The Effect of Bone Anabolic Stimuli on Human Osteoblast to Osteocyte Transition

KATIE WELLDON B.Sc. (Hons)

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School of Medicine

Faculty of Health Sciences

The University of Adelaide

South Australia

Australia

TABLE OF CONTENTS

ABSTRACT

Osteoporosis, a condition defined by a low bone mineral density (BMD) and associated with increased fracture risk, is associated with a decrease in both osteocyte (OY) density and viability. A great deal of evidence implicates OY as central to bone physiology and pathology (1). However, human OY biology in particular is poorly characterised. We previously showed that a variety of bone-acting factors induce a pro-anabolic or pro-catabolic response in human primary osteoblasts (Normal Human Bone-derived Cells, NHBC), concomitant with the acquisition of an OY-like phenotype (2-6). Bone mineralisation, the deposition of calcium and phosphate as calcium phosphate in the form of hydroxyapatite, occurs in lamellar bone concurrent with osteoblast to OY transition (7).

The first aim of the current study was to characterise the role of calcium, a common dietary supplement for the treatment of osteoporosis, in the transition of osteoblasts to OY, using human primary cell models. Secondly, low intensity pulsed ultrasound (LIPUS), an emerging therapy for osteoporosis and fracture repair, was also assessed for its effects on NHBC differentiation into OY. We hypothesised that each of these stimuli would exert a pro-anabolic effect on NHBC differentiation, promoting their transition to OY-like cells.

NHBC were cultured under conditions permissive for *in vitro* mineralisation, in the presence of a wide concentration range of Ca^{2+} (1.8 - 11.8 mM). Experiments were performed in the presence or absence of an inhibitor of the extracellular calcium sensing receptor (CaSR), NPS2390, as we hypothesised that these cells would 'sense' extracellular calcium through this receptor. NHBC tolerated even the highest concentration of Ca^{2+} used. Treatment with Ca^{2+} resulted in a striking dose- and time-dependent increase in *in vitro* mineralisation, associated with an increasing ratio of Ca:P, as determined by electron dispersive spectroscopy (EDS). Levels of mRNAs encoding the OY markers, SOST, E11 and dentin

matrix protein 1 (DMP1), were elevated in the mineralised cultures indicating promotion of osteoblast to OY transition. Gene expression was differentially regulated by Ca^{2+}. The expression of the osteoclast inhibitor, OPG, was dramatically enhanced by calcium. It was found that CaSR mRNA expression was rapidly lost from human trabecular bone *ex vivo* and is not expressed by NHBC. However, NHBC did express the related receptor, GPRC6A. Surprisingly, mineralisation was either unchanged or enhanced in the presence of the calcium sensing receptor inhibitor, NPS2390. Calcium-dependent mineralisation was reversed in the presence of phosphorylated MEPE-ASARM peptides. This study suggests that osteoblast to OY transition, and the concurrent mineralisation of the extracellular matrix, is sensitive to extracellular calcium independent of the canonical CaSR.

LIPUS is transmitted to target tissues as a low pressure acoustic wave (8), and has been shown to improve fracture healing (9-12). NHBC isolated from five donors were grown under conditions permissive for mineralisation and treated with a regimen of LIPUS at 1.5 mHz for 20 min daily for up to 7 days, either pre- or post-onset of mineralisation. The results showed a mild increase in the proliferation of cells in some cases in response to LIPUS treatment. Also, the expression of E11, a gene associated with osteoblast-OY transition, was increased. Cells from some donors responded to LIPUS by releasing measurable prostaglandin E2 (PGE_2), a response also associated with mechanical loading of bone and the effect of LIPUS in other models though there was no significant trend towards increased mineralisation. The results from this study suggest that LIPUS treatment may promote the differentiation of NHBC to a pre- or osteoid-OY-like phenotype. In summary, bone anabolic stimuli either in the form of calcium or LIPUS differentially affect the transition of osteoblasts to OY.

DECLARATION

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CHAPTER 1: Literature Review

1.1 Bone

Bone is a dynamic mineralised connective tissue involved in the regulation of a number of homeostatic systems (13). The cells involved in the maintenance and turnover of bone include bone lining cells, osteoblasts, osteoclasts, and osteocytes (OY). Osteoblasts and osteoclasts are located at the surfaces of bone and together with bone lining cells, another osteoblast-lineage cell type, form the basic multicellular unit (BMU) responsible for the removal and formation of new bone. As osteoblasts synthesise the bone organic matrix, termed osteoid, around 10-20% of these cells become embedded in the bone matrix (14). Thus, the majority of osteoblasts become either inactive bone lining cells or undergo apoptosis (15). Osteoblasts that become surrounded by osteoid undergo transition into phenotypically recognisable pre-OY and then osteoid OY, characterised by the development of dendrite-like cell processes (7, 14, 16-19). As described in detail below, it is the osteoid OY that begins the mineralisation process, at least in lamellar bone, and once completely surrounded by mineral, assumes the role of a mature OY (19).

1.2 Osteocytes

1.2.1 Morphology

OY are the most numerous cell type in bone, making up around 90% of the cell population, with around 20,000 to 80,000 cells/mm^3 in human bone tissue (15, 19, 20). Maturation of the OY is marked by a reduction in the extent of Golgi apparatus and endoplasmic reticulum,

corresponding with a decrease in protein synthesis and secretion, as well as a reduction in cytoplasmic volume (19, 21). The cell bodies of the mature OY are located in small cavities termed lacunae, within the mineralised bone matrix. The mature OY has long branching dendrite-like processes that form a complex interconnected network through a series of channels termed canaliculi (18). The OY processes exhibit a marked polarity, with the majority of processes being located on the side facing the bone surface (17, 22). When bone is cross-sectioned, OY lacunae can be seen arranged in concentric circles in a lamellar (layered) pattern around Haversian canals (containing the blood and nerve supply) and the bone periphery (23, 24). OY are highly interconnected with each other, forming an extensive syncytium. Communication between OY and cells at the bone surface is mediated in part *via* hemichannels and gap junctions formed by complexes of connexin43 (Cx43) and 45 (Cx45) (25, 26). Gap junctions formed by Cx43 connect the OY to each other and allow diffusion of relatively large molecules, as well as ions, metabolites and small signalling molecules (25). Hemichannels are effectively unpaired gap junctions that allow access to molecules from the interstitial space surrounding the cell body or process and are involved in a variety of roles regulating bone cell function (27).

1.2.2 Origin and Biomarkers

As osteoblasts transition to OY and become deeply embedded within the bone matrix, their morphology changes and a suite of genes are switched either on or off (13, 17, 28) (**Figure 1.1**). As only a small percentage of osteoblasts transition to OY, it remains to be determined whether the signal for transition is initiated by the osteoblast or *via* messages from other cell types (13, 17, 19). As osteoblasts mature, there is an initial increase in genes associated with osteoid production, such as type 1 collagen (Col-1). Col-1 is the principal protein constituent

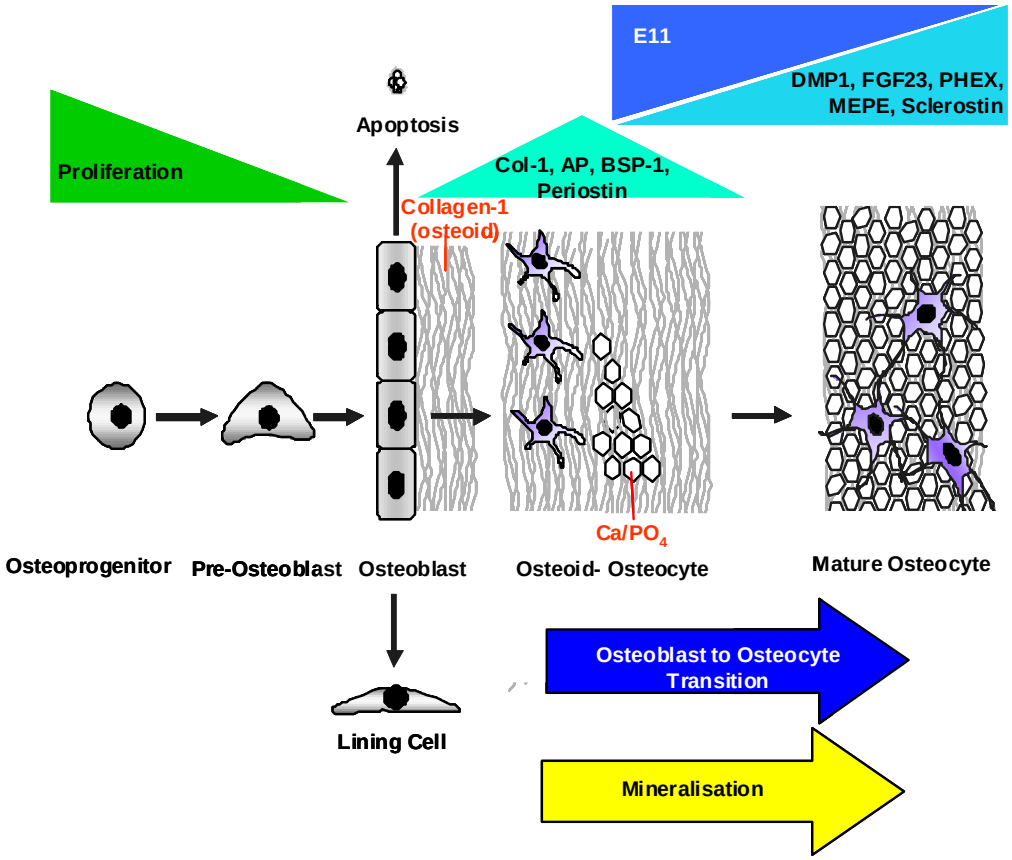

Figure 1.1: Osteoblast transition to Osteocyte

of the bone scaffold, whereas other non-collagenous proteins perform a variety of functions in the regulation of bone mineralisation and homeostasis. For example, bone sialoprotein-1 (BSP-1) and perhaps also DMP1, are involved in mineral crystal nucleation (29). A typical marker of the osteoid-OY in murine models is the increase in the expression of E11 (or podoplanin) (17, 30). E11 is involved in the initiation of formation of the dendrite-like processes (28, 31). E11 expression decreases with OY maturation and, concurrent with this, the expression of mature OY markers increases; these include DMP1, SOST, MEPE and PHEX (13, 17, 32).

Sclerostin is the protein product of the *SOST* gene and has been characterised as an inhibitor of bone formation (33). The mechanism of action of sclerostin has not been fully elucidated but it is generally thought to act as an antagonist of the canonical Wnt signalling pathway (**Figure 1.2**). Wnts are soluble molecules that bind to a receptor complex of low-density lipoprotein receptor related protein 5 or 6 (LRP5/6) and frizzled (Fzd) on the cell surface (34). Binding of canonical Wnts to the LRP5/6/Fzd complex causes inhibition of the enzyme glycogen synthase kinase 3β (GSK3β), allowing cytoplasmic accumulation and nuclear translocation of the signalling molecule β-catenin, which forms an effector complex that initiates (bone anabolic) gene transcription (35). In the absence of Wnt signalling, GSK3β targets β-catenin for ubiquitin-proteolytic degradation (35). Sclerostin is thought to disrupt this cascade by binding to LRP5/6, preventing β-catenin initiation of gene transcription (36, 37). However, in a previous study, we demonstrated that sclerostin can act through alternative pathways, such as the mitogen activated protein kinase (MAPK) pathway (38). In support of this, recent studies have proposed that LRP4 also functions as a sclerostin receptor (39, 40). Nevertheless, *SOST* expression is critical for normal bone growth as its loss of function results in high bone mass disorders such as Sclerosteosis, which gave *SOST* its name, and Van Buchem's disease. Patients with this disorder present with unusually strong bones and an

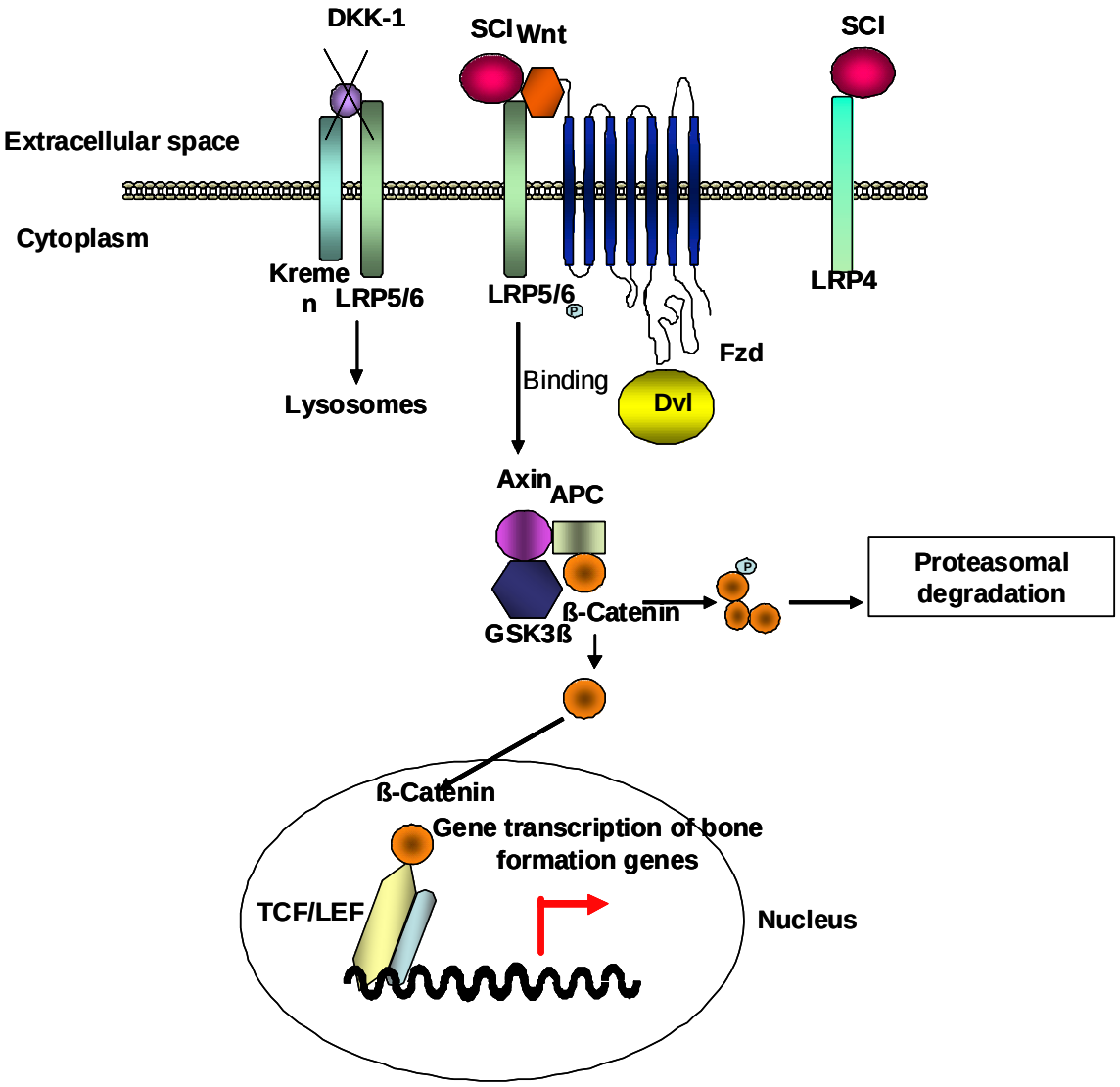

Figure 1.2: WNT signalling pathway (adapted from (41))

impaired ability to swim due to their high bone density (42). Studies carried out in mice where the *SOST* gene was deleted also resulted in increased bone strength and formation (33). The decrease in SOST expression in OY in response to mechanical stimulus *in vivo* highlighted the crucial role of OY in regulation of the catabolism of bone (43).

The gene deletion of DMP1 in mice results in the dysregulation of bone mineralisation (44). DMP1 has been implicated in the maintenance of mineralisation in the canalicular system, along with genes involved in phosphate regulation, such as MEPE (18, 45). Both DMP1 and MEPE are members of the small integrin N-linked glycoprotein (SIBLING) family of proteins. While DMP1 expression is associated, in bone, with a mature OY, MEPE is expressed at levels varying from low in osteoblasts through to high in mature OY (18, 28). The protein PHEX is also expressed at higher levels in the OY lineage and plays a role in systemic phosphate homeostasis (17, 18, 46). The loss of PHEX expression perturbs normal bone mineralisation, resulting in conditions such as X-linked hypophosphataemic rickets (XLH), which is accompanied by a dramatic increase in fibroblast growth factor 23 (FGF23) expression in OY (17, 47). The primary role of FGF23 appears to be the negative regulation of phosphate reabsorption by the renal system and inhibition of renal 1,25-dihydroxyvitamin D3 (1,25D) synthesis (46). Thus elevation of FGF23 in human XLH and in the hypophosphataemic (HYP) mouse, results in decreased phosphate reabsorption, and decreased intestinal phosphate absorption, resulting in severe hypophosphataemia (48, 49). Additionally, recent studies have highlighted the role of the acidic serine aspartate-rich MEPE-associated (ASARM) motif, a unifying feature of the SIBLING family of proteins (50, 51). ASARM peptides are believed to play an important role in the regulation of mineralisation and other important systemic processes. Importantly, ASARM appears to interrupt the PHEX-DMP1 interaction, which leads to an increase in FGF23 expression and therefore negative regulation of mineralisation (50). The complex relationship between

PHEX, DMP1, MEPE, ASARM peptide and FGF23 are still under investigation and appears to be a fertile field in mineralisation research. The expression of these unique OY genes not only provides a set of markers, with which to examine osteoblast to OY transition but also points to the contribution of OY to the regulation of mineralisation and systemic phosphate homeostasis.

1.2.3 Mineralisation

The complex process, by which bone tissue becomes mineralised, is an area of great interest. Traditionally, osteoblasts have been ascribed the role of mineral production, however recent studies have demonstrated that mineralisation occurs simultaneously with osteoblast to OY transition (7, 17, 52). Mineralisation has been studied in the late osteoblast/pre-OY cell line MLO-A5, and has been observed to initiate in a cell-regulated manner associated with the formation of mineralised matrix vesicles or spheres, termed calcospherulites (7). The cell associated calcospherulites eventually bud from the cells and become associated with collagen type 1 fibrils of the osteoid matrix (7, 53). Calcospherulites are spheres of protein-associated calcium phosphate in the form of hydroxyapatite and contain other proteins such as BSP-1, BAG75 and osteopontin (OPN) (29). As the OY continues to produce calcospherulites it becomes embedded within the mineralised osteoid matrix (7, 19). As mentioned above, the MEPE-ASARM, PHEX-FGF23 interaction plays a crucial role in OY regulation of mineralisation. The tight regulation of DMP1-PHEX increases mineralisation and this is interrupted by the ASARM peptide, leads to an increase in FGF23 expression thus decreasing the renal reabsorption of phosphate (46).

1.2.4 Mechanosensation

The remodelling of bone as a result of mechanical forces has been well characterised both *in vitro* and *in vivo* (54, 55). The presence of OY has been demonstrated to be necessary for effective mechanotransduction (1). The longitudinal axis of the OY cell body is aligned with the load-bearing axis in the mineralised bone matrix (24). OY mechanosensors work separately or in partnership to respond effectively to mechano-stimulators that likely include fluid shear stress (FSS), pressure, strain and electrical fields (54, 56). Mechanical strain exerted on the bone translates to movement of fluid from the bone vasculature to the OY canalicular network, creating FSS as well as removing cell waste and providing oxygen and fresh nutrients (57). It has been suggested that fluid flow leads to mechanical deformation of the primary cilium on the surface of the OY, generating an intracellular signalling response (58-60). Other mechanisms of OY mechanotransduction include integrins and integrin-associated proteins, cytoskeleton deformation, and connexins (25, 58, 61). Signals generated by OY mechanotransduction are communicated through the OY network, initiating both local and systemic effects on bone remodelling. For example, mechanotransduction is associated with decreased expression of SOST and Dikkopf-1 (DKK-1), another Wnt inhibitor, in response to loading (62). The involvement of integrin binding in the mechanotransduction activities of OY most probably occurs *via* the movement of fluid through the canalicular network. The fluid causes movement of the integrin tethered to the extracellular matrix, resulting in deformation of the actin cytoskeleton that in turns triggers a chain of events resulting in the release of PGE_2 into the extracellular space and increased intracellular calcium (63).

1.2.5 Apoptosis and Aging

OY number and viability decreases with age, and may negatively impact bone strength by leading to increasing bone fragility and consequent fracture risk (64). The ablation of OY in a mouse model results in fragile bone and resistance to unloading related bone loss (1). Ablation of OY in a transgenic mouse model was achieved using the diphtheria toxin receptor-mediated cell-knockout (TRECK) system and a DMP-1 promoter to specifically target the expression of diphtheria toxin (DT) receptor to OY (1). Apoptotic OY can either become mineralised or leave empty lacunae (65). The apoptosing OY are able, however, to recruit osteoclasts to initiate removal of damaged bone and promote its repair (66). Whether OY themselves are directly involved in repair of microscopic bony lesions, termed microcracks, in addition to sending signals through the OY network to cells at the bone surface to activate remodelling, is still to be determined (67, 68).

1.2.6 Osteocyte Cell lines

Much of the current knowledge concerning OY biology has been generated using the well characterised mouse OY-like cell line, MLO-Y4 (69). MLO-Y4 cells are characterised by the presence of long dendrite-like processes, functional gap junctions and expression of high levels of E11 and OPN mRNA (69-71). The MLO-Y4 line, however, mineralises poorly and expresses low levels of type I collagen and ALP (30, 70). Conversely, the MLO-A5 cell line probably represents a late osteoblast/osteoid-OY phenotype, as they mineralise well and express high levels of OCN, ALP, Col-1, BSP-1 and periostin mRNA (7). Despite their limitations, MLO-Y4 in particular and MLO-A5 cells have greatly facilitated the study of OY. A more recent cell line, IDG-SW3 has recently been established (72) that may prove to

more faithfully replicate primary OY behaviour, since this cell line differentiates, mineralises and results in OY-like cells that express characteristic markers including E11, PHEX, DMP1, SOST, as well as FGF23. It seems important however to take into account the possibility of differences between mouse and human osteoblasts and OY, therefore ideally comparisons should always be made between these models and human primary models, such as NHBC-derived OY-like cells, characterised to a large extent by our previous work (3-5, 38, 51, 73-76).

1.2.7 2-Dimensional (2D) Models

Primary human osteoblasts, termed NHBC, are grown as explant cultures from trabecular bone chips obtained at total hip replacement (THR) surgery. Traditionally, NHBC have been isolated from patients undergoing scheduled THR for end-stage osteoarthritis (OA). Trabecular bone cores from these patients are removed from the proximal femur, a region termed the trochanter. This OA-derived intertrochanteric bone has been considered as a source of 'normal' osteoblasts because firstly, this site is distal from the 'diseased' sub-chondral bone immediately proximal to the affected joint, and secondly because NHBC have been extensively characterised and conform to the expected characteristics of osteoblasts, in that they produce an extensive type I collagenous matrix, which they mineralise, giving rise to a matrix with bone-like mineralised properties (3, 77). It is possible also to derive a similar population of cells from patients undergoing emergency surgery for fractured neck of femur, and in our hands these behave very similarly but are termed neck of femur cells (NOF) and treated as potentially different to NHBC. By immunostaining for the haemopoietic cell surface marker, leukocyte common antigen or CD45, we have demonstrated that NHBC are an osteoblastic population with no contamination of haemopoietic cells (5). Grown over

extended periods of time under conditions permissive to mineralisation, that is in the presence of ascorbic acid, dexamethasone and inorganic phosphate (KH_2PO_4), both NHBC and NOF generate a mineralised matrix (2, 4) and eventually develop an OY-like phenotype (3, 76). While convenient for the purposes of long-term culture, assessment of mineralisation and for the analysis of gene expression, the morphology of the NHBC differentiated in 2D is difficult to assess, given the high cell density in a confluent culture, and of the extensive mineralised matrix present.

1.2.8 3-Dimensional (3D) Models

Primary OY *in vivo* are held in a 3D conformation within the mineralised bone matrix. Standard tissue culture techniques only allow for a 2D environment. Therefore, a variety of techniques have been used to simulate a 3D environment. Cells grown in ceramic particles or Matrigel™ have been used with some success to mimic the highly mineralised bone microenvironment *in vivo* (78, 79). Hydroxyapatite scaffolds have also been implemented to study OY in close to native conformation (80). The difficulty of imaging the cells in these models is, however, a limiting factor. We have established that the use of collagen gels is an effective system for studying OY in a 3D environment (3, 5). The limitation of this model is related to the relative lack of stiffness in comparison to bone. The benefit of the collagen gel system, however, is that it provides the potential to study OY at a variety of stages of osteoblast to OY transition when used in combination with cells harvested from native bone.

1.2.9 Tantalum and Tantalum Mesh

A percentage of prosthetic hip replacements fail after a short time in patients. This is both traumatic and costly for both the patient and the government. Periprosthetic osteolysis leads to loosening of the orthopaedic implant, termed aseptic loosening, and this has been the focus of extensive research as it is the major factor in joint failure. A variety of potential solutions have been developed including a number of inert metal implants such as titanium (Ti), tantalum (Ta) and cobalt chromium and using hydroxyapatite coatings (81-84). Tantalum was demonstrated to be a suitable candidate for use in prosthetic joints but the factors involved in osteoblast response to this metal remained to be investigated (85, 86). We previously conducted studies to examine if all of the above metals were biocompatible in terms of the response to these of NHBC (74). A variety of parameters including cell proliferation, *in vitro* mineralisation, gene expression and cell viability were investigated in this study. The NHBC were able to proliferate and mineralise on the metals as has also been shown in animal studies (85). A number of genes involved in both pro-osteoclastic and osteogenic processes were investigated but no consistent, substrate-dependent differences were seen. The primary interest in this study was to investigate Ta in the form of a trabecular-like mesh (74). Initial studies of the Ta mesh in canine moulds showed osseous integration and mineralisation (87). The aim of trabecular implants is to create a long lasting integrated implant that allows better quality of life. Ongoing studies following patients with Ta mesh implants has demonstrated good initial osteoblast infiltration into the metal trabeculae (88, 89). This is supported by another study we carried out on trabecular mesh (75). NHBC seeded into mesh showed the ability to infiltrate the mesh *in vivo* and mineralise normally. Additionally, the maturation of NHBC on Ta mesh when compared to cells cultured on plastic for some donors showed increase maturity in terms of cell surface STRO-1/alkaline phosphatase expression (77).

Interestingly, cells grown in Ta mesh showed decreased expression of Col-1, which is consistent with an increase in maturational state towards an OY-like phenotype. Thus, Ta mesh potentially provides another 3-dimensional model, which gives an insight into osteoblast to OY differentiation in the context of metal implants.

1.3 Catabolic and Anabolic Agents

Our laboratories previous studies have investigated a variety of both anabolic and catabolic agents, such as vitamin K (3), vitamin D (4), strontium (76) and polyethylene wear particles (5), and have indicated their importance in both initiating the transition of osteoblasts to OY-like phenotypes but also the osteocytic regulation of the bone microenvironment. These previous studies have indicated the importance of OY in the regulation of the bone microenvironment and have piqued interest in other agents already under investigation in both the treatment of osteoporosis and fracture healing such as calcium and LIPUS. Though both these agents have been extensively investigated there is only a small amount known about their effect on OY and therefore this is an area requiring further investigation.

1.3.1 Polyethylene Wear Particles

Wear particles are generated from orthopaedic implants, in particular from the polyethylene (PE) bearing surfaces, by friction at the implant surface during use of the limb (90, 91). These microscopic PE wear particles are thought to be engulfed by macrophages, triggering an immune response resulting in inflammation and eventual lysis of surrounding bone and the subsequent loosening of the prosthesis, a process known as aseptic loosening (92-94). Evidence suggests that not only do macrophages respond to wear particles but so do

osteoblasts at the bearing surface that are exposed to the particles (95, 96). Our group has shown that PE wear particles generate a catabolic response at high doses in differentiating 3D cultures of NHBC, including phagocytosis of PE particles and up-regulation of the markers of bone resorption, RANKL, IL-8 and M-CSF (5).

Matrix metalloproteinases' (MMPs) are involved in degrading the components of extracellular matrix (ECM) (97, 98). Osteoblasts synthesise collagen matrix and other proteins that make up the ECM. Our preliminary data suggest that osteoblasts produce MMPs in response to PE particles (Welldon, Findlay & Atkins, unpublished), although this remains to be confirmed; this response may have implications for both aseptic loosening and the ability of osteoblasts to transition into OY.

1.3.2 Vitamin K

Vitamin K (vit K) found in foods such as soya beans is currently under investigation as a treatment for osteoporosis (99). Current data suggest that vitK2 (menatetrenone) has a protective role in post-menopausal bone loss, alone and in combination with anti-resorptive agents (100). VitK is a known cofactor in the conversion of glutamate residues to γ-carboxyglutamate (Gla) residues in target proteins, such as osteocalcin (OCN), periostin and matrix-Gla protein (MGP), whose Gla residues confer high affinity binding to hydroxyapatite (101). However, our work and that of others, has shown that vit K also acts independently of γ-carboxylation and this may potentially be related to its genomic action as a transcription co-activator of the steroid xenobiotic receptor (SXR) (3, 101). Studies from our laboratory have shown that vitK homologues can promote maturation in NHBC, thereby increasing the number and survival of a pre-OY-like phenotype (3). The involvement of vitK homologues in the transition of osteoblast to OY warrants further investigation.

1.3.3 Vitamin D

Deficiencies in vitamin D_3 result in dramatic effects on the skeleton, including rickets and osteomalacia, diseases resulting from defects in bone mineralisation (102). The tight regulation of vitamin D is essential for maintenance of calcium and phosphate homeostasis. The major circulating metabolite of vitamin D_3 is 25-hydroxyvitamin D_3 (25D), which is converted in the kidney by the enzyme CYP27B1, the 25-hydroxyvitamin D 1α-hydroxylase, to biologically active 1,25D. A novel pathway, by which osteoblasts are able to convert 25D into active 1,25D, indicates the importance of locally produced 1,25D in the regulation of osteoblast activity (4). Promotion of mineralisation and proliferation of NHBC by both 25D and exogenous 1,25D *in vitro* is consistent with the formation of mature OY (4). Vitamin D_3 is therefore a potential player in the initiation and promotion of osteoblast to OY transition.

1.3.4 Strontium

Strontium, a divalent cation, is a bone-seeking agent absorbed at the bone surface in place of calcium. Commercially available as Strontium Ranelate (SR), it is now marketed as an anti-osteoporotic agent. Clinical trials have demonstrated the anti-fracture efficacy of SR in both hip and vertebral fracture in post-menopausal women (103, 104). Rodent models have demonstrated a protective effect of SR on bone loss and increase in bone mass, volume and strength (105, 106). We have identified that SR promotes OY differentiation from NHBC, increasing mineralisation dose-dependently and promoting the expression of markers of OY transition (76). We also showed that SR induces the production of osteoprotegerin (OPG) a critical antagonist of RANKL and inhibitor of osteoclastic activity (76), together suggesting that SR has both anabolic and anti-catabolic modes of action, in keeping with clinical findings

(103, 104). Importantly, together with the studies described above, this work suggests that OY may adopt either an anabolic (as in the case of SR, vitK and 25D) or a catabolic (in the case of PE) phenotype.

1.3.5 Calcium

Approximately 2 million Australians are thought to have osteoporosis a condition associated with a BMD of less than 2.5 standard deviations lower than the mean (i.e. T-score of ≥ -2.5) and this number is projected to increase with an ageing population (107). Calcium supplementation, with and without vitamin D, is a common treatment for osteoporosis, with the average recommended dose ranging from 1200-1500 mg/day. *In vivo*, blood calcium levels for any individual are tightly regulated however the values usually lie within the range 2.2 – 2.6 mM. Individuals are considered hypocalcaemic if the levels are < 2.1 mM and considered hypercalcaemic if the levels are > 2.63 mM. Bone is the major reservoir for calcium and osteoblastic/osteocytic incorporation of calcium into bone matrix is an important homeostatic mechanism (108). Mineralisation, the deposition of Ca^{2+} as hydroxyapatite-like calcium phosphate, occurs in lamellar bone, concurrent with osteoblast to OY transition, as discussed above. Thus, the transition of osteoblast to OY plays an important role in bone health.

It is known that binding of calcium to the G-coupled protein calcium sensing receptor (CaSR) regulates the reabsorption of calcium by the kidney tubule through modulation of circulating parathyroid hormone (PTH) levels (109). A number of other divalent and trivalent cations are also able to activate the CaSR, though their affinity for the receptor is less; these include Sr^{2+}, Mg^{2+}, Al^{2+} and Gd^{3+}. CaSR has been isolated from a number of tissues including kidney,

brain, keratinocytes and bone (110). The presence of CaSR in NHBC if demonstrated would indicate its potential role in calcium homeostasis in bone.

Although a number of groups have demonstrated the presence of CaSR in osteoblasts, others have been unable to do so (111-114). There are a variety of reasons why this may be the case, including the sensitivity and specificity of antibodies used to detect CaSR by techniques such as Western blotting, the level of expression of CaSR by osteoblast or the presence of different isoforms. In addition, it has been proposed that osteoblasts express alternative G-coupled protein receptors able to sense cations (ob.CaSR) (111, 115, 116). The presence of a unique ob.CaSR is supported by the lack of bone remodeling abnormalities or bone phenotype independent of alterations in PTH in the CaSR knockout mouse (117, 118). Levels of calcium between 8-40 mM have been shown to be present at bone resorption sites, far higher than those found in the systemic circulation (119). The higher calcium levels experienced by bone cells would seem to support the potential need for a distinct ob.CaSR (115). A member of the C family of G-protein coupled receptors (GCPRs); GPRC6A is a potential candidate for the ob.CaSR, although it appears to have a lower affinity for calcium (110, 120). Interestingly in the presence of OCN, in conjunction with threshold levels of calcium, GPRC6A is stimulated whereas CaSR is not (110). Thus, the mechanism by which OB and OY sense extracellular Ca^{2+} remains to be fully elucidated. Additionally, the role of OY in calcium homeostasis is unclear, although there is evidence that OY are able to remodel the bone microenvironment, potentially releasing calcium, in a process known as osteocytic osteolysis (121-124).

1.3.6 Ultrasound

Ultrasound is a form of mechanical loading that is transmitted to tissue as high frequency acoustic pressure waves (8, 125). The density of tissue can attenuate the intensity of the signal

received (8). The modes of ultrasound include thermal, caviation and mechanical stimulus (8). Low intensity pulsed ultrasound (LIPUS) of around 1.5 mHz (<100 mW/cm^2) uses intensities that are so low that they are considered to be neither thermal nor destructive (126, 127). LIPUS has been used with success to facilitate fracture healing in non-union tibial fractures (128, 129). Rodent osteoblast models have demonstrated an increased proliferation and mineralisation rate as a result of LIPUS treatment (126, 130-132). Genes involved in promoting mineralisation, such as BSP-1, are up-regulated by LIPUS treatment (133). A number of genes associated with osteoblast maturation have been reported to increase with LIPUS treatment. Genes such as OCN and TNAP both associated with mature osteoblasts have been demonstrated to be up-regulated by LIPUS treatment in both human and rodent cells (133). The gene expression profile in response to LIPUS in mouse osteoblasts was found to depend on the site of cell harvest- whether from long bones, calvaria or mandibles (134). This differential regulation of genes may be related to the variation in loading conditions experienced throughout the skeleton (135, 136).

The activation of the Wnt signalling pathway in the osteoblast cell line MG63 suggests a potential role for OY in LIPUS-stimulated fracture healing (125). The key involvement of OY in mechanotransduction highlights them as a potential target for LIPUS therapy (54). A recent study by Sena *et al.* has demonstrated the stimulation of gap junction regulated intercellular communication by LIPUS in rat bone marrow stromal cells (137). The expression of PGE_2 and COX-2 (the inducible isoform of cyclooxygenase, an enzyme critical in the synthesis of PGE_2) is increased in MC3T3-E1 cells when treated with LIPUS (138, 139). Interestingly, in rodent models where COX-2 has been 'knocked out' the induction of RANKL has been shown to be dependent on COX-2 (140). Borsje *et al.* reported the increase of RANKL and OPG expression following LIPUS treatment (134, 141, 142). The increase in secretion of OPG protein at 0 and 4 hrs post LIPUS treatment in the osteoblast-like cell line SAOS-2

would be expected to be consistent with a reduction in osteoclastogenesis (141). LIPUS has also been shown to have a negative effect on osteoclastogenesis (143). Additionally, LIPUS has been shown to inhibit the myogenic and adipogenic differentiation of C2C12 cells, a mouse pluripotent mesenchymal stem cell line (136).

1.4 Bone Pathologies

1.4.1 Osteoporosis

As mentioned above, approximately 2 million Australians are estimated to have osteoporosis (OP) and this places an enormous burden on both health budgets and the community in terms of social cost. Osteoporosis is associated with a decreased bone density (BMD), which is associated with increased pathological fracture (144). A number of therapeutic agents have been investigated in the treatment of osteoporosis, including agents acting to both decrease bone turnover and promote deposition of mineral. These include vitamin D, vitamin K analogues, bisphosphonates, strontium, calcium, PTH, calcitonin, and bone morphogenetic proteins (BMP)-2 and 7 (145-149). As discussed above, newer therapies have been designed to encourage bone growth, using mechanical stimulation including vibrating platforms and LIPUS (8, 150). Bone strength is determined by a number of factors including the amount of bone, micoarchitecture, mineralisation and geometry (151). The current focus of all OP treatments is to modify osteoclast or osteoblast behaviour (14). However, the OY is by far the most abundant bone cell type and a great deal of evidence implicates these cells as central in directing bone physiology and pathology, secondarily influencing the behaviour of other cell types (66, 67). As discussed above, aging and OP are associated with a decrease in both OY density and viability (64).

1.4.2 Fracture

Fractures are trauma-associated events made more likely in conditions of low bone mass such as OP, or where there is disrupted bone composition as in some osteopetrotic diseases, or as a result of cancer-related bone loss (8, 64, 152, 153). The majority of fractures in otherwise normal bone are able to heal within a relatively short period of time (154). However, a small percentage of fractures take longer than 9 months to heal and are termed non-union (130). Many fractures require treatment with pharmaceutical agents and/or surgical fixation to heal though this is not always successful (155). Microcracks have also been postulated to predispose to fractures (156). The association of apoptotic OY and empty lacunae with fracture has also been investigated (1). The ability of apoptosing OY to recruit osteoclasts has been demonstrated, which points to a role for OY in bone matrix repair (65). Calcium in combination with vitamin D has been used for a number of years as a preventative measure for fractures due to osteoporosis (157). Additionally, the effectiveness of LIPUS as a tool in the expedited repair of non-fractures is being explored and indeed employed (158).

1.5 Scope, Hypotheses, and Aims of Study

In summary, the initial aim of this project was to further characterise the role of anabolic agents in the transition of osteoblasts to OY in our previously established model systems. Here, Ca^{2+} was chosen as the agent of interest. Additionally, an aim of this study was to look at the role of mechanical signals in the regulation of OY activity and viability. For this aspect, LIPUS was chosen. The overarching hypothesis of this study is that both calcium treatment and LIPUS stimulation will promote an OY-like phenotype in human primary osteoblasts (NHBC) and will regulate the subsequent activity and viability of the OY. These data will

elucidate further the role of the OY in the formation and resorption of the mineralised bone matrix. The results of this study will provide rationales for the development of novel clinical therapies for the prevention and treatment of a variety of bone diseases, including osteoporosis.

1.5.1 Hypotheses

We hypothesise that:

1. Bone anabolic agents promote the transition of osteoblasts into OY. Additionally, OY are able to sense and respond to anabolic agents by directly regulating the formation and resorption of bone;
2. Mechanical loading such as LIPUS, plays a role in the regulation of OY activity and viability in the bone microenvironment.

1.5.2 Aims

1. To test the Ca^{2+} on the differentiation of osteoblasts to OY in an *in vitro* model system.

The basis of this study is to establish specifically the effects of Ca^{2+} ($CaCl_2$ at low physiological to high physiological levels (e.g. 1.8 – 11.8 mM) on the differentiation and survival of NHBC in the presence of the agent in the previously established culture systems. OY generated from NHBC will be compared with those in the process of transition and additionally primary bone explants.

2. To investigate the effect of Ca^{2+} via the CaSR on osteoblast to OY transition in an *in vitro* model system.

Treatment of NHBC with calcium, mimicking hypocalcaemic to hypercalcaemic concentrations, has demonstrated an increase in mineralisation with increased cation availability. This study will investigate the possible mechanisms, by which calcium affects mineralisation. The mineralisation initiated by calcium could potentially occur by signalling through the CaSR. Alternatively the passive deposition of calcium may increase local stiffness of the matrix initiating the formation of focal adhesions and FAK signalling promoting an OY-like phenotype. This study will explore the potential pathways of calcium action by inhibiting CaSR with known small molecule inhibitors. The alternative mechanism by which OY respond mechanically to the environmental stiffness generated by passive calcium deposition will also be investigated in this study.

3. To test the effects of LIPUS on osteoblast to OY differentiation as a new anabolic treatment in *in vitro* model systems.

The mechanical loading of bone is integral to the maintenance of bone cell health and viability. LIPUS is thought to apply a form of mechanical loading to the body when used clinically. Clinical use of ultrasound has been effective in accelerating the rate of fracture union (128, 129). This study will investigate (indirectly) the cellular mechanisms, by which ultrasound promotes fracture healing in the previously established culture systems and will be used to investigate the effect on osteoblast to OY transition in relation to proliferation, mineralisation and the influence on the gene expression profile of NHBC-derived OY.

CHAPTER 2: Anabolic effects of calcium on human osteoblasts are independent of the canonical extracellular calcium sensing receptor

OY have been implicated in the regulation of both bone anabolism and catabolism. The study on LIPUS treatment of NHBC under mineralising conditions described in the subsequent chapter demonstrated an effect on the regulation of genes associated with osteoclastogenesis as well as genes associated with osteoblast to OY transition. The transition of osteoblast to OY as described in our prior studies is integral to the anabolic process of mineralisation. Since this transition itself depends on mineralisation surrounding the OY, and on access to sufficient calcium, the effect of extracellular calcium on NHBC maturation was investigated. Both LIPUS and calcium supplementation are currently being used as preventative and therapeutic treatments for osteoporosis.

Anabolic effects of calcium on human osteoblasts are independent of the canonical extracellular calcium sensing receptor

K.J. Welldon, D.M. Findlay, A. Evdokiou, R.T. Ormsby and G.J. Atkins

Bone Cell Biology Group, Discipline of Orthopaedics and Trauma, University of Adelaide, Adelaide, SA, 5000, Australia.

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STATEMENT OF AUTHORSHIP

Anabolic effects of calcium on human osteoblasts are independent of the canonical calcium sensing receptor

WELLDON, K.J. (Candidate)

Performed analysis on the majority of samples, interpreted data, wrote manuscript

I hereby certifv that the statement of contribution is accurate

Signed.. .....Date.25/7/12...

FINDLAY, D.M.

Contributed to planning of article and provided critical evaluation

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the manuscript in the thesis.

Signed. Date25/07/2012

EVDOKIOU, A.

Contributed to planning of article.

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the manuscript in the thesis.

Signed Date25/07/12

ORMSBY, R.T.

Performed analysis on samples

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the manuscript in the thesis.

Signed .....Date25/7/12.....

ATKINS, G.J.

Supervised development of work, helped in data interpretation, manuscript evaluation and acted as corresponding author

I hereby certify that the statement of contribution is accurate and I give permission for the inclusion of the manuscript in the thesis

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Anabolic effects of calcium on human osteoblasts are independent of the canonical extracellular calcium sensing receptor

Katie J. Welldon, David M. Findlay, Andreas Evdokiou, Renee T. Ormsby, Gerald J. Atkins

K.J. Welldon, D. M. Findlay, R. T. Ormsby, G. J. Atkins

Bone Cell Biology Group,

Centre for Orthopaedics & Trauma Research,

Discipline of Orthopaedics & Trauma,

The University of Adelaide,

Adelaide, SA, Australia.

A. Evdokiou

Breast Cancer Research Unit

Basil Hetzel Institute,

Discipline of Surgery,

The University of Adelaide,

Adelaide, SA, Australia

Corresponding author: G. J. Atkins

Discipline of Orthopaedics and Trauma, The University of Adelaide,

North Terrace,

Adelaide, SA 5005, Australia

e-mail: gerald.atkins@adelaide.edu.au

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Disclosures

The authors have nothing to disclose.

Mini-abstract

We assessed the effects of extracellular calcium on human bone-derived osteoblast transition into osteocytes. Calcium treatment strikingly induced matrix mineralisation and the expression of genes associated with mature osteocytes. The expression of CaSR was rapidly lost from human bone cultured *ex vivo* and was not expressed by the resultant osteoblasts. We conclude that human osteoblast to osteocyte transition is sensitive to extracellular calcium independent of CaSR.

Abstract

Introduction

Calcium, in combination with vitamin D, has been shown to be an effective treatment for osteoporosis. Since the mineralisation of bone occurs concurrently with osteoblast to osteocyte transition, we hypothesised that calcium provides an anabolic stimulus in terms of stimulating the differentiation of osteoblasts into osteocytes.

Methods

The effect of calcium on the differentiation of adult human primary osteoblasts (NHBC) was tested *in vitro*. NHBC cultured under conditions permissive for mineralisation in the presence of a range of calcium concentrations (1.8 - 11.8 mM) were assayed for cell-

associated mineral and gene expression associated with osteoblast differentiation and mineralisation.

Results

Treatment with calcium resulted in a striking dose- and time-dependent increase in cell-associated mineralisation. Calcium appeared to promote osteoblast to osteocyte differentiation, as indicated by increased expression of osteocalcin (OCN), E11, dentin matrix protein 1 (DMP1) and SOST mRNA. The expression of the osteoclast inhibitor, osteoprotegerin (OPG), was dramatically enhanced by calcium. It was found that extracellular calcium sensing receptor (CaSR) mRNA expression was rapidly lost from human trabecular bone *ex vivo* and was not expressed by NHBC. However, NHBC did express the related receptor, GPRC6A. Surprisingly, mineralisation was either unchanged or enhanced in the presence of the calcium sensing receptor inhibitor, NPS2390. Calcium-dependent mineralisation was reversed in the presence of MEPE-ASARM peptides.

Conclusion

This study suggests that osteoblast to osteocyte transition, and the concurrent mineralisation of the extracellular matrix, is sensitive to extracellular calcium and this is independent of the canonical CaSR.

Introduction

Calcium, in combination with vitamin D, has been shown to be effective in the treatment of osteoporosis, a condition typically associated with a low BMD, increased fracture risk and a reduced osteocyte number (1-3). Although these treatments are widely accepted, the exact mechanisms, by which these agents act, are yet to be fully elucidated (4, 5). Blood calcium (Ca^{2+}) levels are strictly regulated, with total calcium levels in healthy individuals usually maintained within the range of 2.2 – 2.6 mM and between 1.1-1.3 mM for ionized calcium (6). However, soluble calcium levels in bone interstitial fluid may be higher. For example, calcium concentrations can range from 8-40 mM in the immediate vicinity of the resorbing osteoclast (7-9). Osteocytes are also capable of releasing significant amounts of calcium, demonstrated during lactation (10), suggesting that they are exposed to elevated concentrations of extracellular calcium, however actual levels have not been reported. Osteoid osteocytes appear to mediate calcium phosphate crystal formation in mineralising bone (11-14) and these differentiating osteocytes may also be dependent on the surrounding mineral for the full expression of their phenotype. However, understanding the vital process of mineralisation is challenged by the difficulty of replicating it *in vitro*. We hypothesised that calcium itself would be a regulator of the mineralisation process, and to that extent would act as an anabolic agent in bone. Using a human primary osteoblast model, we found that calcium potently increased calcium phosphate mineral deposition. This response was associated with osteoblast differentiation to an osteocyte-like phenotype. Our results suggest that these effects were independent of the canonical CaSR but a response to calcium may be mediated by an alternative member of the C-family of G-protein coupled receptors, GPRC6A, shown previously to act as an extracellular calcium sensing receptor (15).

Methods

Cell Culture

Normal human bone-derived cells (NHBC), obtained from intertrochanteric trabecular bone samples from 10 female donors (Donors 1-10) undergoing primary hip replacement surgery for osteoarthritis, with informed consent and approval by the Human Ethics Committee of the Royal Adelaide Hospital, were processed for culture and the cells propagated as described previously (16). Briefly, cells were cultured in α-MEM, which contains 1.8 mM calcium, with 10% fetal calf serum FCS, L-glutamine (2 mM), HEPES (1 mM) and ascorbate 2-phosphate (100 μM) at 37°C/5%CO_2, in a humidified incubator. Cells grown to confluence were enzymatically removed from dishes using collagenase, dispase, and/or trypsin, and plated into plastic tissue culture multi-well dishes or flasks. Experiments were performed on cells passaged up to three times. For experimental purposes, cell culture medium contained graded concentrations of additional $CaCl_2$ (Sigma-Aldrich, St. Louis, MO, USA) from 0.1 mM to 10 mM, with or without the CaSR inhibitor NPS2390 (Sigma-Aldrich, St. Louis, MO, USA) (17). Treatments were initiated 24 h after cell seeding and continued for the duration of the experiment. In some experiments, the effect of a tri-phosphorylated peptide representative of the MEPE-ASARM motif (ASARM-PO4) (12, 18) was investigated. In these experiments, NHBC were cultured under standard mineralising conditions for 21 days, whereupon medium was replaced with media containing the indicated concentrations of calcium and ASARM-PO4 (NeoMPS Inc., San Diego, CA), and cultured for a further 7 days.

Cell viability assays

NHBC viability was determined after 7 days of culture using a number of methods, including trypan blue dye exclusion and caspase 3 activity. Additionally, apoptotic nuclei

morphology was determined by 4',6-diamidine-2'-phenylindole dihydrochloride staining (DAPI, Roche Diagnostics, Castle Hill, NSW, Australia), as described previously (19). The cell surface expression of annexin V, an indicator of apoptosis was used in conjunction with staining, using the fluorescent viability stain, 7AAD to investigate the viability of NHBC in increasing concentrations of calcium, as described previously (20).

Mineralisation of Cells

The effect of calcium on NHBC ability to form a mineralised matrix was determined using a method described previously (21). Briefly, NHBC were incubated in triplicate in wells of a 96-well plate (seeded at $8x10^3$ cells/well) in α-MEM containing 10% FCS, dexamethasone (10^{-8}M), KH_2PO_4 1.8mM), and HEPES (10 mM), with varying concentrations of calcium. Medium was replaced every 3-4 days, and incubation continued for up to 6 weeks. Mineralisation was determined using a variation of the Von Kossa technique, as described previously (16). For the purpose of quantification, representative images were obtained, using a Nikon D1H digital camera attached to an inverted microscope (Nikon TE300) with a x20 objective. These were then converted to grayscale, and Image Tool version 2.2 (Molecular Dynamics®, Microsoft) was then used to quantify the amount of mineralisation per image (16).

To determine the composition of the mineralised layer, separate experiments were performed on NHBC grown in 8-chamber slides. NHBC were grown under mineralising conditions for 21 days in the presence of calcein (Sigma-Aldrich) (22) and increasing amounts of calcium. The samples were then fixed with 4% paraformaldehyde and fluorescent labeling visualized using a Nikon confocal microscope. Matched samples were prepared for analysis by critical point drying and electron dispersive X-ray spectroscopy

analysis was performed on a XL-20 scanning electron microscope to determine relative elemental composition of the mineral.

Proliferation rate of cells

Cell proliferation was measured using 5,6-carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Molecular probes, Eugene, OR, USA), a nuclear stain that is partitioned evenly between daughter cells as they divide (16, 23). After being irreversibly labeled with CFSE, NHBC were seeded into tissue culture plastic and cultured as indicated for 7 days (16). The level of CFSE staining was then determined by fluorescence-activated cell scanning analysis (FACS), using a FACStar^{PLUS} flow cytometer (Becton Dickinson, Sunnyvale, CA, USA). The number of cell doublings was determined from listmode data using ModFit LT^{TM} software (Verity Software House, Topsham, NJ, USA), as described previously (16).

Expression of osteogenic and osteoclastogenic genes

Total RNA was extracted from NHBC using Trizol™ (Life Technologies, Gaithersburg, MD) and complementary DNA (cDNA; Superscript III, Promega Corp., Madison, WI) was prepared as described previously (16). Gene expression was analysed by real-time RT-PCR, using the SYBR Green incorporation technique, as we have described previously (24). Relative gene expression between samples was calculated using the comparative cycle threshold (C_T) method (ΔC_T), calculated by the formula $\Delta C_T = 2^{-(CT(x)-CT(GAPDH))}$, where CT(x) is the C_T of the gene of interest, and CT(GAPDH) is the C_T for glyceraldehyde-3-phosphate dehydrogenase, a housekeeping gene we have used previously for examining gene expression in long-term human osteoblast cultures (12, 16, 22). Similar results were obtained when 18S ribosomal RNA (rRNA) was used as a

housekeeping gene (data not shown). Messenger RNA-specific oligonucleotide primers were designed in-house to flank intron-exon boundaries, and were purchased from Geneworks (Thebarton, SA, Australia). Real-time oligonucleotide primers for the amplification of receptor activator of NF-κB ligand (RANKL), OPG, SOST/sclerostin, E11/podoplanin, DMP1, OCN, matrix extracellular phosphoglycoprotein (MEPE), tissue non-specific alkaline phosphatase (TNAP), 18S rRNA and GAPDH were described previously (12, 16, 24-26). Sequences for the amplification of all known isoforms of human CaSR mRNA (100 bp product) were (sense – GAGTATAGTGATGAGACAGATGC), (antisense - ACTCGATCTCCTTGGCAATGCA), and GPRC6A mRNA (104 bp product) (sense – AAATCCAGGAGTGTGTTGGC), (antisense – GACTCCAGGTAAGAGTGTTG).

Expression of secreted OPG

Supernatants were collected at intervals for up to 42 days from cultures under mineralising conditions, with calcium concentrations ranging from 1.8-11.8 mM. Secreted OPG levels in culture supernatants were measured by an in-house enzyme-linked immunoassay (ELISA) using matched antibodies purchased from R&D Systems Inc (Minneapolis, MN, USA) and quantified using a standard curve generated using recombinant human OPG (Peprotech, Rocky Hill, NJ) (16).

Ex Vivo Human Bone Culture

To investigate the expression of CaSR in native bone, human cancellous bone was collected from the proximal femur of female donors at joint replacement surgery. The bone samples were dissected into 3-5 mm^3 pieces, as described previously (25). The pieces were placed into T25 tissue culture flasks and maintained in a non-differentiative,

proliferation medium consisting of α-MEM with 10% FCS, L-glutamine (2 mM), HEPES (1 mM) and ascorbate 2-phosphate (100 μM), but in the absence of either dexamethasone or inorganic phosphate, at 37°C/5%CO_2 in a humidified incubator and the medium was changed every 2-4 days. RNA was extracted using the method described above, at day 0, 3, 7, 14 and 21 from either bone samples or from the plastic adherent cells growing from the bone samples.

Statistical Analysis

One way analysis of variance (ANOVA), followed by Tukey's post-hoc analysis, was used to examine differences in gene expression studies from individual donors' cells. Paired Student's T-tests were used to compare effects in data pooled from multiple donors' cells. A p value < 0.05 was considered to be significant.

Results

In vitro mineralisation in response to calcium

NHBC were cultured under mineralising conditions for up to 4 weeks in the presence of extracellular calcium concentrations ranging from the standard media content of 1.8 mM up to 11.8 mM. Mineralisation of the cell layer was visualised using Von Kossa staining of phosphate. Although there were donor dependent differences in the level of mineralisation, calcium consistently increased mineralisation in all donor cells in a dose and time-dependent manner (Fig. 1A & B). Mineralisation was maximal for all donors' cells tested at 6.8 mM calcium (Fig. 1B). Additionally, analysis of the mineralised layer in cells treated with 1.8 mM and 2.6 mM of calcium by EDS analysis demonstrated Ca:P ratios similar to those found in hydroxyapaptite (Fig. 1C). At the highest concentration of calcium tested, there was increased calcium relative to phosphorus, indicating an altered crystal structure, due possibly to limiting concentrations of inorganic phosphate, which was kept constant in these cultures. In separate experiments, the calcium mimetic, calcein, was used to visualise the relative capacity of cells to incorporate calcium into their matrix at different calcium concentrations. As shown in figures 1 D and E, a higher extracellular concentration of calcium promoted increased calcein uptake. SEM imaging of these samples showed calcospherulite-like structures associated with cell processes, consistent with normal mineralisation and differentiation to osteocyte-like cells (13) at 2.6 mM calcium (Fig. 1 F & G).

Effect of calcium on osteoblast viability and proliferation

The effect of extracellular calcium on cell proliferation and survival of NHBC was investigated over a culture period of 7 days. Treatment with calcium at 1.8 mM, 2.8 mM, 6.8 mM and 11.8 mM showed no effect on the percentage of viable cells, as assessed by

trypan blue dye exclusion, which indicated close to 100% viability (Fig. 2A). Other measures of cell death, including caspase 3 activity, the expression of annexin V on the cell surface and altered nuclear morphology visualised with DAPI staining, all indicated that calcium did not induce apoptosis at any of the concentrations tested (data not shown). The effect of calcium on cell proliferation, examined using the CFSE staining technique, revealed that the percentage of divided cells increased from that at 1.8 mM but was maximal at 2.8 mM calcium (Fig. 2B).

Gene expression effects of calcium

The expression of several osteoblastic genes was examined in NHBC cultured for up to 42 days in media containing a range of calcium concentrations, under conditions otherwise permissive for mineralisation. Consistent with a maturational effect, calcium dose-dependently increased OCN mRNA at early time points (Fig. 3A) and decreased mRNA encoding TNAP (Fig. 3B). Interestingly, the expression of the mineralisation inhibitor, MEPE, was suppressed in response to calcium for all donors' cells tested (Fig. 3C). The expression of the endopeptidase PHEX, a pro-mineralisation gene, was notably increased at the earliest time point assayed and thereafter was suppressed at the higher concentrations of calcium (Fig. 3D). Increased exposure to calcium appeared to alter the kinetics of expression of other osteoblast differentiation-associated genes. Messenger RNA levels of E11, a pre-osteocyte-associated gene, was elevated in early cultures at the highest calcium concentration used and was elevated at later time points at the 2.8 and 6.8 mM calcium concentrations (Fig. 3E). A marked increase in expression of the mature osteocyte-associated genes, *DMP1* (Fig. 3F) and *SOST* (Fig. 3G), was also observed in all donors' cells tested, consistent with osteoblast differentiation into osteocytes. Since the *SOST* gene product, sclerostin, is a potent inhibitor of mineralisation by late

osteoblasts/pre-osteocytes (12), its elevation suggests a negative feedback response by the cells to high doses of extracellular calcium and subsequent mineralisation (see Fig. 1). Overall, these results suggest that increased extracellular calcium levels promote differentiation of NHBC to an osteocyte-like phenotype.

The response of NHBC to calcium was also investigated in terms of a potential effect on bone resorption by analysing genes involved in osteoclastogenesis. As depicted in figure 4A, RANKL mRNA expression was biphasic for some donors early in the time-course, however, in all donors' cell tested RANKL mRNA expression increased late in cultures (Fig. 4A) concomitant with the late phases of mineralisation (see Fig. 1). Calcium triggered a robust, dose-dependent induction of OPG mRNA expression (Fig. 4B), equating to an increased OPG:RANKL mRNA ratio at least up until the 42 day time point, although the latter effect was not strictly dose-dependent (Fig. 4C). Calcium also elicited a strong OPG secretory response (Fig. 4D) for all donors' cells tested. Overall, this gene expression profile is consistent with calcium eliciting a net anabolic effect.

Calcium Sensing Mechanism

The CaSR inhibitor, NPS2390, was used to investigate whether CaSR plays a functional role in calcium-induced mineralisation. Surprisingly, NPS2390 either did not affect the overall level of mineralisation achieved in the cultures or enhanced it (Fig. 5). Interestingly, the expression of CaSR mRNA was undetectable by RT-PCR in any of the donors' cells examined. In separate experiments, examination of human cancellous bone samples, either freshly removed or cultured *ex vivo*, revealed that CaSR mRNA expression declined sharply with time *ex vivo*, and was not detectable in osteoblasts growing from the bone samples (Fig. 6A). In contrast, a related receptor shown to have calcium sensing properties in osteoblasts, GPRC6A (15), showed mRNA expression in

both fresh and cultured bone samples, as well as in NHBC growing from the bone samples under proliferative but not mineralising conditions (Fig. 6B). Interestingly, the maximal expression of GPRC6A at 21 d of culture coincided with maximal expression of the osteoblastic genes, osteocalcin and type I collagen (Fig. 6C-D). These experiments indicate that trabecular bone cultured *ex vivo*, in an unloaded and 'fractured' state, by virtue of surgical removal and dissection with a scalpel blade, undergoes an activation of osteoblastic gene expression, which culminates in osteoblastic cells growing out from the bone and adopting a proliferative phenotype. The lack of expression of CaSR by these proliferative osteoblasts (NHBC), and their expression of GPRC6A, suggests that human primary osteoblasts respond to extracellular calcium independently of the canonical CaSR but that sensitivity to calcium may be mediated by a related receptor, such as GPRC6A. It is possible that CaSR expression in bone *in vivo* is predominantly due to calcium sensitive osteoclasts, but this remains to be confirmed.

We have previously shown that ASARM peptides derived from MEPE are powerful inhibitors of mineralisation by NHBC (12). Since we had observed that calcium dose-dependently inhibited the expression of MEPE mRNA (see Fig. 3C) it was possible that this inhibition allowed the mineralising cells to incorporate additional calcium into the matrix. To test this, we generated mineralised cultures of NHBC and, after onset of mineralisation, replaced media with medium containing various concentrations of calcium in the presence or absence of ASARM-PO4 peptide, at concentrations we have previously shown to inhibit human osteoblast mineralisation (12), and cultured for a further 7 days. As shown in figure 7, mineralisation was enhanced by increased calcium at 2.6 and 5 mM over this period. No effect of ASARM-PO4 was observed in cultures under standard mineralising conditions (1.8 mM calcium), likely since mineralisation did not increase sufficiently over the 7 day period. However, ASARM-PO4 potently abrogated the

increase seen at the two higher concentrations of calcium tested. These data imply that calcium-mediated suppression of MEPE expression may be at least in part responsible for the ability of the cells to increase calcium apposition.

Discussion

Consistent with previous reports (9, 27, 28), this study demonstrates that the process of osteoblast-mediated mineralisation is sensitive to the extracellular concentration of calcium, up to around 4 mM. Also at 2 - 4 mM extracellular calcium, the Ca:P ratio of the mineralised cell layers was similar to that reported in bone (29). The increased incidence of calcospherulite-like structures with calcium supports an effect of calcium in promoting bio-mineralisation (13, 30). Our findings suggest that adult human long-bone derived osteoblasts are able to tolerate even very high concentrations of calcium, which is generally consistent with previous findings, although concentrations of calcium between 10 and 15 mM exhibited a mild negative effect on cell survival in mouse neonatal calvarial cells (9).

Matrix mineralisation is concomitant with osteoblast transition to osteocytes, as elegantly demonstrated by Barragan-Adjemian and colleagues, using the mouse late osteoblast cell line MLO-A5 (13). A suite of gene expression markers are known to be up or down-regulated during this process, and our findings are consistent with this. Calcium induced an increase in OCN mRNA expression and decreased TNAP expression, similar to findings in rat primary osteoblasts and mouse 2T3 cells (31). Decreased alkaline phosphatase activity has also been reported to be associated with the accumulation of calcium in matrix vesicles (31). The biphasic expression of E11 in our cultures, a gene preferentially expressed by pre-osteocytes and thought to be involved in cell process formation (32), was consistent with the promotion of cell differentiation by calcium. Sclerostin/SOST, an important inhibitor of bone formation, is also a marker of mature osteocytes (33, 34). In the current study, high concentration of calcium induced a large and rapid increase in SOST mRNA expression. Since we have shown that sclerostin retards osteoblast differentiation beyond the pre-osteocyte stage (12), it is possible that

calcium up-regulation of SOST serves to extend the pre-osteocyte phase to accommodate further calcium incorporation. In addition, calcium increased the mRNA levels of DMP1, another marker of mature osteocytes and, potentially of relevance here, a nucleator and promoter of mineralisation (35, 36). The calcium-induced increase in PHEX mRNA expression, a gene associated with promotion of mineralisation (37), also suggests a mechanism, by which NHBC could increase mineral incorporation. Intriguingly, exposure of NHBC to increasing calcium levels resulted in a decrease in the expression of MEPE mRNA, in particular between days 21 to 28. The post-translational products of the MEPE gene, phosphorylated MEPE-ASARM peptides, are potent inhibitors of mineralisation (18, 38), including that by human primary osteoblasts (12). In the current study, addition of MEPE ASARM-PO4 peptides to calcium treated cultures completely inhibited the calcium-enhanced incorporation of calcium over a 7-day period, suggesting that the calcium-induced decrease in MEPE mRNA expression may be an important step in the ensuing pro-anabolic response. Calcium also induced RANKL mRNA expression over time. Interestingly, recent studies indicate that RANKL is a functional marker of osteocytes in the adult skeleton (39, 40). Despite this increase in RANKL mRNA expression, the level of expression of the RANKL antagonist, OPG, was also increased in response to calcium and to a much greater extent, consistent with a net pro-anabolic effect. The mechanism, by which osteoblast lineage cells respond to extracellular calcium is still unclear. There is controversy in the field as to whether or not the CaSR is expressed by osteoblasts and osteocytes (6, 41-45). Using oligonucleotide primers designed to amplify all known CaSR isoforms, we were unable to detect CaSR mRNA by real-time RT-PCR in any of our human primary osteoblast cultures. Upon further investigation, we observed a loss of CaSR mRNA expression with time in human cancellous bone cultured *ex vivo* and no detectable expression in the osteoblastic cells actively growing from this tissue,

suggesting that the canonical CaSR is not expressed by adult human cancellous bone explant-derived osteoblasts. The G-coupled receptor GPRC6A has been proposed as an alternative calcium sensing receptor present in osteoblasts and osteocytes (15, 46). Pi and co-workers showed that *GPRC6a-null* mice exhibit low bone mineral density and at least two human polymorphisms in *GPRC6A* are associated with osteopenia (47). In contrast, Wellendorph and co-workers generated a *GPRC6a-null* mouse, which at the age of 13 weeks, under physiological conditions, displayed no skeletal phenotype (48). These authors point to several limitations of their study, including that the role of GPRC6A in bone physiology was evaluated at a single age and in a normal physiological setting and that it cannot be ruled out that involvement of GPRC6A on bone development and/or homeostasis is age or strain dependent (48). The lack of an apparent bone phenotype in the GPRC6A knockout mouse is in contrast to conditional 'osteoblast-specific' CaSR gene knockouts, generated under the control of the 2.3 kb (49) or the 3.6 kb (41) type I collagen α1 promoters, in which severe bone developmental abnormalities were observed. The role of the CaSR in the post-developmental skeleton remains to be determined, since Chang and co-workers reported in abstract no skeletal phenotype when CaSR was conditionally deleted in osteoblasts under the control of the 3.5 kb osteocalcin promoter (50). Interestingly, GPRC6A mRNA expression increased with time in human bone cultured *ex vivo*, as did the expression of other osteoblastic genes including type I collagen, OCN and E11 (E11 data not shown), implying that the expression of GPRC6A is related to proliferating osteoblasts and therefore cells that would give rise to new bone formation. Some of the controversy surrounding GPRC6A and CaSR may stem from different calcium sensing mechanisms being utilised at different stages of osteoblast differentiation. It is also possible that redundancy exists in the calcium sensing mechanism and that each of CaSR and GPRC6A can fulfil this role to a certain extent; a double-gene

knockout model, as proposed previously (48), would be of great future interest. The observed effects of the CaSR inhibitor NPS2390 in our models is consistent with the close structural relatedness within the GPRC family of receptors (51), of which both CaSR and GPRC6A are members, and the previously demonstrated broad activity of NPS2390 across various members of this family, such as the metabotropic glutamate receptor 1 (52), as well as the CaSR (17). It is possible that NPS2390 may also inhibit the activity of GPRC6A, although this remains to be shown. However, we found that NPS2390 resulted in increased calcium apposition, suggesting that in the absence of calcium sensing, the matrix may become inappropriately mineralised.

In summary, our findings are consistent with calcium having an anabolic effect on human osteoblasts, increasing the transition of osteoblasts into an osteocyte-like phenotype. The precise mechanisms governing bone mineralisation remain to be fully elucidated. Our results support a balance between physicochemical mineral deposition and active, cell mediated modulation of mineralisation, consisting of both positive and negative genetic influences. Incorporated into this mechanism appears to be an effect of calcium to promote osteoblast differentiation, a process permissive of mineral incorporation, and which results in a cell phenotype equipped to ‘handle’ the incorporated mineral (11).

Acknowledgements

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Figure Legends

Fig. 1: Effect of extracellular calcium on *in vitro* mineralisation by NHBC over a time course of 42 d. A) Typical staining patterns (Donor 1) for Von Kossa are shown at day 14, 28 and 42 for 1.8 mM, 2.3 mM, 3.8 mM, 6.8 mM and 11.8 mM calcium. B) Quantification (ImageJ) of Von Kossa staining over the calcium dose range from 1.8 mM to 11.8 mM at 28 days. Data shown are means of quadruplicate readings ± SD from Donor 1. Statistical difference to 1.8 mM Ca^{2+} control is indicated by $*p < 0.001$. Similar results were obtained for cells from four other donors (Donors 2-5). C) Analysis of mineral composition by electron dispersive X-ray spectroscopy (EDS) of NHBC cultured in the presence of 1.8 mM, 2.6 mM and 11.8 mM total calcium. Data shown are the means of triplicate readings ± SD from a representative donor (Donor 1). Similar results were obtained for 4 other donors (Donors 2-5). Typical levels of incorporation of calcein into the mineralising layer by NHBC cultured for 21 days in medium containing D) 1.8 mM and E) 2.6 mM total calcium, using confocal microscopy (Donor 1). Typical appearance of cell-associated mineralised nodules by SEM analysis for F) 1.8 mM and G) 2.6 mM calcium (21 days of culture; Donor 1).

Fig. 2: Effect of varying calcium concentrations on cell viability and proliferation. A) NHBC were cultured in 96 well plates with a range of concentrations of calcium for 7 days and cell viability determined using trypan blue dye exclusion.. B) NHBC were labelled with CFSE, cultured for 7 days and analysed by flow cytometry. The percentage of divided cells was calculated using Modfit software, as described in Materials and Methods. Data shown are the means ± SEM of quadruplicate samples. Statistical difference to 1.8 mM Ca is indicated by *** ($p < 0.001$). Data shown were obtained using

cells from Donor 1. Similar results were obtained for cells from the two other donors tested (Donors 2 & 3).

Fig. 3: Effect of varying the calcium concentration on NHBC mRNA expression of osteoblast and osteocyte markers A) OCN, B) TNAP, C) MEPE, D) E11, F) DMP1 and G) SOST. NHBC were grown under mineralising conditions for 42 days, with Ca at 1.8, 2.8, 6.8 and 11.8 mM. RT-PCR was performed in triplicate on total RNA prepared over the time course. Data shown are means ± SEM. a, b, and c indicate significant differences ($p < 0.001$) for 11.8, 6.8 and 2.8, respectively, using cells from Donor 1. Similar results were obtained from 4 donors' cells tested (Donors 2, 3 and 4).

Fig. 4: Effect of Ca on bone resorption-associated gene expression. NHBC were grown under mineralising conditions for 42 days in differentiation media containing Ca^{2+} at 1.8, 2.8, 6.8 and 11.8 mM. RT-PCR was performed in triplicate on total RNA prepared over the time course, for A) RANKL and B) OPG mRNA, normalised to GAPDH mRNA expression levels. C) The OPG:RANKL mRNA ratio for the data shown in A & B. D) Supernatants sampled 3d after feeding in each case were assayed for OPG secretion by an in-house enzyme-linked immunosorbent assay (ELISA) system, as described in the Methods. In all cases data shown are means ± SD. a, b, and c indicate significant differences from control (1.8 mM Ca) ($p < 0.01$) for 11.8, 6.8 and 2.8 mM, respectively, using cells from Donor 1. Similar results were obtained with cells from 3 other donors' cells tested (Donors 2, 3 and 4).

Fig. 5: Effect of the CaSR inhibitor NPS2390 on calcium-induced mineralisation. Cultures of NHBC were grown under mineralising conditions for 14 days in media

containing either 1.8, 2.6 or 5.0 mM Ca^{2+}, in the presence or absence of NPS2390 (10 μM) and then stained with Alizarin Red. Data shown are means ± SD of quadruplicate wells and are representative (Donor 2) from three other donors' cells tested (Donors 1, 3 and 4). Significant differences induced by NPS2390 are indicated by $*p < 0.05$ and $**p < 0.01$.

Fig. 6: Gene expression in human cancellous bone and in explant cultures. Bone samples were cultured *ex vivo* for up to 21 days. At each time point indicated, total RNA was prepared from bone samples or from cells attached to the well, and both were processed for real-time RT-PCR analysis of A) CaSR, B) GPRC6A, C) OCN and D) COLA1 mRNA expression, and results normalised to the expression of GAPDH mRNA. Data shown are means of triplicate reactions ± SD. Similar results were obtained in 3 independent experiments using bone from individual donors (Donors 6-8).

Fig. 7: Effect of ASARM-PO4 peptides on calcium induced mineralisation. NHBC were grown under mineralising conditions for 21 days. Additional calcium to the indicated concentrations was then added for 7 days ± ASARM-PO4. Data shown are means ± SEM. $*p < 0.05$, $**p < 0.01$. Similar results were obtained using cells from 2 donors (Donors 9 and 10).

Figure R1

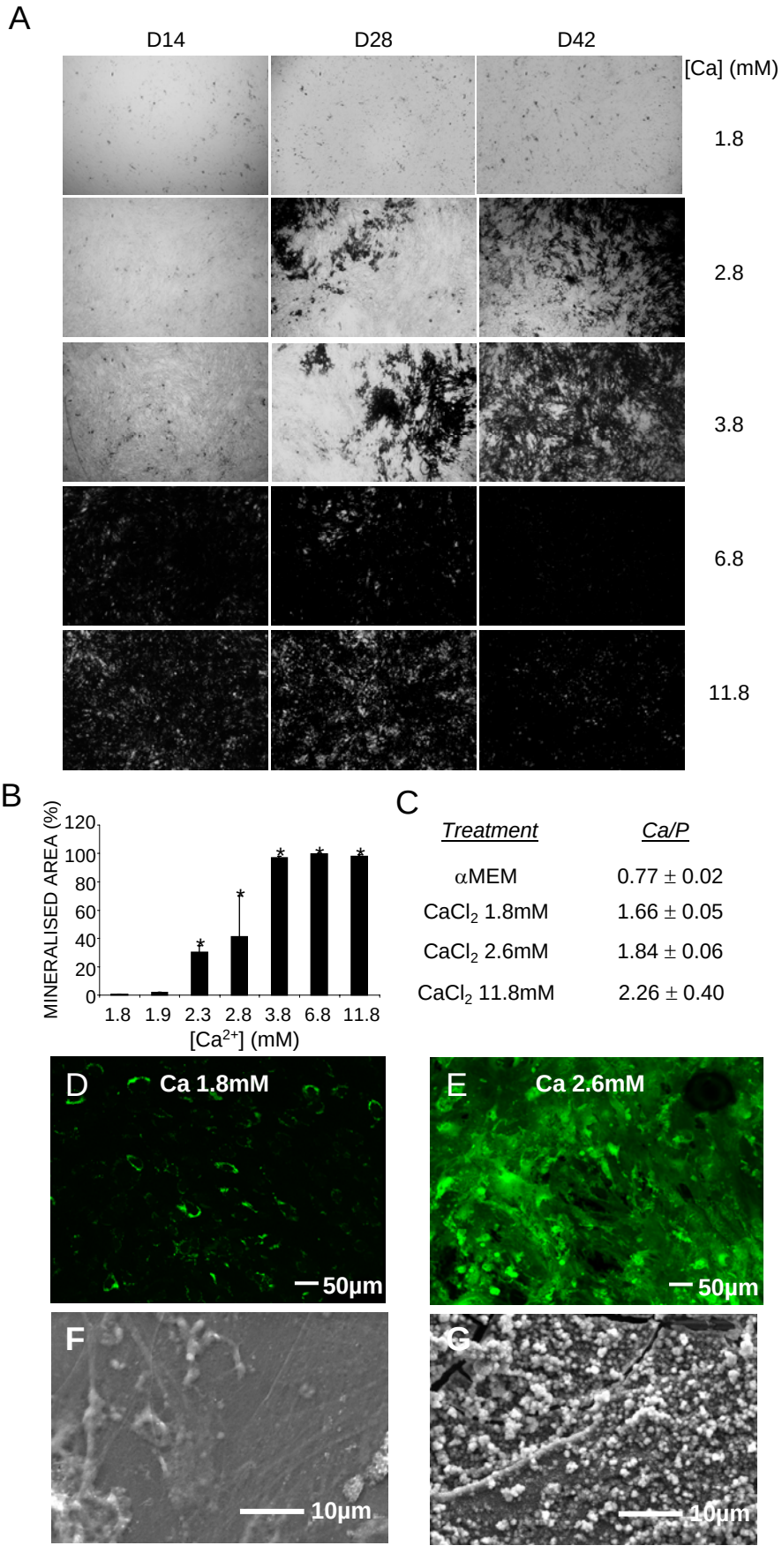

A
D14
D28
D42
[Ca] (mM)
1.8
2.8
3.8
6.8
11.8
B
MINERALISED AREA (%)
120
100
80
60
40
20
0
1.8 1.9 2.3 2.8 3.8 6.8 11.8
[Ca2+] (mM)
C
Treatment
Ca/P
αMEM
0.77 ± 0.02
CaCl2 1.8mM
1.66 ± 0.05
CaCl2 2.6mM
1.84 ± 0.06
CaCl2 11.8mM
2.26 ± 0.40
D
Ca 1.8mM
50µm
E
Ca 2.6mM
50µm
F
10µm
G
10µm

Figure 2

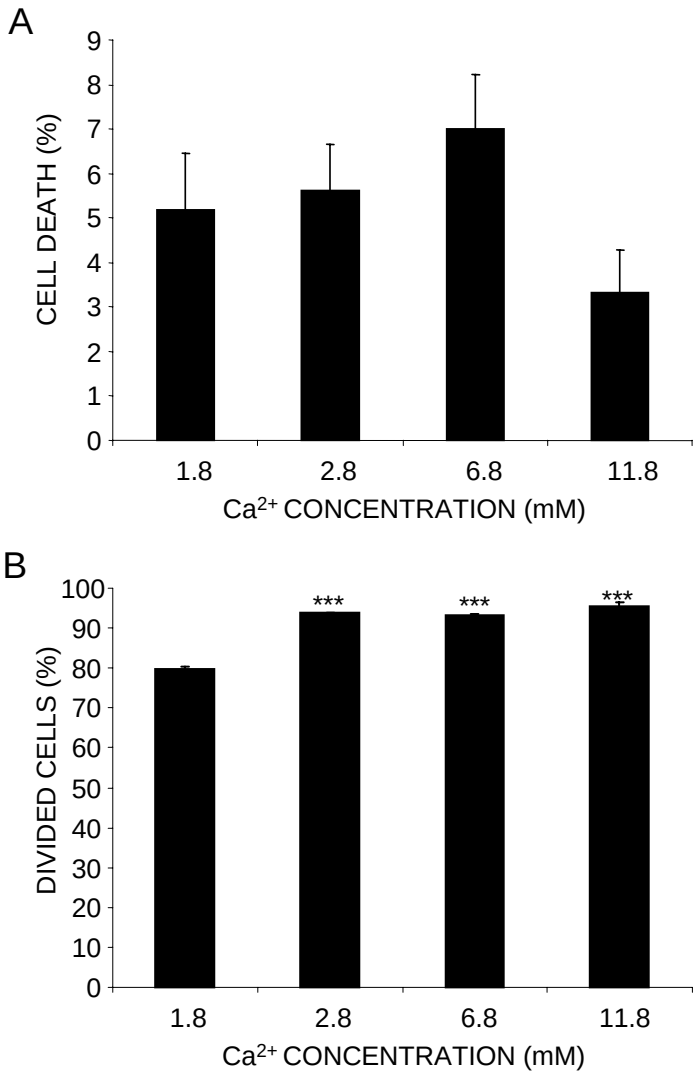

A
CELL DEATH (%)
0
1
2
3
4
5
6
7
8
9
1.8
2.8
6.8
11.8
Ca2+ CONCENTRATION (mM)
B
DIVIDED CELLS (%)
0
10
20
30
40
50
60
70
80
90
100
***
***
***
1.8
2.8
6.8
11.8
Ca2+ CONCENTRATION (mM)

Figure 3

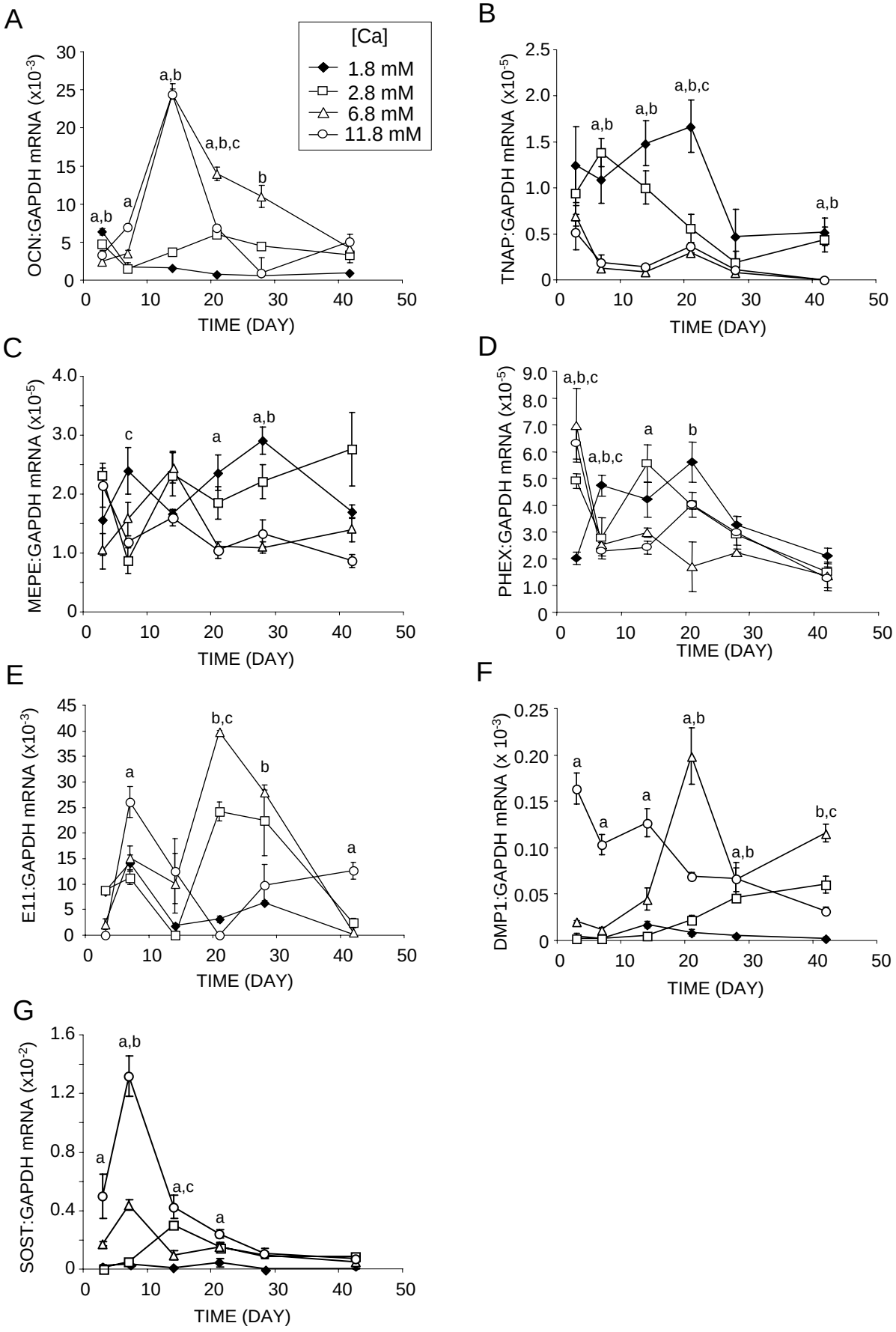

A
[Ca]
1.8 mM
2.8 mM
6.8 mM
11.8 mM
OCN:GAPDH mRNA (x10-3)
TIME (DAY)
B
TNAP:GAPDH mRNA (x10-5)
TIME (DAY)
C
MEPE:GAPDH mRNA (x10-5)
TIME (DAY)
D
PHEX:GAPDH mRNA (x10-5)
TIME (DAY)
E
E11:GAPDH mRNA (x10-3)
TIME (DAY)
F
DMP1:GAPDH mRNA (x 10-3)
TIME (DAY)
G
SOST:GAPDH mRNA (x10-2)
TIME (DAY)

Figure R4

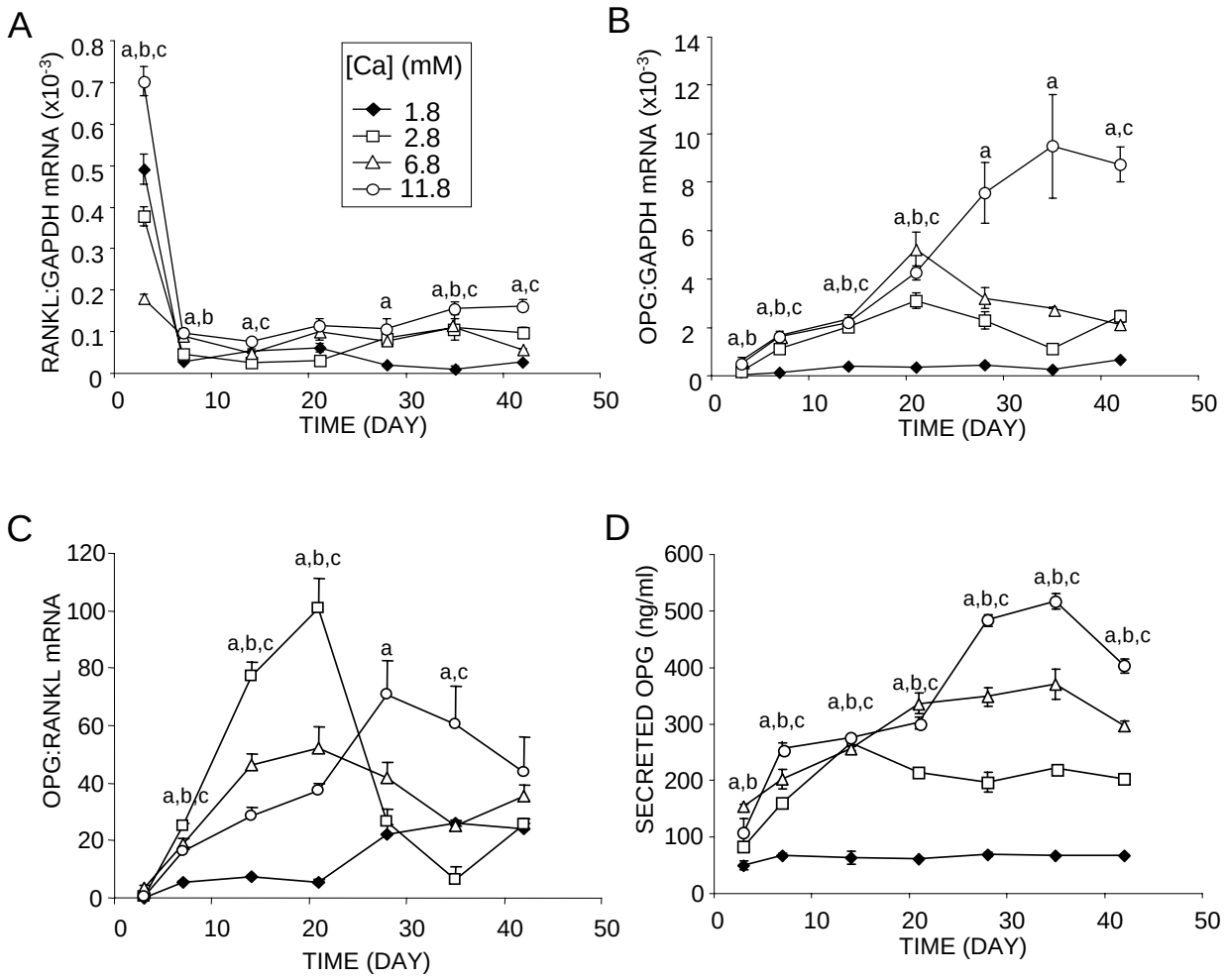

A
RANKL:GAPDH mRNA (x10-3)
TIME (DAY)
[Ca] (mM)
1.8
2.8
6.8
11.8
B
OPG:GAPDH mRNA (x10-3)
TIME (DAY)
C
OPG:RANKL mRNA
TIME (DAY)
D
SECRETED OPG (ng/ml)
TIME (DAY)

Figure 5

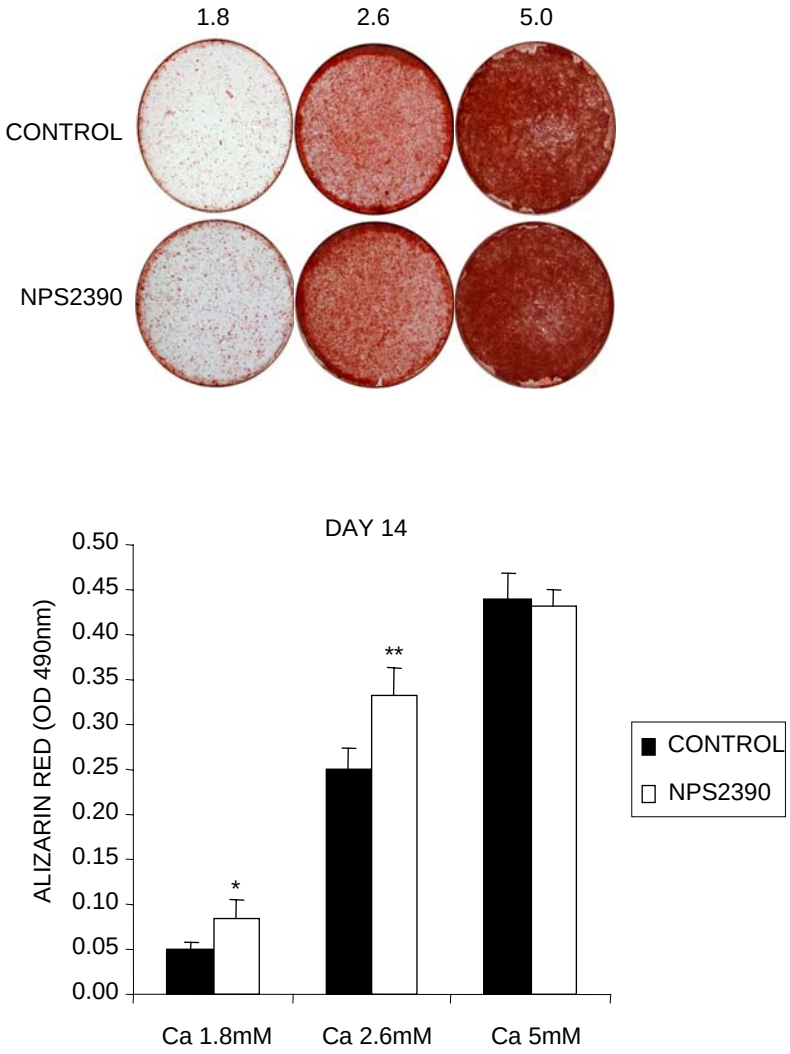

1.8
2.6
5.0
CONTROL
NPS2390
DAY 14
ALIZARIN RED (OD 490nm)
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
**
Ca 1.8mM
Ca 2.6mM
Ca 5mM
CONTROL
NPS2390

Figure R6

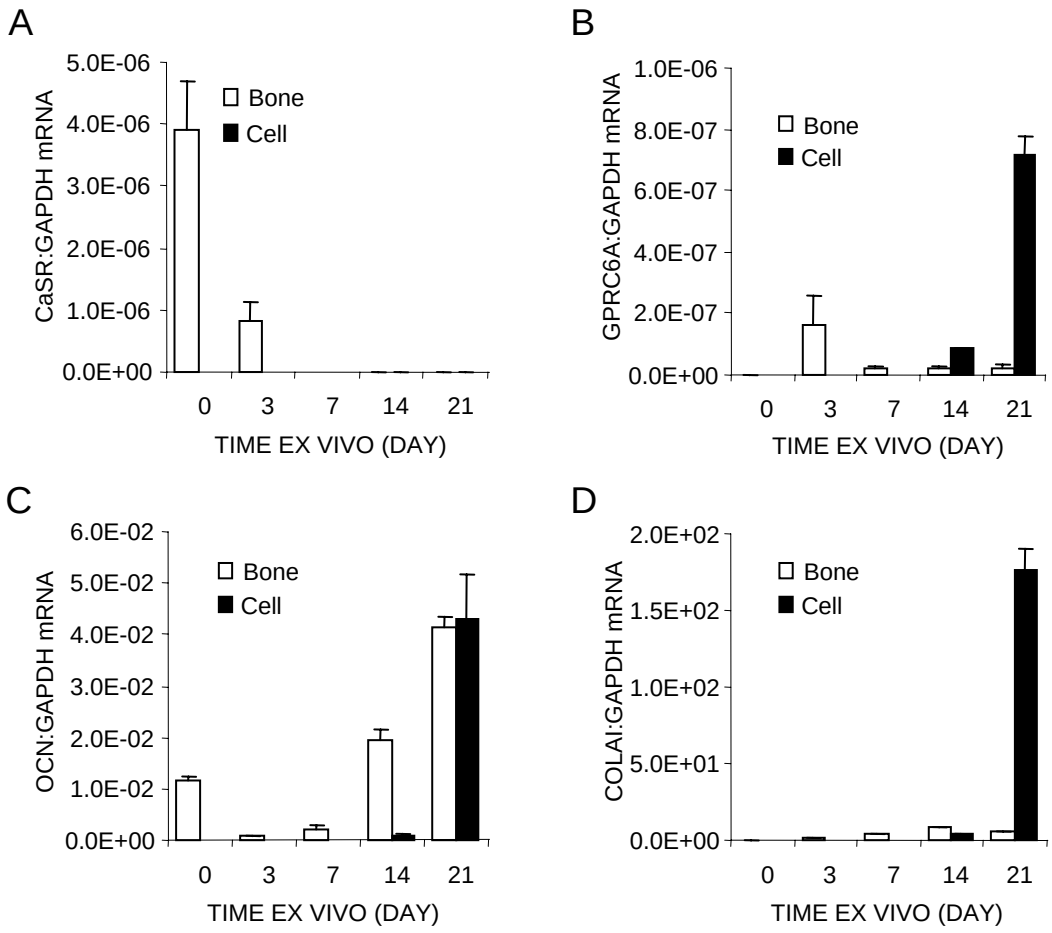
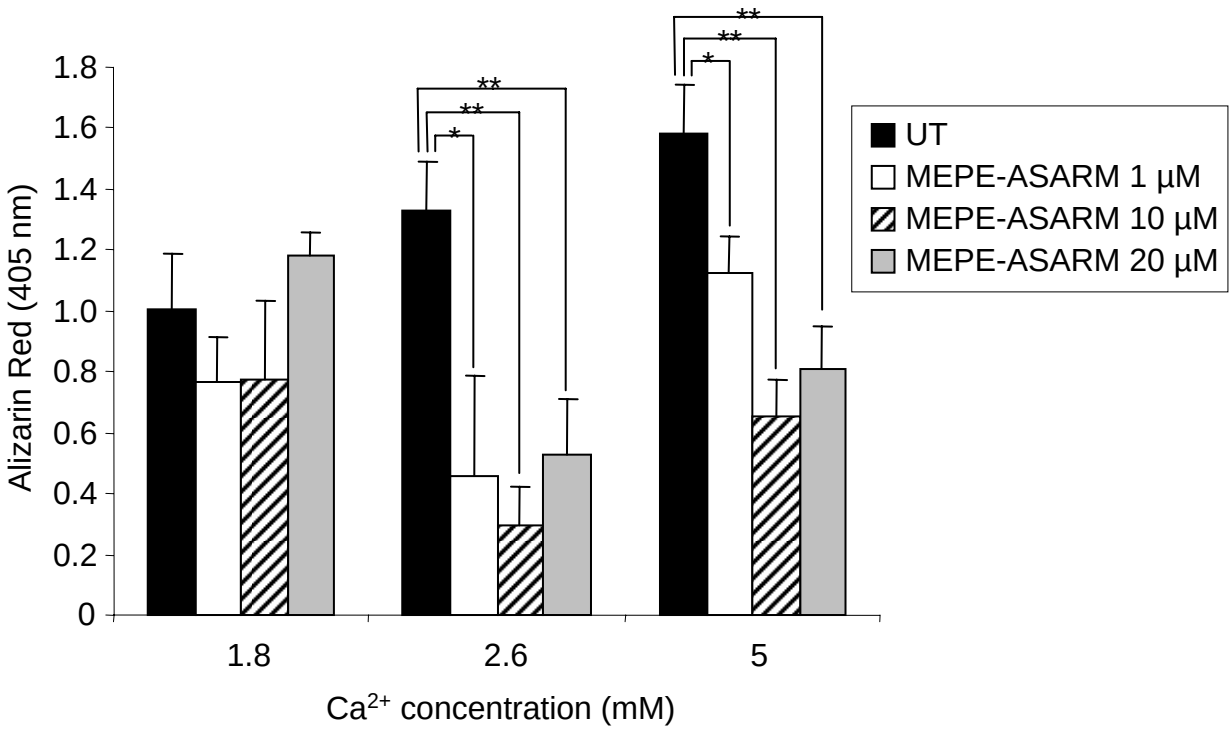

A
5.0E-06
4.0E-06
3.0E-06
2.0E-06
1.0E-06
0.0E+00
CaSR:GAPDH mRNA
Bone
Cell
0
3
7
14
21
TIME EX VIVO (DAY)
B
1.0E-06
8.0E-07
6.0E-07
4.0E-07
2.0E-07
0.0E+00
GPRC6A:GAPDH mRNA
Bone
Cell
0
3
7
14
21
TIME EX VIVO (DAY)
C
6.0E-02
5.0E-02
4.0E-02
3.0E-02
2.0E-02
1.0E-02
0.0E+00
OCN:GAPDH mRNA
Bone
Cell
0
3
7
14
21
TIME EX VIVO (DAY)
D
2.0E+02
1.5E+02
1.0E+02
5.0E+01
0.0E+00
COLAI:GAPDH mRNA
Bone
Cell
0
3
7
14
21
TIME EX VIVO (DAY)

Figure 7


Alizarin Red (405 nm)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
1.8
2.6
5
Ca2+ concentration (mM)
UT
MEPE-ASARM 1 µM
MEPE-ASARM 10 µM
MEPE-ASARM 20 µM
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CHAPTER 3: LIPUS stimulus induces normal human osteoblast transition to an OY-like phenotype

3.1 Introduction

Around the world, millions of traumatic and pathological fractures occur every day and a large number of these will go on to be classified as non-union. Non-united fractures comprise 5-10% of all fractures and are characterised by an impaired ability to heal, and often these take more than 9 months to fuse radio graphically (159). The search for effective treatments for fracture repair and bone growth is ongoing. Many invasive techniques have been investigated and implemented such as bone grafting, plating and surgical screws (129). The aforementioned methods are used in combination with a variety of agents such as growth factors and hormones (8, 129, 159). The development of treatments which are non-invasive, simple and inexpensive to use would be a major breakthrough in the field. Ultrasound is a form of mechanical energy transmitted as acoustic pressure wave that is absorbed at a rate in proportion to the density of the tissue that is being treated (8). The use of low intensity pulsed ultra sound (LIPUS) as a therapeutic tool for treatment of soft tissue is well established (160, 161). Although the level of strain induced by functional load bearing is several orders of magnitude higher than those affected by LIPUS, clinical studies have indicated that it can still affect bone tissue (8, 162, 163). However, the effects of LIPUS and the mechanisms by which it acts are still under investigation in the treatment of bone pathologies such as fracture, femoral head necrosis and osteoporosis (164, 165).

Osteoblasts and osteoclasts locate at the surface of bone to form the basic multicellular unit and are responsible for the repair of bone matrix. Due to their location on the surface of bone, LIPUS treatment may have greater impact on these cell types. As osteoblasts synthesise the

bone organic matrix, termed osteoid, around 10-20% of these cells become embedded and differentiate into osteocytes (OY) (14). The OY bodies are located within lacunae in the bone matrix and are connected to each other by long dendrite-like processes that extend through a network of interconnected canaliculi to form a syncytium (18). The OY are able to sense mechanical loading *via* a number of mechanisms, such as integrin binding in the form focal adhesions on surrounding bone matrix, primary cilia, cytoskeleton deformation, and connexins (25, 58-61). OY mechanosensors work separately or in partnership to respond effectively to mechano-stimulators that likely include fluid shear stress (FSS), pressure, strain and electrical fields (54, 56). Mechanical strain exerted on the bone translates to movement of fluid from the bone vasculature to the OY canalicular network, creating FSS as well as removing cell waste and providing oxygen and fresh nutrients (57). The OY are then able to transmit the signals generated by the mechanical stimulus to the osteoblasts at the cell surface, *via* gap junctions and hemichannels linking the OY syncytium to regulate bone formation (25, 166).

The process of fracture healing involves the proliferation and differentiation of bone cells (126, 158). The number and viability of OY is reduced with age and certain pathology and this is linked to increased fracture risk (1, 64). While a clear role for OY in fracture repair has not been identified, some evidence suggests that the local OY response may be important (64, 167). However, when micro fractures occur, the canalicular system and consequently the OY processes are disrupted and the cells undergo apoptosis (168). The apoptosis of OY triggers the recruitment of osteoclasts and stimulation of bone remodelling (66).

Beyond the bone repair response, mechanical stimulation in addition to bone and therefore OY growth is important for OY viability (167, 169). Therefore, in this study the effect of LIPUS both on NHBC proliferation and differentiation into OY was investigated.

3.2 Methods and Materials

3.2.1 Normal human bone donor cells

All human osteoblast or normal human bone-derived cells (NHBC) for the LIPUS study were obtained from the trabecular bone of five donors, two male and three female, at joint replacement surgery for end stage osteoarthritis, with informed consent and approval by the Human Ethics Committee of the Royal Adelaide Hospital (170, 171). The bone specimens, collected using tube saws, were dissected into 3-5mm fragments and washed extensively with phosphate buffered saline (PBS), pH 7.4. The bone fragments were then placed into 75 cm^2 tissue culture flask (Corning, Co-star Corp. Cambridge MA, USA) and cultured at 37ºC/5%$C0_2$ in α-MEM (Sigma Aldrich Co. Ltd., St Louis, MO, USA) containing 10% FCS (MultiSer FCS, Thermo electron corp., Mel, Aus) and 100 μM L-ascorbic acid (Wako Pure Chemical, Japan). The culture medium was changed every 3-4 days until the cells reached confluence. NHBC generally became confluent in 3-4 weeks post harvest, with some donor dependent variability. Cells were then removed from the flask with a 0.1% (w/v) mixture 1mg/ml each of collagenase (Sigma Aldrich Co. Ltd., St Louis, MO, USA) and dispase (GIBCO, Life Technologies Corp, Japan). Cells were either cryopreserved for storage in a mixture of 1.5% FCS, α-MEM and 1% dimethylsulfoxide (DMSO) (Merck, Dramstadt, Germany) or passaged for experimental use. Cells from up to passage two were used for all experiments.

3.2.2 Cell Culture

All tissue culture was performed in sterile Class II laminar flow hoods. Cell cultures were incubated in a humidified incubator at 37°C with 5% CO_2. Cell suspensions were diluted 1 in 2

with 0.4% (w/v) trypan blue (Sigma Aldrich Co. Ltd., St Louis, MO, USA) in PBS and counted using a Neubauer haemocytometer at 10x magnification on a light microscope (Eclipse TE300, Nikon, Japan). Prior to experimentation, cells were cultured in α-MEM, containing 1.8 mM calcium, with 10% FCS, 2 mM L- glutamine, 1 mM HEPES (supplied by the media production unit, IMVS, SA), 100 μM L-ascorbate 2-phosphate (WAKO Pure Chem, Japan), 100 IU/ml penicillin, and 160 μg/ml gentamicin (supplied by the media production unit, IMVS,SA), at 37°C/5%CO_2 in a humidified incubator (171). The filtered sterile medium was stored at 4°C for no longer than 1 week. Cells were enzymatically removed from flasks by collagenase/dispase digestion for 1 hour. If cells still remained attached after the hour, they were further treated with trypsin (supplied by the Media Production Unit, IMVS, SA) for 5 min after washing with PBS. Cells were resuspended at 2 x 10^6 cells/ml, and seeded at 8 x 10^3 cells/cm^2 into 6 or 48 well plastic tissue culture dishes (NUNC, Denmark) or 25 cm^2 flasks (Corning, Co-star Corp. Cambridge MA, USA) for experimental use. For mineralising cultures, the media was supplemented with dexamethasone phosphate ($1x10^{-8}$ M) (DBL, Mulgrave, Vic, Aus), KH_2PO_4 (1.8 mM) (AJAX chemicals, Sydney, Aus) and 5% FCS as well as L-glutamine, HEPES, L-ascorbate 2-phosphate and antibiotics. Mineralising conditions were initiated 24 h after seeding and maintained for the duration of the experiment. The cell culture medium was replaced at 3-4 day intervals.

Live images of NHBC were taken by phase contrast using a light microscope. Live images of cells were taken at day 0 and 7 for early cultures and day 0, 3, and 7 for mineralising cultures, at 20x magnification.

3.2.3 Specialised Equipment

Low intensity pulsed ultra sound (LIPUS) was administered to cell cultures using a device provided by BTT (Auburn, NSW, Aus) (**Figure 3.1 a**). BTT v2.85 software was used to set up a treatment regimen for test cultures and record data during treatment. The ultrasound probe was mounted in a stable polystyrene platform and placed in a different 37ºC/5% CO_2 incubator from the incubator containing the experimental plates. The bases of the test plates were coated with a standard amount of water soluble transmission gel (Medtel, Lane Cove, NSW, Australia) that had been pre-warmed to 37°C, to ensure even contact and conduction of ultrasound waves during treatment (**Figure 3.1 b**). The tissue culture plates were placed in the same orientation each time, using guides marked on the platform. Each culture was treated for 20 min per day at 1.5 MHz for the duration of the experiment. Treatments were carried out at the same time each day. Total RNA and supernatants were collected 3 h after treatment for each time point and cells were also fixed at this time for Alizarin red staining.

3.2.4 Cell proliferation assay and Flow cytometric analysis

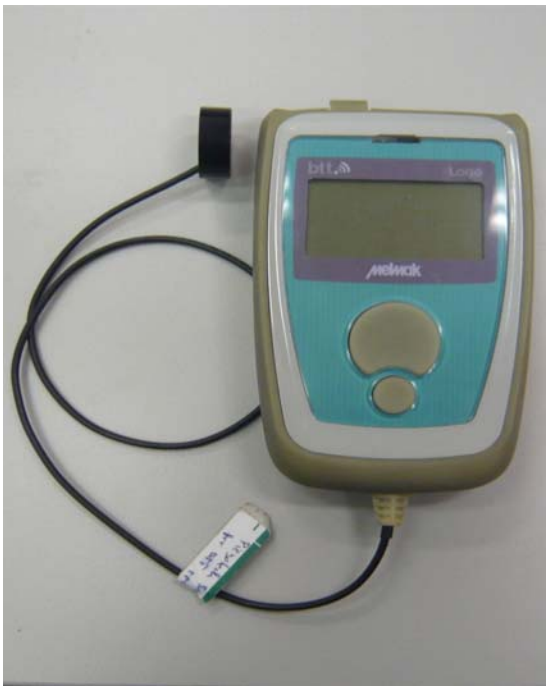
Cell proliferation was measured using 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Molecular probes, Eugene, OR, USA), a nuclear stain that is evenly divided between daughter cells (172), as we have previously described (76). Enzymatic treatment with collagenase and dispase was used to remove the cells from the tissue culture flask. NHBC were resuspended in 1 ml of 0.1% BSA/PBS for CFSE staining. To control for auto fluorescence, 1.5 x 10^5 cells were seeded into one well before staining was commenced. CFSE at a final concentration of 10 μM was then added to the cell suspension and incubated at 37°C for 10 min (171). The reaction was then quenched with the addition of 5 volumes of ice cold standard culture medium and incubated for 10 min on ice. CFSE labelled NHBC

were then seeded at 1.5 x 10^5 cells/well into tissue culture plastic and allowed to attach overnight. A single well of stained cells was treated with colcemid at 300 ng/ml to maintain cells in an undivided state as a control. The following day, NHBC media was changed to mineralisation media (ref. sec 3.2.2). LIPUS treatment was commenced after the media was changed and continued for 20 min at 1.5 MHz for the duration of the experiment. Cells were then removed from the flask by collagenase and dispase digestion and resuspended in FACS fix. Cells were then stored at 4°C until analysis. The level of CFSE staining after 7 days of treatment was determined by flow cytometric analysis using a FACStar ^{PLUS} flow cytometer (Becton Dickinson, Sunnyvale, CA, USA). The number of doublings was determined from the listmode files generated. These where then analysed using ModFit LT^{TM} software (Verity Software House, Topsham, NJ, USA) (76).

3.2.5 RNA extraction and cDNA synthesis

Total RNA was extracted from NHBC using Trizol™ (Life Technologies, Gaithersburg, MD, USA). After supernatant was removed, cells were collected in 1ml of Trizol reagent. The Trizol:Cell mixture was stored frozen at -80°C until required and allowed to reach room temperature before being mixed at a 1:5 ratio with chloroform. After vigorous mixing for 15 seconds the Trizol chloroform mixture was allowed to stand at RT for 2-3 min and then centrifuged at 12,000 rpm at 4°C in an eppendorf microfuge for 15 min. The clear organic phase was removed, avoiding the white DNA layer, and 2 µl of glycogen was added. After inverting several times a 1:1 ratio of isopropanol (CHEM-supply, Bedford, SA, Aus) was added to the organic phase, vortexed and left at -20°C overnight to precipitate. The isopropanol mixture was centrifuged at 12,000 rpm at 4°C for 20 min to pellet RNA the following day. The RNA pellet was washed with 75% Ethanol (Merck, Kilsyth, Vic, Aus) by inverting several

A



B

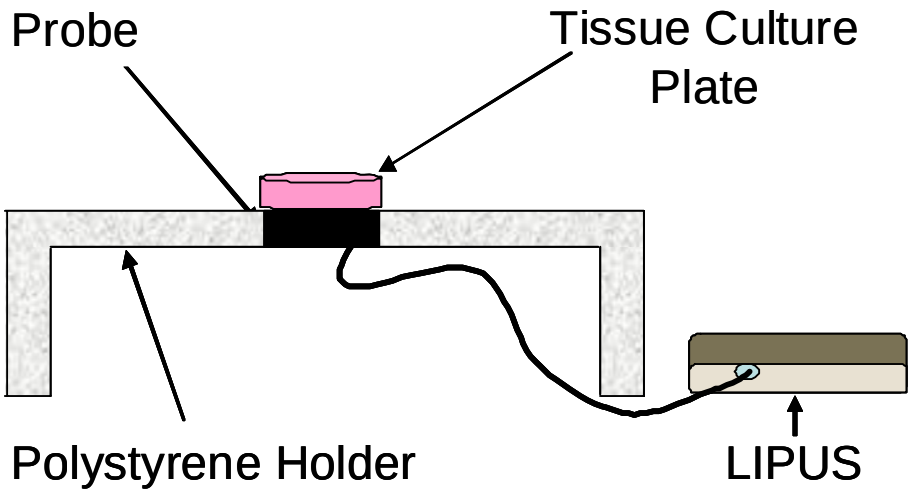


Figure 3.1: The BTT system was used to administer low intensity pulsed ultrasound (LIPUS) at 1.5 mHz (a). The set up of LIPUS system for treatment of cell cultures was placed in an incubator at 37°C/5% CO_2 (b). The incubator used for treatments was different from the incubator in which the experiments and controls were kept during the experiment.

times and centrifuging for 5 min at 12,000 rpm. After the ethanol was removed, the RNA pellets were dried at 60°C in a heating block and resuspended in 20 µl of diethyloxypyrocarbonate (DEPC) (ICN Biomedical Inc, Ohio, USA) H_2O. Before storage at -80C the RNA was heated to 65°C for 2 min. After thawing, 1µl of RNA was analysed using a spectrophotometer (NanoDrop1000, Thermo scientific, Germany) to determine the RNA concentration. For cDNA preparations, 1 µg of RNA was used to make a 20 µl volume with the Superscript III (cDNA; Superscript III, Promega Corp., Madison, WI), Random Hexamers (Geneworks, Thebarton, SA, Australia), First strand buffer (cDNA; Superscript III, Promega Corp., Madison, WI), DTT (cDNA; Superscript III, Promega Corp., Madison, WI), 10 mM dNTPs (Sigma Aldrich Co. Ltd., St Louis, MO, USA) (4). Gene expression was analysed by real-time RT-PCR, using the SYBR Green incorporation technique (4). For each 15 µl, 1 µl of cDNA was mixed with 7.5 µl of UMM (Qiagen Sciences, Maryland, USA) and 0.75 µl of 3' and 5' primers and run on a BIORAD myIQ cycler (BIORAD, Hercules, CA, USA) (4). Relative gene expression between samples was calculated using the comparative cycle threshold (C_T) method (ΔC_T), using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. Oligonucleotide primers were designed in-house to flank intron-exon boundaries, and were purchased from Geneworks (Thebarton, SA, Australia). Real-time oligonucleotide primers for the amplification of RANKL and GAPDH were described previously. Sequences of other primers for real-time PCR used are shown in (**Table 1**).

Table 1

Primer name and Direction (Forward or Reverse)		Sequence (5′-3′)	Product (bp)
Col-1(5)	F	AGGGCTCCAACGAGATCGAGATCCG	225
	R	TACAGGAAGCAGACAGGGCCAACGTCG	
BSP-1(75)	F	ATGGCCTGTGCTTTCTCAATG	123
	R	AGGGATAAAAGTAGGCATGCTTG	
RUNX-2(5)	F	GATGTGCCTAGGCGCATTTCAG	110
	R	AGGGCCCAGTTCTGAAGCACC	
E11(5)	F	ACAGTGAACAGGCATTGGCATCG	138
	R	CTGTGTGCTCCATCCACTTTCTC	
RANKL(4)	F	TCAGCCTTTTGCTCATCTCACTAT	96
	R	CAAGAGGACAGACTCACTTTATGGG	
OPG(5)	F	GCTCACAAGAACAGACTTTCCAG	106
	R	CTGTTTTCACAGAGGTCAATATCTT	
COX-2	F	GGAGAAAACTGCTCAACACCG	118
	R	TATTCACAACGTTCCAAAATCCC	

3.2.6 PGE_2 EIA assay

To determine whether PGE_2 was released in response to LIPUS stimulation, cells were grown under mineralising conditions until the onset of mineralisation. Cells were then treated with a daily regimen of 20 min LIPUS at 1.5 mHz over the remaining course of the experiment. Supernatant was collected from cell cultures 90 mins after LIPUS treatment at day 0, 3, and 7 of LIPUS treatment. Supernatants were analysed using a monoclonal competitive enzyme-linked immunosorbent assay for PGE_2 (Cayman Chemical Company, Ann Arbor, MI, USA). Briefly, plates were pre-coated with goat polyclonal anti-mouse IgG and blocked with a proprietary formulation of proteins with tracer, antibody, and either standard or sample with the appropriate controls. The plate was then washed to remove the unbound reagents with wash buffer provided plus Tween 20 (Sigma Aldrich Chemical Co. Ltd., St Louis, MO, USA). The colour reaction, representing PGE_2, was then developed with Ellman's reagent. The reaction was analysed at 405 nm on an EL808 Ultramicroplate reader (BIOTEK

instruments). The readings were inversely proportional to the amount of PGE_2 present. A standard curve of serially diluted PGE_2 was used to plot a standard curve using linear (y) and log (x) axes and fit data to a 4-parameter logistic equation. The concentration of each sample was determined using the equation obtained from the standard curve plot.

3.2.7 Alizarin Red assay

The effect of calcium on NHBC ability to form a mineralised matrix was determined using a method described previously (77), with some modifications. Briefly NHBC were incubated in triplicate in wells of a 96-well plate (8×10^3 cells/well) in α-MEM containing 10% FCS, dexamethasone (10^{-8} M), KH_2PO_4 1.8 mM), and HEPES (10 mM). Media was replaced every 3-4 days, and incubation continued for up to 21 days. LIPUS treatment commenced after 21 days under conditions determined to be permissive to mineralisation. Cells were treated with LIPUS for 20 min each day at 1.5 mHz. Mineralisation was determined using Alizarin red staining at day 0, 3 and 7. Cells were washed 2 times with PBS and then fixed for 1 hour in 10% formalin (Sigma Aldrich Chemical Co. Ltd., St Louis, MO, USA). After washing once with deionised water (dH_2O), the cells were stained for 5min with 1% Alizarin red (Sigma Aldrich Chemical Co. Ltd., St Louis, MO, USA), 0.2% Ethanol in dH_2O with pH adjusted to 4.1-4.3 using 0.5% ammonium hydroxide (Sigma Aldrich Chemical Co. Ltd., St Louis, MO, USA). The cell layer was then washed in dH_2O until no more red stain was released, and then dried. The cell layers were then photographed using a dissecting microscope at 1.68x (Olympus Corp., Toyko, Japan). The alizarin stain was then extracted with 10% acetic acid (Merck, Kilsyth, Vic, Aus). After 20 min on a rocking platform, the cell layers were removed using a scraper and transferred to 1.5 ml reaction tubes. The samples were then briefly agitated on a Vortex mixer, heated at 85°C for 10 min, then placed on ice for 5 min before being centrifuged at 20,000 xg for 15 min at 4°C. The

sample was then transferred to a fresh tube, avoiding the cell debris. To 200 µl of sample, 75 µl of 10% ammonium hydroxide was added and 150 µl of this mixture was transfer to a 96 well plate for analysis. The absorbance of the samples was then read at 405_{nm} using an EL808 ultramicroplate reader. Relative mineralisation levels were then determined.

3.2.8 Statistical analysis

One way analysis of variance (ANOVA), followed by Tukey's post-hoc analysis, was used to examine differences in gene expression studies from individual donors' cells. Paired Student's T-tests were used to compare effects in data pooled from multiple donors' cells. A p value < 0.05 was considered to be significant.

3.3 Results

3.3.1 In vitro mineralisation in response to LIPUS

NHBC were seeded in conditions previously shown to be permissive for *in vitro* mineralisation (3, 4, 74, 76, 170). The cells were allowed to proliferate and differentiate to the point of onset of mineralisation. At this point, they were treated with LIPUS for either 0, 3, or 7 days. Alizarin red was used to visualise and quantify the changes in mineral deposition induced by LIPUS treatment post differentiation. The level of mineralisation was donor-dependent and the trend in the effect of LIPUS treatment was consistent for at least 3 out of the 5 donors tested (**Figure 3.2)**. In some cases the level of mineralisation was so great that a further increase was perhaps impossible to detect. However, in donors where levels were lower, a subtle though not always statistically significant increase in mineralisation was

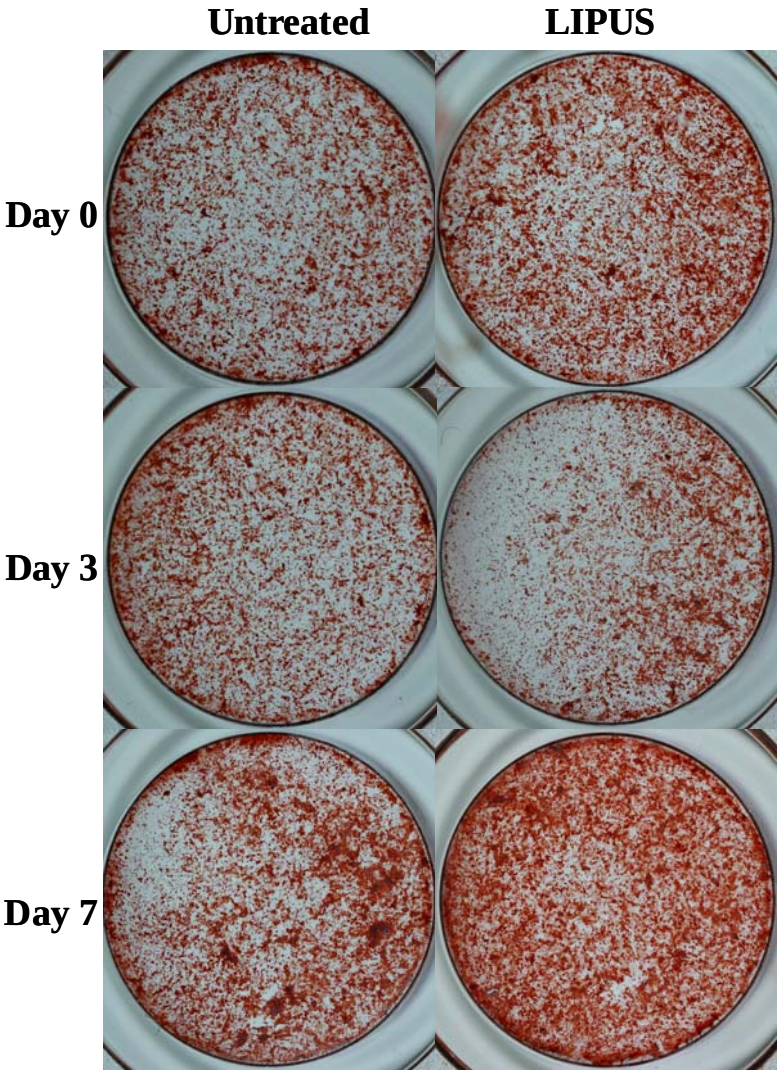


Figure 3.2: The effect of LIPUS on NHBC mineralisation. The NHBC were seeded at 1.5 x10^4 cells per tissue culture well and allowed to adhere. Media were then replaced with standard mineralising media and cells were grown for 21 days until they started to mineralise. Then at day 21 quadruplicate wells were either untreated or treated with LIPUS in a CO_2 incubator at 37°C for 20 min each day and harvested at day 0, 3 and 7 of treatment. Cell layers were then stained with Alizarin red and images taken on a dissecting microscope at 1.68x. Data shown are from a representative donor.

observed. These results indicate a subtle positive trend, albeit not statistically significant, with LIPUS treatment in the propagation and maintenance of mineralisation (**Figure 3.3**). The lack of any negative regulation on already active mineralisation at the very least indicates a non-detrimental effect of LIPUS treatment.

3.3.2 Effect of LIPUS on osteoblast proliferation

The effect of LIPUS on cell proliferation was tested using CFSE staining over a period of 7 days. Cells were irreversibly stained and seeded under mineralising conditions then treated with LIPUS daily before analysis. The number of undivided cells and those that had undergone cell division were tracked by relative intensity of CFSE staining by flow cytometry. The general trend in the cells tested indicated no significant change in proliferation rate with LIPUS treatment (**Figure 3.4**). Donors overall showed a small increase in the number of early cell divisions and consequently a decrease in parent cell population (**Figure 3.5 a & b**). Additionally, trypan blue exclusion showed no difference in viable cell number between untreated controls and LIPUS treated groups (**Figure 3.6**). These data suggest that there may be a subtle proliferative effect of LIPUS, albeit not statistically significant, treatment and no unfavourable effect on NHBC cell proliferation.

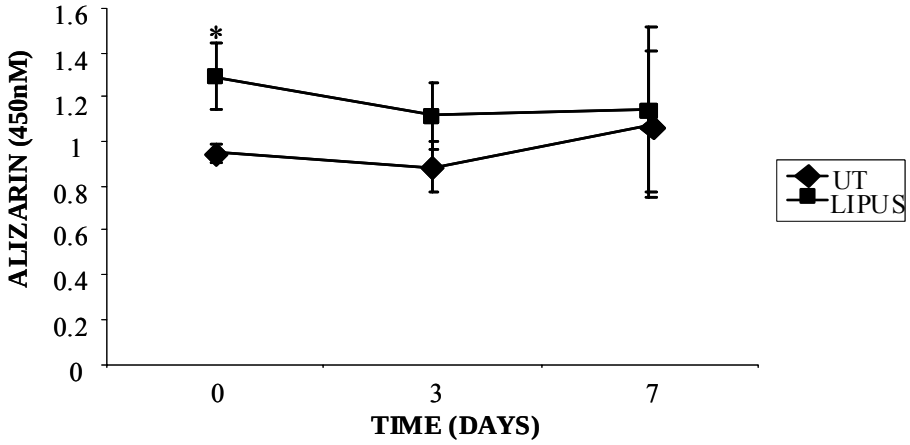


Figure 3.3: The effect of LIPUS on NHBC mineralisation. The NHBC were seeded at $1.5x10^4$ cells per tissue culture well and allowed to adhere. Media was then replaced with standard mineralising media and cells were grown for 21 days until they started to mineralise. Quadruplicate wells were then either untreated or treated with LIPUS in a CO_2 incubator at 37°C for 20 min each day and harvested at day 0, 3 or 7 of treatment. Cell layers were then stained with Alizarin red and extracted as described in the Methods section. Data shown is from pooled data of 3 donors ± SEM *P<0.01.

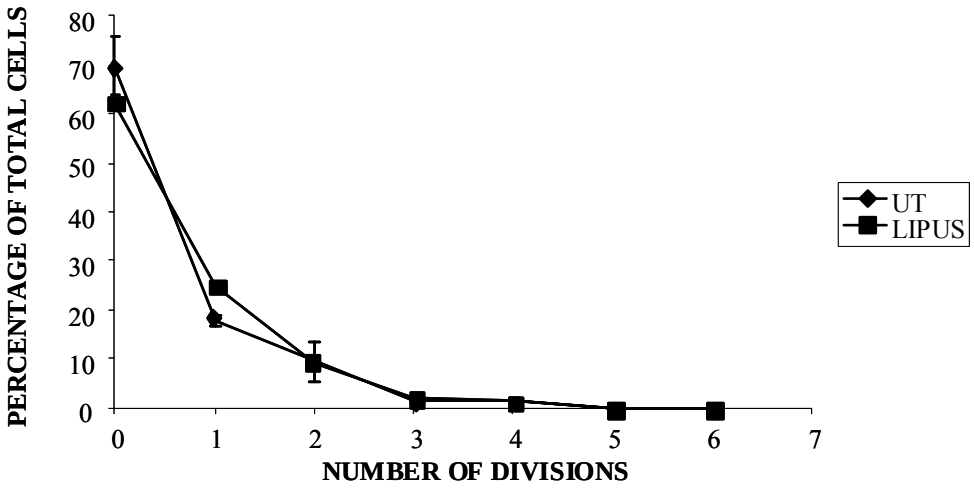


Figure 3.4: The effect of LIPUS on NHBC proliferation rate. The NHBC were labelled with CFSE and seeded at $2x10^5$ cells per T25 tissue culture flask and allowed to adhere. Media was then replaced with standard mineralising media and triplicate flasks were then either untreated or treated with LIPUS in a CO_2 incubator at 37°C for 20 min each day for 7 days with no prior mineralisation period. Proliferation rate was measured using flow cytometer and analysed using verity modfit software. Data shown are from cells from a representative donor ± SEM.

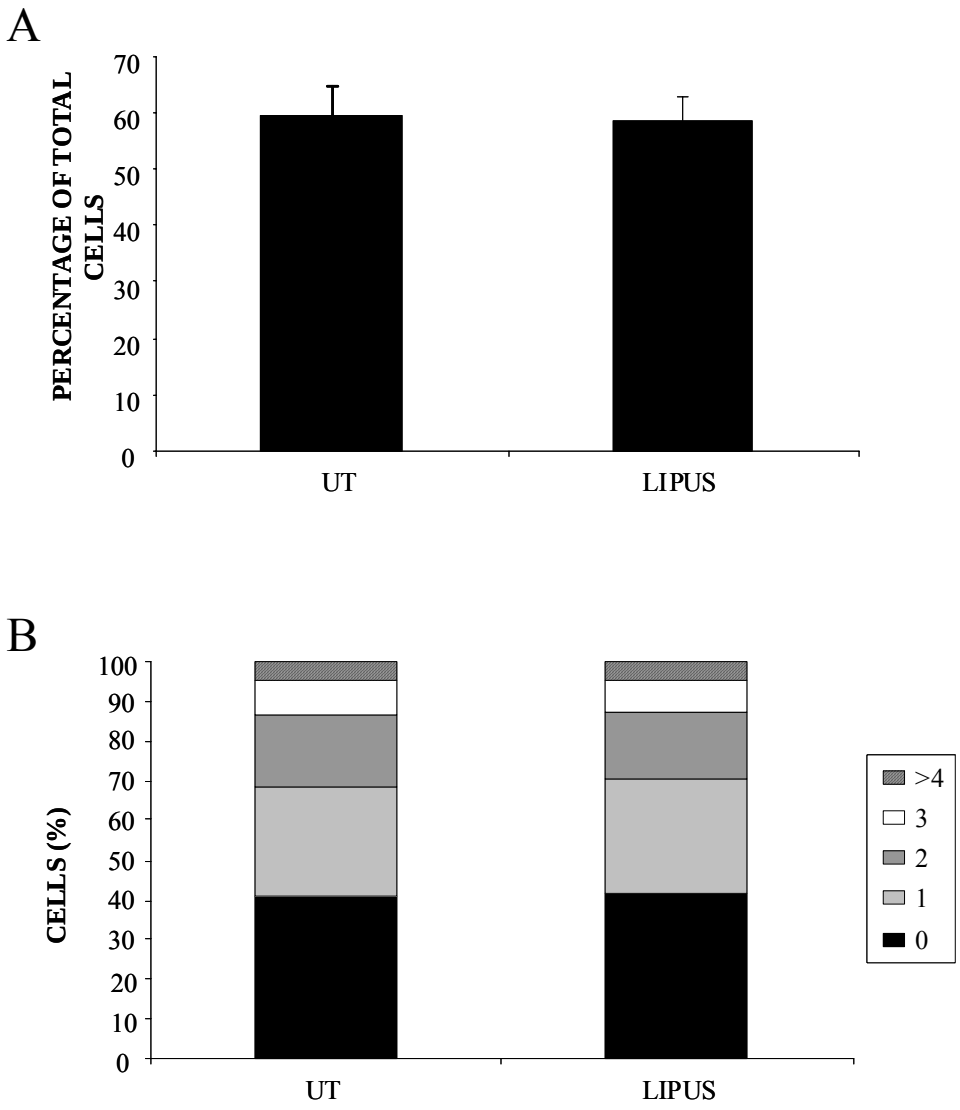


Figure 3.5: The effect of LIPUS on NHBC proliferation rate. The NHBC were labelled with CFSE and seeded at $2x10^5$ cells per T25 tissue culture flask and allowed to adhere. Media was then replaced with standard mineralising media and triplicate flasks were then either untreated or treated with LIPUS in a CO_2 incubator at 37°C for 20 min each day for 7 days prior to mineralisation. Proliferation rate was measure using flow cytometer and analysed using verity modfit software percentage of total cells divided for each treatment (**a**) and percentage of cells per generation (**b**). Data shown are pooled from cells of 5 donors' ± SEM.

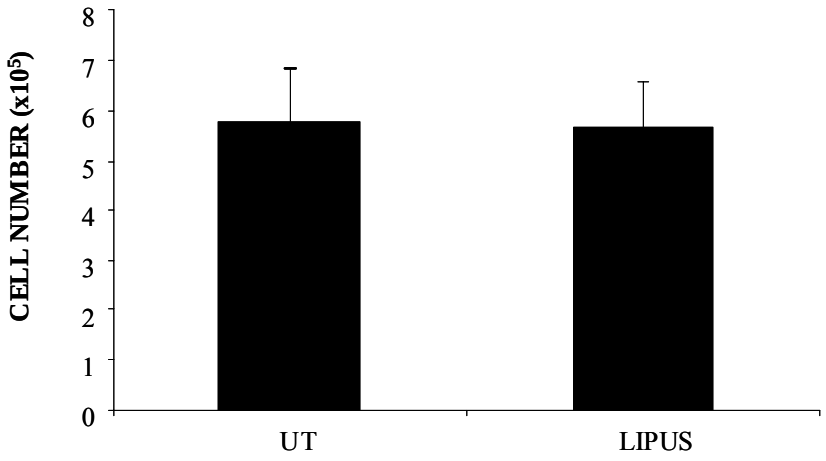


Figure 3.6: The effect of LIPUS on NHBC proliferation rate. The NHBC were seeded at $2x10^5$ cells per T25 tissue culture flask and allowed to adhere. Media was then replaced with standard mineralising media and triplicate flasks were then either untreated or treated with LIPUS in a CO_2 incubator at 37°C for 20 min each day for 7 days with no prior mineralisation period. Proliferation was measured using trypan blue exclusion to count viable cells at day 7 of LIPUS treatment. Data shown are from cells of a representative donor ± SEM.

3.3.3 Morphological Effects of LIPUS

Cell morphology was observed over time by phase contrast light microscopy. Cells were seeded under mineralising conditions and images taken at day 0 of seeding and day 7 of growth, then again at day 21 (**Figure 3.7 a & b**), 24 (**Figure 3.7 c & d**) and 28 (**Figure 3.7 e & f**). Cell layers showed no marked morphological differences between controls and LIPUS-treated cells (**Figure 3.7**). Early time points showed qualitatively consistent levels of proliferation and a fibroblastic morphology for all donors at day 0 (**a & b**) and 7 (**c & d**) (**Figure 3.8**). Mineralisation levels differed between donors' but gross morphology and the appearance of mineralisation was similar between untreated and LIPUS treated cultures.

3.3.4 Effect of LIPUS on gene expression

Gene expression in cells under mineralising conditions was investigated at day 7 of differentiation and again after the onset of mineralisation, at days 21, 24 and 28, using real-time RT-PCR. At the early time point of 7 days, cells from 3 out of 5 donors showed increases in collagen type 1 (Col-1) mRNA expression (**Figure 3.9 a)** and increases in that of BSP-1 (**Figure 3.9 b)** consistent with a maturation effect of LIPUS treatment. The negative effect on Col-1 and increase in BSP-1 was further supported by findings at the later time points where cells where first mineralised then treated for 7 days from day 21 of mineralisation (**Figure 3.10 a & b**). Additionally, increases in RUNX-2 (**Figure 3.11 a**) and E11 (**Figure 3.11 b**) mRNA expression pointed towards the transition of osteoblasts to a preOY-like phenotype with LIPUS treatment. This was further supported by the increase in E11 mRNA at later time points of day 24 and 28 post the onset of mineralisation (**Figure 3.12**).

Additionally, as previously observed in mouse cell lines, we saw an increase in RANKL mRNA expression (**Figure 3.13 a**) for some donors at the early stage of culture. However, only in cells from one donor was an increase in OPG mRNA levels observed. In fact, the majority of donors' cells showed a decrease in OPG mRNA expression at day 7 of treatment (**Figure 3.13 b**).

3.3.5 Effect of LIPUS on PGE_2 release.

OY have been shown to increase the synthesis of PGE_2 in response to mechanical loading of the bone. It has been shown that the release of PGE_2 into the extracellular space and increased expression of COX-2 by bone cells is induced by the movement of fluid through the canalicular network of bone (63). COX-2 is the inducible isoform of the COX family, the key enzymes involved in the conversion of PGE_2. COX-2 has been shown to be increased in human bone cells by the application of mechanical strain in the form of fluid flow (173). In this study, an increase in PGE_2 release was observed for two out of the five donors tested (**Figure 3.14 b**), and also an increase in the expression of COX-2 mRNA (**Figure 3.13 a**). These data are consistent with LIPUS having a mechanostimulatory effect in some instances.

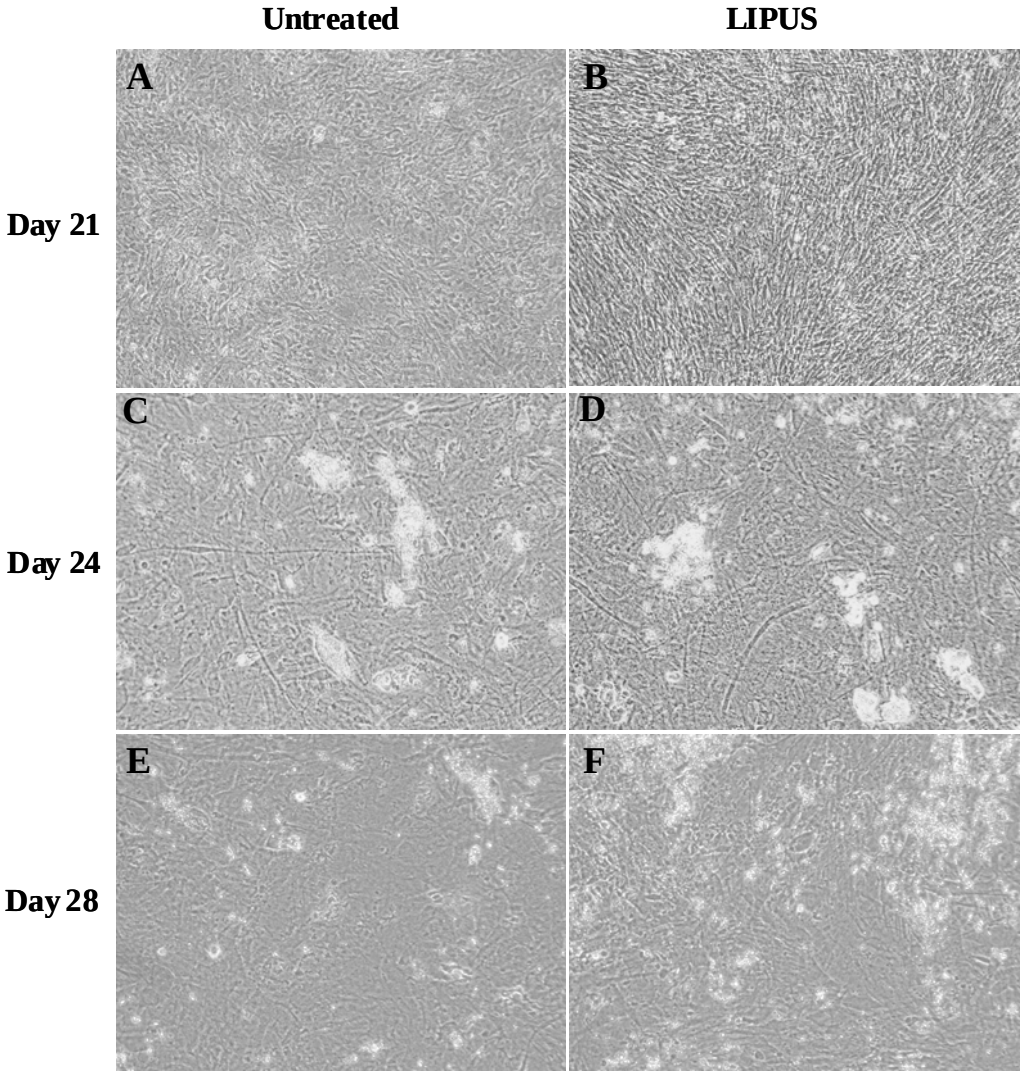


Figure 3.7: The effect of LIPUS on NHBC cell morphology. The NHBC were seeded at 1 x 10^5 cells per tissue culture well and allowed to adhere. Media were then replaced with standard mineralising media and cells were grown for 21 days until they started to mineralise. Then triplicate wells were either untreated or treated with LIPUS in a CO_2 incubator at 37°C for 20 min each day to day 21 (a, b) , 24 (c, d) and 28 (e, f) of culture. Morphological characteristics of NHBC were viewed by phase contrast microscopy at 10x magnification. Data shown are from cells of a representative donor.

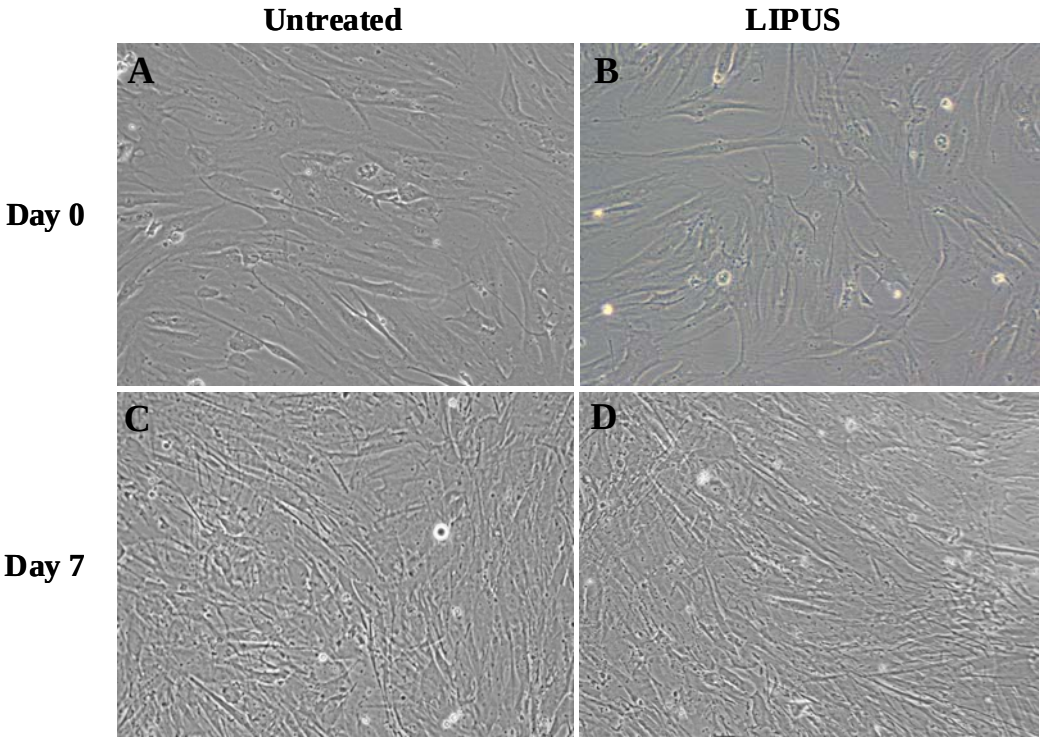


Figure 3.8: The effect of LIPUS on NHBC proliferation rate and morphological characteristics of NHBC viewed by phase contrast microscopy at 10x magnification. Photographic images of NHBC at day 0 (a and b) and day 7 (c and d) of cells grown with and without LIPUS treatment with no prior mineralisation period. Data shown are from cells of a representative donor.

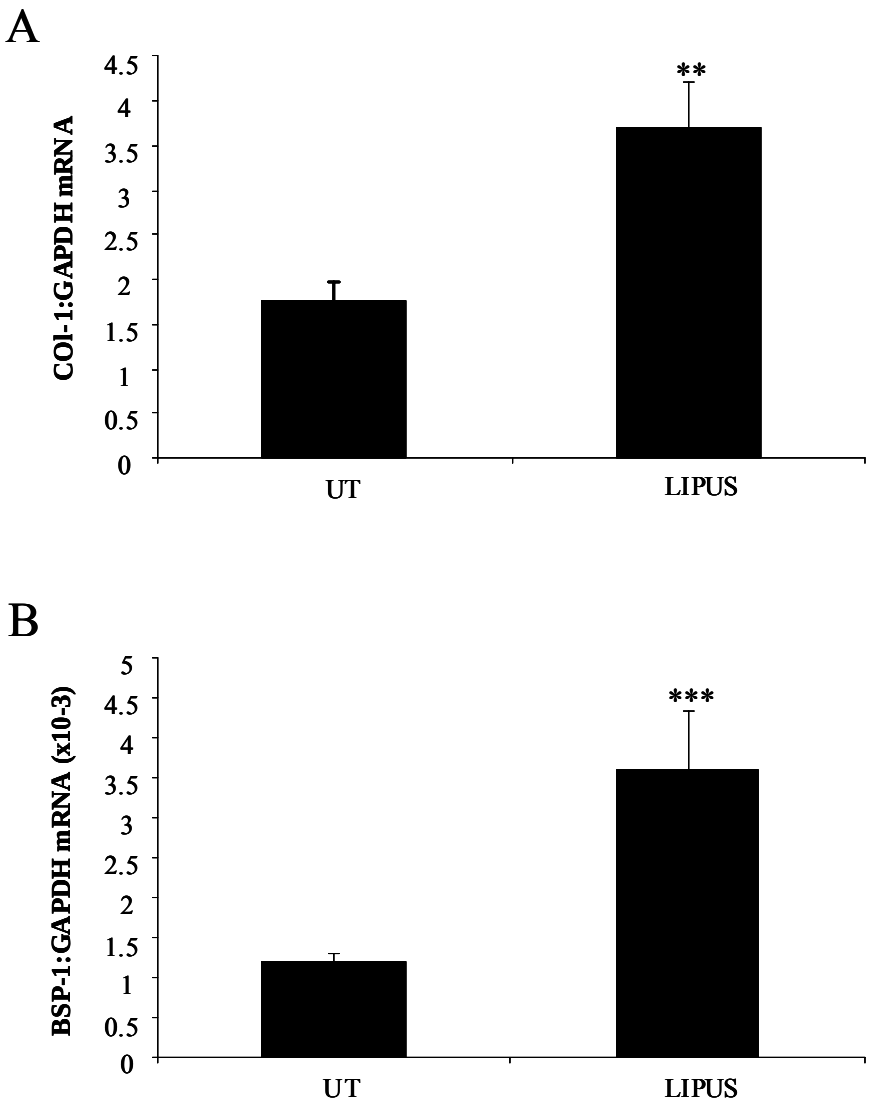


Figure 3.9: The effect of LIPUS on NHBC gene expression. The NHBC were seeded at $2x10^5$ cells per T25 tissue culture flask and allowed to adhere. Media were then replaced with standard mineralising media and triplicate flasks were then either untreated or treated with LIPUS in a CO_2 incubator at 37°C for 20 min each day for 7 days with no prior mineralisation period. RNA was collected from cells and RT PCR performed to amplify mRNA encoding for Col-1 (**a**) and BSP-1 (**b**). Data shown are from cells of a representative donor ± SEM, ** $P<0.01$ and *** $P<0.001$.

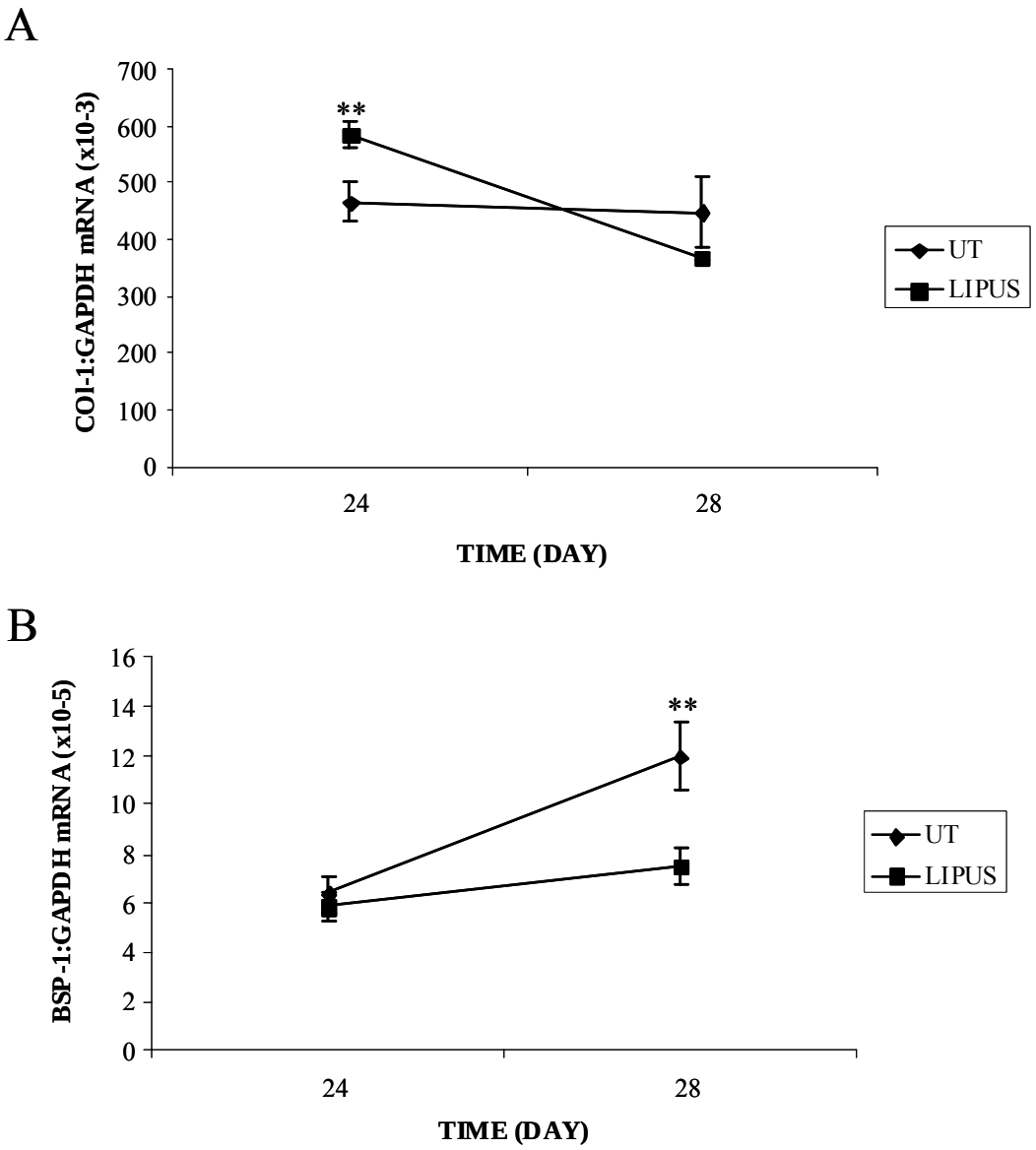


Figure 3.10: The effect of LIPUS on NHBC gene expression. The NHBC were seeded at 1 x 10^5 cells per tissue culture well and allowed to adhere. Media was then replaced with standard mineralising media and cells were grown for 21 days until they started to mineralise. Then triplicate wells were either untreated or treated with LIPUS in a CO_2 incubator at 37°C for 20 min each day for a further 3 and 7 days. RNA was collected from cells and RT PCR performed for Col-1 (a) and BSP-1 (b). Data shown are from cells of a representative donor ± SEM ** P<0.01**.**

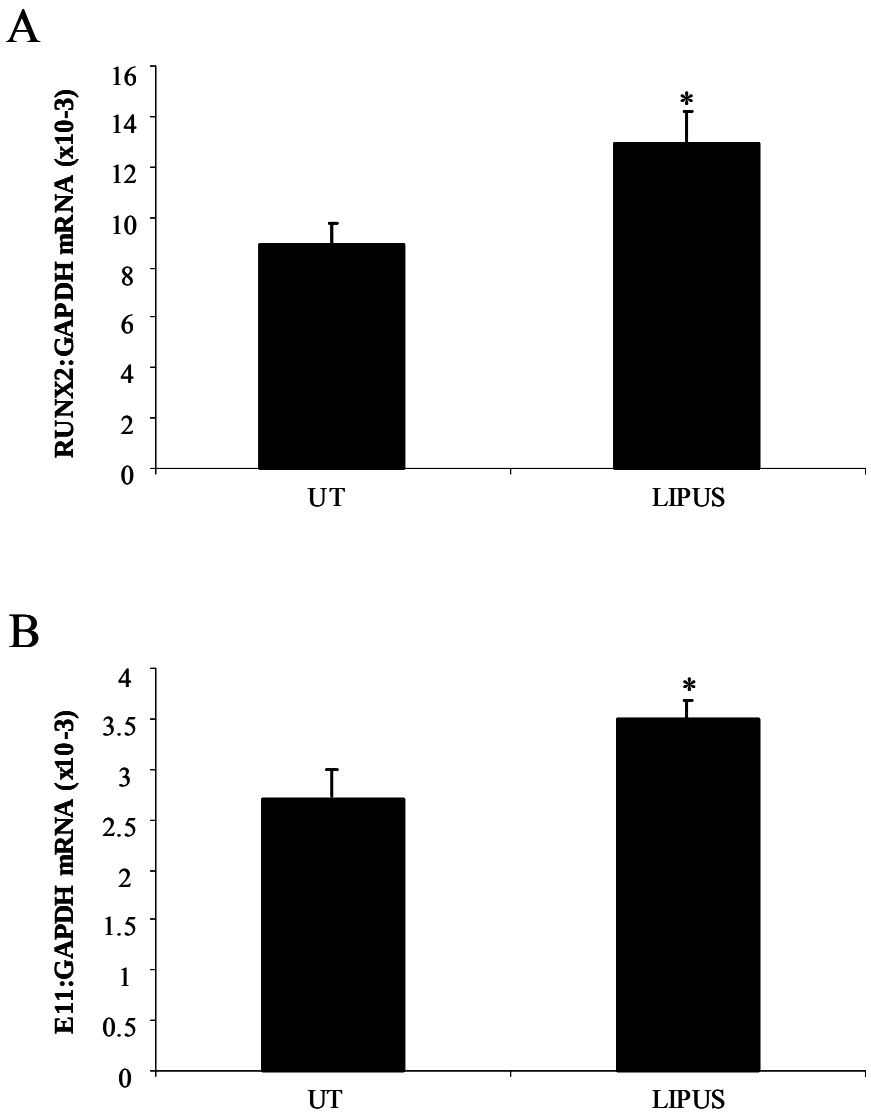


Figure 3.11: The effect of LIPUS on NHBC gene expression. The NHBC were seeded at $2x10^5$ cells per T25 tissue culture flask and allowed to adhere. Media were then replaced with standard mineralising media and triplicate flasks were then either untreated or treated with LIPUS in a CO_2 incubator at 37°C for 20 min each day for 7 days with no prior mineralisation period. RNA was collected from cells and RT PCR performed for RUNX2 (**a**) and E11 (**b**). Data shown are from cells of a representative donor ± SEM * $P<0.05$.

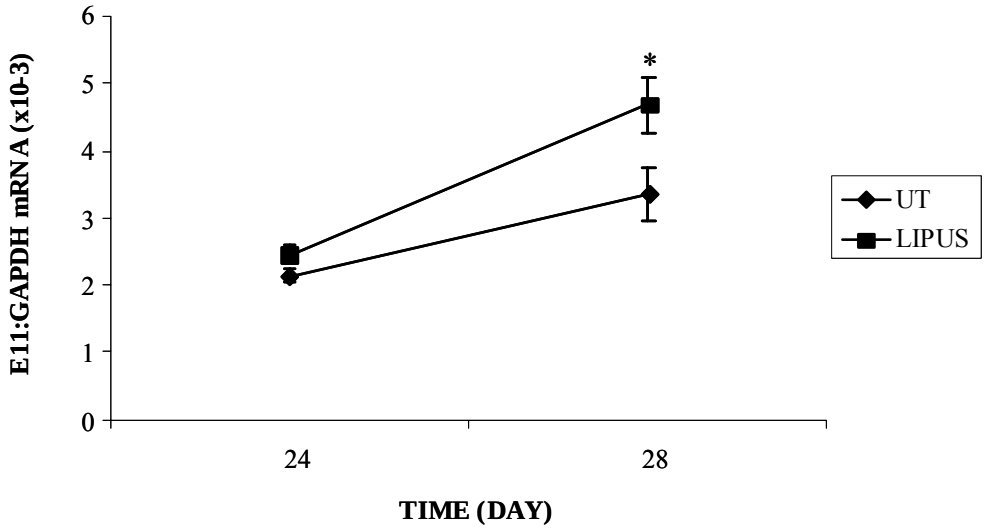


Figure 3.12: The effect of LIPUS on NHBC. The NHBC were seeded at 1 x 10^5 cells per tissue culture well and allowed to adhere. Media were then replaced with standard mineralising media and cells were grown for 21 days until they started to mineralise then triplicate wells were either untreated or treated with LIPUS in a CO_2 incubator at 37°C for 20 min each day for a further 3 and 7 days. RNA was collected from cells and RT PCR performed to amplify mRNA encoding E11. Data shown are from cells of a representative donor ± SEM *P<0.05.

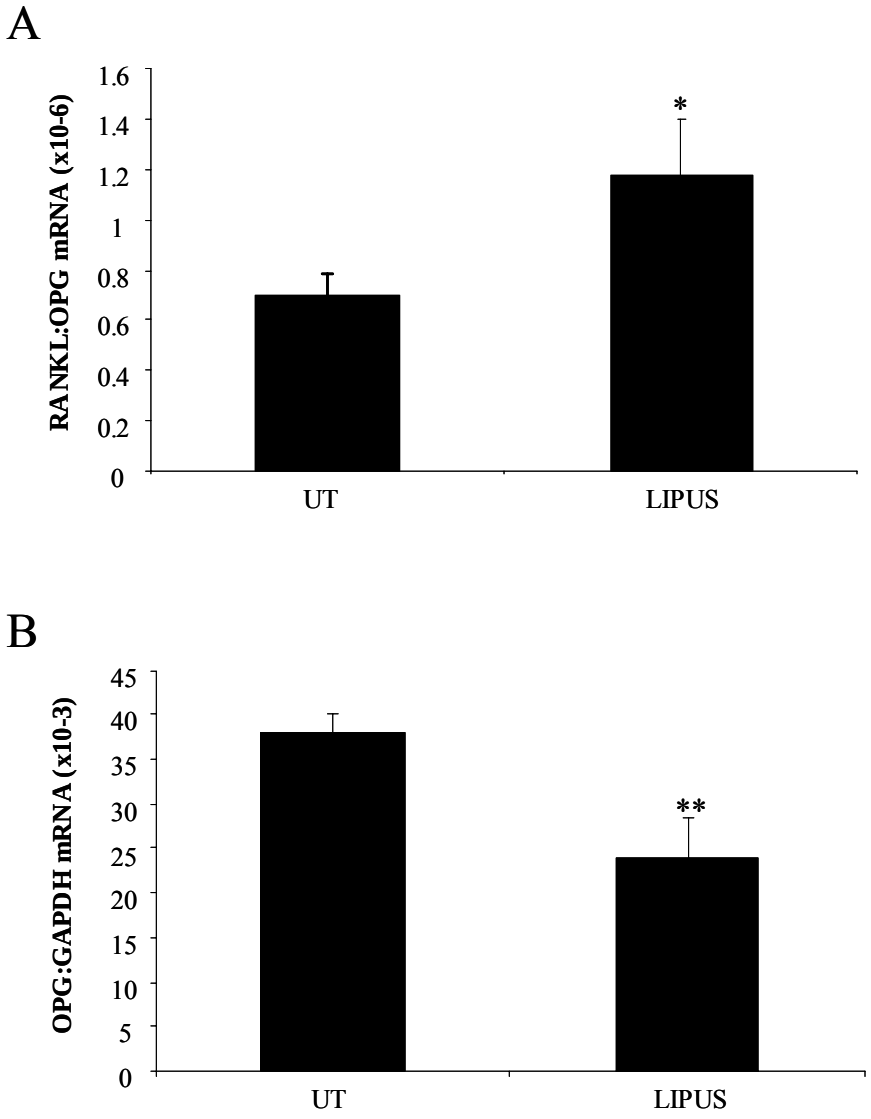


Figure 3.13: The effect of LIPUS on NHBC gene expression. The NHBC were seeded at $2x10^5$ cells per T25 tissue culture flask and allowed to adhere. Media were then replaced with standard mineralising media and triplicate flasks were then either untreated or treated with LIPUS in a CO_2 incubator at 37°C for 20 min each day for 7 days with no prior mineralisation period. RNA was collected from cells and RT PCR performed to amplify mRNA encoding RANKL (a) and OPG (b). Data shown are from cells of a representative donor ± SEM, * $P<0.05$ and ** $P<0.01$.

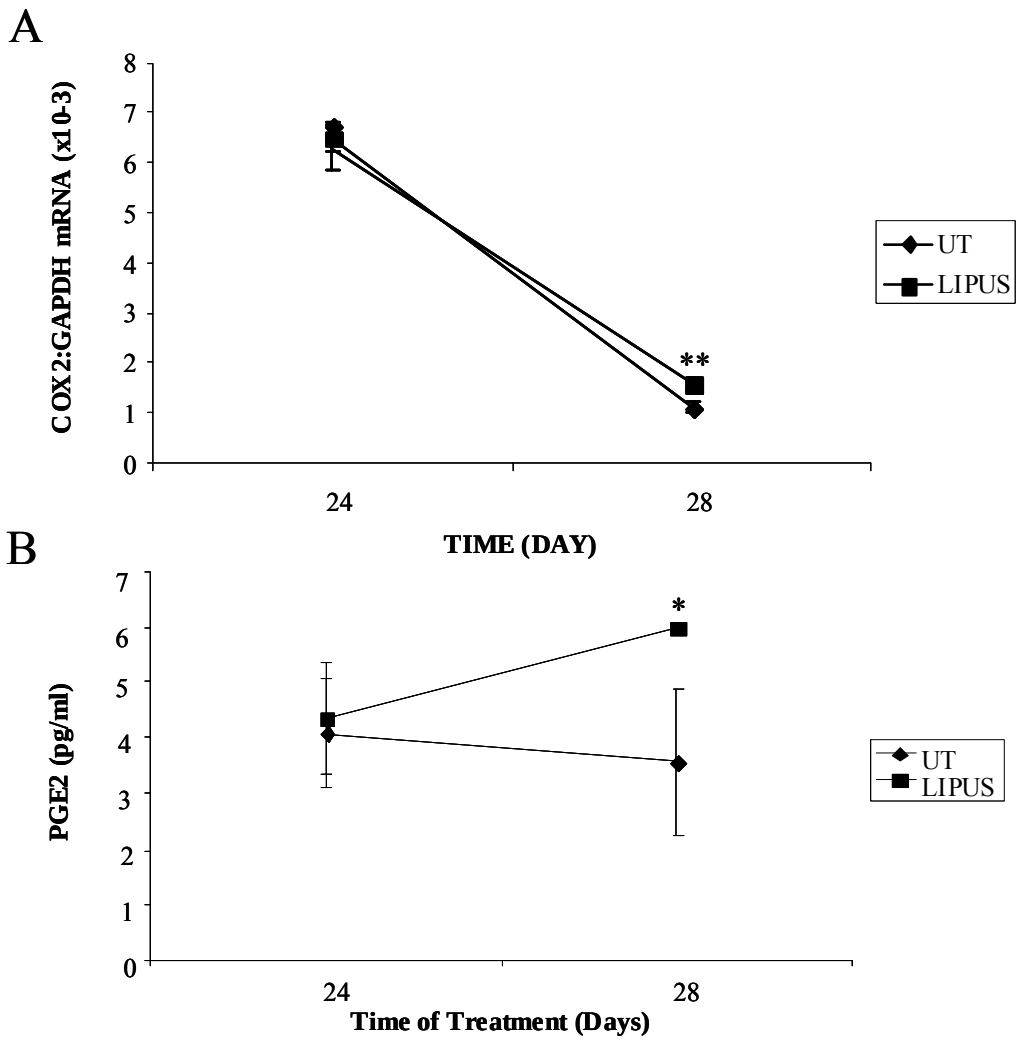


Figure 3.14: The effect of LIPUS on NHBC gene expression. The NHBC were seeded at 1 x 10^5 cells per tissue culture well and allowed to adhere. Media was then replaced with standard mineralising media and cells were grown for 21 days until they started to mineralise then triplicate wells were then either untreated or treated with LIPUS in a CO_2 incubator at 37°C for 20 min each day for a further 3 and 7 days. Supernatant and RNA were collected from cells at each time point. RT PCR performed to amplify mRNA encoding COX2 and an EIA assay for PGE2 was performed as described in the methods section. SEM, *P < 0.05 and ** P<0.01

3.4 Discussion

Given the current and potential clinical applications of LIPUS and the emerging central role of OY in bone homeostasis, investigation of the response to LIPUS of human osteoblasts and OY-like cells is important both for understanding its mechanism of action and for determining its potential application. In this study, the effect of LIPUS was investigated on both proliferating and mineralising cultures of NHBC.

An important aspect of bone anabolism is the extent of osteoblast proliferation. There are conflicting results where the effect of LIPUS on proliferation of cells is concerned, as previous studies have shown either no effect (126) or increases in various cell types (133, 158, 174). A study by Hasegawa *et al*., using human cells derived from fracture haematoma sites, showed no increase in proliferation of cells by LIPUS treatment but increases in their differentiation (158). Other studies using human mesenchymal stem cells, rat chondrocytes and cells collected from rat fracture sites showed an increase in differentiation with LIPUS treatment (9-12). The advantage of the CFSE technique is that it enables identification of doublings of a minor percentage of cells within a larger population and can therefore detect subtle effects. Using this sensitive technique we observed only a mild proliferative effect of LIPUS in some of the donors' NHBC preparations tested, albeit not significant, but this is however consistent with a pro-anabolic response.

Studies in both human derived cells and rodent cell lines have demonstrated an increase in mineralised surface with LIPUS treatment (126, 130, 132). In this study, NHBC grown under conditions permissive for mineralisation and then treated with LIPUS increased their rate of mineral apposition in some cases. The rate of mineral apposition varied between donors and probably reflected their differentiation state. Cells from three out of five donors showed increased BSP-1 mRNA expression in cultures treated with LIPUS, consistent with onset of

mineralisation and differentiation of an osteoblast to an OY-like phenotype (126). Cells from three out of five donors showed a subtle increase in Col-1 at early time points with a subsequent decrease with the onset of mineralisation with LIPUS treatment. The lack of obvious differences in Col-1 expression may be explained by findings that LIPUS affects collagen matrix cross-linking in MC3T3-E1 cells but not necessarily the overall expression of Col-1 (127). A decrease in Col-1 expression is indicative of osteoblast differentiation and propagation of mineralisation.

A number of genes regulate the differentiation of osteoblasts to mature OY and a number of these have been shown previously to be activated by LIPUS treatment (133, 139). The trend of Alkaline phosphatase (ALP) levels being elevated at early time points in this study (data not shown) suggests that LIPUS may affect the early stages of osteoblast differentiation, as indicated by other studies (126, 158, 165). E11 (podoplanin), a marker of OB differentiation to the pre- or osteoid-OY, is involved in the initiation of formation of the dendrite-like processes (28, 31). LIPUS induced a significant increase in E11 mRNA expression in NHBC at both early stages of growth and in late cultures with the onset of mineralisation. As mineralisation is initiated by cell and cell process associated matrix vesicles (calcospherulites) the formation of cell processes is a crucial part of differentiation and maturation of OY (7). We have previously shown that the pro-mineralisation effect of vitamin K was due in part to increased cell process-associated mineralised vesicles in NHBC grown in 3D cultures (3).

The formation or removal of bone is tightly regulated with the balance between RANKL and its antagonist OPG regulating this process (175). These molecules are able to either stimulate or block, respectively, the action of osteoclasts in the process of bone remodelling. Borsje *et al*. reported an increase in RANKL and OPG expression 4 hours after LIPUS treatment and this was supported by findings in both MC3T3-E1 cells and primary mouse osteoblasts (134, 141, 142). In the experiments reported here, a subtle donor-dependent increase in RANKL

mRNA expression was seen, which was accompanied by either no effect or a decrease in OPG mRNA expression after 7 days of LIPUS treatment. Only cells from one donor showed a significant increase in OPG expression at this early time point. This may point to a role for LIPUS in early cultures in the regulation of osteoblast and OY control of bone resorption. The regulation of RANKL but not of OPG identified here is reminiscent of findings in similar cells subjected to another form of mechanical stimulation, stretching, where RANKL expression increased but there was no effect on OPG expression levels (176).

OY form gap junctions of connexion 43/45 allowing cell-cell communication and the propagation of signals through the interconnected network to the bone surface (25, 166). A recent study by Sena *et al*. has demonstrated the stimulation of gap junction regulated cellular communication by LIPUS in rat bone marrow stromal cells (137). PGE_2 is released in response to mechanical stimulus and the expression of PGE_2 and COX-2 are increased in response to LIPUS in MC3T3-E1 cells (138, 139). Interestingly, in rodent models where the COX-2 gene was deleted, the induction of RANKL was shown to be COX-2-dependent (140). The reduction in cell proliferation and increase in differentiation of MC3T3-E1 *via* soluble mediators such as PGE_2 in conditioned media harvested from LIPUS treated OY-like MLOY4 supports a role for OY in the response to LIPUS stimulation (177). In the current study, an increase in PGE_2 secretion was observed in two out of the five donors in later cultures, with a weak trend to increased COX-2 mRNA expression. This general lack of response, in conjunction with donor dependency, suggests that the major affects of LIPUS is not on mature OY in this model system. The lack of effect on the COX-2/PGE_2 pathway could be as a result of the rather stiff nature of the tissue culture plastic used, since LIPUS has been shown to be effective in inducing mechanical signals during fracture healing but not in intact rat bone, which has reduced flexibility (178).

In summary, the overall changes seen in this study were consistent with LIPUS having a mild proliferative and differentiative effect on NHBC. The increased expression of E11, a marker of differentiation, increased proliferation and mineralisation combine to point to LIPUS as a potential pro-anabolic agent in bone. These data are consistent with LIPUS promoting transition of osteoblasts to pre-OY. The current study was carried out on cells in a *in vitro* culture systems and are therefore by their very nature in isolation from the myriad of cell types that are also involved in regulating bone metabolism *in vivo*. Hence, though these results are significant in furthering our understanding of the effect of LIPUS on OY mediated regulation of the bone microenvironment LIPUS's effect on blood flow and of cells actively involved in fracture healing must not be overlooked. It is unlikely therefore that the effect of LIPUS is confined to one particular cell type but is rather a multi-system affecter and therefore the effect on OY may be only part of the story.

CHAPTER 4: General Discussion and Future Directions

4.1 General Discussion

Over the last decade, much interest has been focussed on the multifunctional OY. We and others have demonstrated the importance of this OY-like cells in the regulation of both pro-anabolic and pro-catabolic pathways in bone (76). The insight gleaned into the OY's role in maintaining a healthy and fully functional bone microenvironment is crucial, not only in the design but also the use of a variety of therapeutic agents. In the two studies described in this thesis, OY-like cells are shown to be involved in pro-anabolic responses. Additionally, OYs have a role in catabolic responses, as very recently reviewed (124). The density of OY within the bone (up to 90% of bone cells) as well as their combined vast surface area (>1200 m^2 in the average human skeleton) makes them a potential major player in bone health (179) and important targets in regard to the development of drugs and therapies. Both LIPUS and calcium supplementation are easily delivered and extremely economical current therapies. The use of calcium supplementation and LIPUS treatment are currently focussed on osteoporosis prevention or treatment of complications associated with osteoporosis. Therefore the increasing prevalence of osteoporosis in society and the associated economic burden made them ideal subjects for our studies especially in relation to the OY.

Though calcium has been accepted for many years as an appropriate supplement for the treatment of osteoporosis in both men and post-menopausal women, there is now some contention over its potential contribution to increasing the risk of cardiovascular adverse events. Current studies have indicated that there is a potential correlation of calcium and vitamin D supplementation with an increased risk of myocardial infarction and stroke though no subsequent increase in mortality was observed (180, 181). Other studies have found no

relationship between calcium supplementation and increased cardiovascular risk (182). However, it is clear that further studies need to be conducted to fully elucidate the potential role of calcium in cardiovascular events. Interestingly, increased risk of cardiovascular events with calcium supplementation may not be dose-dependent but rather related to abrupt increases in plasma calcium concentration, rather than total calcium load ingested (180). Therefore investigation into the influence of the manner in which calcium is administered may avert the potential cardiovascular risk.

With the aging population comes the increased prevalence of fragility fracture, due in most cases to osteoporosis (151). This increased fracture risk has been demonstrated to be inextricably linked to the viability of OY (64). Calcium, in combination with vitamin D3, has been shown to be an effective treatment for osteoporosis (183). Additionally, studies including our own have demonstrated the tolerance of NHBC to high levels of extracellular calcium (184). The novelty of our study lies in the investigation of the OY's role in terms of gene expression and functional characterisation of their role in mineralisation in our culture system under conditions permissive to mineralisation. Though other studies have shown that calcium initiates the differentiation of osteoblast, they have stopped short of looking at the regulation of osteocytic genes and features (185). Our studies have clearly demonstrated a consistent up-regulation of genes associated not only with the regulation of mineralisation but additionally genes associated with differentiation of osteoblasts to an OY-like phenotype. We demonstrated in previous studies that many pro-anabolic/pro-osteogenic factors promote osteoblast transition, such as strontium ranelate, analogues of vitamins K and D, and 3-dimensional (3D) conformation (2, 4-6). The support of osteoblast to OY transition is of major importance in the preservation of a viable population of OY to maintain bone integrity and decrease fracture risk (64, 66). The regulation of osteoclast activity by OY is crucial for a reducing fracture risk and, potentially, executing and controlling fracture repair (65, 66). Two

very recent studies where RANKL was conditionally deleted in OY under control of the *Dm-1* promoter resulted in an osteoporotic phenotype, demonstrating the critical role of OY in controlling osteoclastic resorption in the mature skeleton (186, 187). Our findings indicating potential regulation of osteoclastic function by the OY-like cells within our mineralising cultures with the addition of increasing concentrations of extracellular calcium supports the role of OY in regulation of both pro-catabolic and pro-anabolic actions on bone.

A crucial process for the generation of OY from an immature cell is the proliferation of osteoblasts. As only 10% of the osteoblast population go on to become OY it is important that a large pool of osteoblasts is propagated (14). These studies of the action of extracellular calcium showed a subtle increase in cell proliferation. Our findings are consistent with findings by a number of other groups in both primary human osteoblasts and cell lines (184, 185). Additionally, not only extracellular calcium supplementation but also LIPUS treatment has been shown to potentially influence osteoblast differentiation (165).

The focus of the majority of LIPUS studies has been on investigating the treatment's efficacy on non-union fracture healing (129, 158, 165). No studies have concentrated directly on the mechanical stimulus properties of LIPUS in terms of osteoblast to OY transition. As the presence of functional OY is a critical determining factor of fracture risk, the insights of this study are of potential importance in illuminating the role of OY (64). The maturation of osteoblasts in response to LIPUS stimulation *in vivo* and *in vitro* has been well documented though there is some debate as to whether there is a significant effect when it is used on intact bone (8, 130, 141, 142). In osteoporosis, a significant loss of functional OY is related to age, sex, nutritional state, hormone levels and genetic factors (188-190). Any treatment that can increase OY number or survival is therefore potentially important in the treatment of this complex disease (191).

The role of OY in sensing mechanical strain has undergone intense investigation over the last decade. Bonewald *et al*. have elegantly demonstrated the response of OY to mechanical stimulus using the cell line MLO-Y4 (54, 69). In a mouse model where OY were ablated, not only was an osteoporosis-like condition induced, but the bone response to mechanical loading was lost (1). The induction of PGE_2 release has been demonstrated to be crucial to the response of OY to mechanical stimuli (63).

Sclerostin (SOST/SCL) is not only a marker of the mature OY but also a crucial regulator of bone formation (192). The up-regulation of SCL by the addition of calcium maybe involved in increasing calcium incorporation into the matrix by extending the duration of the pre-OY stage of differentiation . We have published that SCL targets pre-OY/OY and acts as a master regulator of mineralisation, by up-regulating the expression of MEPE and down-regulating the expression of PHEX (51). MEPE on the other hand is a critical negative regulator of bone mineralisation, achieved by the post-translational production of ASARM peptides (193). Calcium dose-dependently down-regulated the expression of MEPE in contrast to the increased expression of the other OY markers. The resulting decrease in ASARM peptides apparently accounted for the ability of the cells to increase their level of mineralisation, since addition of MEPE-ASARM peptides completely reversed the pro-mineralisation effect of Ca^{2+}. These results are consistent with mineralising pre-OY controlling the degree of mineral deposition by modifying MEPE expression. In this case then, it appears that the increase in SCL expression is an indication of OY maturation and perhaps a counter-regulatory signal to the Ca-induced reduction in MEPE mRNA expression. Together, the findings from this study illustrate the complex nature of OY control of mineral handling (194). Studies have also indicated that OYs may be able to directly influence their immediate environment by osteocytic osteolysis (16, 67, 121, 124). Interestingly in LIPUS treatment, though no regulation of SOST/SCL was observed (data not shown), there was regulation of RANKL and

OPG gene expression which is supported by the findings of others (141, 142). Taken together with increased expression of E11 (a marker of pre-OY) and release of PGE_2 by some donors' cells in response to LIPUS, these data would seem to support a definite role for OY regulation of both the pro-catabolic and pro-anabolic responses in bone. Studies combining these two treatments should be further investigated and will be discussed in greater detail in future directions.

Current studies have indicated that there is an important role for PHEX, DMP1, and ASARM peptides in the regulation of FGF23 and modulation of energy metabolism in regards to the mineralisation process (50). As we have previously noted, DMP1 is also a marker of mature OY and we have demonstrated here its regulation by increased extracellular calcium (3). Additionally, we have shown the induction of MEPE mRNA expression. The correlation of decreased MEPE RNA expression with the onset of mineralisation in osteoblasts is supported by our calcium study. The increase in markers of maturation such as COL-1 in early cultures of osteoblast with LIPUS and extracellular calcium indicates the role of mineralisation in differentiation. Again the early up-regulation of genes such as BSP-1, which are involved in the nucleation of mineralisation associated with calcospherulites, reinforces the importance of these in the anabolic process of bone formation (29).

An area of this study that has opened up a plethora of questions is the role of OY in sensing calcium. This study clearly demonstrates that the NHBC, which our published studies indicate acquire an OY-like phenotype with extended culture, are able to respond to calcium at both a genetic and phenotypic level (2, 5, 6). However, how they are able to sense and respond to calcium is still not clear. My study looking at the expression of the CaSR in human bone chips harvested at joint replacement surgery demonstrated a loss of CaSR mRNA over a very short time in *ex vivo* culture, and NHBC derived from these bone chips never expressed CaSR mRNA. This could be due to a variety of factors such as the absence of hormonal signals such

as PTH and vitamin D_3 or disruption of the neural and/or vascular systems. As mentioned previously there is also some debate as to whether in fact osteoblasts do express the true CaSR but that they instead express an alternative ob.CaSR receptor (111). GPRC6A is a possible candidate which we have shown is expressed in NHBC explant cultures (110). The addition of GCP inhibitor NP2390 to mineralised cultures resulted in increased calcium apposition, further suggesting that an important aspect of calcium sensing by osteoblasts is to negatively regulate mineralisation, perhaps through the expression of genes such as *SOST* and regulation of the PHEX/MEPE axis. Initial studies have shown that NHBC are indeed able to respond to increased levels of extracellular calcium and that this is to a certain extent maybe energy dependent, though this area requires further investigation. However there is a possibility that once the process of mineralisation has been initiated, it may in part, passively continue when there is a high amount of extracellular calcium. In support of this, sodium azide, a metabolic poison, increased the degree of mineralisation (data not included here) showing that calcium apposition once instigated, is a passive process and needs to be actively controlled.

In summary, these studies are consistent with the OY-like cells being integral to the control of both pro-anabolic (i.e. regulation of bone mineralisation) and pro-catabolic (i.e. regulation of RANKL and OPG expression) processes within in bone, and that two potentially important regulators of this process are calcium and mechanical stimuli (in this study in the form of LIPUS). The complex interplay of MEPE, PHEX and DMP1 and FGF23 has been highlighted as an important pathway by which the OY-like cells regulate mineralisation. The results here also imply that a pro-anabolic response will also impact the pro-catabolic pathway(s), in particular by concomitant effects on the RANKL/OPG pathway. Based on my findings, further investigation into the mechanism by which osteoblasts respond to extracellular calcium is warranted.

4.2 Future Directions

Though calcium has been used as a therapeutic tool in the treatment and prevention of osteoporosis for a long time, the exact mechanism by which it acts has remained to be fully elucidated. We have determined a role for extracellular calcium in the propagation of mineralisation and consequently the enhancement of osteoblast transition to OY-like cells. The tolerance of high levels of calcium by NHBC OY-like cells was also established in this study.

The addition of extracellular calcium to the cultures in this study was either continuous or post-onset of mineralisation. This allowed us to look at the response of normal human bone cells at different states of differentiation. There is always a certain amount of donor-dependent variability when using NHBC, though in this study we saw a consistent overall response to extracellular calcium within the donors tested. Future studies to further this work would include investigation not only at different stages of osteoblast transition to OY but also pulsed doses of extracellular calcium mimicking isolated sporadic remodelling events such as would occur in the case of fracture healing. The collagen gel model, previously developed in our laboratory, for investigation of osteoblast differentiation to OY (3, 5) provides a unique opportunity to investigate cells with an osteoid OY phenotype. It would also be informative to investigate the effect of extracellular calcium on osteoclast-OY interaction, given the effects of calcium on the RANKL/OPG axis in the current study and the critical importance of RANKL expression by OY in the adult skeleton (187, 195).

Interestingly, we demonstrated the loss of CaSR in bone chips with time in culture and the presence of the GCP family member GPRC6A. This could be in part due to the loss of native conformation as well as extracellular influences such as PTH, and vitamin D3 from the vascular system. In normal physiology extracellular calcium would never be the only stimulus

acting upon the cells but would be in combination with a variety of other factors. These factors would include signals generated by the bone cells and/or factors arriving via the circulation. Factors arriving by the circulation would include PTH, sex steroids, 25D and 1,25D, though previous work in our lab has shown that 1,25D can be generated in the bone microenvironment (4). Therefore, future studies would also need to incorporate these additional factors into the bone chip assay to examine the influence on CaSR expression. Interestingly, studies have demonstrated that the expression of factors such as FGF23 (Atkins, Welldon, Ito, unpublished observations) and calcitonin receptor (148, 196) are also lost when cells are cultured outside of the native bone environment. Furthermore, cells cultured in 3-dimensions within collagen gels with extracellular calcium could be used to investigate the possible recovery of the expression of functional CaSR or alternatively the presence of alternative ob.CaSR receptors.

The mechanism, by which mineral incorporation is initiated, also requires further investigation. The mode by which mineralisation is propagated appears to have both a passive and as well as an active aspect. The mechanism that is set into motion by the stimulus of extracellular calcium may be dependent on the differentiation state of the cells upon which it is acting. Therefore early in transition this process is quite likely to be energy dependent and therefore possibly related to the cells' ability to sense extracellular calcium. However, after the onset of mineralisation the acquisition of mineral may to a certain extent may be a passive process once the crystalline seed is present. Again the relationship of osteoblast to OY transition is closely intertwined with this process and the further investigation of this will shed light upon this complex relationship.

As previously discussed, calcium is not acting in isolation, but in the presence of many other factors. The importance of the OY-like cell in the activation of pro-anabolic and pro-catabolic response in the bone microenvironment has been highlighted in this study exploring their

interaction with calcium. OY as well as being involved in the anabolic process of bone formation have also been proposed to have an important role in the catabolic process of bone resorption (195). The means by which they can contribute to either of these processes may be in part be related to their ability to sense load applied to bone. Utilising the mechanism by which OY act upon the bone microenvironment in response to mechanical load is proving to be a fertile area of research. One such mechanical stimulus that was encompassed in this study was LIPUS.

The orientation of cells in this study may have affected their ability to respond to LIPUS stimulation. *In situ*, OY are orientated along the plane of mechanical stimulation within the lacunae, with processes extending along the canalicular network (24). As fluid is forced through the network due to mechanical stimulus, it results in the movement of integrin attachments and focal adhesions, put down by OY and the subsequent deformation of the actin cytoskeleton (61). A suite of genes have been identified to be affected by LIPUS in the human cell line SAOS2. These include a number of members of the integrin family as well as cytoskeletal genes (18, 59, 61). In 2-dimensional tissue culture plates, the cells are unable to send out processes in all directions therefore limiting there ability to respond to mechanical stimuli. Future directions for this work would include the use of 3-dimensional culture described previously (5). The collagen gel culture allows the cells to assume a more OY-like morphology. Studies carried out using ceramic scaffolds have shown increases in stress-signalling mediators (pERK-1/2) and adhesion proteins (vinculin and α6β4 integrin) in osteoblast precursor cells during the early phases of cell attachment when treated with LIPUS (197). Ceramic blocks inserted into rabbit femurs also showed increased in-growth of osteoblasts and a greater amount of mineralised tissue *in vivo* (174). This is supportive of the further investigation of osteoblasts and OY under 3-dimensional conditions looking at proliferation, mineralisation and differentiation in response to LIPUS.

Another method to explore in future would include the use of LIPUS in *ex vivo* bone assays. This approach would solve the problems of cell orientation as well as enabling a study of a mixed population of cells to be investigated. In order to investigate the effect of LIPUS treatment under conditions of normal loading, the ZETOS system could be employed. In the ZETOS system, bone samples experience both fluid shear stress and mechanical loading (198).

Additional studies could look at the combined effect of extracellular calcium with LIPUS treatment. LIPUS may increase the apposition rate of extracellular calcium. The combination of therapies may have an additive effect to enhance the transition of osteoblast to OY as LIPUS and calcium are potentially acting *via* different pathways to initiate mineralisation and subsequent differentiation of cells. The effect of both of these factors on OY-like cells on survival and regulation of pro-anabolic and pro-catabolic processes would further illuminate how these cells contribute to a healthy bone microenvironment.

In summary, our findings are consistent with both LIPUS and calcium having an anabolic effect on human osteoblasts, thereby increasing the transition of osteoblasts to OY and therefore the prevalence of an OY-like phenotype. Further investigation of LIPUS' anabolic effects on OY would be greatly enhanced by the use of 3D models systems and co-cultures to better mimic their native environment. Additionally, the mechanisms, by which calcium exerts these effects, remain to be elucidated. However, calcium sensitivity appears to be independent of the canonical CaSR and instead may involve an alternative receptor, possibly the GPRC6A.

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