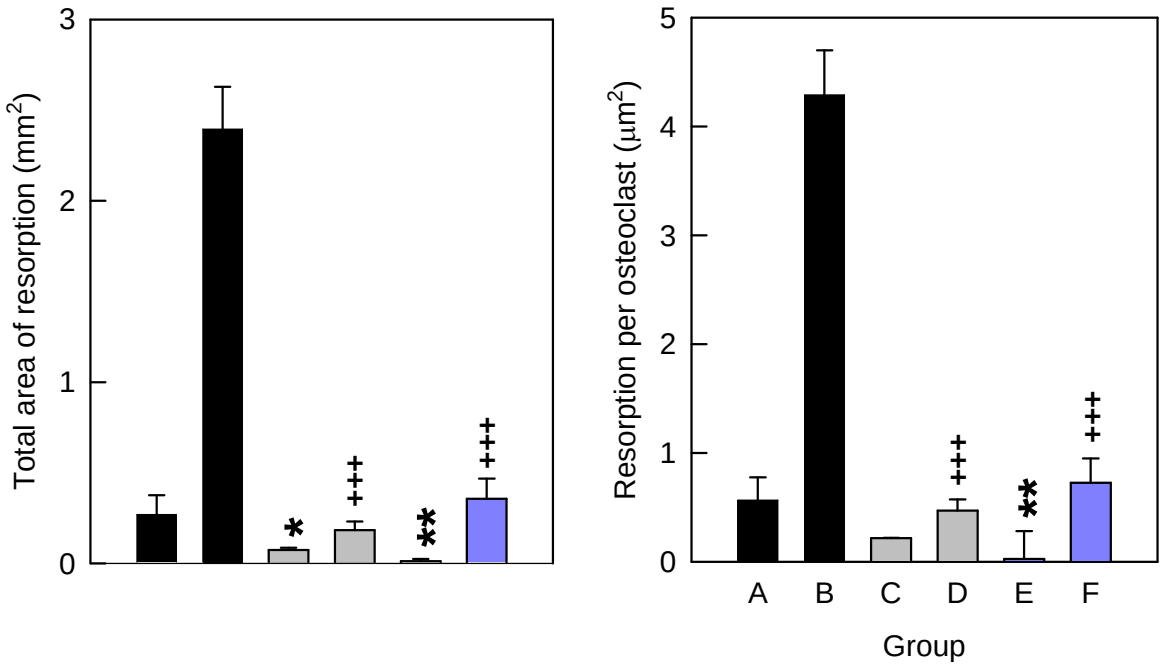


Figure 6.6 B *Effect of pH and co-culture with human breast cancer cells on osteoclastic resorption*

Osteoclasts were generated for 5 days at pH >7.3 from mouse marrow using M-CSF and RANKL. Human breast cancer cells were then added for a further two days, without direct contact with the osteoclasts, to determine their effects on acidification and resorption. Co-culture of osteoclasts with either MDA-MB-231 or MCF-7 breast cancer cells significantly decreased resorption.

A, osteoclast formation at pH 7.34 (days 1-7).

B, osteoclast formation at pH 7.34 (days 1-5); resorption stimulated (days 5-7) by acidification with 10meq/L H^+ to pH 7.17.

C, osteoclast formation at pH 7.32 (days 1-5); co-culture with MDA-MB-231 cells (days 5-7), with addition of 10meq/L OH^- to prevent self-acidification (pH maintained at 7.29).

D, osteoclast formation at pH 7.32 (days 1-5); co-cultured with MDA-MB-231 cells (days 5-7), resulting in self-acidification to pH 6.98.

E, osteoclast formation at pH 7.31 (days 1-5); co-culture with MCF-7 cells (days 5-7), with addition of 10meq/L OH^- to prevent self-acidification (pH maintained at 7.30).

F, osteoclasts allowed to form at pH 7.31 (days 1-5), then co-cultured with MCF-7 cells (days 5-7), resulting in self-acidification to pH 7.02.

(statistics: * compared to osteoclasts at pH 7.34 in column A, + compared to osteoclasts at pH 7.17 upon termination of culture in column B. (*n* = 10, * = p <0.05, ** = p <0.001 *** = p <0.001).

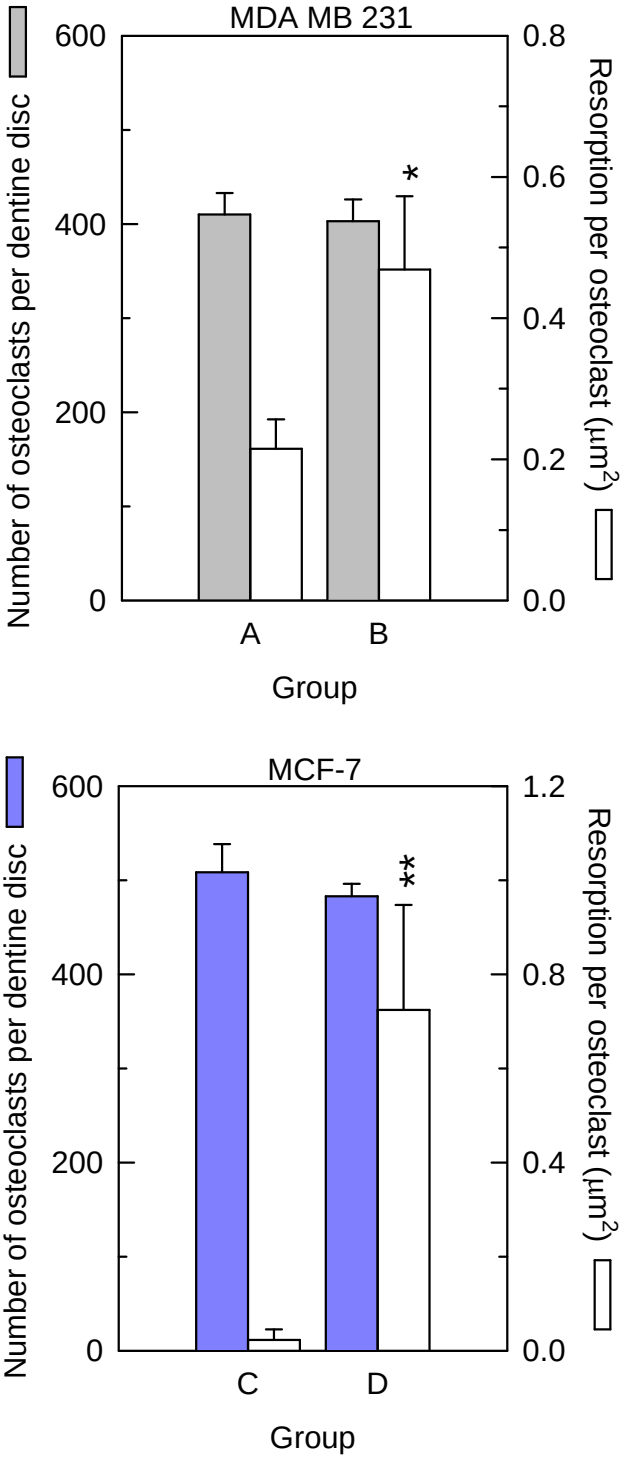


Figure 6.6 C *Acid activation of osteoclasts when co-cultured with human breast cancer cells.*

Osteoclasts in co-culture with breast cancer cells resorb significantly more when the system was allowed to self-acidify when compared to those at physiological pH.

A, osteoclast formation at pH 7.32 (days 1-5); co-culture with MDA-MB-231 cells (days 5-7), with addition of 10meq/L OH^- to prevent self-acidification. (pH maintained at 7.32).

B, osteoclast formation at pH 7.32 (days 1-5); co-culture with MDA-MB-231 cells (days 5-7), resulting in self-acidification to pH 6.98.

C, osteoclast formation at pH 7.31 (days 1-5); co-culture with MCF-7 cells (days 5-7), with addition of 10meq/L OH^- to prevent self-acidification. (pH maintained at 7.31).

D, osteoclast formation at pH 7.31 (days 1-5); co-culture with MCF-7 cells (days 5-7), resulting in self-acidification to 7.12.

(n = 10, * = $p < 0.05$, ** = $p < 0.001$)

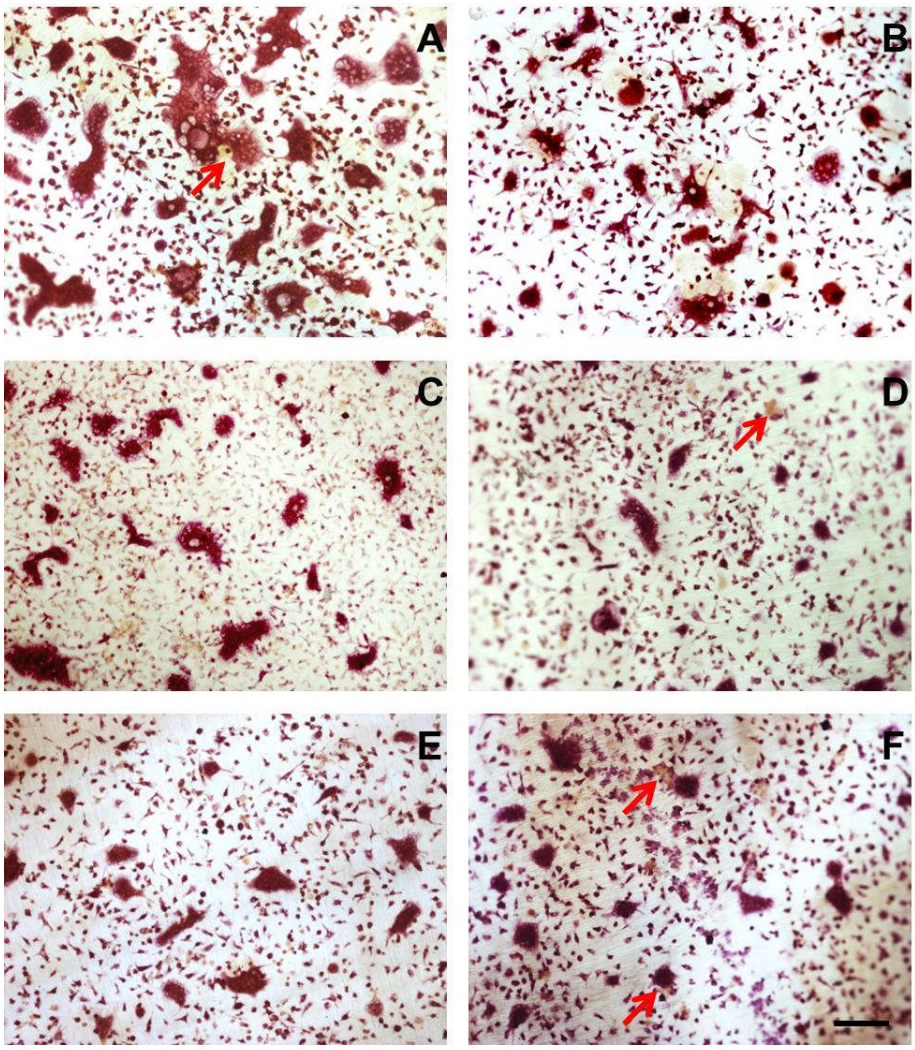


Figure 6.7 *Formation of and resorption by osteoclasts is reduced when co-cultured with MDA-MB-231 or MCF-7 cells*

A, osteoclast formation at pH 7.34 (days 1-7).

B, osteoclast formation at pH 7.34 (days 1-5); resorption stimulated (days 5-7) by acidification with 10meq/L H^+ to pH 7.17.

C, osteoclast formation at pH 7.32 (days 1-5); co-culture with MDA-MB-231 cells (days 5-7), with addition of 10meq/L OH^- to prevent self-acidification (pH maintained at 7.29).

D, osteoclast formation at pH 7.32 (days 1-5); co-cultured with MDA-MB-231 cells (days 5-7), resulting in self-acidification to pH 6.98.

E, osteoclast formation at pH 7.31 (days 1-5); co-culture with MCF-7 cells (days 5-7), with addition of 10meq/L OH^- to prevent self-acidification (pH maintained at 7.30).

F, osteoclasts allowed to form at pH 7.31 (days 1-5), then co-cultured with MCF-7 cells (days 5-7), resulting in self-acidification to pH 7.02.

Osteoclasts: purple/red; resorption trails/pits: orange. Red arrows highlight some of the smaller resorption pits. Scale bar = 100µm.

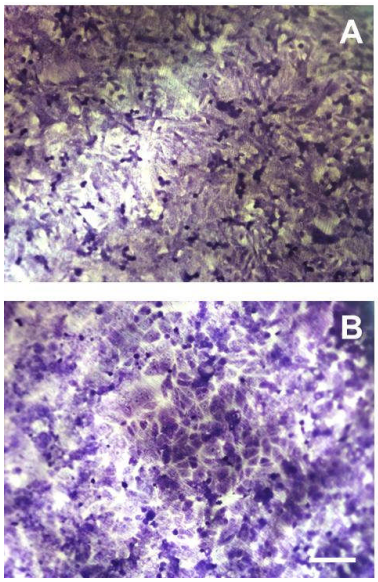


Figure 6.8 *Cancer cell control discs*

MDA-MB-231 cells (**A**) and MCF-7 cells (**B**) were cultured on dentine discs for 7 days then fixed with glutaraldehyde and stained with toluidine blue. Scale bar = 100µm.

Direct co-culture of MDA-MB-231 and MCF-7 human breast cancer cells with marrow derived mouse osteoclasts

Osteoclasts were cultured directly with cancer cells to determine if cell-to-cell contact between the two cell types affected osteoclast formation or resorption. The cancer cells were able to out-compete the osteoclasts for space on the dentine so barely any osteoclasts formed. (**Fig. 6.9**)

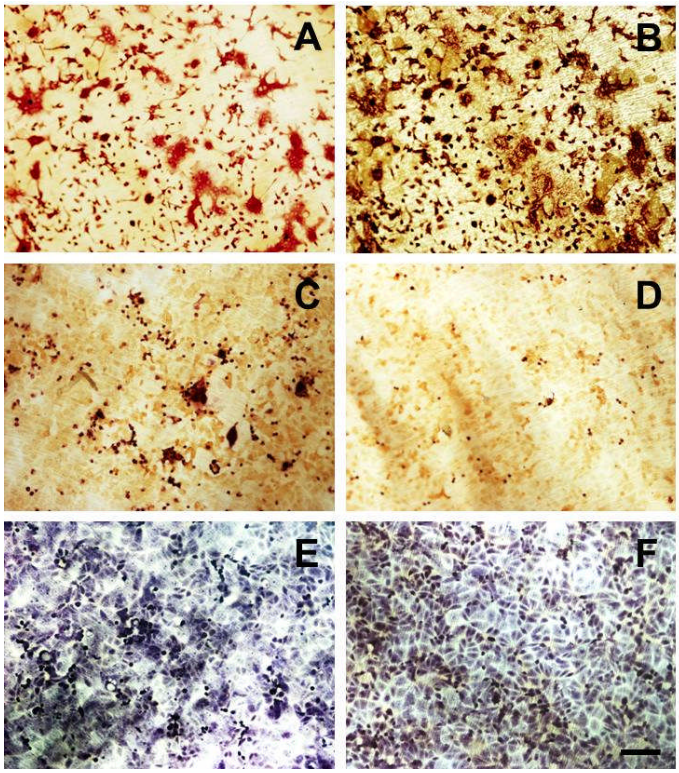


Figure 6.9 *Direct co-culture of osteoclasts with breast cancer cells*

Murine marrow derived cells were cultured in the presence of M-CSF and RANKL for 7 days, and with MDA-MB-231 or MCF-7 breast cancer cells. TRAP staining (red/purple) revealed barely any TRAP positive cells when co-cultured directly with MDA-MB-231 cells (**C**) and none when co-cultured directly with MCF-7 cells (**D**). Staining with toluidine blue demonstrated MDA-MB-231 cells (**E**) and MCF-7 cells (**F**). Osteoclast only controls under transmitted light (**A**) and reflected light to view resorption trails (orange) (**B**). Scale bar = 100µm.

Expression of genes, important in osteoclastogenesis and resorption, by MCF-7 and MDA-MB-231 breast cancer cells

qRT-PCR was performed on cDNA generated from RNA extracted from MCF-7 and MDA-MB-231 cells. Cells were investigated for *RANKL*, *M-CSF*, *OPG*, *IL-6*, *TNFα*, *TGFβ1* and *TGFβ2*. RANKL expression was not detected in either cell type. Results revealed that M-CSF was only expressed by MDA-MB-231 cells. As such, the relative amounts compared between cell types cannot be carried out, therefore the PCR products were run on a gel and visualised using ethidium bromide and UV (**Fig. 6.10**). Additionally, *IL-6* was shown to be expressed only by MDA-MB-231.

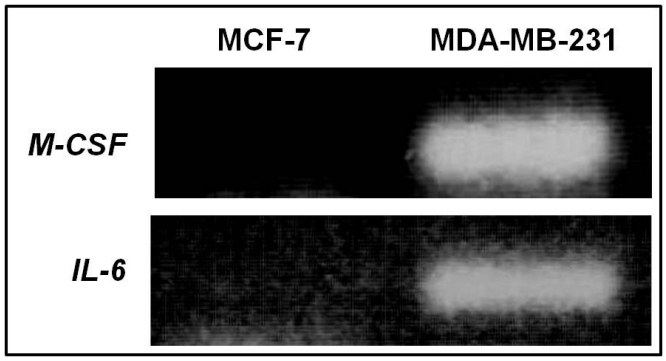


Figure 6.10 *Expression of M-CSF and IL-6 by MCF-7 and MDA-MB-231 cells*

qRT-PCR revealed that MCF-7 cells do not express mRNA for M-CSF or IL-6, in comparison; MDA-MD-231 cells did express both M-CSF and IL-6, as shown by bands present at 100bp and 99bp.

Other genes investigated all demonstrated differences in expression levels between the two cell types. *OPG* was expressed 10.95-fold higher in MDA-MB-231 cells compared to MCF-7 cells. *TNFα* was decreased in MDA-MB-231 cells by 16% compared to MCF-7. Both *TGFβ1* and *TGFβ2* were increased in MDA-MB-231 cells compared to MCF-7s, 4.89- and 49.51-fold, respectively (**Fig. 6.11**).

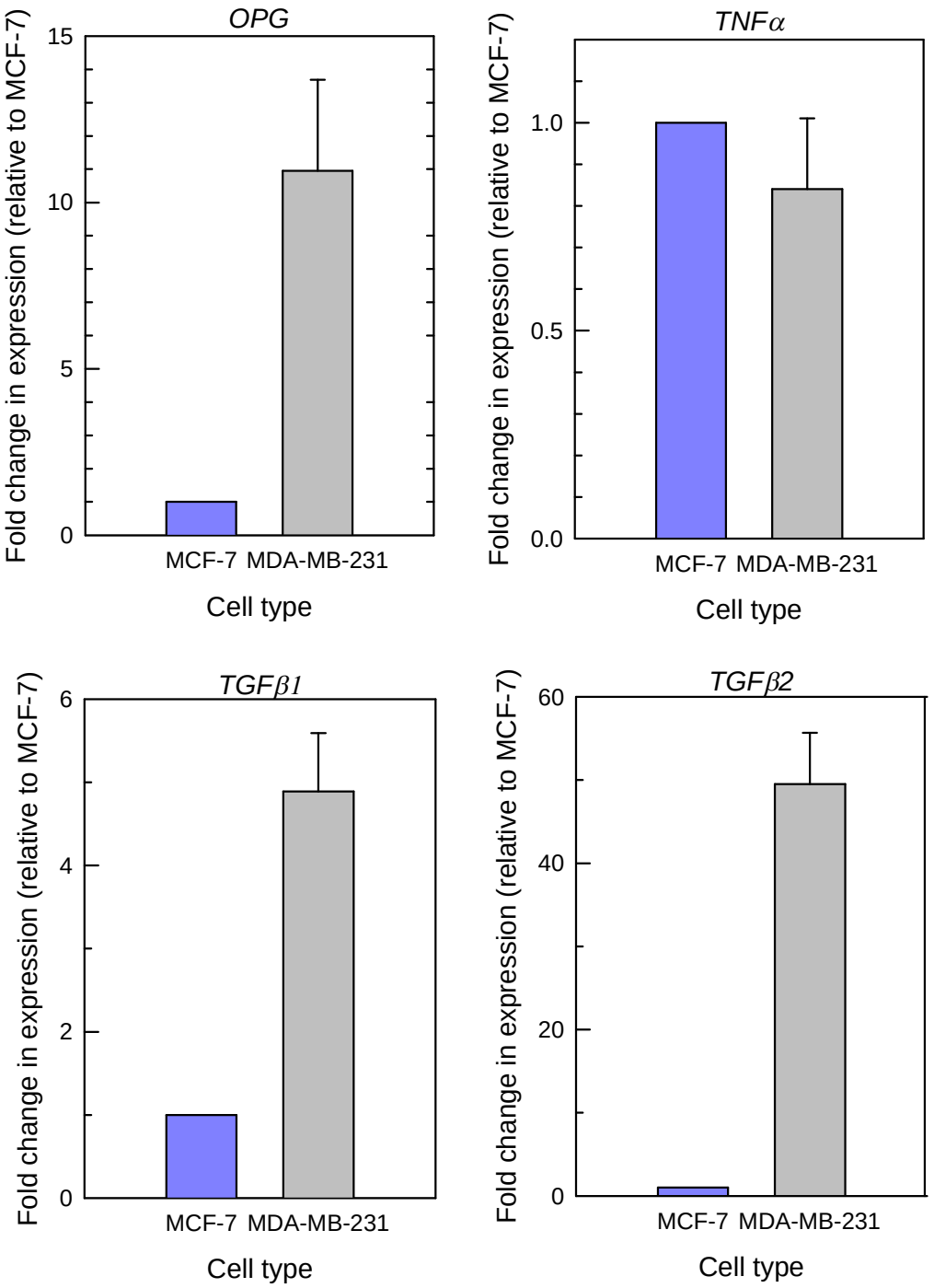


Figure 6.11 *Expression of genes involved in osteoclastogenesis by MDA-MB-231 and MCF-7 cells*

qRT-PCR revealed that *OPG, TGFβ1 and TGFβ2* expression is 10.95-, 4.89- and 49.51-fold higher, respectively, in MDA-MB-231 cells compared to MCF-7s. It also demonstrated that *TNFα* expression is decreased by 16% in MDA-MB-231 compared to MCF-7s.

Discussion

Breast cancer is the most common type of cancer in the UK (Key *et al.* 2001), and the second most common in Europe (Ferlay *et al.* 2010), although survival rates are increasing year on year (Peto *et al.* 2000; Jemal *et al.* 2010). This, however, means that more people are living with the complications that accompany breast cancer. A common consequence of breast cancer is metastasis, particularly to bone, where osteolytic bone disease occurs. Osteolytic bone disease results in increased osteoclast activation and therefore greater bone resorption, and localised bone destruction.

The current model of cancer metastasis and subsequent bone destruction is known as the 'vicious cycle of bone metastases'. The model proposes that once cancer cells have metastasised to bone, they produce a large number of growth factors and cytokines including PTHrp, TGFβ and IL-11. The model states that these growth factors and cytokines are able to stimulate osteoclastic resorption of bone, therefore resulting in release of growth factors ordinarily trapped within the bone matrix. Once released into the bone/cancer microenvironment, they stimulate the growth of further cancer cells and hence the cycle continues (Kingsley *et al.* 2007).

This model, however, fails to take into account the effect of acid on bone cells. Acid has been known to have a detrimental effect on bone for many years; it prevents bone formation (mineralisation) by osteoblasts and causes done destruction by stimulating osteoclast activity. Some authors are now beginning to take account of acidic microenvironment in which the vicious cycle of bone metastases takes place (Kingsley *et al*. 2007) but rarely consider the direct action of low pH on osteoclasts in this location, instead placing more significance on the many growth factors and cytokines found in this locale.

One of the initial aims of this thesis set out to examine whether two of the key cytokines implicated in this model (activin A and BAFF) could stimulate osteoclasts to resorb at physiological pH. Activin A recently came to attention as

a cytokine of interest following cytokine profiling of patients suffering from multiple myeloma and associated osteolysis. Activin A was the only cytokine found to be significantly increased in patients with osteolysis compared to those who had no osteolytic lesions (Vallet *et al.* 2010). It was found that activin A at concentrations up to 100ng/ml was unable to stimulate osteoclastic resorption at physiological pH; furthermore it was unable to increase the amount of resorption carried out when osteoclasts had been initially stimulated with exogenous H^+ (**Figs. 6.2 and 6.3**).

Currently, the precise role of activin A is unknown, with many laboratories reporting contradictory stimulatory and inhibitory effects of activin A on osteoclast activity (Fuller *et al.* 2000; Gaddy-Kurten *et al.* 2002; Nicks *et al.* 2009; Leto 2010; Vallet *et al.* 2010); interestingly, none of these groups reported the pH of cultures, so increased resorptive activity as a result of acid activation cannot be ruled out. Recent work by our collaborators has shown that activin A actually inhibits osteoblastic differentiation and tumour burden (Chantry *et al*. 2010). In animals in which activin A activity was blocked, bone mass was increased *in vivo*; *in vitro*, blocking activin A resulted in increased osteoblast differentiation and mineralisation. Moreover, osteoclasts from these animals in which activin A was blocked were unaffected, both in number and activity (Chantry *et al.* 2010); these data are consistent with the results presented in this thesis. However as stated, other groups have reported contradictory results in which *in vivo* mouse studies using an activin A soluble decoy receptor show decreased osteoclast number (Vallet *et al.* 2010); additionally, human trials with a similar activin A decoy receptor resulted in decreased serum levels of C-terminal type 1 collagen telopeptide (CTX) and TRAPC-5b levels (Ruckle *et al.* 2009), both indicative of decreased resorption levels. Taken together these results indicate that *in vivo*, activin A can affect osteoclast formation and resorption, though neither study reveals the mechanism by which this may be occurring.

B-cell activating factor (BAFF) is often produced by cancer cells that go on to cause osteolytic bone disease, including multiple myeloma. (Tai *et al.* 2006);

(Podar *et al.* 2009). BAFF also supports the growth of cancer cells within the bone marrow microenvironment (Breitkreutz *et al.* 2008). Patients with multiple myeloma have serum levels of BAFF approximately 4-fold higher than healthy individuals (Moreaux *et al.* 2004). It was found that BAFF at concentrations up to 200ng/ml was unable to stimulate osteoclasts to resorb when at physiological pH (**Fig. 6.4**). Furthermore, BAFF did not cause any change in the amount of resorption carried out by osteoclasts which had already been stimulated to resorb by the addition of exogenous H^+ (as HCl) to the system.

It has been reported that within the bone microenvironment, BAFF acts to promote survival of cancer cells themselves via a paracrine action (Neri *et al.* 2007; Baud & Karin 2009). Hemingway *et al*. have demonstrated that BAFF could stimulate osteoclastic resorption via a RANKL-independent mechanism; however, osteoclasts treated with M-CSF and BAFF only resorbed 0.2% the area of osteoclasts treated with both M-CSF and RANKL and the authors acknowledge that BAFF is not an efficient inducer of osteoclast generation (Hemingway *et al.* 2011). If BAFF only accounts for 0.2% of the normal amount of resorption that occurs it is unlikely that blocking it would have been responsible for the large changes in resorption reported by other groups. Furthermore, the pH of these cultures was not controlled and osteoclasts are able to self-acidify their medium *in vitro*, so resorption might have been a consequence of acid stimulation. The *in vivo* effects of BAFF are less clear; it has been shown that a mouse model of multiple myeloma administered with an anti-BAFF neutralising antibody had decreased osteoclast recruitment and activity (Neri *et al.* 2007).

Results I have presented indicate that any effects that activin A and BAFF might have on osteoclasts must occur via indirect means. Thus, future work could involve co-culturing osteoclasts with osteoblasts or stromal cells in order to determine whether activin A or BAFF are able to mediate effects on osteoclasts via these cell types and therefore an indirect mechanism. The murine osteoclast isolation method used to investigate the effects of activin A and BAFF involved removal of the majority of stromal cells and isolating just the haematopoietic cells

of the mouse marrow. These are the cells which gave rise to osteoclasts following addition of M-CSF and RANKL, thus providing a means to determine direct effects of test compounds on osteoclasts.

The rest of the work presented in this chapter involved the co-culture of osteoclasts with breast cancer cells. My results show that when osteoclasts were co-cultured with either MDA-MB-231 or MCF-7 human breast cancer cells for 5 days, osteoclast formation was significantly reduced in both cases (**Fig. 6.6 A**). This was surprising, as it was hypothesised that cancer cells would stimulate osteoclast formation. There are two possible explanations for the decreased osteoclast number firstly that the decreased number of osteoclasts were the result of the cancer cells using up the nutrients and therefore inhibiting osteoclast formation; a second more likely explanation is due to the increased levels of OPG produced by both of the cancer cell lines. Effects of co-culture on osteoclast activity are discussed later.

qRT-PCR revealed that, although neither of the breast cancer cell types express *RANKL*, both express *OPG* (**Fig. 6.10**). *OPG* mRNA was expressed 11-fold higher in MDA-MB-231 cells compared to MCF-7 cells, which correlates with the greater reduction in osteoclast number formed when co-cultured with MDA-MB-231 cells compared to MCF-7 cells. Expression of *OPG* by these cancer cell lines is in agreement with work published by (Holen *et al.* 2005; Rachner *et al.* 2009). It should be noted that the MDA-MB-231 and MCF-7 cells were grown in RPMI, the qRT-PCR should be repeated on cells that have been grown in MEM as the co-culture experiments in order to confirm that the effects are the same.

In patients with breast or prostate cancer that has metastasised to the bones, higher levels of serum OPG have been recorded in comparison to those without metastases (Dougall & Chaisson 2006). In mice injected with cancer cells, immediately followed by administration with OPG, reduced numbers of osteoclasts resulted and osteolytic lesions did not form unlike animals injected with cancer cells only (Zhang *et al*, 2001). Given that neither MDA-MB-231 cells nor MCF-7 cells produced RANKL but did produce OPG, the

(exogenous)RANKL:OPG ratio would have been altered. This would have prevented differentiation of pre-osteoclasts into mature bone resorbing osteoclasts. This could account for the decrease in osteoclast numbers recorded compared to osteoclast-only control groups.

Correspondingly, when cancer cells were added for only the final 48 hours of culture (i.e., to determine their effect on osteoclast activation rather than formation), no reduction in osteoclast number was observed, which adds further weight to the theory that the OPG produced by the cancer cells themselves is responsible for inhibition of osteoclast formation. These data show that the *in vivo* effects of cancer on bone cells are likely to be a complex system of inhibitory and stimulatory factors.

The effect of co-culture on osteoclast activity (i.e., resorption) was also investigated. Osteoclasts co-cultured with MDA-MB-231 cells and MCF-7 cells resorbed significantly less at both physiological and acidic pHs when compared to osteoclast only controls at the equivalent pH (**Fig. 6.6 B**). Within the 'vicious cycle' model, much emphasis has been placed on growth factors and cytokines produced by cancer cells that go on to stimulate osteoclast activity (Guise 2000; Roodman 2004; Rose & Siegel 2006; Kingsley *et al.* 2007; Clezardin 2011). qRT-PCR has demonstrated that MDA-MB 231 cells express *IL-6*, *TGFβ1*, *TGFβ2* and *TNF*α and MCF-7 cells express *TGFβ1*, *TGFβ2* and *TNFα* (**Figs. 6.10** and **6.11**). Results presented in this chapter however, suggest that these factors have not stimulated osteoclasts to resorb to the same extent that acid can (**Fig. 6.6 C**), however, when osteoclasts were co-cultured with MDA-MB-231 cancer cells at physiological pH, there was 10 times the amount of resorption than MCF-7 cells. This provides evidence that the different combination of growth factors produced by each cell type in addition to the acid produced by these cells contributes to the overall stimulatory effect of osteoclast activity.

Owing to the high metabolic activity of osteoclasts, they are able to self-acidify their own culture medium. This therefore means that the cytokines and growth factors implicated in the 'vicious cycle' may not be solely responsible for

stimulating osteoclasts to resorb but rather, they augment resorption when osteoclasts were stimulated to resorb by acid. This is true of RANKL; acid potentiated the small stimulatory effect of RANKL at pH 7.41 to cause a ~20-fold increase in resorption in osteoclasts treated with 100ng/ml RANKL at pH 6.98 (Orriss & Arnett 2012).

It should be noted that only mRNA expression has so far been investigated, and any effect on protein would need to be determined to confirm these findings. Additionally, there are some differences in expression of cytokines detected compared with reports in the literature. MCF-7, MDA-MB-231 and another breast cancer cell line, T-47D, account for more than two thirds of all breast cancer research listed in PubMed (Lacroix & Leclercq 2004). It is proposed that the very nature of cancer cells means that these cell lines *in vitro* are likely to undergo genotypic and phenotypic alterations resulting from long term cell culture (Lacroix & Leclercq 2004). Given that individual laboratories use slightly different conditions to maintain cell lines and to conduct experiments, cell lines evolve differently, characteristics diverge and as such become a distinct sub-population of the original cell line result (Lacroix & Leclercq 2004). Differences in expression I have reported here may be a result of this divergence.

As previously stated, qRT-PCR revealed a lack of *RANKL* but the presence of *OPG* expression by both of the cell lines investigated. In addition to the clear inhibitory effect that OPG has on osteoclast formation, as discussed previously, it is also an important regulator of resorption. OPG has been found to block cancer induced skeletal destruction (Honore *et al.* 2000; Zhang *et al.* 2001; Theoleyre *et al.* 2004). Therefore the lack of resorption I saw when osteoclasts were cultured with breast cancer cells may be a result of increased OPG levels. If this is the case, it would indicate the 'vicious cycle' model might not take into account other inhibitory factors that might be present in the micro-environment.

Other factors known to be inhibitory to osteoclasts and therefore provide opportunities for further work include IFN-γ, which inhibits bone resorption *in vitro* (Gowen & Mundy 1986). Additionally IL-4 has been shown to inhibit both

osteoclast formation and resorption (Shioi *et al.* 1991; Bendixen *et al.* 2001; Mangashetti *et al.* 2005). Oestrogen, as outlined in Chapter 1, is a major inhibitor of osteoclast formation (Roodman 2004).

An important observation made when analysing the results of the co-culture experiments independently of osteoclast-only control groups was that more resorption was carried out by the osteoclasts when the pH of the medium was allowed to drop compared to those that had been maintained at a physiological pH (**Fig. 6.6 C**), highlighting the role of low pH as a stimulator of osteoclastic resorption. Importantly the pH values at which resorption occurred for MDA-MB-231 cells and MCF-7 cells (6.98 and 7.12) were both within the pathophysiological range. pHs of cancer *in vivo* have been measured in a 27 day tumour where hypoxia or anoxia were also present and pH was consistently found to be below 7.0. Distance from the nearest blood vessel also influenced the interstitial pH within the tumour; 20µm from the blood vessel gave a pH of 7.2, 50µm gave a pH of 7.04, at 150µm from the blood vessel the pH was recorded as 6.9, and at 300µm the pH was 6.7. In some large tumours in which some areas had become avascular, interstitial pH was recorded as low as 6.6 (Helmlinger *et al.* 1997). This indicates that pH values generated by the co-culture system *in vitro* are comparable to those achieved in the *in vivo* disease situations which are being investigated.

Work presented in this chapter was dependent on *in vitro* modelling of a disease state. One of the most obvious challenges of *in vitro* modelling is that cell culture provides conditions are optimised for growth, whereas *in vivo* conditions are dictated by a particular person's physiology. Environmental changes to which the cancer cells are exposed to *in vivo* may well result in both phenotypic and genotypic changes to the cancer cells which in vitro modelling cannot replicate (van Staveren *et al.* 2009). The model I have developed to co-culture breast cancer cells with osteoclasts has some advantages, most notably, that the model allows osteoclasts to be cultured on dentine, on which they are able to resorb and therefore behave as they would *in vivo*; this is clearly more

realistic than using plastic tissue culture dishes, which osteoclasts cannot resorb. Furthermore, in this model, the pH was closely monitored, whereas in current systems, this important modulator of osteoclast activity is not considered. Achieving the desired pH without altering it artificially did require a careful balance between amount of medium used to keep the cells alive and functioning normally and seeding enough cancer cells to allow these cells to acidify the medium themselves as they would *in vivo*. Repeating the experiments with increased culture volumes and artificially altering the pH might yield different results but this would mean that cells are further removed from conditions comparable to those within the body.

In vivo, tumours are often hypoxic, which in some cases can promote tumour growth (Knowles & Harris 2001; Harris 2002), and this model has not accounted for this. Different results may have been obtained if a hypoxic environment had been established. Hypoxia has well documented effects on osteoclasts; *in vitro*, hypoxia has been reported to double osteoclast formation from human peripheral blood mononuclear cells and increase their resorption 10-fold (Utting *et al.* 2010). Incorporating hypoxia experimentally provides an opportunity to build upon and improve this model. It is also worth noting that this work has only been carried out on two human breast cancer cell lines: MDA-MB-231 and MCF-7; however, the majority of all breast cancer research carried out worldwide is carried out on these cells lines (in addition to one other) (Lacroix & Leclercq 2004). This work should be repeated on additional breast cancer cell lines to determine whether this effect is universal or not. It would also be advantageous to repeat this on other cancer cell types that metastasise to bone i.e., prostate cancer (Langley & Fidler 2011) and lung adenocarcinoma (non-small cell lung cancer) (Nguyen *et al.* 2009; Yamashita *et al.* 2012).

Finally, it must be noted that this system was heterologous; using human breast cancer cell lines and primary murine osteoclasts. This work should be repeated in a homologous system using primary human osteoclasts, derived from

peripheral blood mononuclear cells in order to confirm that results presented here are the same.

In conclusion, it has been shown that many of the intricacies of the vicious cycle model of cancer metastases to bone may not be fully appreciated and, although simplifications and modelling need to be made to study these systems *in vitro*, the actual *in vivo* system should always be considered. I have also highlighted the importance of monitoring pH of *in vitro* experiments as acidosis has profound effects on osteoclast activity.

Chapter 7

General discussion and future work

The negative effects of acid on the skeleton have been known for many years (Goto 1918). For a long time it was thought that the negative effects were a simple physico-chemical effect, whereby excess acid in the body dissolved the bone which is a large store of base in order to balance pH within the body. This would function to maintain pH within the narrow physiological limits (normal blood pH 7.34-7.45) that are required to support life. However in 1986 an important observation was made, whereby it was noted that osteoclasts were actually stimulated to resorb bone when the pH dropped slightly but was still within normal physiological limits (Arnett & Dempster 1986). This indicated that drops in pH had a direct effect on osteoclasts.

It was another nineteen years before it was reported that acidosis also had a direct negative functional effect on osteoblasts. Acid prevents mineralisation both physico-chemically and by suppressing alkaline phosphatase expression. The effect is selective as the growth of cells and collagen production are not affected (Brandao-Burch *et al.* 2005b).

The above observations led to attempts to elucidate the mechanisms by which osteoclasts and osteoblasts were able to detect acidosis; this has been one of the key focuses of this thesis. pH within the body is one of the most tightly controlled of all the homeostatic mechanisms (Frassetto *et al.* 1996). Acidosis will typically result in impaired cellular function. One of the consequences of severe changes in pH is to amino acids and therefore proteins. Fluctuations away from the physiological norm result in protein denaturation (Dzakula *et al.* 1991) and as a consequence reactions would cease and life would not continue. Presumably this has been the case since the beginning of cellular life, therefore even the most primitive cells would have needed a way to sense the pH (Johnson 1998; Padan 2011). As cells evolved

into multicellular organisms it is likely that various different single celled organisms that were absorbed into the larger organism each had their own acid sensing receptor(s) (Padan 2011). This may help account for the many different types of acid sensing receptor expressed by mammalian cells. Thus, understanding the mechanisms by which bone cells sense acid is a potentially difficult task. It is worth pursuing however, as it may be possible to design a drug to the particular acid-sensor on osteoclasts for example, in order to block the receptor so that protons cannot be detected and therefore the osteoclasts are not stimulated to resorb bone. This approach could potentially offer new therapeutic targets for bone loss disorders such as osteoporosis.

Work by past members of the Arnett laboratory investigated the acid-sensing receptors OGR1, TDAG8, GPR4 and ASIC1a. Osteoclasts isolated from *Ogr1*$^{-/-}$ and *Tdag8*$^{-/-}$ mice had the same response to acid as those from wildtype animals and from that it is inferred that they are able to sense protons in the extracellular environment (Gasser *et al.* 2006). mRNA from GPR4 could not be detected on osteoclasts, and mRNA for ASIC1a was down-regulated in acid activated osteoclasts (Brandao-Burch 2005a), suggesting that these proteins are unlikely to act as acid-sensors in osteoclasts.

The focus of work presented in Chapter 5 was the TRP family of ion channels. TRPV1 is well a well-documented acid-sensing receptor which also detects the hot ingredient in chilli peppers, capsaicin, as well as temperatures above 43ºC (Benham *et al.* 2003; Clapham 2003; Desai & Clapham 2005; Montell 2005; Voets *et al.* 2005; Nilius *et al.* 2007; Venkatachalam & Montell 2007). As described in Chapter 5, I found that both osteoclasts and osteoblasts express TRPV1. I therefore tested the hypothesis that capsaicin might stimulate bone resorption by cultured osteoclasts, including in non-acidified conditions. However, this was not the case. Capsaicin was similarly without effect on osteoblast function. Immunofluorescence appeared to show that TRPV1 expressed by osteoclasts was located intracellularly; therefore, the lack of effect could be a result of capsaicin being unable to interact with TRPV1 at

the cell membrane. This immunofluorescence needs to be repeated, ensuring that the osteoblast cell membrane is intact to confirm the cellular location of TRPV1 by osteoblasts. The TRPV1 antagonist SB366791 inhibited bone nodule formation up to concentrations of 2μM, although not significantly. It is possible that there is a biphasic effect, and a wider concentration range would need to be tested to investigate this further. Given that SB366791 is an antagonist of TRPV1, it is unexpected that it would inhibit bone formation at pH 7.4, rather it was expected to antagonise the effect of acid and, therefore, bone nodule formation would happen at pH 6.9. The effect of SB366791 on bone nodule formation at physiological pH warrants further investigation.

Agonists and antagonists for the channels TRPV4, TRPV3 and TRPM8 were either without effect or cytotoxic; therefore leading to the conclusion that the TRP family of ion channels are probably not acting as the sole acid-sensing receptors in osteoclasts or osteoblasts.

It should, however, be taken into consideration that the system used for this investigation was optimised to result in maximal osteoclast formation and activity with RANKL. Lack of stimulatory effects seen may be because maximal stimulation had already been reached and, therefore, any additional effects that might have been observed may have been masked. To clarify whether TRP agonists have a stimulatory affect it would be prudent to repeat these investigations using a lower concentration of RANKL.

Osteoclasts obtained from *Trpv1*$^{-/-}$ null mice were still able to resorb and therefore respond to acid, indicating that protons must have been sensed by another acid-sensing receptor. Resorption by osteoclasts, however, was reduced by approximately 31% compared to osteoclasts isolated from wildtype animals. This might provide evidence that TRPV1 does contribute in part to acid-sensing by osteoclasts.

Work carried out by other groups investigating the role of the TRP receptors on bone cells have presented contradictory results, whereby

capsaicin has inhibited resorption by osteoclasts (Takita *et al.* 2007; Kobayashi *et al.* 2012). In both cases it is proposed that capsaicin is able to attenuate osteoclastic resorption by signalling through TRPV1 on osteoblasts which in turn down-regulates production of RANKL (Takita *et al.* 2007; Kobayashi *et al.* 2012). Co-culture experiments of osteoblasts with osteoclast precursor cells could be performed in order to determine if the lack of effect seen in this study was due to capsaicin exerting its effects via TRPV1 on osteoblasts rather than on osteoclasts directly.

The functions of several other classes of acid sensing proteins in bone cells may also be worth investigating further. Of the ASICs, ASIC3 is activated within the physiological range that osteoclasts are activated (Birdsong *et al.* 2010) and it is expressed by human osteoclasts (Jahr *et al.* 2005). Additionally TWIK-1, TALK-1, TASK-1, -2 and -3 are all activated within the physiological range (Duprat *et al.* 1997; Holzer 2009). Any of these may be a candidate proton sensor in osteoclasts or osteoblasts.

Since pH sensing is critical to cell survival (Frassetto *et al.* 1996; Padan 2011), it may be that bone cells have evolved 'degenerate' acid sensing mechanisms, involving many different pH cell surface sensors. Protons are also able to enter the cell; extracellular decreases in pH resulted in fast drops in intracellular pH when cultured in bicarbonate/CO_2 buffer (Arnett, personal communication). Such redundancy may suggest that it will be a difficult problem to solve. Blocking or knocking out a single channel may have limited effects.

Chapter 4 demonstrated that acid has additional direct effects on osteoblasts, whereby it prevents mineralisation of the organic matrix in a manner that is not physico-chemical. The focus of work in this chapter was to elucidate the mechanism by which osteoblasts were prevented from mineralising the matrix. It is known that the key regulator of mineralisation anywhere in the body is the ratio of phosphate to pyrophosphate (Fleisch *et al.* 1966). Pyrophosphate as a by-product of many cellular reactions is present, and acts to inhibit mineralisation of the soft tissue. In mineralised tissues,

however, pyrophosphate is hydrolysed by the actions of enzymes including alkaline phosphatase to generate phosphate (Fleisch *et al.* 1966; Meyer & Nancollas 1973; Addison *et al.* 2007). Under acidotic conditions, alkaline phosphatase activity was significantly decreased (Brandao-Burch *et al.* 2005b), which would be expected to shift the balance in favour of more pyrophosphate in the extracellular fluid surrounding the osteoblasts.

The investigations in Chapter 4 focussed on other enzymes that generate pyrophosphate and phosphate, and of those examined, the pyrophosphate producing E-NPP1 was found to be up-regulated in osteoblasts that had been cultured in acidotic conditions. However, I found that long term acidosis, in addition to causing an increase in pyrophosphate generation, also resulted in significantly increased phosphate generation. Thus, the overall Pi:PPi ratio remained the same; indicating that acidosis was not preventing mineralisation by ultimately altering the critical Pi:PPi ratio. As other members of the E-NPP and E-NTPdase families were either unaffected or down-regulated in osteoblasts cultured at pH 6.9, it may be that another phosphate producing enzyme was responsible for the increases in phosphate generation.

In order to understand further the mechanism by which acid inhibits mineralisation by osteoblasts, it would be worth determining whether PHOSPHO 1 is affected by acid conditions, especially given that the optimum activity of PHOSPHO 1 is reported to occur around pH 6.7 (Roberts *et al.* 2004). In *Phospho 1*$^{-/-}$ animals, bones are negatively affected; there is reduced mineralisation of the trabecular bones and an absence of secondary ossification centres in some animals (Yadav *et al*. 2011). Furthermore, TNAP activity in hypertrophic chondrocytes is reduced. These observations suggest that PHOSPHO 1 has a role in producing phosphate for proper bone production (Yadav *et al.* 2011). pH regulation of the function of the enzyme ANK, which additionally contributes to maintaining levels of PPi both intra- and extra-cellularly (Ho *et al*. 2000) would also be worthwhile.

It would appear that the activities of E-NPP1, TNAP, PHOSPHO 1 and ANK are intrinsically linked in order to regulate mineralisation *in vivo* (Yadav *et al.* 2011). Investigating the more specific effects of acid on PHOSPHO 1 and ANK individually may help to elucidate the exact parts played by each enzyme in order to determine the role that acid has. Additional information about the role of E-NPP1 in mineralisation will come from experiments with primary osteoblasts derived from *E-npp1*$^{-/-}$ mice in our laboratory.

Furthermore, because the Pi:PPi ratio was not altered to prevent mineralisation as hypothesised, the question remains as to whether E-NPP1 in mature osteoblasts might have another role. Overexpression of E-NPP1 by muscle, adipose tissue and fibroblasts has been demonstrated in insulin-resistant individuals. E-NPP1 overexpression inhibits insulin-induced activation of the insulin receptor (Goldfine *et al.* 1998; Goldfine *et al.* 1999; Maddux *et al.* 2006). In addition, it has been postulated that bone is able to function as an endocrine gland and plays a part in glucose metabolism (Ferron *et al.* 2010). Therefore, the increase in E-NPP1 expression seen during this study may not only play a part in regulation of mineralisation but might instead have a role in energy metabolism.

There are a number of other factors that have a role in aberrant ossification, including members of the BMP family. Unregulated BMP signalling is involved in Fibrodysplasia ossificans progressive (FOP), which is characterised by progressive heterotopic bone formation. Mutations in the regulatory domain of the BMP type 1 receptor lead to aberrant pathway activation, the receptor is hypersensitive to BMP ligands and in some cases is activated even in their absence (Fukuda *et al.* 2009). The activation of the BMP signalling pathway leads to heterotopic bone formation. Increased ossification of the soft tissue can also occur following traumatic injury such as amputation. Recent work has implicated excessive BMP2 stimulation in the soft tissue as stimulating osteogenic activity and the cause of ossification (Wosczyna *et al.* 2012). In both cases the excess BMP stimulation and signalling has caused

increased ossification. It may be worth investigating if decreased BMP signalling results in decreased mineralisation and whether acidosis has any effect on this signalling pathway in osteoblasts.

Chapter 6 investigated the role that the acid conditions of the tumour microenvironment might have in the role of 'the vicious cycle of bone metastases', whereby cancer cells that have metastasised to bone cause osteolytic bone disease (Kingsley *et al.* 2007). This model, however, fails to take into account the stimulatory effect that acid has on osteoclasts; which is surprising given that the tumour microenvironment is known to be acidotic (Fukumura & Jain 2007). The interactions between cancer cells and genuine osteoclasts have so far been poorly investigated. Work presented in Chapter 6 involved development of a new *in vitro co*-culture model to study the osteoclasts and the breast cancer cell lines MDA-MB-231 and MCF-7 but without the cells coming into contact so that the different cell types could be analysed separately.

Surprisingly cancer cells were found to inhibit both formation and activation of osteoclasts. Whether inhibitory effects were specific to the cell lines I used or whether they represent a more general phenomenon remains to be investigated. Work by (Pederson *et al.* 1999) has shown that different combinations of growth factors produced by MDA-MB-231 cells can have both inhibitory and stimulatory effects on osteoclastic resorption.

Overall, these findings were unexpected in view of the widely held supposition that cancer cells stimulate osteoclast formation and activity. However, when cancer cells were allowed to acidify cultures there were striking increases in resorption pit formation. It was observed that when co-cultures were clamped at pH 7.3, there was minimal resorption; however, when osteoclasts were co-cultured with osteoclasts, there was 10 times the amount of resorption than when osteoclasts were co-cultured with MCF-7 cells. This provides evidence that a factor other than pH could be having a stimulatory effect on osteoclast activity. A number of osteolytic factors reported to be produced by the MDA-MB-231 cell line are listed earlier and any one of these

may be responsible for the stimulation of resorption. The effect of each of these cytokines and growth factors on osteoclast formation and activity can be investigated separately from one another using the same experimental setup as that for determining the effects of activin A. Furthermore, investigations using conditioned medium from the cancer cells on osteoclast formation and activity could be performed without addition of exogenous M-CSF or RANKL which might help to elucidate whether factors produced by the cancer cells are having an effect on osteoclast function.

Differences from the anticipated results compared to those observed here may be because acidosis did not cause the effects as hypothesised. There are a number of reasons why effects on osteoclasts are contradictory to those expected, whether inhibitory effects were due to inhibitory cytokines or depletion of essential nutrients in the culture medium remains to be investigated. Also, this work was carried out in a heterologous system, and it needs to be repeated in a homologous cell culture system.

In vivo, the situation is clearly more complex still. Acidosis occurs hand in hand with hypoxia (Brahimi-Horn *et al.* 2007), and this would be expected to have profound effects on both osteoclast and osteoblast activity (Arnett *et al.* 2003; Utting *et al.* 2006; Knowles *et al.* 2010; Utting *et al.* 2010). Additionally, there are the many growth factors (such as TGFβ, BMPs) trapped within the bone matrix; following resorption of the bone by osteoclasts these are released into the bone/cancer microenvironment. Once released from the bone matrix these factors are then able to bind to cell-surface receptors on the cancer cells and osteoclasts where they may have a stimulatory effect.

The bone cancer metastases microenvironment is a complex one and provides a multi-factorial problem that needs to be studied further in order to elucidate more precisely the role of acid. However, I have shown that acid does have stimulatory effects on osteoclast activity when co-cultured with breast cancer cells and so the effects of acid in the vicious cycle model should be

taken into consideration when trying to understand the intricacies of this system. A revised schematic for the 'vicious cycle' model can be seen in figure 7.1.

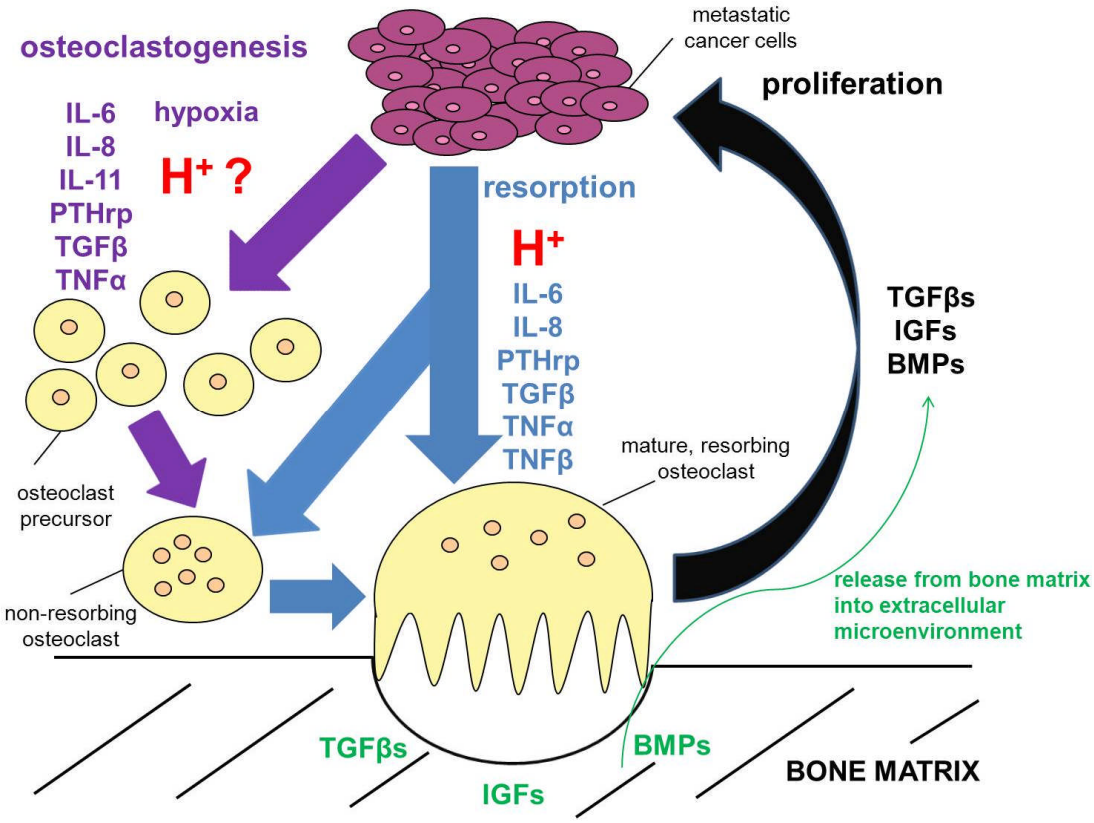


Figure 7.1 *The vicious cycle of bone cancer metastases*

Metastatic cancer cells in the bone microenvironment release cytokines and growth factors that promote osteoclastogenesis and/or resorption (Garrett e*t al* 1987; Bendre *et al* 2003; Kudo *et al* 2003; Kingsley *et al* 2007; Yamashita *et al* 2007; Mohammad *et al* 2009). Although not specifically addressed in the thesis, hypoxia will contribute to osteoclast formation (Utting *et al* 2010). Acid produced by the cancer cells will stimulate resorption. Resorption by osteoclasts will release growth factors (TGFβs, IGFs and BMPs) ordinarily trapped within the bone matrix into the extracellular microenvironment where they are able stimulate further cancer cell proliferation (Kingsley et al 2007).

In conclusion, it is clear that acid has profound effects on both osteoclasts and osteoblasts which are detrimental to bone. The reciprocal effect of increased osteoclastic bone resorption and decreased bone formation provide an evolutionary 'fail-safe' mechanism to buffer life threatening decreases in overall bodily pH in times of disease. However, in situations such as osteoporosis for example, understanding the mechanism more thoroughly may provide useful drug targets. The work presented in this thesis provides evidence to rule out the TRP super-family of ion channels as the acid-sensor, there are still several other possible candidates that may be worth investigating.

Furthermore, it has been shown that E-NPP1 plays an important role in regulation of mineralisation of bone subjected to low pH levels still within the pathophysiological range. Although more work is required to understand the mechanism more thoroughly in relation to the other enzymes, specifically PHOSPHO 1 and ANK, this may provide a potential therapeutic target to prevent bone loss.

These data also indicate that acidosis is a key factor in 'the vicious cycle of bone metastases'. More research is required to understand exactly the interplay between acidosis and other factors implicated in this model, particularly growth factors and cytokines as well as hypoxia. This presents a substantial challenge to model *in vitro*.

Overall, the experimental data presented in this PhD thesis demonstrate that extracellular pH is a fundamental regulator of bone cell function.

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Appendix I

Primer sequences

Gene product	Primer sequences (5'-3')	Product size (bp)	Annealing temperature (˚C)
β actin	S - GTTCGCCATGGATGACGAT AS - TCTGGGTCATCTTTTCACGG	332	53
Alkaline phosphatase	S – CTCATTTGTGCCAGAGAA AS - GTTGTACGYCTTGGAGAC	238	50
TRPV3	S – GACTTCAAGACCCAGAATGA AS - ATATGCAGCAGTGTATGCAG	368	53
TRPV4	S – AGAACACCAAGTTTGTCA AS - GTTGTAAACCAGGATCTCCA	311	53
TRPM8	S – GACCTATGGAACGTTATGGA AS - ATAGATGACAGAGCGGAAGA	330	53
E-NPP1	S – GTCAGTATGCGTGCTAAC AS - TGGCACACTGAACTGTAG	309	49
E-NPP2	S – GCCCTCCGTTAATCATCT AS - GCAGAGAAAGCCACTGAA	399	51
E-NPP3	S – GCATGCAGAGGAATTGTC AS - TGGGAACGGTGTATGAAC	296	53
E-NTPdase2	S – CCAGCTATGCAAATGAAC AS - AACACCCCTTCATCCTGT	256	56

E-NTPdase3	S – CAGCCAAACCTTCAGATG AS - TGTGCCACAGGTTCTTCT	356	53
E-NTPdase4	S - AGGCAGTTGTGGAAGTCA AS - CAGAAATGGAGCATCAGG	362	53
E-NTPdase5	S – TAGCTTGGGTTACCGTGA AS – CTCCTTCCAACCATCTTG	315	53
E-NTPdase6	S – GGGATGACTGTGTTTCCA AS - TTGTCATCCTCAGCAGGT	322	53

Table I.I *Primers used for amplification of cDNA by RT-PCR (Rattus norvegicus)*

Gene product	**Primer sequences (5'-3')**	**Product size (bp)**	**Annealing temperature (˚C)**
GAPDH	S – CTCACTCAAGATTGTCAGCA AS - GTCATCATACTTGGCAGGTT	346	53
TRPV3	S – GAACGTCTCCAAAGAAAGTG AS - CTTAAGCCAGGCATCTACAC	345	53
TRPV4	S – ACCACAGTGGACTACCTGAG AS - TCATGAAGAGCAGGTACACA	367	53

Table I.II *Primers used for amplification of cDNA by RT-PCR (Mus musculus)*

Gene product	**Primer sequences (5'-3')**
β actin	S – GATCATTGCTCCTCCTGAGC AS - ACATCTGCTGGAAGGTGGAC
M-CSF	S – GCAAGAACTGCAACAACAGC AS - TCACTGCTAGGGATGGCTTT
RANKL	S – AAGGAGCTGTGCAAAAGGAA AS - GGCCAGATCTAACCATGAGC
OPG	S – AGGAAATGCAACACACGACA AS - CCTGAAGAATGCCTCCTCAC
IL-6	S – AAAGAGGCACTGGCAGAAAA AS - TTTCACCAGGCAAGTCTCCT
TNFα	S - TCTTCTGCCTGCTGCACTT AS - GCCAGAGGGCTGATTAGAGA
TGFβ1	S – GTGGAAACCCACAACGAAAT AS - CGGAGCTCTGATGTGTTGAA
TGFβ2	S - ACTGTCCCTGCTGCACTTTT AS - GTGCCATCAATACCTGCAAA

Table I.III *Primers used for amplification of cDNA by qRT-PCR (Homo sapiens)*

Appendix II

Abbreviations

µg	Microgram
µM	Micromolar
4αPDD	4 α-phorbol 12,13-didecanoate
ABAM	Antibiotic-antimycotic
ADAM	A disintegrin and metalloproteinase
AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride
ALP	Alkaline phosphatase
AMG 6880	(2*E*)-3-[2-piperidin-1-yl-6-(trifluoromethyl)pyridin-3-yl]-N-quinolin-7-ylacrylamide
AMG 7472	5-chloro-6-{(3R)-3-methyl-4-[6-(trifluoromethyl)-4-(3,4,5-trifluorophenyl)-1Hbenzimidazol-2-yl]piperazin-1-yl}pyridin-3-yl)methanol
AMG 9810	(2*E*)-*N*-(2,3-Dihydro-1,4-benzodioxin-6-yl)-3-[4-(1,1-dimethylethyl)phenyl]-2-propenamide
Aml3	Acute-myeloid-leukaemia protein 3
ANK	Progressive ankylosis protein
Ap_nA	Diadenosine polyphosphates
ASIC	Acid-sensing ion channel
$Atf^{-}/^{-}$	Activating transcription factor 4 knockout
ATF4	Activating transcription factor 4
ATP	Adenosine triphosphate
BAFF	B-cell activating factor
BCTC	4-(3-Chloro-2-pyridinyl)-*N*-[4-(1,1-dimethylethyl)phenyl]-1-piperazinecarboxamide
BDNF	Brain derived neurotrophic factor
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
BSP	Bone sialoprotein

Ca^{2+}	Calcium
Cbfa1	Core-binding factor alpha 1
CCL4	Chemokines (C-C motif) ligand 4
CDK	Cyclin-dependent kinase
cDNA	Copy deoxyribonucleic acid
CO_2	Carbon dioxide
Col 1	Type 1 collagen
CREB2	cAMP-response element-binding protein
CTX	C-terminal telopeptide
DAPI	4',6-diamidino-2-phenylindole
DC-STAMP	Dendritic cell specific transmembrane protein
dH_2O	Deionised water
Dlx	Distal-less homeobox
DMEM	Dulbecco's modified Eagle's medium
DMP-1	Dentine matrix protein 1
ECM	Extracellular matrix
E-NPP	Ecto-nucleotide pyrophosphatase/phosphodiesterase
E-NTPdase	Ecto-nucleoside triphosphate diphosphohydrolases
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte macrophage-colony stimulating factor
GRO	Growth related oncogene
H^+	Proton
H_2CO_3	Carbonic acid
H_2O_2	Hydrogen peroxide
HBSS	Hank's balanced salt solution
HCl	Hydrochloric acid
HCO_3^-	Bicarbonate ion
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMRC	Her Majesty's Revenue and Customs
HRP	Horseradish peroxidise

IFN-γ	Interferon gamma
IgE	Immunoglobulin E
IGFBP	Insulin-like growth factor-binding protein
IHH	Indian hedgehog
IL	Interleukin
IP-10	Interferon gamma induced protein 10
KO	Knockout
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LIF	Leukaemia inhibitory factor
LPA	Lysophosphatidic acid
LRP	Low-density lipoprotein receptor-related protein
MAFP	Major acidic fibroblast-growth-factor stimulated phosphoprotein
M-CSF	Macrophage-colony stimulating factor
MEM	Minimum essential medium
meq/L	Milliequivalents per litre
MFR	Macrophage fusion receptor
$MgCl_2$	Magnesium chloride
MGP	Matrix gla protein
MIP	Macrophage inflammatory protein
ml	Millilitre
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
Msx	MSH homeobox homologue
NADH	Nicotinamide adenine dinucelotide, reduced
NaOH	Sodium hydroxide
NAP-2	Neutrophil-activating protein 2
NDP	Nucleotide diphosphate
ng	Nanogram
nM	Nanomolar
NMP	Nucleotide monophosphate

NPPase	Nucleotide pyrophosphatase phosphodiesterase
NPPα	Nucleotide pyrophosphatase phosphodiesterase α
NPPβ	Nucleotide pyrophosphatase phosphodiesterase β
NPPγ	Nucleotide pyrophosphatase phosphodiesterase γ
NTP	Nucleotide triphosphate
NTPPPH	Nucleotide triphosphate pyrophosphohydrolase
Ocn	Osteocalcin
OC-STAMP	Osteoclastic stimulatory transmembrane protein
OH^-	Hydroxide ion
OPG	Osteoprotegerin
OPGL	Osteoprotegerin ligand
OPLL	Ossification of the posterior longitudinal ligament of the spine
Osf2	Osteoblast specific factor 2
Osx	Osterix
Pax1	Paired box gene 1
PBMNC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PC-1	Plasma cell differentiation antigen 1
pCO_2	Carbon dioxide partial pressure
PCR	Polymerase chain reaction
PD- Iα	Phosphodiesterase-Iα
PDGF-B	Platelet-derived growth factor-B
PD-Iβ	Phosphodiesterase-1β
PGE_2	Prostaglandin E_2
Pi	Inorganic phosphate
PLAP-1	Periodontal ligament associated protein-1
PMSF	Phenyl methyl sulfonyl fluoride
pNPP	p-nitrophenylphosphate
pNP-TMP	p-nitrophenyl thymidine 5’-monophosphate
pO2	Oxygen partial pressure
PO_4^{3-}	Phosphate

PPi	Pyrophosphate
PTHrp	Parathyroid hormone related peptide
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative real time polymerase chain reaction
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RIPA	Radio immunoprecipitation
RNA	Ribonucleic acid
Runx2	Runt-related transcription factor 2
$Runx2^{-/-}$	Runt-related transcription factor 2 knockout
SA-CAT	Stretch-activated cation channel
SB366791	N-(3-methoxyphenyl)-4-chlorocinnamide
SIRPα	Signal regulatory protein-alpha
SOST	Sclerostin
TGFβ	Transforming growth factor beta
TIMP	Tissue inhibitor of metalloproteinases
TNAP	Tissue-nonspecific alkaline phosphatase
TNF	Tumour necrosis factor
TNFSF11	Tumour necrosis factor ligand super family member 11
TRANCE	Tumour necrosis factor-related activation-induced cytokine
TRAP	Tartrate resistant acid phosphatase
TRPA	Transient receptor potential ankyrin
TRPC	Transient receptor potential canonical
TRPM	Transient receptor potential melastatin
TRPM8	Transient receptor potential Melastatin type 8
TRPML	Transient receptor potential mucolipin
TRPN	Transient receptor potential no mechanopotential
TRPP	Transient receptor potential polycystin
TRPV	Transient receptor potential vanilloid
TRPV1	Transient receptor potential vanilloid type 1
$TRPV1^{-/-}$	Transient receptor potential vanilloid type 1 knockout
TRPV3	Transient receptor potential vanilloid type 3

TRPV4	Transient receptor potential vanilloid type 4
VEGF	Vascular endothelial growth factor
VSCC	Voltage-sensitive calcium channel
WT	Wildtype

Appendix III

Publications

Papers

Patel JJ, Utting JC, **Key ML**, Orriss IR, Taylor SEB, Whatling P, Arnett TR. 2012. Hypothermia inhibits osteoblast differentiation and bone formation but stimulates osteoclastogenesis. *Exp. Cell. Res*. **318**: 2237-2244

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