Function of cells producing type I collagen: potential influence of the vasculature

Sarah Elizabeth Brooks Taylor

A thesis submitted for the fulfilment of the degree of

Doctor of Philosophy

Department of Cell and Developmental Biology

University College London

2011

Declaration of Authorship

I, Sarah Taylor, declare that this thesis titled 'Function of cells producing type I collagen: potential influence of the vasculature' and the work presented in it are my own. I confirm that:

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

The interplay of fundamental extracellular factors involved in the control of cells producing type I collagen is still not fully understood. Bone formation by osteoblasts is known to be dependent on a good vascular supply and to be inhibited by hypoxia. This strongly suggests that oxygen carried by the blood acts as a key regulator of connective tissue cell function. To investigate further the role played by vascular oxygen supply in connective tissues, calvarial osteoblasts and dermal fibroblasts were grown under hypoxic (2% oxygen) and normoxic conditions (20% oxygen). Hypoxia caused striking decreases in growth and collagen formation by both cell types, along with increased expression of VEGF mRNA by fibroblasts. A novel assay using osteoblasts isolated from neonatal rat long bones was developed to examine more closely the bone formation process. These osteoblasts were characterised alongside autologous calvarial cells. *In vivo*, limb bones and flat bones form by distinct developmental processes; unexpectedly, osteoblasts from both sources behaved similarly in culture, with no clear differences in growth, gene expression or bone formation. As part of the characterisation of the long-bone-derived osteoblasts, the direct effects of PTH were examined. The mechanism for the anabolic response of bone *in vivo* to PTH treatment has yet to be fully elucidated. Unexpectedly, PTH exerted powerful inhibitory effects on cell growth, differentiation and bone formation. Moreover, mRNA expression of sclerostin, a key endogenous inhibitor of bone formation, was downregulated by PTH treatment. These findings indicate that the *in vivo* anabolic response to PTH involves additional factors that override its direct inhibitory action on osteoblasts. Collectively, the results presented in this thesis strengthen the notion that the production of collagen by connective tissue cells is dependent on oxygen and the vascular supply, and implicate the vasculature in mediating the effects of anabolic hormones on bone.

Acknowledgements

There are many who deserve my gratitude: friends, family and colleagues, who have supported me and enabled me to carry out my PhD. Beyond these, there are a few whom I would like to acknowledge explicitly.

During my PhD I have had the privilege to be a part of a dynamic research team, to carry out my research with freedom and responsibility, and to present my work at various international conferences. For this I express my gratitude to Tim Arnett. Tim has been an inspiring supervisor with a contagious enthusiasm about what he does. I have learnt to greatly appreciate his dedication and eye for detail, especially when it came to him proofreading my abstracts, papers and this thesis.

Many thanks also go to Isabel Orriss and Andrea Brandao-Burch for their guidance and valuable discussions; to Mark Turmaine for his help in the electron microscopy suite and Mary Rahman for sharing her molecular biology expertise.

Finally, I must acknowledge Michelle Key and Jessal Patel for making my time in the lab as fun as it was.

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Chapter 1

Introduction

The ability to adapt to a changing local environment is fundamental for the survival of mammalian cells. A good vascular network enables adaptation by feeding cells with an adequate oxygen supply and nutrients, providing factors required for growth, activity and sustainability, and removing the by-products of these processes.

Vascularisation of osseous tissue has been long implicated in the successful growth and regeneration of this tissue. The early observations of Albrecht von Haller in 1763 first suggested a relationship may exist between the vascular system and osteogenesis, implying that blood vessels are needed for bone formation. Since this initial hypothesis was proposed much work has followed, with details of the circulation in and around bone as we understand it today described in the last century (Trueta *et al.*, 1953; Brookes *et al.*, 1957). The current focus of research in this area is on the relationship between osteogenesis and angiogenesis and the coupling mechanisms that exist between these two processes.

This thesis is ultimately concerned with the role of the vasculature in the formation of collagenous connective tissues and the relationship between these tissues and the vascular supply in mature organs, particularly skin and bone. The main focus of this introduction will be on fibroblast and osteoblast differentiation, formation and production of extracellular matrix. The different mechanisms for the formation of bone will be outlined and some of the key factors in the relationship between osteogenesis and angiogenesis highlighted.

Mesenchymal stem cells

There are multiple different types of connective tissues within the mammalian body that derive from mesenchymal stem cells (MSCs). MSCs have the ability to self-multiply and are multipotent, thus can differentiate into a wide variety of different cell types including: adipocytes, osteoblasts, chondrocytes, myocytes and fibroblasts (Baksh *et al.*, 2004; Chamberlain *et al.*, 2007) and the tooth specific cells, odontoblasts and cementoblasts (Bluteau *et al.*, 2008).

The commitment of MSCs to a specific lineage is dependent upon the presence of specific transcription factors whose expression is tightly regulated in order to maintain strict control over the differentiation pathways. The key factors involved in chondrocyte, fibroblast, osteoblast, osteocyte and adipocyte differentiation from MSCs are outlined in Figure 1.1 (overleaf).

Fibroblasts

Fibroblasts are the principal cellular component of connective tissues, which function to establish, maintain and modify the extracellular matrix (Sorrell *et al.*, 2009). The differentiation of fibroblasts from MSCs has, until recently, been poorly understood. Earlier this year, it was reported that connective tissue growth factor (CTGF) was sufficient to cause differentiation from MSCs into fully functional fibroblasts capable of producing type I collagen. Further, these fibroblasts were terminally differentiated and as such, had lost the ability to differentiate into non-fibroblastic lineages (including osteoblasts, adipocytes and chondrocytes) (Lee *et al.*, 2010). CTGF is produced by human umbilical vein endothelial cells (Bradham *et al.*, 1991) and by skin fibroblasts after activation with TGF-β (Grotendorst, 1997); environments in which fibroblast differentiation would be required for tissue formation in the developing embryo and in wound healing.

Despite commitment to the fibroblastic lineage, terminally differentiated fibroblasts maintain a remarkable diversity; fibroblasts from different anatomical sites display individual transcriptional patterns (Chang *et al.*, 2002) and consequently distinct phenotypes result.
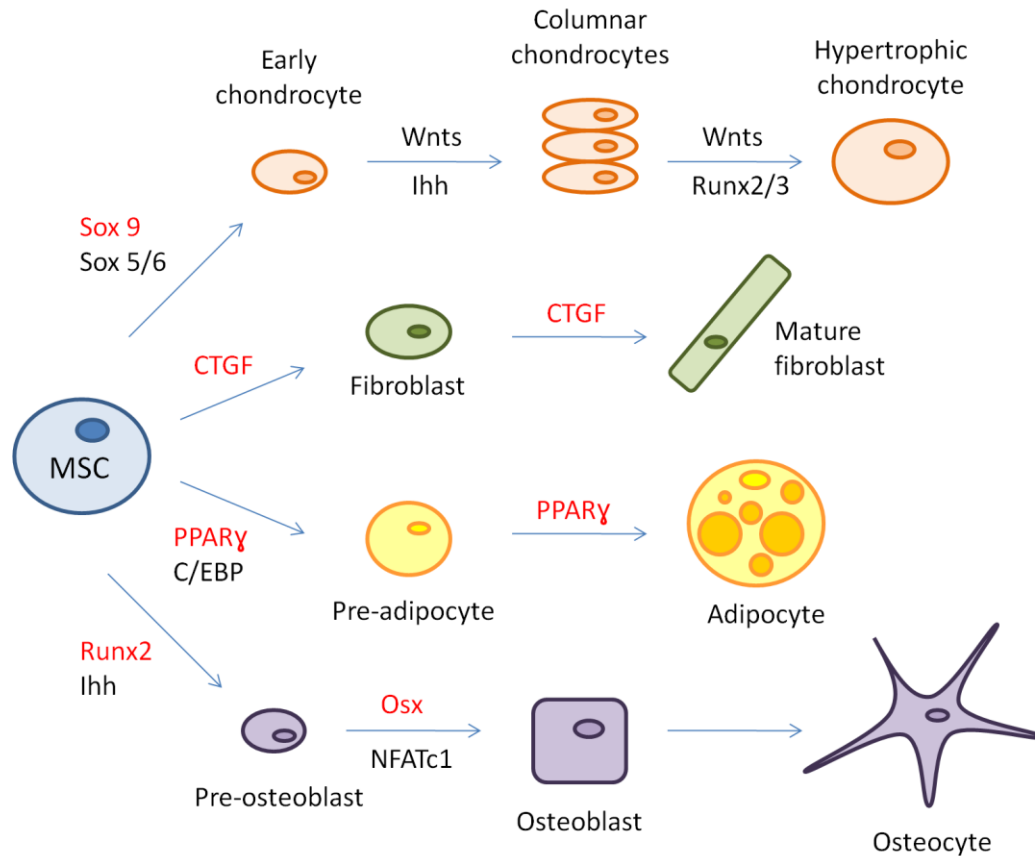
Figure 1.1. *Differentiation of mesenchymal stem cells into chondrocytes, fibroblasts, adipocytes and osteocytes*

Stages of cellular development are indicated beneath each cell type. Some of the key transcription factors involved in differentiation are indicated; 'master' regulators of commitment to specific lineages are shown in red. For details of abbreviations please see Appendix II. Adapted from (Caplan *et al.*, 2001; Takada *et al.*, 2009).

Chondrocytes

Chondrocytes are the cartilage producing cells. The synthesis of cartilage is required for endochondral bone formation and the formation of articular cartilage

that covers the ends of bones. Cartilage is composed of 70-80% water which allows it to act as a 'shock absorber', providing cushioning at joints. The organic component of cartilage is mostly type II collagen fibrils and aggrecan. Aggrecan combines with hyaluronic acid, forming large noncovalent structures which attract cations and water molecules. The resulting tissue is highly hydrated and thus is an ideal material for resisting compression. The majority of mature articular cartilage is poorly vascularised, consequently if damaged it has a striking inability to heal (Newman, 1998). However, capillary systems are present in the cartilage at the ends of newly forming long bones; vascularisation of the chondro-osseous junctions of growth plates is essential for bone formation (Gerber *et al.*, 1999).

Sox9, the major regulator of chondrogenic differentiation, initiates the differentiation of chondrocytes from MSCs (Bi *et al.*, 1999). Sox5 and 6 are also thought to play key roles in the differentiation process; investigations have suggested that mutations in either gene can result in severe skeletal malformations (Lefebvre *et al.*, 2001) indicating the importance of proper cartilage formation in skeletal development.

Osteoblasts

The formation of the mineralised extracellular matrix that comprises bone is the responsibility of the osteoblast. Early differentiation of osteoblasts from MSCs is governed by Runx2, the 'master switch' for osteoblast transcription (Schroeder *et al.*, 2005), initiating the differentiation of osteoblasts from MSCs and activating osterix. Osterix is the transcription factor required for osteoblast maturation (Nakashima *et al.*, 2003); its activation stimulates the expression of a wide range of bone matrix genes (Kurata *et al.*, 2007).

Mature osteoblasts are characterised by their high expression of alkaline phosphatase and their ability to secrete osteoid, the unmineralised bone matrix, and matrix vesicles. The osteoid secreted by osteoblasts consists of type I collagen, the major organic component of bone comprising ~90% of the total bone protein. Osteoid also contains osteocalcin (a bone-specific, calcium-binding protein) and chondroitin sulphate which is thought to function to control the water content of bones and is likely to be involved in regulating the formation of collagen fibres subsequent to mineralisation (Young *et al.*, 2006). Maintenance of the bone water content at an appropriate level is essential to the structural integrity and elasticity of bone and for mineral homeostasis, allowing ion exchange on bone surfaces that are relatively removed from the circulation (Timmins *et al.*, 1977).

Osteocytes

Osteocytes are the most abundant cellular component of mammalian bone, making up 95% of all bone cells (Franz-Odendaal *et al.*, 2006). Mature osteoblasts that become trapped in the mineralised matrix and cease forming bone are the terminally differentiated osteocytes. Osteocytes have long cytoplasmic extensions which cover a vast surface area allowing them to perform a multipurpose role in bone biology. These cellular projections are located in thin canaliculi in the mineralised matrix and allow osteocytes to form connections via gap-junctions with neighbouring cells and with the bone surface (Aarden *et al.*, 1994; Doty, 1981). These cytoplasmic extensions function for communication and nutrient exchange, in the early stages of the osteocyte development they are mainly directed towards the bone surface. As the cell becomes more engulfed in the mineralised matrix some of these processes are directed towards blood vessels to maintain cellular nutrition (Palumbo, 1986).

Early work suggested the role of osteocytes was to induce osteolysis (Belanger, 1969), but more recent evidence points towards the role they play being related to mineral homeostasis and strain detection (Cullinane, 2002; Lanyon, 1993), essential for the mechanical adaptation of bone.

Collagen

Collagen is an insoluble fibrous protein secreted by connective tissue cells. In humans there are at least 19 different gene products for collagen, each giving rise to a different type as a result of different amino acids being incorporated into the helical structure. The different collagen types are distinguished by their ability to associate into fibrils, form sheets or cross-link with other collagen molecules (Lodish *et al.*, 2000); it is the variation in size and orientation of collagen molecules that gives rise to the varied morphologies of connective tissues.

The structure of collagen

The basic structural unit of collagen consists of three amino acid chains (α chains) characterised by the repeating 'gly-X-Y' triplet, where X and Y can be any amino acid but X is often proline and Y is often hydroxyproline (Berg *et al.*, 1973). The characteristics of these amino acids are responsible for the polypeptide chains spontaneously forming left-handed alpha helices. The high prevalence of glycine is required in order for the chains to twist enough to form a helix. The small hydrogen side chain of the glycine residues are accommodated in the interior of the helix, whereas the larger ring structures of the proline side chain are found protruding from the helix; their presence allows sharp turns in the polypeptide chains, contributing to helical aggregation. The polypeptide

chain is subject to multiple post-translational modifications. Certain prolyl and lysyl residues are hydroxylated, the extent of which depends on the species, cell and tissue type and may be subject to change depending on developmental status, age or pathological conditions (Lehmann *et al.*, 1995; Kowits *et al.*, 1997).

Hydroxyproline is believed to be the key to the stability of the triple helical structure; it was originally hypothesised that the -OH group allowed increased hydrogen bonding and thus conferred stability. However, more recently it has been shown that the hydroxyl group confers stability through stereo-electronic effects and that its hydration provides little benefit in terms of structural stability (Kotch *et al.*, 2008). The hydroxylase enzymes require ferrous ions, ascorbic acid, α-ketoglutarate and molecular oxygen for activity (Hutton *et al.*, 1967). A lack of any one of these cofactors results in delays or reductions in the formation of the collagen molecule. This is clearly exemplified by the prevalence of scurvy when the diet is low in vitamin C. Lack of ascorbate results in reduced proline hydroxylation and consequently reduced collagen biosynthesis which leads to characteristically poor wound healing associated with the disease (Chojkier *et al.*, 1983). The dependence of the hydroxylase enzymes on molecular oxygen is of particular relevance to this thesis; reduced oxygen levels result in a diminished activity of the proline and lysyl hydroxylases and as a result aggregation of the triple helical structure is impaired (Towe, 1970). The role of oxygen in connective tissue formation is investigated in Chapter 3.

In a second stage of post-translational modifications some of the hydroxylysyl residues are glycosylated, the exact function of this is unclear but it has been suggested that they may play a role in fibrillar organisation (Brinckmann *et al.*, 1999) and contribute to collagen stability (Bann *et al.*, 2000b).

Disulphide bond formation between alternative C- and N-terminal regions of the propeptide sequences aligns the three chains, forcing the central portions

of the chains to associate and twist into the characteristic right handed triple helix of the procollagen molecule. The procollagen molecule is transported out of the cell, into the extracellular space where it is subject to cleavage at the N- and C-termini. It is the cleavage of the C-terminal propeptide that is the stimulus for the initiation of fibril formation (Kadler *et al.*, 1987); once cleaved the fully mature collagen molecule can form. Lysyl oxidases act to form reactive aldehydes which allow spontaneous cross-linking between individual fibrils and thus the formation of strong, stable collagen fibres.

Type I collagen is a fibrillar molecule that aggregates to form fibres found in skin, bone, dentin and tendon. It is the most abundant protein in the body and the main constituent of the vertebrate endoskeleton. The most usual molecular form of type I collagen is a heterotrimer of two α1 and one α2 chains wound around each other to form a right handed triple helix. In the full collagenous structure the fibrils in adjacent collagen molecules are displaced by approximately one quarter of their length, resulting in a staggered array that appears striated when viewed by transmission electron microscopy after heavy metal staining (Figure 1.2).

Mineralisation of the collagenous matrix

Following the deposition of collagen in the extracellular space, the mineralisation process begins. The mineral deposits in bone are composed of calcium and phosphate which are deposited as hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) crystals. Matrix vesicles are found in the extracellular fluid of the mineralising tissues and form by budding and then 'pinching off' from the outer cell membranes of osteoblasts, chondrocytes, odontoblasts and tenocytes (Anderson, 2003). They contain high levels of enzymes that can increase local concentrations of phosphate ions, including: alkaline phosphatase,

pyrophosphatase and ATPases (Ali *et al.*, 1970) and as such are capable of initiating the synthesis and deposition of the mineral component of skeletal tissues. In the growth plate of growing animals the mineralisation process begins within the matrix vesicles where mineral crystals begin to form, and is followed by their penetration of the vesicular membrane and exposure to the extracellular fluid. The conditions (i.e. pH, availability of calcium and phosphate ions) of the extracellular fluid govern further mineral crystal growth (Anderson, 1995). In mature bone the mineralisation process is somewhat different. Here, the staggered array of the collagen fibres forms grooves which provide the nucleation sites for calcium phosphate crystals (Mann, 1988).
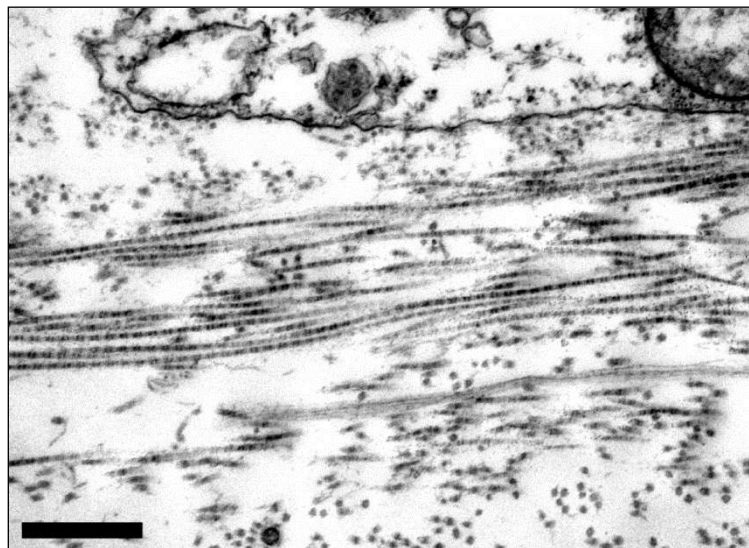
Figure 1.2. *Type I collagen fibres viewed by transmission electron microscopy.*

Collagen fibres formed by long bone osteoblasts after 28 days in culture in DMEM supplemented with 10nM dexamethasone and 50 µg/ml ascorbate. See Chapter 2 for details of transmission electron microscopy procedure.

Striated banding patterns can be clearly seen. Collagen fibres that have been cut in transverse section appear as dots. Scale bar = 500µm.

Mineralisation of the extracellular matrix is regulated by the availability of substrates required for the formation of mineral (i.e. calcium and phosphate ions) and is inhibited by the accumulation of other local factors. Pyrophosphate has long been known to be a physiochemical inhibitor of calcification (Fleisch *et al.*, 1962) and more recent *in vitro* studies have revealed that extracellular nucleotides (Orriss *et al.*, 2007) and acidic conditions (Brandao-Burch *et al.*, 2005) also prevent mineral from being laid down. These findings emphasise the importance of the vasculature in the removal of by-products of cellular functions in the formation and maintenance of bone.

It has been suggested that the mineralisation process is regulated at a local level by matrix gla protein (MGP). MGP is expressed by mature osteoblasts and chondrocytes and inhibits mineralisation of the extracellular matrix around these cells (Murshed *et al.*, 2004). Although MGP$^{-/-}$ mice are normal at birth, severe, lethal arterial calcification occurs within weeks (Luo *et al.*, 1997), suggesting that MGP is a key regulator in the mineralisation process. However, this does not appear to hold true in humans where a lack of functional MGP results in Keutal syndrome, where sufferers experience calcification of cartilage but not arteries (Schinke *et al.*, 2000), indicating that additional mineral-binding proteins must play a role in the mineralisation process.

Skin

The skin is the body's largest organ by weight and by covering the entire body surface provides a protective barrier against pathogen invasion. The skin has a rich vascular supply; a microcirculatory bed feeds the dermis, with capillaries penetrating into the epidermal layer (Yen *et al.*, 1976). In addition to its oxygen and nutrient providing roles, this highly dense capillary network functions to

control body temperature; vasodilation and constriction alter the blood flow through these vessels allowing heat loss from the body to be regulated (Charkoudian, 2003; Fortney *et al.*, 1985). Normally, the skin receives approximately 5-10% of cardiac output in temperate conditions but this can be increased to up to 50-70% under severe heat stress (Rowell, 1977). In very cold conditions blood flow to some body extremities including the finger tips, ears and nose, is almost entirely cut off in order to conserve heat. The skin is capable of withstanding this severe reduction in blood supply for relatively long periods of time but eventually, if not restored, frostbite ensues as a result of both freezing tissue and depleted oxygen supply resulting in severe local hypoxia (Gage *et al.*, 1970).

Bone

Bone is a highly specialised, mineralised connective tissue, the main function of which is structural, whereby the skeleton supports and protects vital organs and tissues and acts as a system of levers for muscles to work from, allowing movement. However, the skeleton is more than just a supportive structure; bone is a dynamic tissue with a high metabolic rate, tightly regulated by a variety of hormones and by mechanical stresses placed upon it. Bone is the predominant store of calcium and phosphate ions in the body. It contains about 99% and 80-90% of the body totals of calcium and phosphate ions respectively, providing a reservoir that is key to whole body chemical homeostasis (Copp *et al.*, 1963; Flynn, 2003). Haematologically, the skeleton is of importance too; haematopoietic stem cell (HSC) differentiation and maturation takes place in the red marrow in the flat bones and at the ends of long bones. The skeleton has also recently been implicated in endocrine regulation and thus energy metabolism (Lee *et al.*, 2007).

During development of the mammalian foetus bone can be formed in one of two ways.

Intramembranous ossification

The flat bones of the skull, the mandible, the sternum and the clavicle, are formed by the *de novo* synthesis of bone. MSCs in the mesenchyme condense and form vascularised layers of connective tissue. Upon stimulation by Runx2, MSCs within these condensed 'nodules' differentiate directly into osteoblasts and begin producing collagen. Mature osteoblasts secrete osteoid into the extracellular space which undergoes mineralisation, forming bone.

The exact vascularisation patterns of flat bones are still disputed but it is agreed that the blood supply is extensive. The blood supply differs between thin and thick parts of the bone; bone thinner than 0.4mm is fed by the periosteum, whereas thicker areas of bones develop distinct periosteal, cortical and bone marrow networks similar to that of long bones (Pannarale *et al.*, 1997).

Endochondral ossification

The majority of bones in the body including the long bones of the limbs, small bones with marrow cavities and the vertebrae, form via endochondral ossification. In this case, MSCs differentiate into chondroblasts forming a cartilagous model of the skeleton which is later replaced by mineralised tissue.

In the early development of endochondrally formed bones, high expression of Sox9 induces MSCs to differentiate into chondrocytes. These chondrocytes form a hyaline cartilage model of the limb bud which is surrounded by a dense layer of fibrous connective tissue, the perichondrium. Chondrocytes

within the perichondrium undergo hypertrophy which induces cellular production and secretion of vascular endothelial growth factor (VEGF), triggering the onset of angiogenesis and vascular invasion of the model of bone (Karsenty, 2001).

Runx2 stimulation causes terminal differentiation of MSCs into preosteoblasts which mature, begin producing type I collagen and secrete osteoid. Newly synthesised osteoid matures in the extracellular space for several days after which time mineral deposition begins. Continuous mineral accumulation on the newly forming bone surface results in the formation of a ring of bone around the cartilage model. The cavities within bone are generated when cartilage at the centre of the diaphysis (the middle section of the long bone) begins to disintegrate as chondrocytes undergo apoptosis. Disintegrating cartilage is replaced by trabecular bone. This centre of primary ossification is the point from which bone growth extends outwards to the ends of bones (where, in long bones secondary centres of ossification are established). Long bones continue to grow in this manner until their genetically predetermined length is reached and only a thin strip of cartilage remains at either end; this cartilage (the epiphyseal growth plate) persists until the bone reaches its full adult length and is then replaced with mineralised tissue.

The final stage in the bone formation sees the cessation of cartilage growth and fusion of the growth plate with the end of the bone. Further growth occurs at a much reduced rate and is seen appositionally, whereby bone thickness is increased by osteoblast activity on the surface of the bone and osteoclast activity inside the lumen, creating space for more marrow.

The fully mature bone consists of two main types of osseous tissue. The outer cortex of bone, the cortical bone, is mainly comprised of hydroxyapatite and is organised in osteons; concentric rings of tightly packed bone around a central canal (Haversian canal) which supplies the tissues blood flow and nerve

supply (Trias *et al.*, 1979). The spongy interior matrix (the trabecular or cancellous bone), contains the organic component which includes the collagenous fibres, bone cells and the majority of blood vessels that supply bone and is filled with marrow. As cortical bone contains most of the mineral in the skeleton it is much denser than trabecular bone, contributing significantly more to the total skeletal weight (Gong *et al.*, 1964).

Regulation of osteogenesis

Indian hedgehog/parathyroid hormone related protein signalling

Chondrocyte hypertrophy is controlled by indian hedgehog (Ihh) and parathyroid hormone related protein (PTHrP), a structurally similar but functionally different member of the parathyroid hormone family. Ihh promotes chondrocyte hypertrophy and as such is key to the endochondral bone formation process: Ihh-deficient mice have decreased chondrocyte proliferation and display severe abnormalities in long bone development (St-Jacques *et al.*, 1999). Conversely, PTHrP delays endochondral ossification. This is achieved by its direct inhibitory effects on chondrocyte hypertrophy (Chung *et al.*, 2001) and by inhibiting Runx2 (Guo *et al.*, 2006).

Bone morphogenic proteins and fibroblast growth factor

The TGFβ superfamily consists of multifunctional peptides that control proliferation, differentiation and other functions in many cell types. The bone morphogenic protein (BMP) family is a member of this superfamily and plays a key role in the induction of growth in both bone and cartilage (Massague *et al.*, 2000). The BMPs promote mesenchymal condensation and chondrocyte

differentiation by maintaining the expression of the Sox transcription factors (Yoon *et al.*, 2004). In addition to this, a positive feedback loop exists between BMPs and Ihh, and as such they work together to upregulate chondrocyte proliferation (Minina *et al.*, 2001). Specific BMPs have direct effects on osteoblasts, BMP2 has been reported to stimulate osteoblast precursor lineage commitment, increased alkaline phosphatase activity and osteocalcin production *in vitro* (Yamaguchi *et al.*, 1996).

Fibroblast growth factor (FGF) acts antagonistically to the BMPs, inhibiting chondrocyte proliferation and thus the balance of BMP and FGF signalling dictates the rate of chondrocyte development (Minina *et al.*, 2002).

Wnt signalling

The canonical Wnt signalling pathway plays a central role in controlling embryonic bone development and mass (Westendorf *et al.*, 2004; Chen *et al.*, 2009). Wnts are secreted glycoproteins that bind to receptor complexes composed of Lrp5/Lrp6 and the Frizzled protein. Lrp5 is present in bone on the surface of osteoblasts in endosteal and trabecular bone (Koay *et al.*, 2005) and is critical to proper bone formation; $Lrp5^{-/-}$ mice are viable and fertile but exhibit low bone mass which often results in multiple fragility fractures and can lead to severely shortened life spans (Kato *et al.*, 2002).

Canonical Wnt signalling is also implicated in joint formation (Hartmann et al., 2001) and cartilage development. It has been shown that β-catenin mediated wnt signalling is required for chondrocyte maturation, with misexpression of the Frizzled protein resulting in delayed chondrogenesis and consequently, long bone formation (Hartmann *et al.*, 2000).

Sclerostin

Naturally occurring inhibitors of Wnt signalling have dramatic effects on bone formation, as exemplified by the osteocyte secreted protein sclerostin (SOST). SOST is present in high concentrations throughout the canalicular system and acts to inhibit osteoblastic bone formation (Winkler *et al.*, 2003). SOST negatively regulates bone formation by binding to the Lrp5/Lrp6 coreceptor and antagonising the normal Wnt response (Semenov *et al.*, 2005; ten Dijke *et al.*, 2008).

SOST is almost exclusively expressed by osteocytes and has inhibitory effects on bone by preventing the differentiation of osteoblastic cells (van Bezooijen *et al.*, 2004) and promoting their apoptosis (Sutherland *et al.*, 2004), thus playing a key regulatory role in bone remodelling. Loss of the sclerostin protein results in sclerosteosis, a bone remodelling disorder that arises from progressive bone thickening and overgrowth but has little or no effect on resorption. Sufferers experience increased bone density at multiple sites, but specifically in the clavicles and the skull with noticeable effects seen in the calvaria and mandible (Sugiura *et al.*, 1975). Sclerosteosis is inherited as an autosomal recessive trait, which results from mutations arising in the van Buchem disease-gene region of chromosome 21 (Balemans *et al.*, 1999). Heterozygous carriers of the disorder are clinically normal, although some may show age-related radiographic evidence of calvarial thickening (Gardner *et al.*, 2005).

Osteogenesis and angiogenesis

Bone receives approximately 7-8% of cardiac output which is supplied to developing bones by the rich capillary network that surrounds bone in the periosteum. Without this highly vascular environment osteoblasts do not differentiate from MSCs and bone formation does not occur. Fully formed bones have a rich vascular supply, nutrient arteries enter the diaphysis of long bones though canals which traverse the cortical bone. After reaching the medullary cavity these arteries then divide, with arteriole branches feeding the epiphysis and metaphysis (Brookes, 1963; Wheeless, 2010). Earlier in life, before these arteries have fully developed the blood is supplied to bone through small vessels that penetrate the cortical bone connecting the marrow space and the periosteum (as seen in Figure 1.3). As bone ages medullary atherosclerosis results in a return to the reliance on periosteal blood supply. Consequently, medullary ischemia and reduced marrow arterial pressure result (Bridgeman *et al.*, 1996).

Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is thought to be the key pro-angiogenic stimulus in normal skeletal development and fracture repair. VEGF and its receptor are expressed by both osteoblasts and osteoclasts, indicating that bone formation, remodelling and angiogenesis are tightly coupled processes (Wang *et al.*, 2007; Towler, 2008; Street *et al.*, 2002; Tombran-Tink *et al.*, 2004).

VEGF induces capillary invasion of the perichondrium and plays a key role in skeletogenesis in both intramembranous and endochondral ossification *in vivo*. Using mice lacking some of the allelic variants of VEGF, Zelzer *et al.* (2002) have shown that both intramembranous and endochondral bone

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formation require VEGF with reductions in mineralisation observed in both bone formation processes in the knockout mice compared with the wild-types. However, they also found that the most notable differences displayed between the transgenic animals and the wild-types was in the vascularity of the long bones. Mice lacking fully functional VEGF displayed delayed recruitment of blood vessels into the perichondrium and delayed invasion of the primary ossification centre (Zelzer *et al.*, 2002).

1 mm
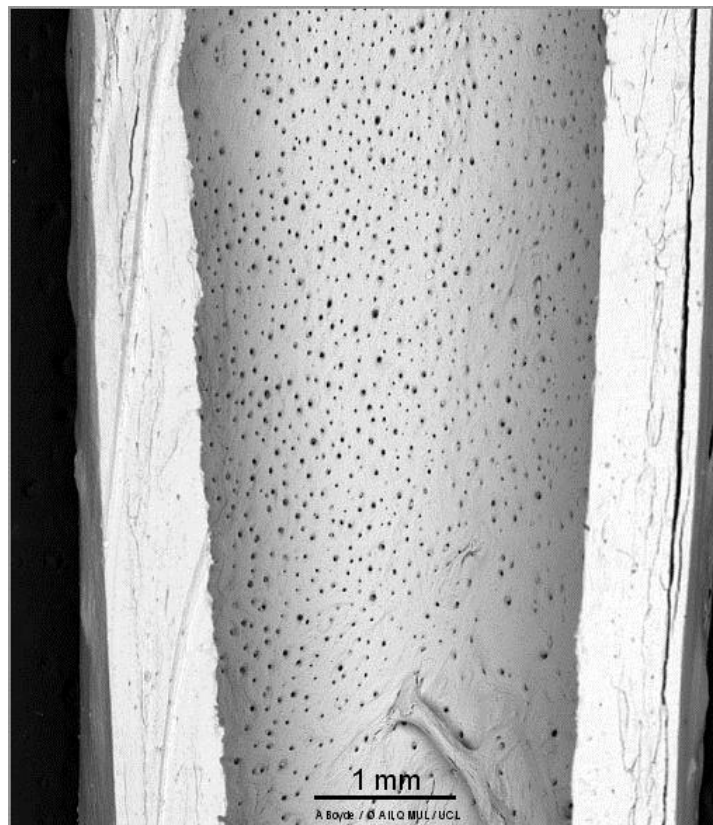
Figure 1.3. *Vascular channels in the shaft of a rat femur.*

Scanning electron microscope image of vascular channels penetrating the inner surface of cortical bone. Channels appear as pores within the bone.

Reproduced with the kind permission of Professor Alan Boyde, QMUL, London.

Bone remodelling

The synchronised activity of osteoblasts and osteoclasts allows bone to be modelled and remodelled, enabling the skeleton to fulfil its structural and homeostatic roles. Bone remodelling serves to repair micro-cracks, maintaining strength and structural integrity and providing a continual reservoir of calcium and phosphorus for mineral homeostasis which is continuously adapting to the systemic needs of the body. Under normal physiological conditions bone resorption and bone formation are balanced, allowing skeletal regeneration to take place. Trabecular bone has a much higher rate of turnover than cortical bone, with 26% and 3% respectively being replaced annually (Sandhu, 2003).

At discrete regions in the skeleton, termed the basic multicellular units (BMU), the remodelling process is initiated by stimulated osteoclasts binding to and resorbing the bone surface. The resorption phase is followed by proliferation and differentiation of MSCs into osteoblasts, which form new osseous tissue in the excavated pit. The formation of osteocytes completes the remodelling cycle and leaves the bone in a quiescent state (Figure 1.4).

Osteoclasts

Osteoclasts are usually quite large, multinucleate cells which contain a high number of mitochondria (Ch'uan, 1931). Functionally, osteoclasts act to resorb bone by excavating pits or trails known as Howship's lacunae. They associate tightly with the bone surface, forming a sealed zone, within which resorption takes place. The osteoclast membrane in contact with the bone surface, known as the ruffled border, is the site from which the osteoclast secretes the bone degrading components. Hydrogen ions are actively pumped across the membrane to dissolve the crystallised hydroxyapatite which makes up the mineral phase of the bone tissue. Osteoclasts actively synthesise lysosomal

enzymes that are capable of degrading the organic component of bone, the collagenous matrix. The cysteine-rich protease, cathepsin K, is secreted from the ruffled border and is collagenolytic (Troen, 2004). Tartrate resistant isoenzyme of acid phosphatase (TRAP 5b) is also secreted by actively resorbing osteoclasts and is thought to modulate osteoclast adhesion by dephosphorylation of osteopontin and bone sialoprotein (Ek-Rylander *et al.*, 1994)

Osteoclast formation is initiated by receptor activator of nuclear factor β (RANK) ligand and macrophage colony-stimulating factor (M-CSF), secreted by osteoblasts and MSCs, respectively. These factors cause monocytic cell fusion, resulting in osteoclast formation and subsequent maturation (Teitelbaum, 2000).

The RANKL / OPG system

Receptor activator of nuclear factor κB ligand (RANKL) and its decoy receptor osteoprotegrin (OPG) are cytokines of the tumour necrosis factor (TNFα) superfamily and play a key role in the regulation of bone turnover. RANKL is expressed on the surface of preosteoblastic cells and its receptor, RANK, on the surface of preosteoclastic cells; upon their interaction, the differentiation and activation of preosteoclasts are induced. RANKL also has a positive role when it binds to RANK expressed on mature osteoclasts, promoting cellular survival (Glantschnig *et al.*, 2003). OPG is a soluble protein also secreted by osteoblasts and is the natural decoy receptor for RANKL, preventing its interaction with the RANK receptor and potently inhibiting bone resorption *in vivo* (Hofbauer *et al.*, 2000; Khosla, 2001). Further evidence for the pro-osteoclastogenic nature of RANKL has been shown in mice with disrupted copies of the RANKL gene; these mice completely lack osteoclasts as a result of an inability of osteoblasts to support osteoclastogenesis. Consequently they experience severe

osteopetrosis and a defect in tooth eruption (Kong *et al.*, 1999). The ratio of RANKL : OPG is therefore directly responsible for controlling the maturation and activation of osteoclasts and thus bone resorption. The majority of factors affecting the resorption rate mediate their effects by causing an alteration in one or both of these cytokines.
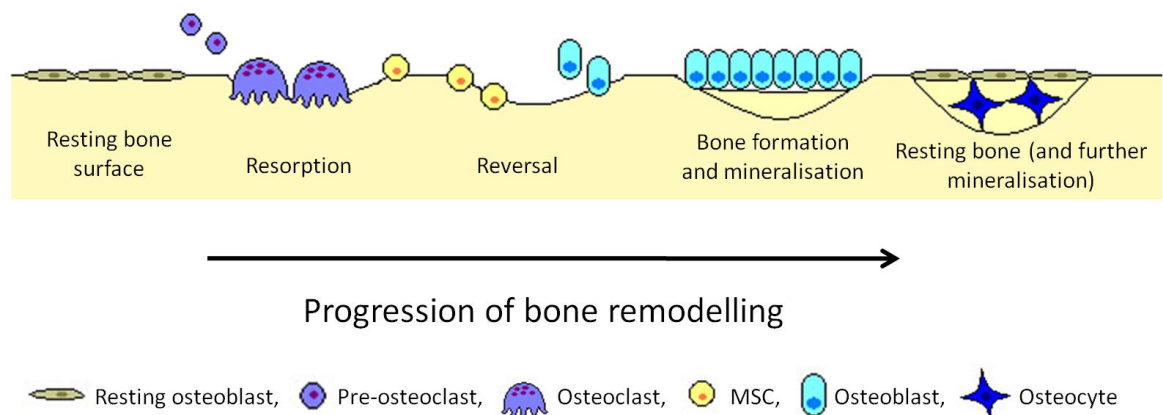
Figure 1.4. *The bone remodelling cycle.*

The arrival of pre-osteoclasts at the bone surface from the marrow initiates the bone remodelling cycle, their maturation and activation leads to resorption of the mineralised matrix. In the reversal phase, osteoblast precursors proliferate and differentiate into fully functional osteoblasts and deposit new bone matrix which is subsequently mineralised. Maturation of the osteoblasts into osteocytes ends the cycle and restores bone to its resting state. Adapted from (Hill, 1998).

Factors affecting bone remodelling

The balance between bone resorption and formation is maintained by a multitude of factors which have positive and negative effects on the differentiation, formation and growth of bone cells. Some of the factors that are relevant to this thesis are discussed below.

Oestrogen and testosterone

The sex steroids have huge effects on bone in both men and women. The most significant sex hormone for bone is oestrogen whose anti-resorptive effects have been long known (Riggs *et al.*, 1969). Oestrogen has a protective effect on bone, increasing OPG release from osteoblasts and inhibiting osteoclast formation (Riggs *et al.*, 2002), thus maintaining the strong bone matrix. Oestrogen inhibits osteoclast resorption even when only present at low levels but severely depleted levels that occur in post-menopausal women can result in increased bone resorption and osteoporosis commonly develops (Heshmati *et al.*, 2002).

Diminishing testosterone levels in aging men have been associated with bone loss by similar mechanisms; when these men were treated with testosterone, reductions in bone remodelling and increases in trabecular bone density were seen (Katznelson *et al.*, 1996). However, as the age related changes in sex hormones in men are much less than those seen in post-menopausal women, associated bone loss is much less.

Parathyroid hormone (PTH)

It has long been known that PTH is the major peptide regulator of calcium and phosphate homeostasis (Albright *et al.*, 1928). PTH is synthesised by the parathyroid gland as a 115 amino acid precursor polypeptide, which is cleaved and secreted as an 84 amino acid polypeptide. Small decreases in blood serum calcium levels are sensed by the calcium sensing receptors (CASR) on the chief cells of the parathyroid glands and PTH 1-84 is released into the circulation (Collip, 1924).

PTH is known to elicit its effects on bone in a number of different ways. PTH binds to its receptor (PTHR1) on osteoblastic and stromal cells, inducing RANKL and inhibiting OPG secretion. This promotes the fusion of osteoclastic precursors resulting in activated cells which resorb bone and consequently calcium is released, increasing the amount in the circulation (Lee *et al.*, 1999).

It was initially thought that PTH had no direct effect on bone resorption by acting on osteoclasts alone and significant changes in resorption were only seen when osteoclasts were cultured in the presence of osteoblast-like cells (McSheehy *et al.*, 1986). As such, it was a long held belief that osteoblast mediated effects on osteoclast activity were responsible for the catabolic effects observed *in vivo* (Ma *et al.*, 2001; Lee *et al.*, 1999). However, more recent reports have shown that direct stimulation of osteoclast activity by PTH does occur; PTHR1 is expressed by activated, resorbing osteoclasts (Hoylans *et al.*, 1999) and upon PTH stimulation osteoclastic resorption has been seen to be increased by 2-3-fold in cultures where no osteoblasts were present (Dempster *et al.*, 2005).

PTHR1 receptors are also present in the kidney, where PTH has multiple effects. Here, PTH enhances the active reabsorption of calcium and increases the secretion of phosphate (lowering the amount of calcium bound to phosphate in the circulation and increasing free calcium) (Agus *et al.*, 1973). The effect of PTH on the kidney is also essential for the hydroxylation of vitamin D, converting it from its inactive to its active form which then acts on the small intestine to increase calcium uptake from dietary sources (Dowdle *et al.*, 1960) (Figure 1.5).

$\downarrow [Ca^{2+}]$

Parathyroid glands

$\uparrow$ PTH

$1,25\text{-}(OH)_2\,D_3$
(Activated vitamin D)

Kidney
Vitamin D hydroxylation
$\uparrow Ca^{2+}$ reabsorption
$\uparrow PO_4^{3-}$ excretion

Small intestine
$\uparrow Ca^{2+}$ absorption

Bone
$\uparrow$ osteoclast activity

$\uparrow [Ca^{2+}]$
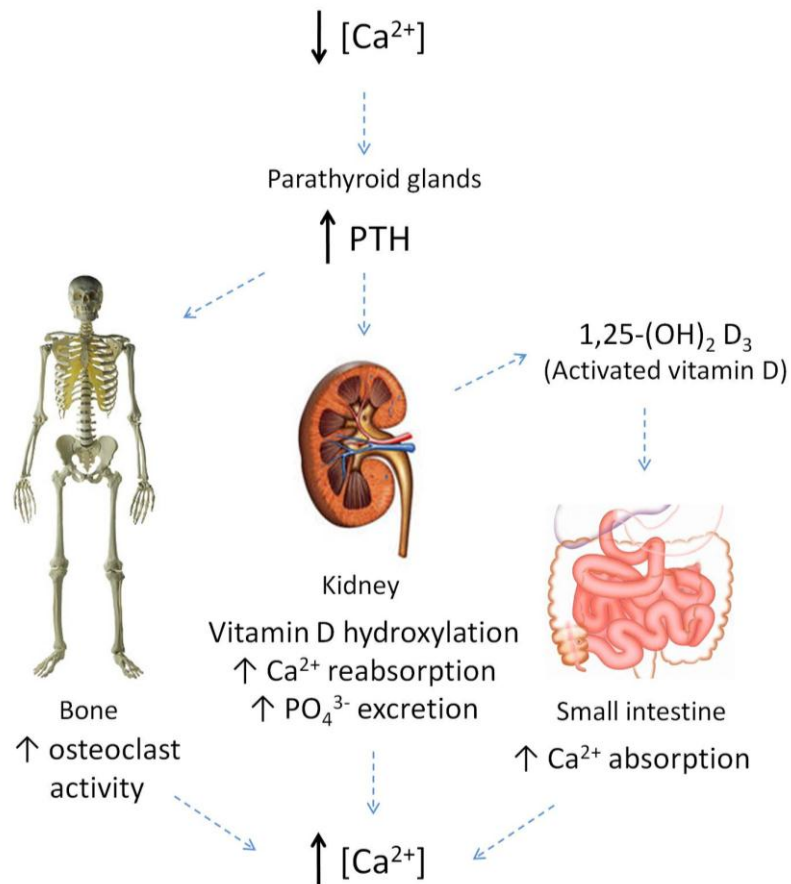
Figure 1.5 . *Whole body calcium regulation by PTH.*

Decreases in serum blood calcium triggers the release of PTH into the bloodstream by the parathyroid glands. PTH modulates calcium activity by causing increased bone resorption, increased reabsorption by the kidneys and the activation of vitamin D which can then act on the small intestine to increase calcium absorption. As a result, blood calcium levels are raised. The system self-regulates via negative feedback mechanisms.

Images taken from http://www.dorlingkindersley-uk.co.uk/, http://www.fotosearch.com and http://anatomy.med.umich.edu/.

The concentration of blood serum calcium is regulated via negative feedback mechanisms which inhibit the secretion of PTH by the parathyroid glands when blood calcium levels are raised, thus maintaining blood serum calcium within narrow limits. The thyroid gland works synergistically with the parathyroid gland to maintain tight regulation of blood calcium levels. CASRs

are also present on the parafollicular cells of the thyroid gland; when blood serum calcium is high, increased secretion of calcitonin is triggered (Foster *et al.*, 1964). This has the opposite effects to those described above and results in a decrease in blood serum calcium.

1,25-dihydroxyvitamin D_3 and calcium

1,25-dihydroxyvitamin D_3, (1,25-$(OH)_2D_3$) the biologically active form of vitamin D, also plays a key role in mineral homeostasis. Vitamin D can be obtained from a small range of foods in the diet with high levels of the vitamin found in liver and oily fish. As these foods are not eaten in significant amounts, foods such as milk, breakfast cereal and margarine are often fortified (Calvo *et al.*, 2004) to increase dietary vitamin D intake. However, the majority of vitamin D in the body is produced in the skin, where ultraviolet light from the sun converts the provitamin into the natural form of vitamin D (Loomis, 1967). This form of vitamin D is transported to the liver where it is hydroxylated to its inactive precursor. Upon stimulation by PTH, 25-vitamin D_3 is transported to the kidney where it is further hydroxylated to its hormonally active form, 1,25-$(OH)_2D_3$ (Jones *et al.*, 1975).

Like PTH, vitamin D is essential to the regulation of calcium homeostasis in the body. 1,25-$(OH)_2D_3$ facilitates intestinal absorption of calcium and regulates the transcription of osteocalcin (Skjodt *et al.*, 1985) which is required for mineralisation by osteoblasts. The importance of vitamin D is exemplified by the skeletal defects arising from a deficiency which include rickets in children and osteomalacia in adults.

'Environmental' factors and bone turnover

The local environment surrounding tissue plays a key role in its function. The vasculature is responsible for the supply of nutrients required for growth and survival as well as the removal of waste products produced by actively metabolising cells and as such is fundamental to cellular homeostasis.

pH

There are numerous potential causes for changes in the pH tissues are exposed to. Acidic conditions may arise locally as a result of tissue hypoxia caused by infection, fractures, inflammation or tumours (Arnett, 2010); or systemically due to kidney failure, respiratory disease, diet (rich in protein or carbonated drinks), ageing and the menopause, to name a few.

The skeleton acts as a reserve base for the body which is called upon to buffer systemic changes in pH if the kidneys and lungs are unable to excrete sufficient H^+ and CO_2 (Arnett, 2008). Alterations in the concentration of hydrogen ions in the local bone environment can have profound effects, with slightly acidic conditions inhibiting the mineralisation phase of bone formation (Brandao-Burch *et al.*, 2005) and switching on osteoclastic resorption resulting in bone loss (Arnett *et al.*, 1986).

Temperature

Decreases in core body temperature have also been seen to have an effect on bone formation. This commonly occurs in the elderly population, where core body temperatures of $35.5^{o}C$ are common as a result of the reduced ability of the body to produce heat and reductions in peripheral vasoconstriction (Collins *et al.*, 1977). *In vitro* experiments have shown that mild hypothermia inhibits

osteoblastic bone formation and promotes osteoclastogenesis (Patel *et al.*, 2009).

Oxygen tension

Bone is capable of sustaining a relatively high metabolic rate (e.g. compared with cartilage) owing to its rich vasculature supply. The blood provides a constant source of nutrients and oxygen which allows bone to remodel and repair. Physiologically, the pO_2 in the majority of tissues is in the region of 5-12% with arterial blood having a pO_2 at the upper end of this range at ~ 12% and venous blood at the lower end, at ~ 5%. The bone marrow cavity is sinusoidal and thus contains a mixture of venous and arterial blood. The pO_2 of bone marrow aspirated from normal, healthy volunteers has been measured at ~ 6.8% (Harrison *et al.*, 2002).

Reduced blood supply to bone can occur in a number of common situations including: ageing (Katschinski, 2006), trauma or fracture, inflammation or inflammatory disease such as rheumatoid arthritis (Firestein, 1999), smoking (Jensen *et al.*, 1991), tumours (either a primary bone tumour or secondary metastasis), diabetic ischemia, or due to prolonged corticosteroid/glucocorticoid use. When the blood supply is reduced or disrupted, oxygen levels fall and hypoxia can occur.

Oxygen sensing

It is critical that cells can sense and respond to alterations in the local oxygen concentration as it is essential for a wide range of developmental, physiological and pathological processes. The effects of hypoxia are mediated by the hypoxia inducible factor 1α (HIF-1α), a transcriptional activator of oxygen responsive

genes (Figure 1.6). In normoxic conditions both HIF1α and the von Hippel Lindau protein (pVHL) are hydroxylated by prolyl hydroxylases (PHD) allowing them to interact. pVHL forms part of an E3 ubiquitin ligase: once the interaction between HIF1α and pVHL has taken place the complex is rapidly ubiquitinated and targeted for degradation by the cellular proteosome.

The prolyl hydroxylase enzymes belong to a family of oxygen, iron and 2-oxoglutarate-dependent dioxygenases and as such, their activity is significantly decreased in hypoxia (D'Angelo *et al.*, 2003). It has been suggested that the absolute requirement for oxygen by the HIF PDH enzymes directly implicates them in cellular oxygen sensing (Jaakkola *et al.*, 2001). The interaction between pVHL and the HIF-1α subunit is regulated through hydroxylation of a specific proline residue (HIF-1α P564); in the absence of oxygen, the HIF-1α subunit is stabilised and accumulates in the cytoplasm. HIF-1α then diffuses into the nucleus, where it forms a complex with the HIF-1β subunit and recruits transcriptional co-activators such as p300/CBP for full transcriptional activity. This complex binds to the hypoxia response element (HRE) in the promoter region of a wide range of target genes required for the cellular adaptation to oxidative stress and induces their transcription (Figure 1.6.). These target genes include genes involved in angiogenesis (in particular VEGF), erythropoiesis, glucose metabolism, apoptosis, cell proliferation and survival, pH regulation and proteolysis (Carroll *et al.*, 2005). VEGF is involved in mediating the cellular response to hypoxia, with chronic hypoxia seen to induce angiogenesis in rat skeletal muscle in response to increased stabilisation of HIF1α (Deveci *et al.*, 2002). Increased VEGF production in response to HIF1α is also believed to be a key factor in the coupling of osteogenesis to angiogenesis (Riddle *et al.*, 2009; Wang *et al.*, 2007).
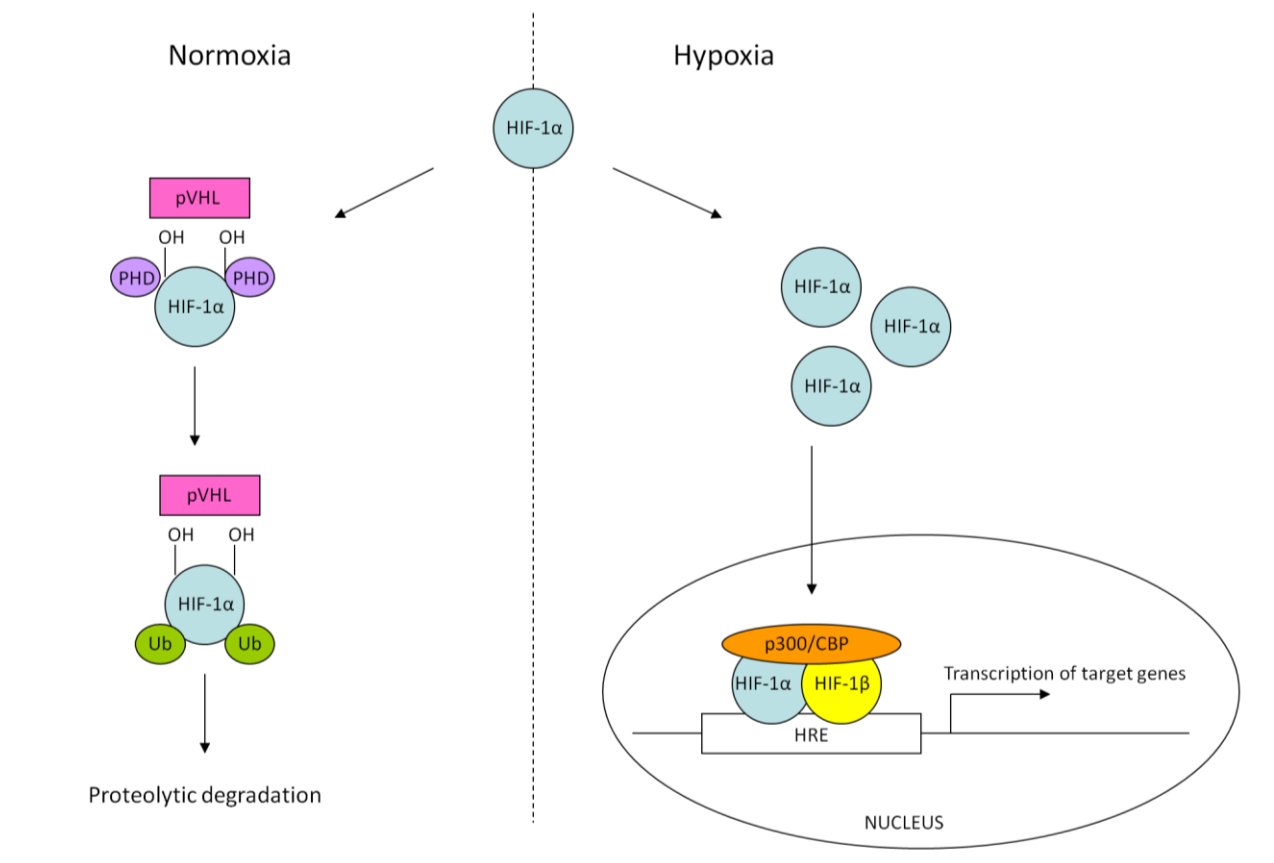
Figure 1.6. *HIF-1α regulation by proline hydroxylation.*

In normoxia HIF-1α is hydroxylated, polyubiquitinated, recognised by the proteosome and subsequently degraded. In response to hypoxia, proline hydroxylation is inhibited and thus HIF-1α is stabilised and accumulates. It translocates to the nucleus where it forms a complex and initiates transcription of oxygen responsive genes. Abbreviations: pVHL = von Hippel Lindau protein, PHD = prolyl hydroxylase, Ub = ubiqutin, CBP = CREB-binding protein. Figure adapted from (Ke *et al.*, 2006).

It is noteworthy to mention that although they belong to the same family, the prolyl hydroxylases (PHDs) involved in the modification of HIF1α are cytoplasmic proteins and differ from the collagen modifying PHDs. HIF hydroxylation is carried out by PHDs 1-3, PHD2 is thought to be the key oxygen sensing enzyme (Berra *et al.*, 2003). However, collagen modifications are catalysed by a homologous family of PHDs which are found in the endoplasmic reticulum. The difference in the nature of the protein surrounding the proline

residues to be hydroxylated means that PHDs 1-3 recognise and bind to different substrates to collagen modifying PHDs and as such it has been shown that recombinant collagen PHD isoforms have no activity on HIF substrates (Bruick *et al.*, 2001).

The effect of hypoxia on bone

It is well documented that hypoxia has a stimulatory effect on bone marrow derived cells of the monocyte-macrophage lineage (Roiniotis *et al.*, 2009), which are very closely related to osteoclasts. These cells appear to be well adapted to working in hostile conditions, a likely reflection of their physiological function and anatomical locations. *In vitro* investigations have shown that hypoxia causes profound stimulation of osteoclast formation, resulting in resorption of significant amounts of bone before undergoing what would normally be considered to be a premature death (Arnett *et al.*, 2003).

This is in stark contrast to the effect of hypoxia on osteoblasts, whose proliferation and differentiation, including collagen forming ability are severely inhibited in low oxygen conditions (Utting *et al.*, 2006). This is believed to be a result of the effect that hypoxia has on Runx2 whose expression is significantly decreased and consequently transcription of osteoblastogenic genes including: osteocalcin, osteopontin, bone sialoprotein, osteoprotegrin and collagenase, are reduced (Park *et al.*, 2002). Upon reoxygenation, bone forming ability is restored, suggesting that the cells adopt a dormant state until more favourable conditions return (Utting *et al.*, 2006).

Pathologies

Soft tissue wounds

Fibroblasts are essential to the wound healing process of soft tissues; the mechanisms for their localisation to the site of damage are poorly understood but it has been proposed that chemokines and cytokines are upregulated at the site of injury and released into the circulation. This causes MSCs to downregulate adhesion molecules that keep them attached at their niche site in the bone marrow (Liu *et al.*, 2009). Circulating MSCs are then 'homed' from the blood to the target tissue and undergo *in situ* proliferation and maturation into fully functional cells that integrate into the injured tissue, contributing to the healing process.

Damage to soft tissue often cannot be repaired perfectly by the body and as a result scars are formed. The rapid deposition of new collagen fibres by connective tissue fibroblasts results in tissue that is thicker and denser than its surroundings. Scar tissue has limited functionality compared to healthy tissue; it is poorly vascularised and consequently appears pale and its extreme density means that movement and sensation are restricted.

Fractures

The coupled relationship between osteogenesis and angiogenesis is highlighted at a fracture site, where a decrease in vascular supply is followed by an increase in osteoclast number in order for the dead and necrotic bone tissue to be resorbed. In these conditions bone can become severely hypoxic; early work performed on rabbit models of fracture healing showed oxygen levels as low as 0.8% O_2 in bone four days post fracture, rising to only 3.8% O_2 in newly formed bone fourteen days post fracture (Brighton *et al.*, 1972). Platelets are some of

the first cells to localise to a site of skeletal injury and play a key role in initiating the process. Platelets secrete platelet-derived growth factor (PDGF) which is believed to act additively with hypoxia to increase VEGF mRNA expression (Bouletreau *et al.*, 2002). VEGF stimulates angiogenesis and thus restores the blood flow to the damaged bone. PDGF also has potent mitogenic effect on cells of MSC origin (Zhang *et al.*, 1991), resulting in increases in osteoblast proliferation, collagen synthesis and bone formation. Newly formed bone is subjected to extensive remodelling, when osteoclast activity is seen to peak again (Schell *et al.*, 2006). Should the blood supply to the fracture not be adequately restored non-union fractures can result.

Metabolic diseases of bone

When the balance between osteoblastic bone formation and osteoclastic bone resorption is upset due to impaired bone formation or enhanced resorption, bone loss results. Two diseases in which bone cell activity is affected leading to potentially detrimental skeletal effects that are relevant to this thesis are described below.

Osteoporosis

Osteoporosis is the most common age-related disorder of bone. It is estimated to affect 75 million people in Europe, the USA and Japan, with a lifetime risk of fracture estimated to be ~50% for women and ~20% for men over 50 suffering from the disease (van Staa *et al.*, 2001). Osteoporosis is characterised by low bone mass which occurs mainly as a result of increased osteoclastic activity. The reasons for the onset of osteoporosis are multifactorial: a family history of

the disease, poor diet lacking calcium and vitamin D, smoking, age-related reductions in the sex hormones (Aloia *et al.*, 1985), lack of weight-bearing exercise (Giangregorio *et al.*, 2002), glucocorticoid use and excessive alcohol consumption (Bilezikian, 1999) are all contributory factors. As has already been discussed, decreases in oestrogen production by the ovaries of post-menopausal women and age related losses in testicular function and thus decreases in testosterone in men, both contribute significantly to loss of bone density. Consequently, the protective roles the sex hormones have on bone are significantly reduced and increases in bone resorption are observed. This results in the trabeculae within bone becoming very thin and deterioration of the microarchitectural structure, leaving the bone very susceptible to fracture.

Glucocorticoid use is a well known inducer of osteoporosis. Although low levels are required for normal cell function and are essential for bone formation *in vitro*, raised levels often lead to osteopaenia and if treatment is prolonged, osteoporosis. This is believed to be a result of glucocorticoid treatment attenuating osteoblast differentiation, promoting apoptosis of osteoblasts and osteocytes (Weinstein *et al.*, 1998) and increasing the life span of osteoclasts (Jia *et al.*, 2006).

Hyperparathyroidism

Over-secretion of PTH by the parathyroid glands is caused by overactivation or enlargement of one or more of the glands. Primary hyperparathyroidism is a relatively common endocrine disease with an incidence of between 1 in 500 and 1 in 1000, the majority of individuals affected are older, post-menopausal, women (Silverberg *et al.*, 2008).

In primary hyperparathyroidism, increased PTH secretion is usually a result of enlargement of one of the glands due to adenoma formation.

45

Secondary hyperparathyroidism usually occurs as a result of renal failure, where decreased serum calcium causes increased PTH secretion by the parathyroid glands. As a result, blood calcium levels are raised and hypercalcaemia results. Hyperparathyroidism is easily detectable by measuring blood serum calcium but if undiagnosed and untreated then osteoporosis, recurrent kidney stones and osteitis and osteoclastic cysts (which can cause significant bone pain) can arise (Keating, 1961).

Current therapies

The majority of current therapies for the treatment of metabolic bone disease work by inhibiting osteoclast activity. The front line therapies for the treatment of osteoporosis are the nitrogen-containing bisphosphonates: Aledronate, Risedronate and Zoledronate. These drugs reduce bone turnover by binding to the mineralised bone surface and inhibiting activity of mature osteoclasts, resulting in increased BMD and reduced fracture risk (Russel, 2006).

Other therapies that are becoming increasingly popular target key factors that modulate bone cell activity. Denosumab, a monoclonal human antibody that specifically targets RANK ligand, has recently been approved by the U.S. food and drug administration (FDA) for treatment of postmenopausal women with osteoporosis who are at increased risk of fracture (Rizzoli *et al.*, 2010). Denosumab effectively mimics the function of OPG, binding to RANKL and results in decreased osteoclast formation (Kostenuik, 2005). Clinical studies have shown significantly greater increases in hip BMD in patients treated for 12 months with Denosumab compared to those treated with Aledronate (Brown *et al.*, 2009).

Targeting naturally occurring inhibitors of bone formation also results in increased bone mass. The inhibition of the production of sclerostin leads to enhanced canonical Wnt signalling and thus increase bone formation (Baron *et al.*, 2007). A sclerostin monoclonal antibody (Scl-Ab) has recently been generated and has been shown to increase bone mass and strength by stimulating new bone formation in an ovariectomy-induced bone loss rat model (Li *et al.*, 2009).

As bone formation and resorption are interconnected processes, inhibition of resorption is accompanied by decreases in bone formation. Bone loss diseases result when the balance between these two processes is upset. Anti-resorptive therapies prevent further bone loss and thus aid in fracture prevention but do not rebuild bone that was lost before the onset of treatment. Consequently, the most effective treatments for bone disease must inhibit excess resorption and increase bone formation.

Anabolic effects of PTH on bone

PTH has been developed as a drug for the anabolic treatment of metabolic bone disease. The naturally occurring hormone has been generated as a recombinant, containing the 34 amino acids present at the N-terminal end of the naturally occurring protein, PTH 1-34. All the structural elements required for binding to PTHR1 are present in this fragment and thus the drug binds with high affinity.

The effects of using PTH as an anabolic drug were recognised over three decades ago (Reeve *et al.*, 1980). However, the understanding that this anabolic action, i.e. promoting bone growth and turnover, is dependent on the duration and pattern of exposure had not been well documented until relatively

recently (Frolick *et al.*, 2003; Ishizuya *et al.*, 1997; Poole *et al.*, 2005). Continuous exposure to PTH *in vivo* results in catabolic effects on bone, with increases in bone resorption and concomitant raised serum calcium. Conversely, when administered intermittently profound anabolic effects are seen; recombinant human PTH (hPTH) (Teriparatide, Forteo™) is widely used as a drug for the treatment of osteoporosis, preventing fractures by increasing BMD (Neer *et al.*, 2001; Dempster *et al.*, 2001; Alexander *et al.*, 2001; Brommage *et al.*, 1999). Initial increases in bone formation by intermittent PTH treatment are followed by increases in remodelling, thus, this treatment is most effective when used in combination with an anti-resorptive therapy. In addition to its use as a preventative measure for fractures, intermittent PTH treatment has recently been shown to improve bone volume and mechanical properties of healing fractures and in bones subjected to distraction osteogenesis (Aleksyniene *et al.*, 2009).

Despite the use of intermittent PTH as an anabolic treatment for low bone mass, the mechanism by which this occurs remains elusive. Furthermore, an explanation for the differential effects of intermittent and continuous PTH exposure has yet to be proposed.

Thesis aims

The aim of this thesis was to explore some of the fundamental regulators of cells of MSC origin and to critically examine factors which have an effect on their ability to produce and excrete extracellular matrix. The emphasis has been on the effects of these regulators on collagen and bone formation with a particular focus on the role of oxygen and, by implication, the vasculature in these processes.

The work started with investigations into the effects of hypoxia on osteoblasts and dermal fibroblasts, examining their differentiation, growth, collagen forming ability and adhesive properties in normoxia and hypoxia (Chapter 3). This study and the majority of *in vitro* research in this field have been carried out on primary cells using osteoblasts derived from the calvaria of neonatal rats. Given the differences between the bone formation processes and blood supply to bones in these discrete anatomical locations, the relevance of using calvarial-derived cells as a representative model of *in vivo* bone formation was questioned. To address this, a new method for isolating osteoblasts using cells derived from the long bones of neonatal rats was developed and these cells were characterised alongside analogous calvarial cells (Chapter 4). Using these long-bone-derived cells, the direct effect of PTH on osteoblasts *in vitro* was investigated. The relationship between PTH treatment and sclerostin was also examined and the direct effect of endogenous sclerostin on the activity and bone forming ability of osteoblasts was observed (Chapter 5). Using the findings from these studies, a general theory that could help to explain the actions of some of the major bone regulatory factors is proposed (Chapter 6).

Chapter 2

Materials and methods

Reagents

Tissue culture media, sera and buffers were purchased from Invitrogen (Paisley, UK). The main buffer used was 1X Dulbecco's phosphate buffered saline (PBS) which contained: 200mg/L potassium chloride (KCl), 200mg/L potassium phosphate monobasic (KH_2PO_4), 8000mg/L sodium chloride (NaCl) and 2160mg/L sodium phosphate dibasic (Na_2HPO_4-$7H_2O$). 1X Hanks' balanced salt solution (HBSS) which contained: 400mg/L KCl, 60mg/L KH_2PO_4, 350mg/L sodium bicarbonate ($NaHCO_3$), 8000mg/L NaCl, 48mg/L sodium phosphate dibasic (Na_2HPO_4) anhydrous, 1000mg/L D-glucose and 10mg/L phenol red was used for making up enzyme solutions.

Bulk liquids (organic solvents, acids and bases) were purchased from VWR International Ltd (Leighton Buzzard, UK). Molecular biology reagents were purchased from Promega, UK (Hampshire, UK), and all primers were from MWG Biotech (Ebersberg, Germany). All other chemicals were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated. The Melinex used was a kind donation from Dupont Teijin Films (HiFi Industrial Film Ltd, Stevenage, UK). A sample of the sclerostin protein was generously supplied by Dr. Christine Halleux (Novartis AG, Basel, Switzerland).

Cell culture

Osteoblast calvarial bone formation assay

Osteoblasts were obtained by sequential enzyme digestion of calvarial bones excised from 2-day-old Sprague-Dawley rats. The enzyme digest involved a three step process; 1% trypsin in PBS for 10 minutes to digest the outer protein layer; 0.2% collagenase type II in Hanks balanced salt solution (HBSS) for 30 minutes to weaken the collagen structure and removed the unwanted collagenous matrix; 0.2% collagenase type II in HBBS for 60 minutes to release osteoblasts from the bone. The first two digests were discarded and cells liberated by the third digest were washed and plated into a $75cm^2$ flask in Dulbecco's modified essential medium (Gibco, Paisley, UK) supplemented with 10% foetal calf serum, 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin (complete mixture abbreviated to 'DMEM'). Cells were cultured for 3 days in a humidified atmosphere of 5% CO_2 - 95% air at 37°C, until confluent. Upon confluency, cells were sub-cultured into 6, 12 or 24-well trays in DMEM supplemented with 10nM dexamethasone, 50 µg/ml ascorbate and 2 mM β-glycerophosphate (complete mixture abbreviated to 'supplemented DMEM'). Dexamethasone is required for osteoblastic differentiation from mesenchymal stem cell precursors; ascorbate as an enzyme cofactor for collagen synthesis and β-glycerophosphate as a phosphate source, enabling osteoblasts to deposit hydroxyapatite mineral. Cells were cultured for up to 28 days with half media changes performed every 3-4 days. Mineralised bony structures typically developed from day 10, giving rise to a dense, organised, trabecular-shaped network by days 21-28. Further details of *in vitro* bone formation assays are given in Chapter 4.

Fibroblast collagen formation assay

Fibroblasts were isolated from the dermis of 2-day-old Sprague-Dawley rats. Skin flaps (~1cm^2) were removed from the flanks of neonatal Sprague-Dawley rats killed by cervical dislocation and washed in PBS. The keratinised outer layer of the skin was removed and discarded and the dermis was subjected to a single collagenase digest (0.2% collagenase type II in HBSS for 60 minutes at 37°C). The cells were washed and plated into a 75cm^2 flask in DMEM. Cells were cultured for 3 days in a humidified atmosphere of 5% CO_2 - 95% air at 37°C, until confluent. Upon confluency, cells were sub-cultured into 6 or 24-well trays in DMEM containing 5% FCS and supplemented with 50 µg/ml ascorbate.

Generation of a hypoxic environment

Isolated cells were allowed to settle in tissue culture plates for 24 hours before being exposed to hypoxic conditions. Tissue culture plates were placed in humidified air-tight polycarbonate enclosures (Fibox Ltd, Cleveland, UK) and exposed to 2%, 5%, 12% or 20% O_2 / 5% CO_2 / N_2 balance (BOC, London, UK) through openings drilled in the lid for 2 minutes. Holes were sealed with PVC electrical tape (Temflex™ 1500, 3M, Bracknell, UK) and boxes placed in a 37°C incubator (Figure 2.1.). Daily gassing of the cells in these air-tight boxes ensured culture medium was maintained at, or around the desired oxygen concentration (Figure 2.2.).

As hypoxia can cause acidification of cell cultures, an excess of medium was used to provide additional buffering capacity and half media changes were performed every 3-4 days. Culture medium pH was regularly monitored using a blood-gas analyser (see later) but was not found to differ significantly between cultures exposed to different oxygen tensions.
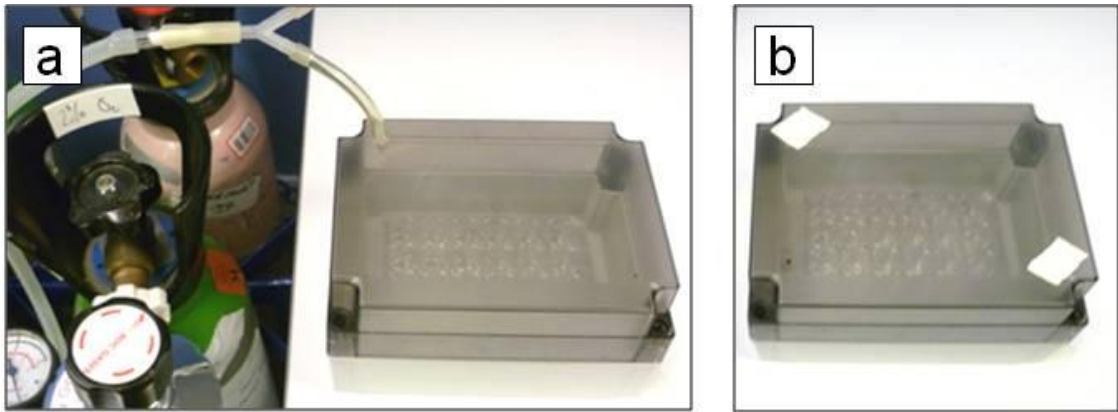
Figure 2.1. *Experimental set up for generating a hypoxic environment.*

a. 24-well trays were placed in humidified, air-tight polycarbonate enclosures and gassed for 2 minutes through openings drilled in the lid with 2%, 5%, 12% or 20% O_2 / 5% CO_2 / N_2 balance custom gas mixtures.

b. Gassing inlet and outlets were then sealed with vinyl tape and boxes placed in a 37°C incubator.
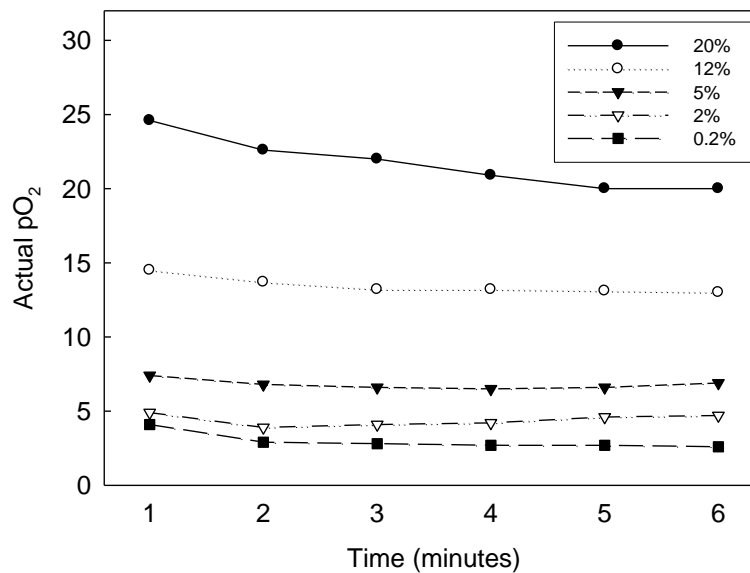
Figure 2.2. *Oxygen concentration of the medium measured 24 hours post-gassing indicates maintenance of a hypoxic environment over the course of the experiment.*

Flasks containing DMEM were gassed at 20%, 12%, 5%, 2% or 0.2% O_2 / 5% CO_2 / balance N_2 and culture medium oxygen concentrations were measured 24 hours later using the FOXY probe. Readings were taken every minute after insertion of the probe. After six minutes readings remained unchanged, suggesting equilibrium between the probe and culture medium had been reached. Adapted from (Utting, 2006).

Staining

Alizarin red staining

Osteoblast cultures were terminated after 14 – 21 days in culture by fixing cell monolayers in 2.5% glutaraldehyde for 5 minutes. Mineralised structures were visualised by staining with 1% w/v alizarin red S in PBS for 5 minutes. Monolayers were washed three times in 50% ethanol to remove excess stain and air-dried before quantification of bone area. The plates were imaged at 800 dpi using a high-resolution flat-bed scanner (Epson Perfection Photo 3200, Epson) in reflected light mode. Images of individual wells were converted to greyscale then digitised, before being subjected to automated analysis (Scion Image software, Scion Corporation; http://www.scioncorp.com), using constant 'threshold' and 'minimum particle' levels to determine the number and plan surface area of mineralised bony structures (Figure 2.3.).
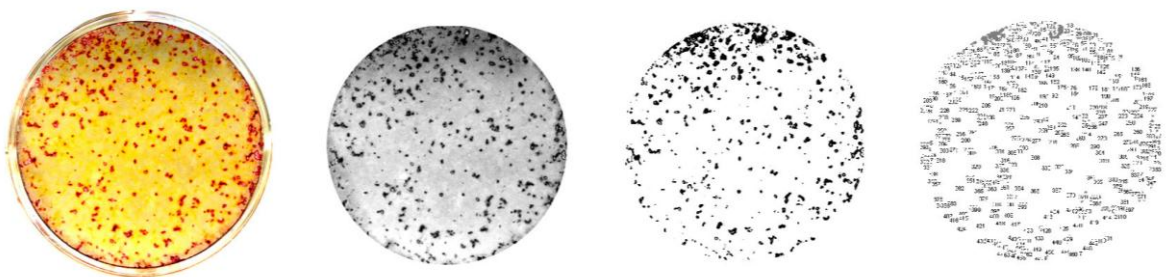
Figure 2.3. *Stages of the image analysis process.*

Alizarin red stained cell well monolayers were imaged using a high-resolution flat-bed scanner. Images were converted to greyscale and standard threshold values applied to create digital images from which extracted data can be analysed.

Masson's trichrome stain for deposited collagen

After 14 days of culture in 6-well trays, cell layers were washed in PBS and fixed in Bouin's solution overnight at room temperature. Monolayers were washed in running water and then stained in Weigert's hematoxylin A solution for 10 minutes. Cells were thoroughly washed in tap water and stained in 0.5% acid fushin / 5% glacial acetic acid (0.5 grams acid fushin, 5ml glacial acetic acid and 100ml dH_2O) for 5 minutes, rinsed in distilled water and treated with phosphomolybidic acid solution for 5 minutes. This solution was then removed and cells stained for 5 minutes with 2% methyl blue / 2.5% glacial acetic acid (2 grams methyl blue, 2.5ml glacial acetic acid and 100ml dH_2O). After rinsing with distilled water cells were treated with 1% acetic acid for 2 minutes before dehydration in a graded ethanol-water series and air drying. Plates were visualised under an Olympus IMT-2 inverted microscope and images taken using a Zeiss AxioCam MRc5 digital camera.

Assays

Alkaline phosphatase assay

Osteoblasts were cultured for 7 or 14 days in 24 well trays as previously described. Culture medium was removed and monolayers washed in PBS. Cells were scraped into 500µl of distilled water (dH_2O) and lysates transferred to 1.5ml Eppendorfs which were sonicated on ice for 10 minutes. Cell debris was pelleted by centrifugation at 2500g, 4°C for 10 minutes. 100µl of supernatant was removed from each sample and added to a 96 well tray along with 100µl of alkaline phosphatase reagent (Biotron Diagnostics, Inc., CA, USA). Absorbance at 405nm was measured immediately and after 1, 3 and 5 minutes incubation at

37°C in the dark. Enzyme activity was determined by the rate of product formation relative to total protein (see Bradford assay, below).

Soluble collagen assay

Cells were grown for 7 or 14 days in 24 well trays with test substances present. 24 hours prior to performing the assay, media was removed from the wells and replaced with 500µl of supplemented DMEM (5% FCS), 200µM β-aminoproprionitrile (βAPN) and test substances. After 24 hours of incubation (at 37°C 5% CO_2 - 95% air), the Sircol soluble collagen assay (Biocolor Ltd, County Antrim, UK) was performed as per manufacturer's instructions. This assay measures the amount of soluble collagen released into the culture medium during cell growth and maintenance by exploiting the binding of the sirius red dye to the [Gly-X-Y]$_n$ motif found in collagen fibrils. A standard curve was prepared using known concentrations of collagen and 100µl of unused culture media from which values of collagen in solution were obtained colorimetrically by measuring absorbance at 570nm. Amounts of collagen produced were normalised for total protein which was assessed using the Bradford assay (with collagen production expressed as a percentage of total protein).

Bradford assay for determination of total protein

A standard curve was prepared using bovine serum albumin (BSA) (0-8µg) with 50µl dH_2O and 200µl Bradford reagent. The same quantities of dH_2O and Bradford reagent were also added to 3µl of test samples. After 5 minutes at room temperature samples were read at 450nm and 570nm and protein levels established.

Cell number and viability assay

Cells were cultured in 24-well trays and cell number was measured after 4, 7, 10 and 14 days of culture using the CytoTox 96® non-radioactive cytotoxicity assay (Promega UK, Southampton UK). This assay quantitatively measures cellular lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released on cell lysis and shows good agreement with non-viable cell number (Racher *et al.*, 1990). LDH oxidises lactate into pyruvate, generating NADH, which is then used to convert a tetrazolium salt into a red formazan product. The amount of colour formed is proportional to the number of lysed cells. One hour prior to assaying, osteoblasts were switched to serum free DMEM containing lysis buffer (1% Triton X-100 in water); following sample collection the assay was performed as per manufacturer's instructions. A standard curve for determination of cell numbers was constructed using cells seeded at 10^3 to 10^6 per well. Manual cell counts were performed in parallel for assay validation.

To investigate cell viability the same assay was performed. One hour prior to assaying, osteoblasts were switched to serum free DMEM and following sample collection the assay was performed as per manufacturer's instructions. To determine the viability of the cells the following equation was used:

$$\% \ loss \ in \ cell \ viability = \frac{(sample \ absorbance \ - \ blank)}{(lysed \ sample \ absorbance \ - \ blank)} \ x \ 100$$

Cellular ATP release

Cells were cultured in 12-well trays and ATP release was measured after 7 and 14 days using CellTiter-Glo® Luminescent Cell Viability Assay (Promega UK, Southampton, UK). Low concentrations of ATP inhibits *in vitro* bone formation (Hoebertz *et al.*, 2002); measuring the amount of ATP released by cells in

response to a specific condition/treatment provides an insight as to whether this is the mechanism by which bone formation is being inhibited. The reagent inhibits endogenous ATPases and provides luciferase/luciferin for ATP measurement. Luciferase catalyses the reactions:

$$Luciferin + ATP \rightarrow Luciferyl\ Adenylate + PPi$$

And

$$Luciferyl\ Adenylate + Oxygen \rightarrow Oxyluciferin + AMP\ (with\ the\ production\ of\ light)$$

The high efficiency of this reaction and the long half-life (generally greater than 5 hours) of the luminescent signal allows this assay to be based on the assumption that the luminescent signal generated in proportional to the amount of ATP present.

To perform the assay, 50µl of culture media was removed from each well in the tissue culture plate and added to 50µl of CellTiter-Glo® Luminescent Cell Viability reagent and allowed to stand for 10 minutes at room temperature. Samples were then read using the GloMax® 20/20 luminometer (Promega UK Ltd). A standard curve for determination of amount of ATP released was constructed using ATP from 100pM to 1µM diluted in serum free media. The remaining cell monolayers were assayed for cell number and viability as described previously.

Microscopy

Transmission electron microscopy (performed with the help of Mark Turmaine, Dept of Cell & Developmental Biology, UCL)

Osteoblasts were cultured on Melinex film (Dupont Teijin Films, UK), cut into 1x1 cm discs in 12-well trays. After 28 days of culture, cell layers were fixed in 2.5% glutaraldehyde for 48 hours. Monolayers were washed in 0.1M phosphate buffer for 10 minutes and then post-fixed in 1% osmium tetroxide / 0.1M cacodylate buffer (pH 7.3) for 1 hour at 4°C in the dark. This was followed by staining with 0.5% uranyl acetate / dH_2O at 4°C for 30 minutes. After rinsing with dH_2O, specimens were dehydrated in a graded ethanol-water series and infiltrated with Agar 100 resin mix overnight. Ultra thin sections were cut at 70-80nm using a diamond knife on a Reichert Ultracut E microtome. Sections were collected on Formvar® coated slot grids (Agar Scientific, Essex, UK) and stained with uranyl acetate and lead citrate. Imaging was performed using a JEOL JEM-1010 transmission electron microscope (JEOL (UK) Ltd., Herts, UK) and photographs taken using an 11 megapixel SC1000 Orius® TEM CCD camera (Gatan UK, Abingdon, UK).

Molecular biology

Total RNA extraction and complementary DNA strand synthesis

Cells were cultured in 6-well trays for up to 14 days in supplemented DMEM; monolayers were washed briefly in PBS and the total RNA was extracted at days 7 and 14 using TRIzol® reagent (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Before first strand complementary DNA (cDNA) synthesis, extracted RNA was treated with RNase-free DNase I (35 U/ml) for 30 minutes at 37°C. The reaction was terminated by heat inactivation at 65°C for 10 minutes. Total RNA was quantified spectrophotometrically by measuring absorbance at 260nM (NanoDrop™ ND1000 Spectrophotometer, Thermo Fischer Scientific, Epsom, Surrey, UK). For each sample, 1µg of DNase-treated total RNA was used as a template for first strand synthesis in a 20µl reaction also containing 0.5µg oligo dT, 3mM $MgCl_2$, 0.5mM dNTPs, 20U recombinant RNasin® ribonuclease inhibitor, ImProm-II® 5x reaction buffer and 200U ImProm-II reverse transcriptase. The reaction mix was annealed for 5 minutes at 65°C followed by extension at 50°C for 60 minutes and inactivation at 70°C for 15 minutes. cDNA was stored at -20°C until amplification by the polymerase chain reaction (PCR).

Polymerase chain reaction

cDNA derived from cultured cells was amplified by PCR in 25µl reactions containing 0.5µg cDNA, 0.2mM dNTP, 1.5mM $MgCl_2$, 0.2µM of both sense and antisense primers and 1U Taq DNA polymerase in thermophilic DNA polymerase 10x buffer. PCR was performed in accordance with the manufacturer's instructions, with cycles of denaturation at 95°C for 30 seconds, annealing for 30 seconds (see Appendix I, Table 1 for annealing temperatures), extension at 72°C

for 45 seconds and reaction termination at 72°C for 5 minutes. For analysis, PCR products were fractionated on a 1% agarose gel labelled with 0.3µg/ml ethidium bromide run at 100mA for ~30 minutes and visualised by UV transillumination. cDNA levels were normalised to β-actin expression.

Quantitative real-time PCR (qRT-PCR)

Calvarial and long bone osteoblast cDNA (25ng/µl) was amplified by qRT-PCR using SYBR® Green Jumpstart™ Taq ReadyMix™ (Sigma, Poole, Dorset, UK). qRT-PCR was performed using a Bio-Rad Chromo4 PTC-400 real-time detector according to manufacturer's instructions with initiation of the reaction (94°C for 2 minutes) being followed by 40 cycles of denaturation (94°C for 15 seconds), annealing (55 or 60°C for 1 minute), elongation (72°C for 1 minute) and detection. Data was analysed by relative quantification of the target gene transcript in comparison with a reference gene transcript using the Pfaffl method (Pfaffl, 2001). The reference gene used for the experiments performed in this thesis was β-actin. All reactions were carried out in triplicate using cDNAs derived from 3 different osteoblast cultures. Primer pairs are displayed in Appendix I, Table 2.

Enzyme-linked immunosorbent assays (ELISAs)

Detection of apoptosis

Apoptosis was assessed using an ELISAPLUS cell death detection kit (Roche Diagnostics Ltd, East Sussex, UK) according to the manufacturer's protocol. Apoptosis is characterised by membrane blebbing, cytoplasmic condensation and the activation of endogenous Ca^{2+} and Mg^{2+} dependent endonucleases

which cleave double stranded DNA at internucleosomal linker regions, generating mono- and oligonucleosomes. During apoptosis, this DNA degradation occurs several hours before breakdown of the plasma membrane and histone DNA fragments (i.e. the mono- and oligonucleosomes) accumulate in the cytoplasm. This assay uses mouse monoclonal antibodies to measure histone DNA accumulation which is assumed to be proportional to apoptotic cell death.

Briefly, cells were cultured for 14 days in the presence of test substances. They were then lysed and mononucleosomes and oligonucleosomes released into the cytoplasm were detected by measuring the absorbance at 405nm and 490nm. The specific enrichment of mononucleosomes and oligonucleosomes released into the cytoplasm was calculated using the following formula:

$$Enrichment\ Factor\ (EF) = \frac{mU\ of\ sample\ (treated\ cells)}{mU\ of\ corresponding\ negative\ control\ (untreated\ cells)}$$

Where mU = absorbance x 10^{-3}

Quantitative determination of VEGF

Cellular VEGF release was examined using a rat VEGF ELISA kit (Invitrogen, CA, USA) and was performed as per manufacturer's instructions. This assay measures the amount of both natural and recombinant rat VEGF protein that has been released into the tissue culture medium. Cells were cultured for 14 days in the presence of test substances after which time the media was collected in pyrogen/endotoxin-free tubes and stored at -20°C until required for analysis. This assay uses a highly purified rat anti-VEGF antibody which binds the VEGF protein present in the sample and thus the intensity of the final coloured product

is directly proportional to the concentration of rat VEGF present in the original sample.

Monitoring cell culture homeostasis

Blood gas analyser

Culture medium pH, pCO_2 and pO_2 were monitored using a blood gas analyser (ABL 705, Radiometer, Crawley, UK). The blood gas analyser automatically equilibrates two buffer solutions (high and low pH) by means of pure air and pure CO_2, these solutions are used for electrode calibration, set to occur every 2 hours. The blood gas analyser also performs an internal calibration and thus a high degree of accuracy is achieved.

Oxygen measurement using fibre optic oxygen (FOXY) probe

Oxygen levels in the hypoxic range in media were measured using a FOXY-R stainless-steel 1/16" OD fibre probe with FOXY-AF silicone overcoat and LS-450 blue LED light source connected to a USB2000-FL spectrometer (all from OceanOptics, Duiven, Netherlands). A pulsed blue LED sends light at ~475nm to an optical fibre which carries it to the tip of the probe which contains a ruthenium complex immobilised in a hydrophobic gel. The light from the LED causes the ruthenium compound to fluoresce and emit light at approximately 600nm. The excitation of the ruthenium compound is quenched by non-radiative transfer of energy to oxygen molecules; the degree of quenching is proportional to the partial pressure of oxygen in the gel, which is in dynamic equilibrium with the sample. The energy released is detected and converted to a digital signal by

the spectrometer and analysed using software that was provided with the spectrometer on a PC.

The system was calibrated using 0% O_2 (7.5% w/v sodium hydrosulphite ($Na_2S_2O_4$) in PBS) and 20% O_2 (PBS) solutions. The probe was left to equilibrate for a few minutes until a stable reading was attained and then washed briefly before measuring experimental samples. Due to difficulties with sterilising the probe, oxygen measurements could only be taken at the end point of experiments.

Statistics

Multiple comparisons were performed by one-way analysis of variance (ANOVA) using GraphPad InStat (version 3.06, GraphPad software Inc.), using the Bonferroni correction. Unless otherwise stated, representative data are presented as means $\pm$ one standard deviation (SD) for 5 or 6 replicates. All experiments were performed in at least triplicate. Significance was assumed to be $P<0.05$, where $P<0.05$ = *, $P<0.01$ = ** and $P<0.001$ = ***.

Chapter 3

Hypoxia inhibits collagen producing cells

Introduction

The biosynthesis of the collagen triple helix is dependent upon the availability of an adequate oxygen supply (Hunt *et al.*, 1969). One obvious explanation for this is that the prolyl hydroxylase enzymes involved in the conversion of proline to hydroxyproline are oxygen dependant (Yen *et al.*, 1979). Hydroxylation of proline residues provides increased stability by serving as glycosylation and cross linking sites which allow individual polypeptides to associate and form the basic collagen unit, the triple helix (Bann *et al.*, 2000a; Mizuno *et al.*, 2003). Thus *de novo* collagen synthesis has an absolute requirement for oxygen.

As has already been described (Chapter 1), in normal, healthy tissue, both bone and skin have extensive blood supplies which serve the local environment with nutrients and oxygen, and remove the by-products of cellular respiration. In osseous tissue, capillary networks penetrate throughout trabecular bone and the osteons of cortical bone carry the blood vessels that feed more densely packed areas. The skin has a dense capillary supply; the majority of these blood vessels are concentrated within the dermal layer of the skin and play an important role in thermoregulation. However, disruption to the capillaries feeding these tissues, as is seen following a fracture or wound, can result in local hypoxia.

The oxygen tension in a wound reflects a balance between oxygen delivery and oxygen consumption; this drops to its lowest in the first day post trauma (Chang *et al.*, 1983), when the cells in the wound have an increased

metabolic demand and the blood supply to the injured area is reduced to prevent excessive bleeding. The oxygen tension in the centre of soft tissue wounds remains low during the healing process; restoration of normal oxygen levels does not begin until at least 8 days post-trauma and is still not complete 14 days post-trauma (Remensenyder *et al.*, 1968). In order for successful wound healing to occur, the blood flow to the affected area must be restored so that the amount of oxygen available to the cells is sufficient for collagen synthesis. Progression of wound healing *in vivo* suggests collagen deposition is related to the oxygen tension of the wound (Tandara *et al.*, 2004) and thus the effects of chronic hypoxia on the ability of osteoblasts and fibroblasts to differentiate, proliferate and form collagen in low oxygen environments merits further investigation.

The majority of studies on the effects of hypoxia on osteoblasts have focused on the gene expression profiles of these cells when exposed to low oxygen tensions for relatively short periods of time (up to 96 hours). In these conditions mRNA has been shown to be decreased for a wide range of osteogenic markers including: Runx2, type I collagen, alkaline phosphatase and osteocalcin (Warren *et al.*, 2001; Park *et al.*, 2002; Liu *et al.*, 2007), with concomitant increases in mRNA expression of VEGF (Steinbrech *et al.*, 1999b; Akeno *et al.*, 2001). It has been proposed that this response is mediated by the hypoxia inducible factor (HIF), whose upregulation in hypoxia is known to initiate transcription of a wide range of genes mediating adaptation and survival (Ke *et al.*, 2006; Schipani *et al.*, 2008).

Similar, short-term exposure studies have been carried out with dermal fibroblasts and hypoxia but the results are less conclusive. Increases in the expression of stress-responsive proteins have been observed in response to hypoxia on human dermal fibroblasts, but the same study found no effects of hypoxia on proliferation (Boraldi *et al.*, 2007). As was seen in osteoblast studies,

short term cultures in hypoxia resulted in notable increases in VEGF mRNA expression and concomitant downregulation of Col1α1 mRNA expression by human dermal fibroblast-like cells (Steinbrech *et al.*, 1999a), suggesting that hypoxia mediates angiogenesis required for tissue repair. Others have found that hypoxia causes increases in collagen synthesis in renal fibroblast-like cells resulting in fibrogenesis (Norman *et al.*, 2000). As such, further work is clearly needed in order to clarify the exact response of fibroblasts to hypoxic insult.

The effects of long term hypoxia on cells producing type I collagen have been somewhat more neglected. Previous work done by our group has begun to examine the long term effects of hypoxia on osteoblasts showing dramatic inhibition of bone formation by cells grown in low oxygen tensions for 7 or 14 days (Utting *et al.*, 2006). The authors suggested that hypoxia induces a dormant state of 'suspended animation' on osteoblasts, resulting in gradual decreases in cellular activity and bone forming ability with decreasing oxygen tension, which is relieved upon reoxygenation. However, there has been little work on the effects of chronic hypoxia on the ability of the fibroblasts of the dermis to differentiate, proliferate and form collagen.

Given the similarities in the behaviour displayed by fibroblasts and osteoblasts to short term hypoxia, it is plausible that fibroblasts would behave in a similar manner to osteoblasts when exposed to long term hypoxia. One study has shown that long term hypoxia suppresses *de novo* elastin production by rat lung fibroblasts (Berk *et al.*, 2005), indicating an inhibitory mechanism similar to that displayed by osteoblasts. However, overgrowth of connective tissue, as occurs in tissue fibrosis, is associated with pathological and disease states where the oxygen tension in the local area is low. Chronic progression of fibrosis results in excessive deposition of extracellular matrix components without resolution (Mutsaers *et al.*, 1997), indicating that even in the lowest oxygen

tensions where collagen formation is likely to be severely inhibited, over-production can still occur. It has been suggested that hypoxia may cause an upregulation of connective tissue growth factor (CTGF), the trigger for fibroblastic differentiation from MSCs, resulting in skin fibrosis. CTGF upregulation has been associated with fibrotic lesions in skin scleroderma and renal fibrosis (Hong *et al.*, 2006; Higgins *et al.*, 2004). This paradox raises questions relating to the behaviour of fibroblasts exposed to long term hypoxia and their ability to differentiate from MSCs, proliferate into fully functional cells and produce collagen.

In this chapter I investigate further the effect of hypoxia on osteoblasts, in particular the effect of low oxygen tension on the expression of some previously unstudied genes, including sclerostin. I then explore the effects of long term hypoxia on collagen production by rat dermal fibroblasts and examine the effects of low oxygen on the adhesive properties and proliferation of these different cell types.

Methods

Materials and methods specific to this chapter are described here, all other methods used are described in Chapter 2.

Adhesion studies

To examine the effect of hypoxia on the adhesion of fibroblasts and osteoblasts to plastic, cells were seeded at 1×10^5 cells per well in 6-well plates and allowed to settle for 6 or 96 hours. After this time period had elapsed the medium was removed from the tissue culture plates and the cells were gently washed in PBS. The osteoblasts and fibroblasts were then trypsinised (0.25% trypsin for 10 minutes at 37°C) and blinded manual cell counts were performed. Manual cell counts had to be performed rather than using the LDH assay; the LDH assay measures the amount of LDH released by the cell upon cell lysis. In hypoxic conditions increased anaerobic respiration results in upregulation of LDH enzyme activity (Robin *et al.*, 1984) and mRNA expression (Firth *et al.*, 1994) and as such, would give inaccurate results in low oxygen conditions.

These experiments were designed based on the principle that in the first 6 hours after plating, cells would attach to the tissue culture plate and a cell count would allow the effect of hypoxia on cellular adhesion to be assessed. After this time, the cells would begin proliferating and thus a cell count performed 96 hours after plating would allow the extent of proliferation under hypoxic conditions to be examined.

Results

Osteoblasts and hypoxia

Hypoxia exerted dramatic inhibitory action on the bone forming ability of osteoblasts. Eight-fold decreases in the amount of bone formed by osteoblasts grown in long-term hypoxia (2% O_2) were observed when compared with those grown in normoxia (20% O_2) (Figure 3.1). Gene expression studies using RNA isolated after 7 and 14 days of culture revealed that exposure to hypoxia caused retardation in the differentiation of osteoblasts, with delays in the onset of expression of osteocalcin (OCN), a key marker of late osteoblast differentiation. Expression of RANKL mRNA was also delayed in osteoblasts exposed to hypoxia. The expression of OPG, the natural decoy receptor for RANKL, appeared to be relatively unaffected by hypoxia (Figure 3.2).

As a key factor in the inhibition of osteoblast function *in vivo*, the potential role of sclerostin in mediating the osteoblastic response to hypoxia was investigated. Sclerostin (SOST) expression appeared to be downregulated by hypoxia, a likely consequence of its increased expression with increasing osteoblastic differentiation. Dentine matrix protein 1 (DMP1), a marker not only for odontoblasts (George *et al.*, 1995), but also for osteocyte formation (Toyosawa *et al.*, 2001) was run alongside SOST and showed clear downregulation (Figure 3.2.), indicating that hypoxia slows the differentiation and maturation of osteoblasts.
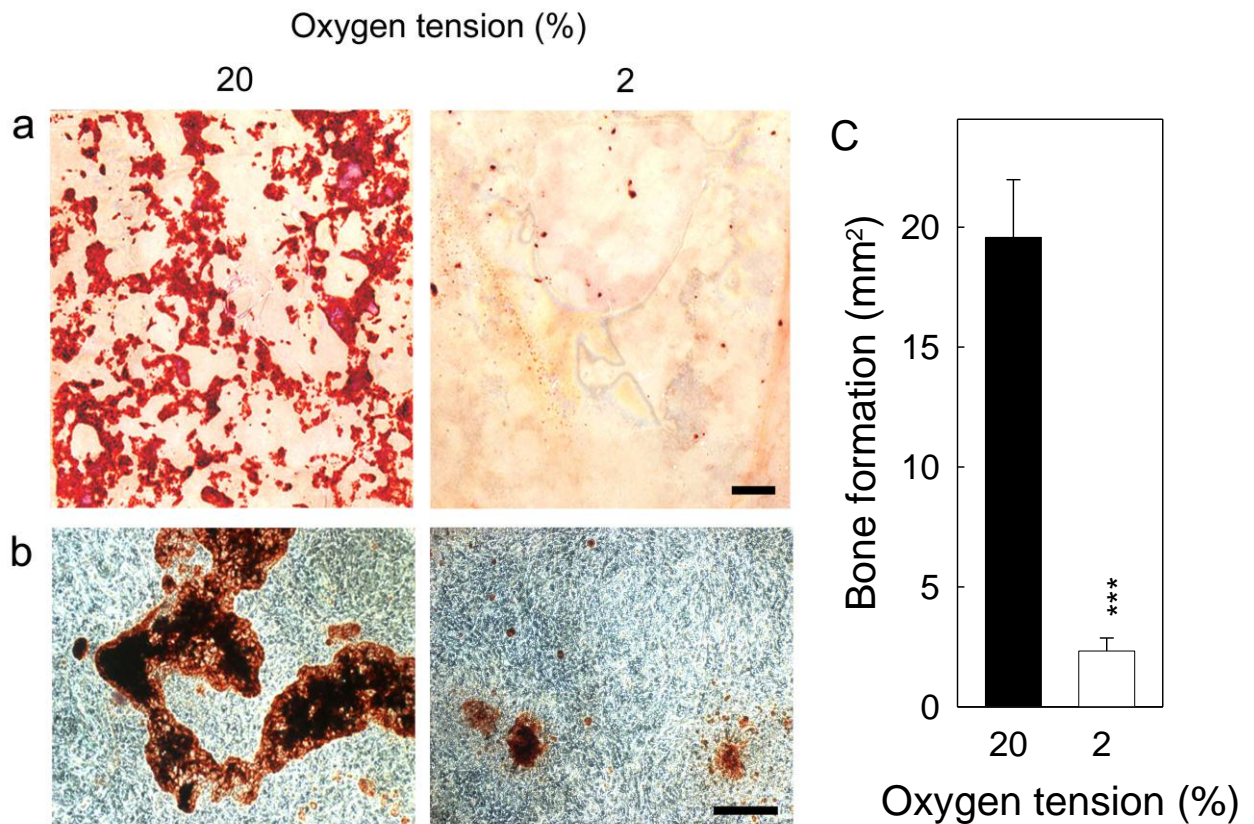
Figure 3.1. *Hypoxia inhibits osteoblast bone forming ability.*

Calvarial osteoblasts from neonatal rats were cultured in supplemented DMEM in 2% or 20% oxygen for 14 days.

a. Scanned images of alizarin red stained mineralised structures formed by osteoblasts revealed strong inhibition of bone formation in hypoxic conditions. Scale bar = 1mm.

b. Alizarin red stained bony structures viewed by phase contrast microscopy showed that hypoxia affects the size, as well as number of trabecular structures formed. Scale bar = 0.2mm.

c. Quantification of bone formation in these cultures by image analysis of alizarin red stained monolayers, revealed that long term exposure to hypoxia causes a 8-fold decrease in bone formation. ***, significantly different from 20% O_2 control, P<0.001.
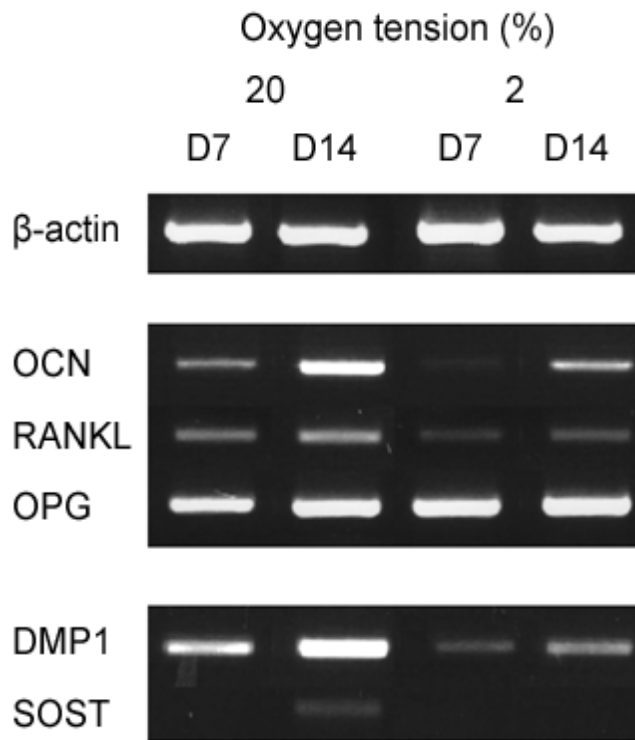
Figure 3.2. *Hypoxia causes changes in gene expression by osteoblasts.*

Osteoblast RNA was extracted after 7 and 14 days of culture in supplemented DMEM in 2% or 20% oxygen and mRNA expression levels assessed using RT-PCR. Hypoxia (2% O_2) caused retardation of osteoblastic differentiation, as displayed by the late expression of mRNA for OCN and RANKL. Expression of OPG was relatively unaffected by hypoxia. DMP1 and SOST (osteocyte markers) were also downregulated by hypoxia suggesting a delay in osteoblast maturation in these conditions. OCN = osteocalcin; RANKL = RANK ligand; OPG = osteoprotegrin; DMP1 = dentine matrix protein 1; SOST = sclerostin.

Dermal fibroblasts and hypoxia

To investigate whether the inhibitory effect of hypoxia on osteoblasts was cell-specific or a general trait common to other collagen producing connective tissue cells, fibroblasts isolated from the dermis of neonatal rat pups were cultured in a gradation of oxygen tensions. Increasing inhibition of growth and collagen forming ability were apparent with decreasing oxygen tension (Figure 3.3). Closer examination in hypoxia (2% O_2) and normoxia (20% O_2) revealed that collagen production by these fibroblasts was affected by hypoxia in a similar way to bone formation by osteoblasts; hypoxia caused severe inhibition of collagen deposition over the course of the culture (Figure 3.4.a. and 3.4.b.) and a significant, 4-fold decrease in production of soluble collagen (Figure 3.5.).

Previous work by this research group has shown osteoblastic expression of VEGF to be strongly upregulated by exposure to long term hypoxia and mRNA for Col1α1 to be relatively unaffected (Utting, 2006). Similar trends were observed when expression of these genes by dermal fibroblasts exposed to the same conditions was examined (Figure 3.6). The expression of Col1α1 mRNA showed a possible mild downregulation in hypoxic condition, while VEGF was strongly upregulated by low oxygen conditions.
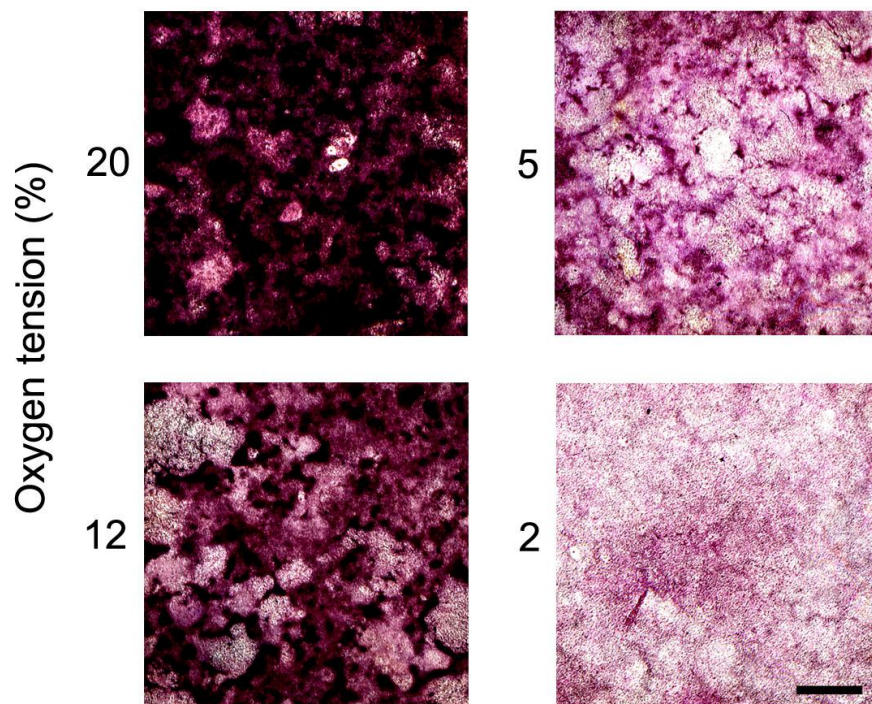
Figure 3.3. *Decreasing oxygen tension inhibits fibroblast collagen production.*

Scanned images of Masson's trichrome-stained dermal fibroblasts cultured for 14 days in DMEM supplemented with 5% FCS and 50 μg/ml ascorbate in 20%, 12%, 5% and 2% oxygen. Images display a general overview of the inhibitory effects of hypoxia on collagen production by dermal fibroblasts, with the darker purple areas indicating more collagen deposition. Scale bar = 2mm.
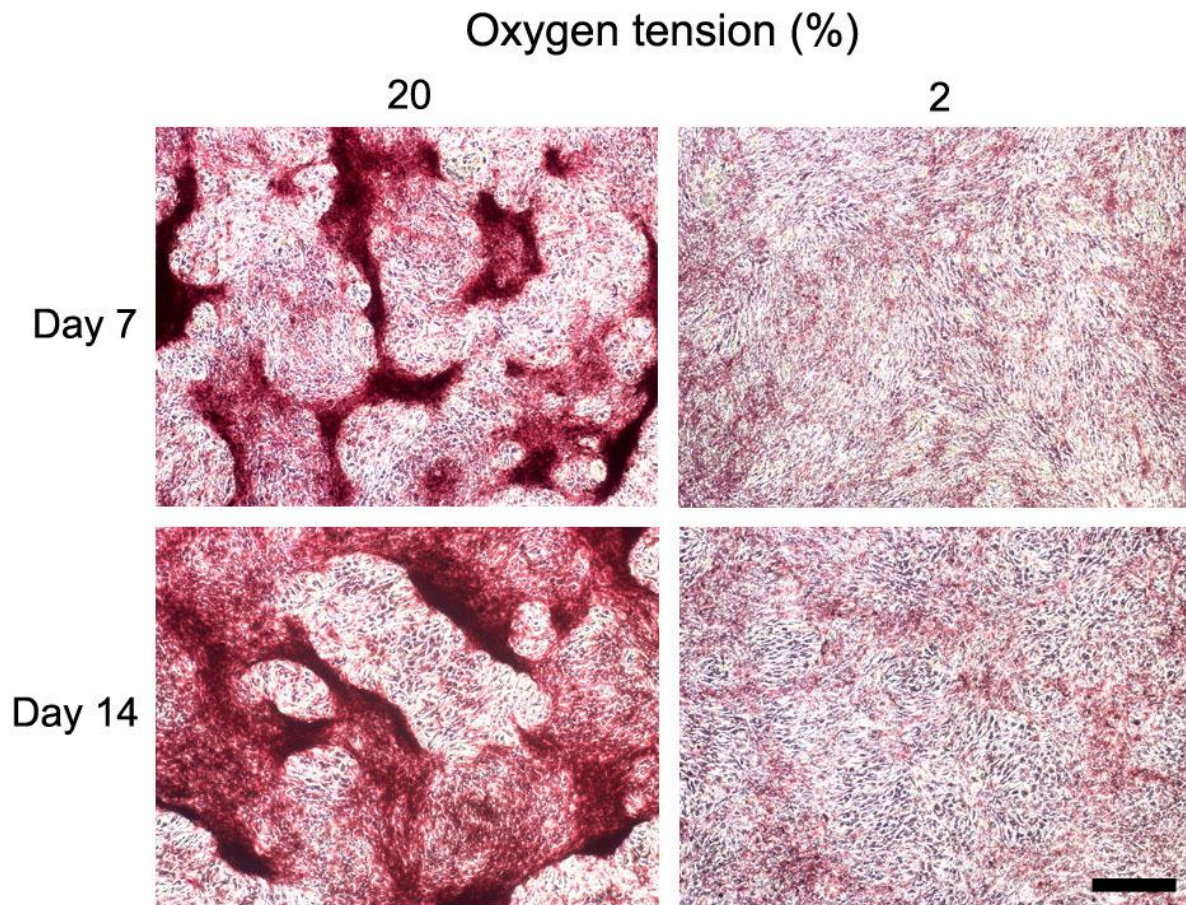
Figure 3.4.a. *Hypoxia inhibits collagen deposition by dermal fibroblasts.*

Rat dermal fibroblasts were grown for 7 and 14 days in DMEM supplemented with 5% FCS and 50 µg/ml ascorbate in 20% and 2% oxygen. Collagen deposits were stained using Masson's trichrome and viewed by reflected light microscopy. Scale bar = 0.5mm.
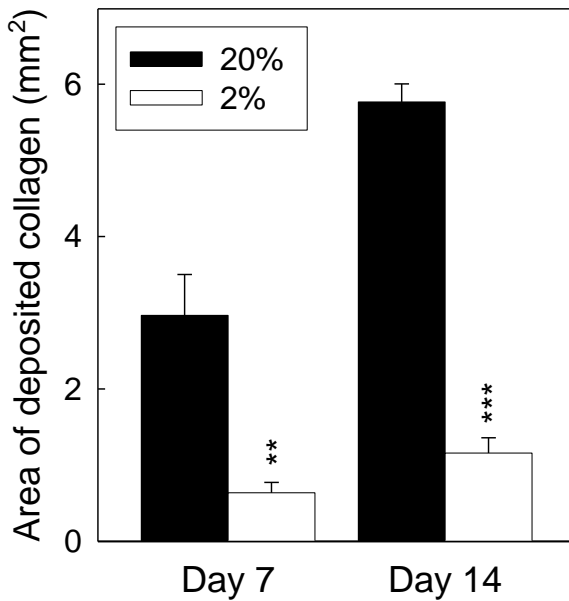
Figure 3.4.b. *Quantification of deposited collagen formation.*

Rat dermal fibroblasts were cultured for 7 and 14 days in DMEM supplemented with 5% FCS and 50 µg/ml ascorbate in 20% and 2% oxygen. Masson's trichrome stained monolayers were quantified by image analysis.

, significantly different from day 7 20% control, $P<0.01$. *, significantly different from day 14 control, $P<0.001$.
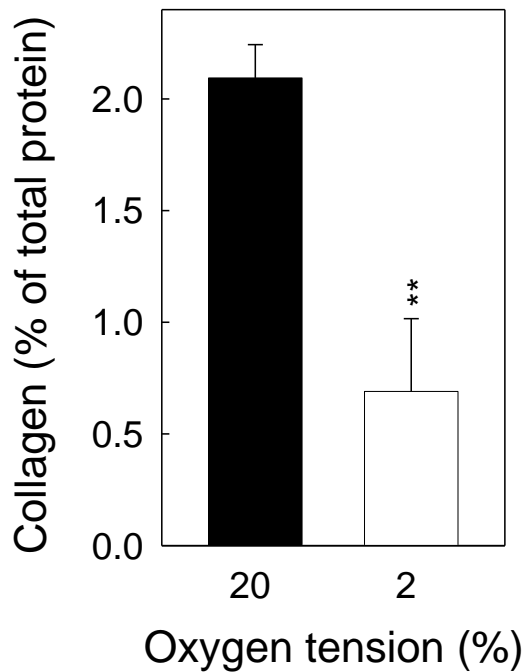
Figure 3.5. *Hypoxia inhibits soluble collagen production by dermal fibroblasts.*

Rat dermal fibroblasts were grown for 14 days in DMEM supplemented with 5% FCS and 50 µg/ml ascorbate in 20% and 2% oxygen. Production of soluble collagen was measured relative to the total cell protein and was seen to be significantly inhibited by low oxygen. **, significantly different from 20% O_2 control, $P<0.01$.

Oxygen tension (%)

20   2

β-actin

Col1α1
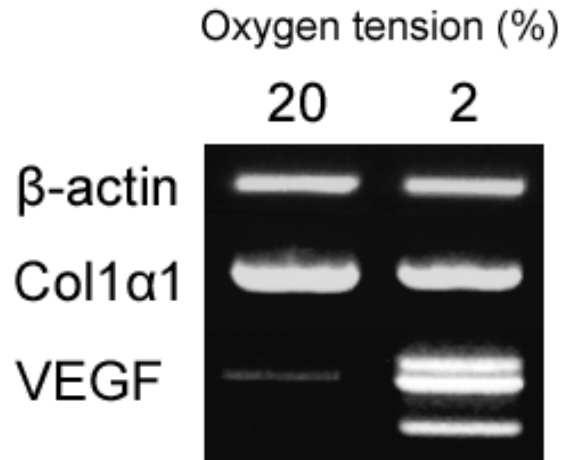
VEGF

Figure 3.6. *Exposure of fibroblasts to hypoxia strongly upregulates VEGF mRNA but does not affect Col 1α1.*

RNA was extracted from dermal fibroblasts grown for 10 days in DMEM supplemented with 5% FCS and 50 µg/ml ascorbate in 20% and 2% oxygen. mRNA expression for type I collagen (Col1α1) was relatively unaffected by hypoxia but expression of mRNA for vascular endothelial growth factor (VEGF) was strongly upregulated. The three bands present represent three mRNA splice variants for VEGF.

Hypoxia does not affect adhesion of osteoblasts or fibroblasts to plastic

Unpublished data from our laboratory suggest hypoxic conditions may promote formation of increased numbers of small colonies in culture from osteoblasts and fibroblasts (K. W. Colston, personal communication), and it was hypothesised that this may be related to the ability of these cells to adhere. To investigate this further, suspended rat calvarial osteoblasts and rat dermal fibroblasts were allowed to sediment onto plastic surfaces in hypoxic and normoxic conditions for 6 hours (the time for initial adhesion of cells to the plastic tissue culture plates to occur). Parallel cultures were maintained for 96 hours by which time cell proliferation would be expected to have commenced. There was no significant difference in cell number after 6 hours of exposure to hypoxia for either cell type when compared to normoxic controls, suggesting hypoxia does not affect the ability of these cells to adhere to plastic. However, hypoxia caused significant

decreases in cell number in both fibroblastic and osteoblastic cultures after 96 hours of exposure to hypoxia (Figure 3.7.).
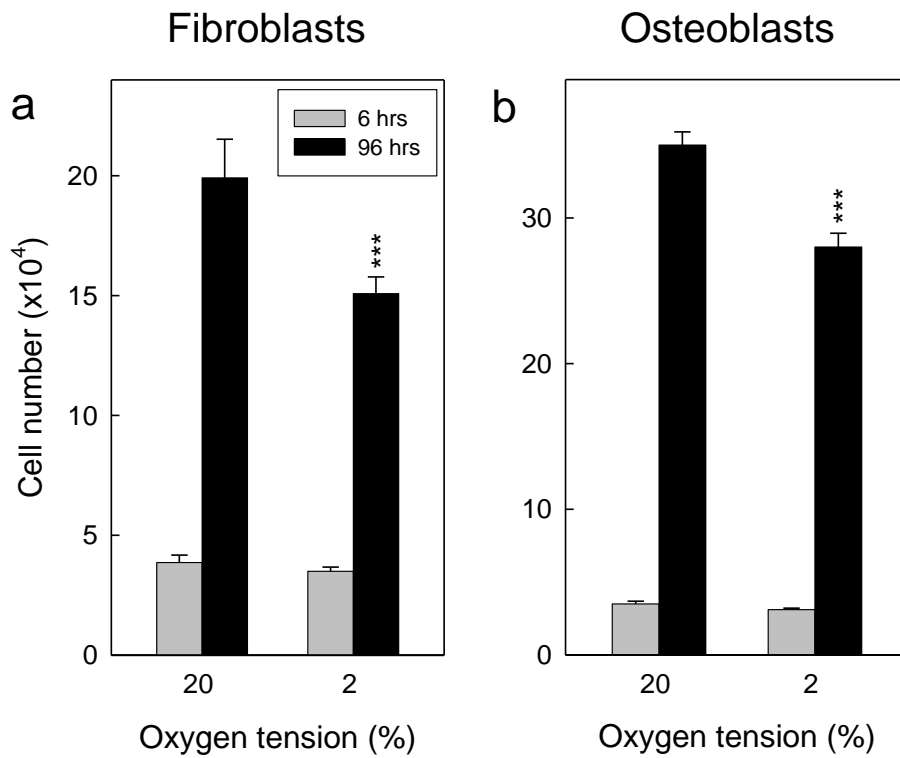
Figure 3.7. *Hypoxia inhibits proliferation but not adhesion of collagen producing cells.*

Dermal fibroblasts were allowed to adhere and proliferate for 6 and 96 hours in DMEM supplemented with 5% FCS and 50 µg/ml ascorbate in 20% and 2% oxygen before blinded manual cell counts were performed.

a. Dermal fibroblasts. b. Calvarial osteoblasts. ***, significantly different from 20% O_2 control, P<0.001.

Discussion

The work presented in this chapter shows that hypoxia inhibits the function of osteoblasts and fibroblasts. The production of collagen by both cell type was particularly affected, with strong inhibition of both bone formation by osteoblasts and collagen deposition by fibroblasts observed. As these cell types are key to the reparative processes in both fracture and wound healing respectively, their adaptation to low oxygen environments is essential to the restoration of healthy tissue. These data provide evidence that both osteoblasts and fibroblasts adapt to low oxygen tensions by regulating cellular differentiation and proliferation.

Before growth, differentiation and collagen formation can occur, cells must adhere to the surface on which they will develop and proliferate, yet the effects hypoxia has on cellular adherence have been paid little attention. *In vivo* studies of low oxygen environments suggest that adherence would be reduced in low oxygen tensions; decreased cell-cell and cell-extracellular matrix interactions as a result of hypoxia are regarded to be the first steps in the progression of tumour metastasis (Kunz *et al.*, 2003). The findings presented in this chapter suggest that hypoxia has little to no effect on adhesion of either osteoblasts or fibroblasts to plastic but does have an inhibitory effect on subsequent proliferation of these cells. However, although decreased, both fibroblasts and osteoblasts retained some ability to proliferative when cultured for 96 hours in hypoxia. While the studies presented here cannot draw conclusions relating to the fate of cells, previous studies indicate that osteoblasts do not undergo apoptosis when cultured in long term hypoxia (Utting *et al.*, 2006). Due to the similar behaviour that is exhibited by fibroblasts and osteoblasts, it is hypothesised that fibroblasts would not undergo apoptosis either, rather, like osteoblasts, fibroblasts display decreased differentiation and adopt a state of cellular quiescence in order to survive.

The method used to generate the hypoxic environment may provide some answers to the reason for this. While cultures were maintained at the desired oxygen tension by gassing the boxes daily (Figure 2.2.), cells were exposed to atmospheric oxygen for a short period of time when media changes were performed. While this short 'burst' of oxygen exposure was not sufficient to induce collagen formation, it may be sufficient to allow cellular proliferation and prevent cell death. However, as the *in vivo* environment is unlikely to remain at very low oxygen tensions for prolonged periods, even in pathological situations, the system used provides a more accurate reflection of the *in vivo* environment than one where cells would be continuously exposed to hypoxia.

The production of extracellular matrix by osteoblast and fibroblasts was significantly decreased by hypoxia. Despite collagen protein production by dermal fibroblasts being severely inhibited by long term hypoxia, mRNA expression revealed only marginal decreases when cells were cultured in low oxygen. This unexpected result could be explained by the oxygen dependence of the collagen modifying enzymes. The prolyl hydroxylases (PHDs), which post-translationally modify the collagen polypeptide chains have decreased activity in hypoxia (D'Angelo *et al.*, 2003; Nakayama, 2009). PHD2 (which is involved in the HIF pathway) is believed to act as the cellular oxygen sensor (Berra *et al.*, 2003). The findings presented in this chapter indicate that the collagen modifying PHDs may also act as cellular oxygen sensors by modulating cellular activity, i.e. collagen production, in response to changing oxygen levels. While collagen mRNA is still synthesised in hypoxic conditions, the PHD enzymes have limited functionality. As such, formation of the stable collagen triple helix does not occur and reduced collagen protein production is observed. This would suggest that collagen synthesis is governed at the translational, rather than the transcriptional level in low oxygen environments. These findings are redolent of the effects of ascorbate deficiency (an additional cofactor required for PHD

activity), which are exemplified by scurvy where decreased collagen synthesis and secretion occur (Kipp *et al.*, 1996; Peterkofsky, 1991). However, it has been reported that ascorbate deficiency also results in significant reductions in collagen mRNA expression which is restored upon ascorbate replenishment (Spanheimer *et al.*, 1986).

Previous reports have suggested that short term hypoxia results in increased collagen formation in the early stages of wound healing due to dermal fibroblasts upregulating TGFβ (Falanga *et al.*, 1991). However, the experiments described in this chapter suggest that collagen production by dermal fibroblasts is inhibited in long term hypoxia and thus it is possible that the effects seen in the early stages of wound healing are produced by cells migrating to the site of injury. Evidence supporting this hypothesis has been published, suggesting that pericytes, relatively undifferentiated cells associated with the walls of blood vessels, migrate from the vessel wall to hypoxic areas (Gonul *et al.*, 2002). Pericyte proliferation is stimulated by hypoxia (Yamagashi *et al.*, 1999) and the increased production of type I collagen by these cells is known to result in fibrosis (Lin *et al.*, 2008).

Cells migrating from other parts of the vessel wall may also play a role in the increased production of collagen in the dermis in hypoxic conditions. While pericytes are located in the basement membrane of the vessel (Joyce *et al.*, 1985), adventitial fibroblasts surround the outer walls of blood vessels. Adventitial fibroblasts, like pericytes, are activated by both short term and long term hypoxia (Das *et al.*, 2001). Both adventitial fibroblasts and circulating fibrocytes have been seen to play a role in the vascular remodelling process in hypoxic conditions (Stenmark *et al.*, 2002; Frid *et al.*, 2006). However, the collagen producing ability of these cells is not discussed in the aforementioned reports. Based on the ideas described above relating to the role of oxygen in collagen formation by these cells it would seem likely that hypoxia would also

inhibit collagen formation by adventitial fibroblasts. As such, attempts were made to isolate and culture cells from the adventitia of rat arteries in order to examine their behaviour when exposed to hypoxia *in vitro*, however, these endeavours proved unsuccessful. Further work examining the collagen forming ability of adventitial fibroblasts would be useful in ascertaining whether their migration to the dermis is responsible for the stimulation of collagen synthesis that is seen in response to short term hypoxia and if fibroblast migration could be linked to fibrosis.

One of the key genes that is upregulated in hypoxia is vascular endothelial growth factor (VEGF), which is secreted by a variety of cells to increase angiogenesis and thus blood flow to the hypoxic area (Shweiki *et al.*, 1992). It has been shown that osteoblasts are one of the cell types which secrete and upregulate VEGF in response to low oxygen (Steinbrech *et al.*, 1999b). Investigations described in this chapter reveal that dermal fibroblasts also upregulate VEGF mRNA in response to hypoxia. Taken together these data suggest that *in vivo* the hypoxic area that develops in and around a wound causes dramatic upregulation of VEGF expression by cells in the locality, resulting in vascular remodelling and ultimately leading to reoxygenation of the area. This increase in oxygen relieves the inhibition of collagen formation by fibroblasts and allows wound healing to progress.

The effects of long term hypoxia on the expression of selected osteoblastic genes was also examined. Increases in sclerostin (SOST) mRNA have been seen to follow VEGF expression in healing fractures (Genetos *et al.*, 2008) implying that hypoxia-induced VEGF expression may be regulating sclerostin. Thus, it is possible that sclerostin could be involved in mediating the inhibitory effect of hypoxia on osteoblasts. Decreased SOST mRNA expression by osteoblasts exposed to hypoxia was observed in this study, which is in good agreement with a report published since these experiments were performed

(Genetos *et al.*, 2010). However, further investigations by Genetos *et al.* led them to conclude that this is mediated through enhanced antagonism of BMP signalling, independent of VEGF, suggesting that the parallel expression of VEGF and SOST seen in healing fractures is coincidental. The results in this chapter suggest SOST expression by osteoblasts is inhibited in hypoxia. It is likely this is a result of decreased differentiation of osteoblasts in hypoxia, a hypothesis supported by the decreased expression of OCN and RANKL in hypoxic conditions. In a normoxic environment, RANKL is expressed by maturing osteoblasts (Atkins *et al.*, 2003), and osteocalcin by mineralising osteoblasts (Desbois *et al.*, 1995), thus their downregulation in hypoxia indicates delayed differentiation.

The experiments described here suggest that dermal fibroblasts respond to hypoxia in a similar way to other mesenchymal stem cell progenitors such as osteoblasts *in vitro* (Utting *et al.*, 2006). These findings strongly reinforce the idea that collagen production is an oxygen dependent process and help to explain the relationship between the highly vascular nature of connective tissues such as the dermis, bone and periodontal ligament, which have high rates of turnover and repair, compared with poorly vascularised tissues, including tendon and cartilage, which show limited regenerative capabilities. Further investigation of the mechanisms underlying the response to hypoxia of collagen-producing cells is certainly warranted, in particular quantitative analysis of fibroblastic collagen mRNA expression of cells grown in hypoxia compared to normoxia. However, the present results provide strong evidence for the oxygen dependence of the wound healing processes in skin and bone.

Chapter 4

A novel method for the isolation of rat long bone osteoblasts

Introduction

In vitro, osteoblast cell culture systems are used to investigate the aetiology of bone diseases and compare the behaviour of bone cells in normal and pathophysiological states. The *in vitro* system provides controlled environments and thus allows direct examination of the effects of test substances or factors on cell growth, proliferation and bone forming ability in an environment that is free from the influence of other cell types which would normally be present in bone.

The classical method for the isolation of osteoblasts for *in vitro* studies uses cells derived from rodent calvarial bones (Yagiela *et al.*, 1977). Several other methods for the isolation of osteoblasts have been developed as alternatives to the calvarial model but none have proved as successful. Attempts have been made to isolate osteoblasts from human trabecular bone (Robey *et al.*, 1985). Although using cells derived from a human source would seem more physiologically relevant than using cells from rodents, the methods used to obtain these cells often have limitations. Human trabecular bone samples can be obtained from surgical waste, however, these samples are regularly sourced from older patients where the number of mesenchymal stem cells (MSCs) with osteogenic potential is decreased (D'Ippolito *et al.*, 1999) and the total cell number achieved at confluency is reduced (Evans *et al.*, 1990). Consequently, *in vitro* cultures using these cells proceed slowly and yield relatively little bone growth when compared with cells obtained from young rats. A bone formation

assay using osteoblasts derived from avian tibia has also been developed (Gay *et al.*, 1993), however, these cells were seen to differentiate and grow more rapidly than those derived from rodent calvaria. This is a likely reflection of the faster bone turnover (and thus more rapid fracture healing) seen in birds compared to mammals (Bennett *et al.*, 1992) and as such differences exist, it may be wise not to use cells from avian sources as a model for a mammalian system.

The rat provides a convenient animal model for the study of bone formation and function. Neonatal pups are readily available to the majority of scientists and are convenient to work with as the rat has a relatively short gestation period (approximately 3 weeks) and produces large litters (on average 8-10 pups). Fragments of rat tibia have been used in attempts to culture long-bone-derived cells but this method of outgrowth is slow with bone formation not evident until 48 days after isolation (Stringa *et al.*, 1995). Consequently, the protocol using the neonatal rat calvaria seems to provide the best model for the study of primary osteoblastic cells to date. The calvariae from these animals provide a plentiful source of young, vigorous, osteoblast precursors which are relatively easy to harvest and culture. Furthermore, these cells exhibit osteoblastic behaviour and produce mineralised bone-like structures after 15 days of growth in supplemented media (Bellows *et al.*, 1986), indicating their suitability for a study model. However, this method is not without its drawbacks; *in vivo* formation of the flat bones, including the skull, mandible, scapula and clavicle, differs from the majority of the other bones in the body. As described in the introduction to this thesis, the flat bones of the body form via intramembranous ossification; MSCs differentiate directly into osteoblasts and bone is synthesised *de novo*. The majority of the other bones in the body including those of the long bones and the vertebrae, form by endochondral (or intracartilaginous) ossification. MSCs differentiate into chondroblasts which form

a cartilaginous model of the skeleton which is replaced by bone after blood vessel invasion and osteoblast proliferation. In addition to these discrete formation mechanisms, the blood supplies to the long bones and flat bones are different; during formation long bones develop their own vascular supply with arteries penetrating the bone (Shim *et al.*, 1968), whereas the flat bones of the skull are, in the most part, reliant on the vascular networks of the periosteum (Pannarale *et al.*, 1997).

Osteoporosis, one of the main focus diseases for bone research, often manifests with fractures in the hip (femoral head) or vertebrae (Center *et al.*, 1999), both bones which form by endochondral ossification. Furthermore, the flat bones, unlike the long bones, do not require a daily input of mechanical loading or strain to maintain their integrity (Lanyon *et al.*, 1984; Carter, 1984). Diseases of malnutrition which result in skeletal abnormalities also exhibit predominant effects in the long bones (Nakamoto *et al.*, 1979). Rickets, which arises due to limited vitamin D / calcium in the diet, results in a failure or delay in calcification of newly forming bone at long bone physes and is often accompanied by osteomalacia in adulthood (Alman *et al.*, 2006). The most apparent effects of rickets are observed in the lower limbs where bowing of the legs is observed resulting in a 'waddling-gait'.

The pronounced differences in the mechanisms of bone formation between the skull (where cells are often obtained from for *in vitro* study) and the long bones (where some of the most profound effects of bone diseases are observed) raise questions relating to the pathophysiological relevance of using a primary *in vitro* culture system of cells derived from the calvaria. While the studies described here have gone some way in providing alternative models for investigation, none has gone so far as to comparatively analyse their findings with those from calvarial cells cultured in a similar manner to validate findings

from previous *in vitro* calvarial studies. To address this, I developed a new method for the isolation and culture of rat osteoblasts derived from the long bones of neonatal animals. In this chapter, I describe the method used for the successful harvesting and culture of these cells. These long-bone-derived osteoblasts are then characterised alongside analogous calvarial cells, with critical examination of their histology, bone forming ability, gene expression profiles and responses to exogenous hormones.

Methods

Materials and methods specific to this chapter are described here; all other methods used are described in Chapter 2.

Long bone osteoblast isolation

Primary osteoblasts were isolated from the long bones of 2-day-old neonatal Sprague-Dawley rats killed by cervical dislocation. The animals' limbs were removed and the long bones excised. The paws were cut off and the skin and flesh scraped away using a scalpel. The bones were washed in PBS and cleaned further to ensure all unwanted connective tissue had been removed. The epiphyses were cut away to remove the growth plate and chondrocytic cells, and the diaphyses was fragmented. These fragments were vortexed in PBS and then allowed to settle before removing the supernatant containing the marrow and remnants of connective tissue. The washed bone fragments were then subjected to sequential enzyme digestion using a three step process (1% trypsin in PBS for 10 minutes; 0.2% collagenase type II in Hanks balanced salt solution (HBSS) for 30 minutes; 0.2% collagenase type II in HBSS for 60 minutes), with the first two digests being discarded. The cells released from the final digest ($\sim 5 \times 10^6$ / animal), were washed and plated into a 75cm^2 flask in DMEM (see Chapter 2 for media details). Cells were pre-cultured for 3-4 days in a humidified atmosphere of 5% CO_2 - 95% air at 37°C, until confluent. Upon confluency, cells were sub-cultured into 6, 12 or 24-well trays in supplemented DMEM, this was regarded as day 1 of culture. Mineralised bony structures typically developed from day 10, giving rise to dense, organised trabecular-shaped networks by days 21-28. Half media changes were performed every 3-4 days.

Results

Long bone and calvarial osteoblasts form similar bony structures in vitro

Growth of osteoblasts isolated from both the calvaria and long bones of neonatal rat pups proceeded over a similar time scale; bony structures formed were of a similar size, shape and exhibited similar trabecular-like structures when grown for up to 21 days in media supplemented with 10nM dexamethasone, 50 µg/ml ascorbate and 2mM β-glycerophosphate (Figure 4.1.).

Closer examination by electron microscopy revealed cells of comparable size and shape depositing collagen and forming extracellular mineralised structures after 28 days in culture (Figure 4.2).

Osteoblasts from autologous calvaria and long bones display similar characteristics in culture

Cell number was estimated using lactate dehydrogenase release by osteoblasts; both calvarial and long bone cells showed comparable increases in cell number over the course of the culture (assessed after 4, 7, 10 and 14 days of growth), indicating the rate of cell proliferation is similar for both cell types, with cell number peaking at day 10 for both types of osteoblast (Figure 4.3). Increases in cell number were accompanied by increases in collagen deposition and bone formation. Progression through the stages of differentiation, proliferation, growth and bone formation by long bone osteoblasts are displayed in Figure 4.4.

Calvarial OB   Long bone OB
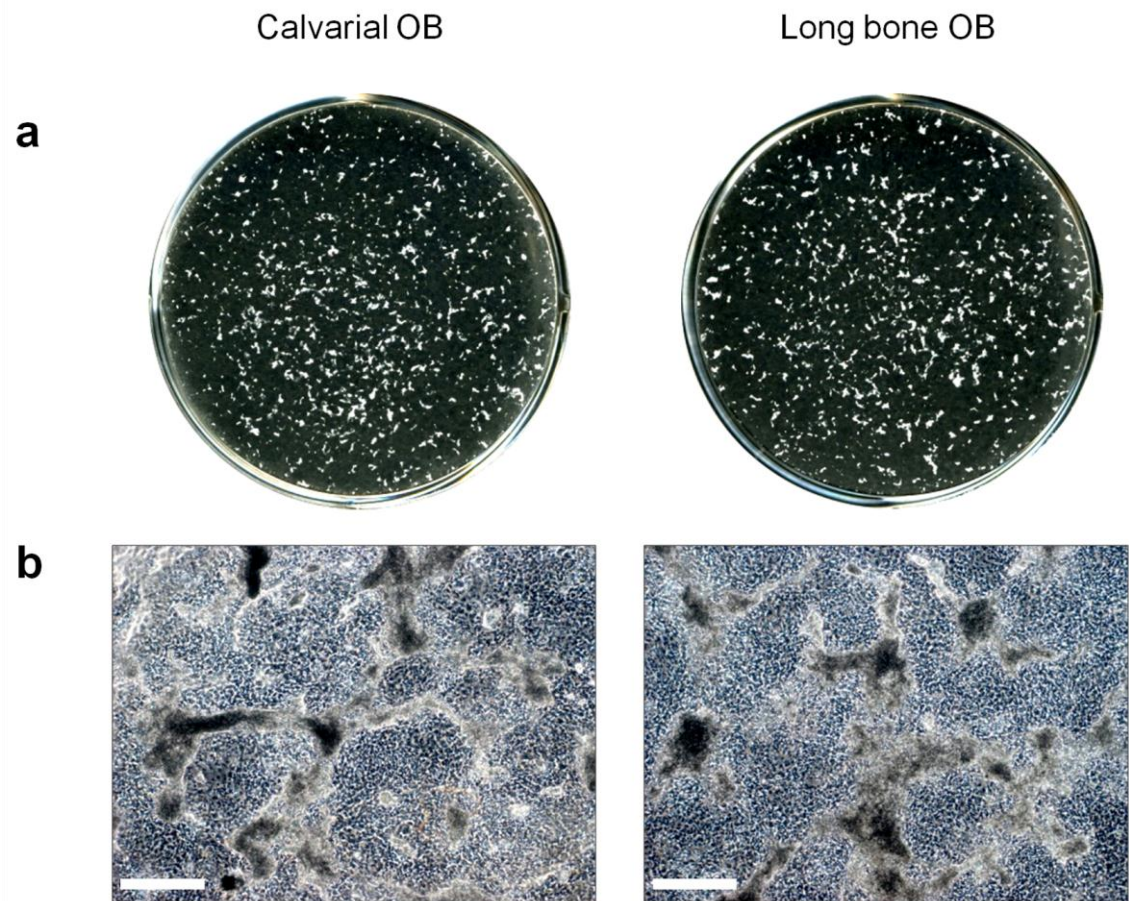
Figure 4.1. *Bone formation by calvarial and long bone osteoblasts.*

Osteoblasts were grown in supplemented DMEM for 21 days.

a. Unstained images of bone formed by osteoblasts viewed by reflected light. Bony structures appear as white features. Well width = 3.5cm.

b. Phase contrast microscopy of unstained trabecular like structures viewed by at x4 magnification. Trabecular-like structures appear grey against confluent cell background. Scale bar = 1mm.
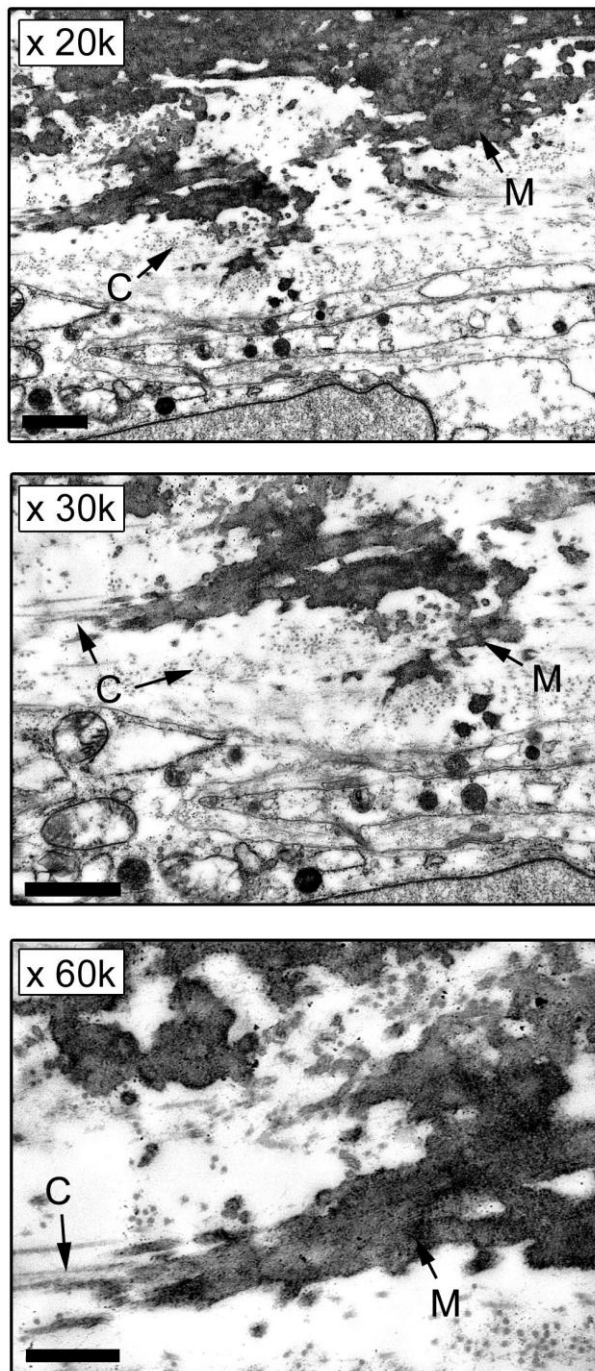
Figure 4.2. *TEMs of the extracellular matrix produced by long bone osteoblasts.*

Osteoblasts were grown in supplemented DMEM for 28 days and then viewed by transmission electron microscopy (TEM). Collagen fibres can be seen longitudinally and in transverse section (appear as dots) indicated by C. Specific mineralisation of the collagenous extracellular matrix is indicated by M. Magnification at x20k and x30k scale bar = 1μm, x60k scale bar = 500nm.
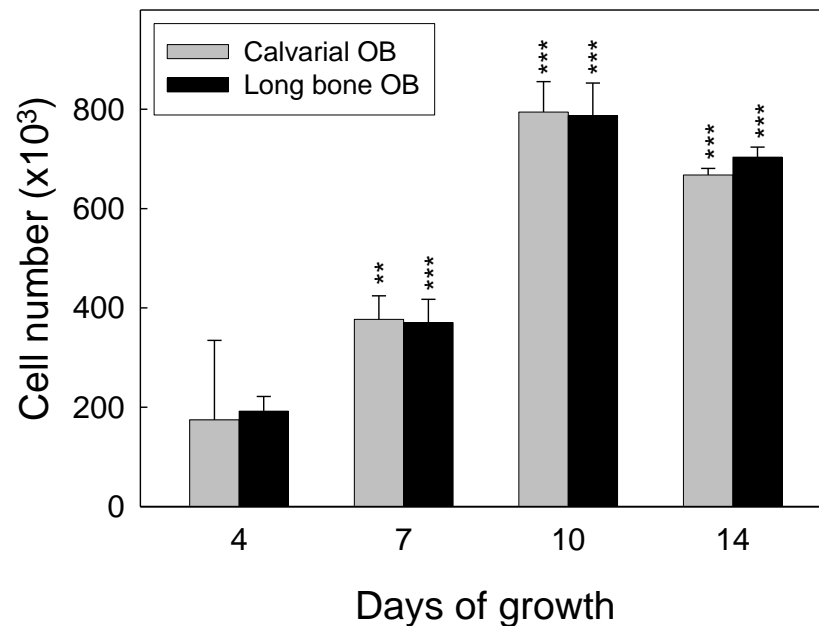
Figure 4.3. *Calvarial and long bone osteoblasts showed parallel increases in cell number over the course of the culture.*

Osteoblasts were grown in supplemented DMEM and cell number assessed by osteoblastic LDH release. At the four time points examined both calvarial and long bone osteoblasts displayed similar cell number suggesting equivalent proliferation rates. **, significantly different from cell number at day 4, $P<0.01$. *** = $P<0.001$.

To assess the dependence of osteoblast differentiation, proliferation and growth on the different supplements in the media, cells were cultured in media with each of the key supplements needed for osteogenesis removed in turn. Removal of any one of these components resulted in a loss of bone forming ability for both calvarial and long bone osteoblasts (Figure 4.5.a.). Alkaline phosphatase (ALP) activity also showed similar trends for both cell types upon removal of culture medium additives. ALP activity was significantly decreased in the absence of dexamethasone or in the absence ascorbate. The role of dexamethasone in osteoblast differentiation is well known and as such decreases in ALP activity would be expected in its absence. However, decreases in ALP activity in response to the removal of ascorbate1 would

suggest that ascorbate may also be playing a role in the differentiation of osteoblasts in addition to it being a cofactor for collagen formation. The removal of β-glycerophosphate resulted in an approximate 1.5-fold increase in ALP activity for both calvarial and long bone osteoblasts, it is likely that the removal of the phosphate source required for mineralisation causes enzyme upregulation in order for phosphate groups to be cleaved from other intracellular sources (Figure 4.5.b.).

β-glycerophosphate concentration

Rat calvarial and long bone osteoblasts were cultured for 14 days before staining with alizarin red to demonstrate mineral deposition. For further assessment of the cellular effects of this deposition, long bone osteoblasts were cultured for 28 days and then prepared for transmission electron microscopy (TEM). In the absence of β-glycerophosphate (β-GP) bone mineralisation failed to occur due to insufficient inorganic phosphate with widespread, unmineralised collagenous matrix evident in the phase contrast and TEM images. Bone formed in the presence of 2mM β-GP displayed typical 'trabecular' morphology and mineralisation was confined to these structures. In the presence of 10mM β-GP, widespread, non-specific deposition of bone mineral occurred across the cell monolayer, with inhibition of normal matrix deposition. Intracellular deposition of mineral that appeared to be causing damage to cell membranes and organelles was also evident (Figure 4.6.).
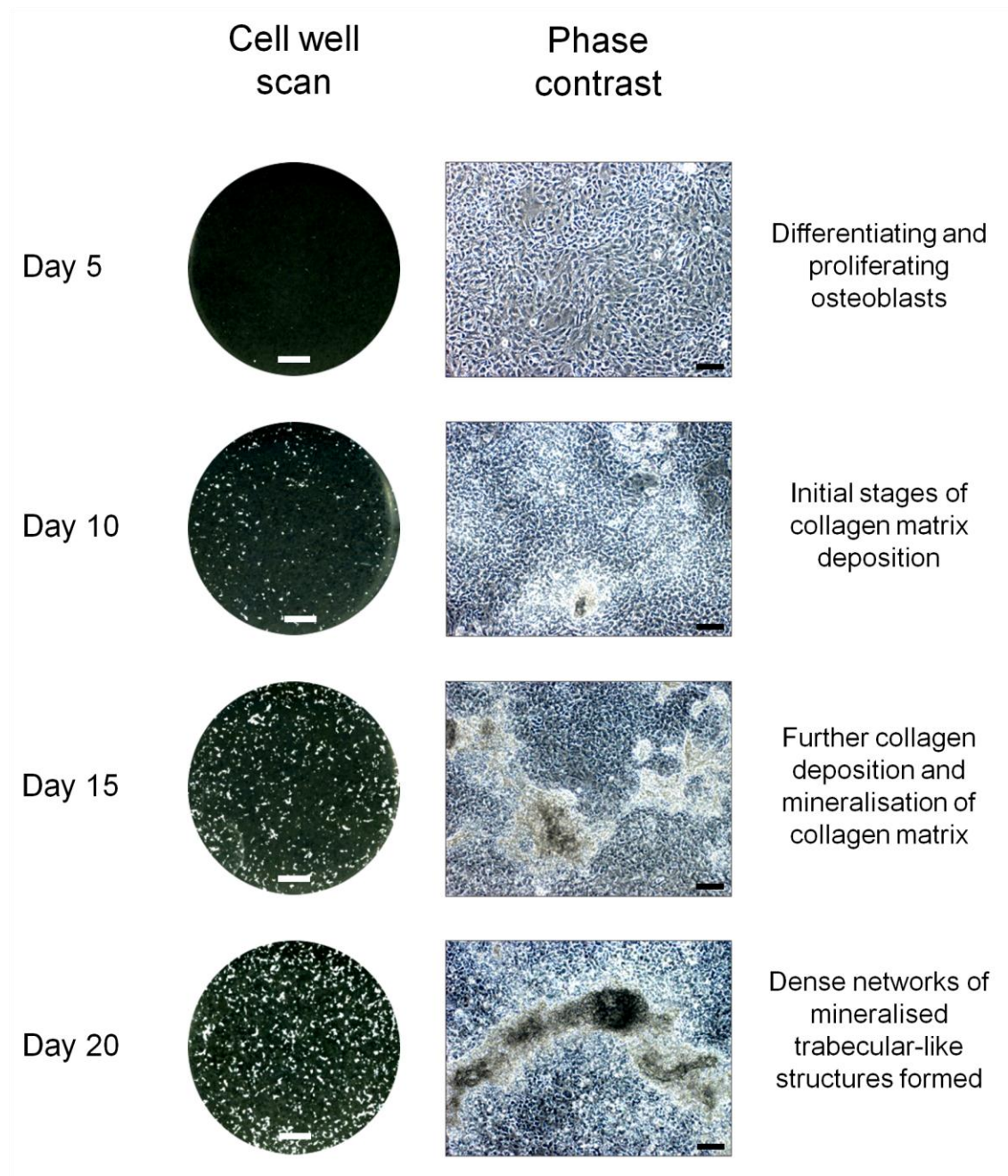
Figure 4.4. *Collagen deposition and bone formation by long bone osteoblasts at selected time points.*

Osteoblasts were grown for the indicated time period in supplemented DMEM. Unstained structures were scanned and viewed by phase contrast microscopy at x4 magnification.

Cell well scan scale bar = 50mm. Phase contrast scale bar = 100µm.
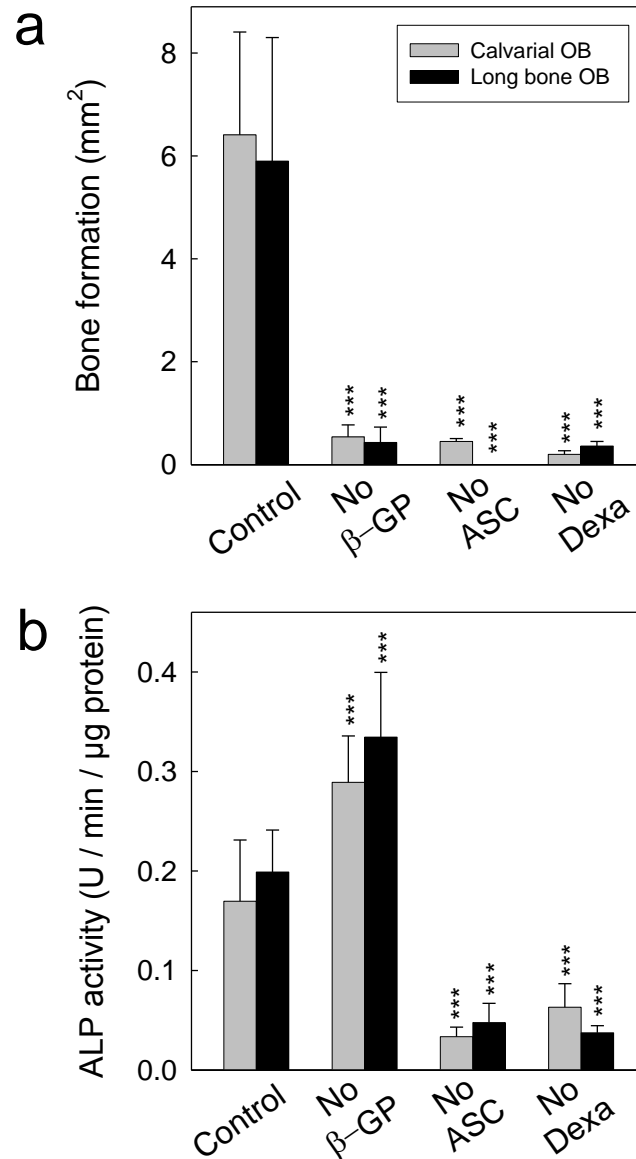
Figure 4.5. *Requirement of calvarial and long bone osteoblasts for key osteogenic culture medium supplements.*

Osteoblasts were grown for 14 days in control media supplemented with 2mM β-glycerophosphate (β-GP), 50µg/ml ascorbate (ASC) and 10nM dexamethasone (Dexa). Cells were also cultured alongside in media with each of these additives removed in turn.

a. Full supplementation of the culture media is required for bone formation by both calvarial and long bone osteoblasts.

b. Alkaline phosphatase (ALP) activity is significantly increased in the absence of β-glycerophosphate but significantly decreased upon removal of ascorbate or dexamethasone from the media. ***, significantly different from control, P<0.001.
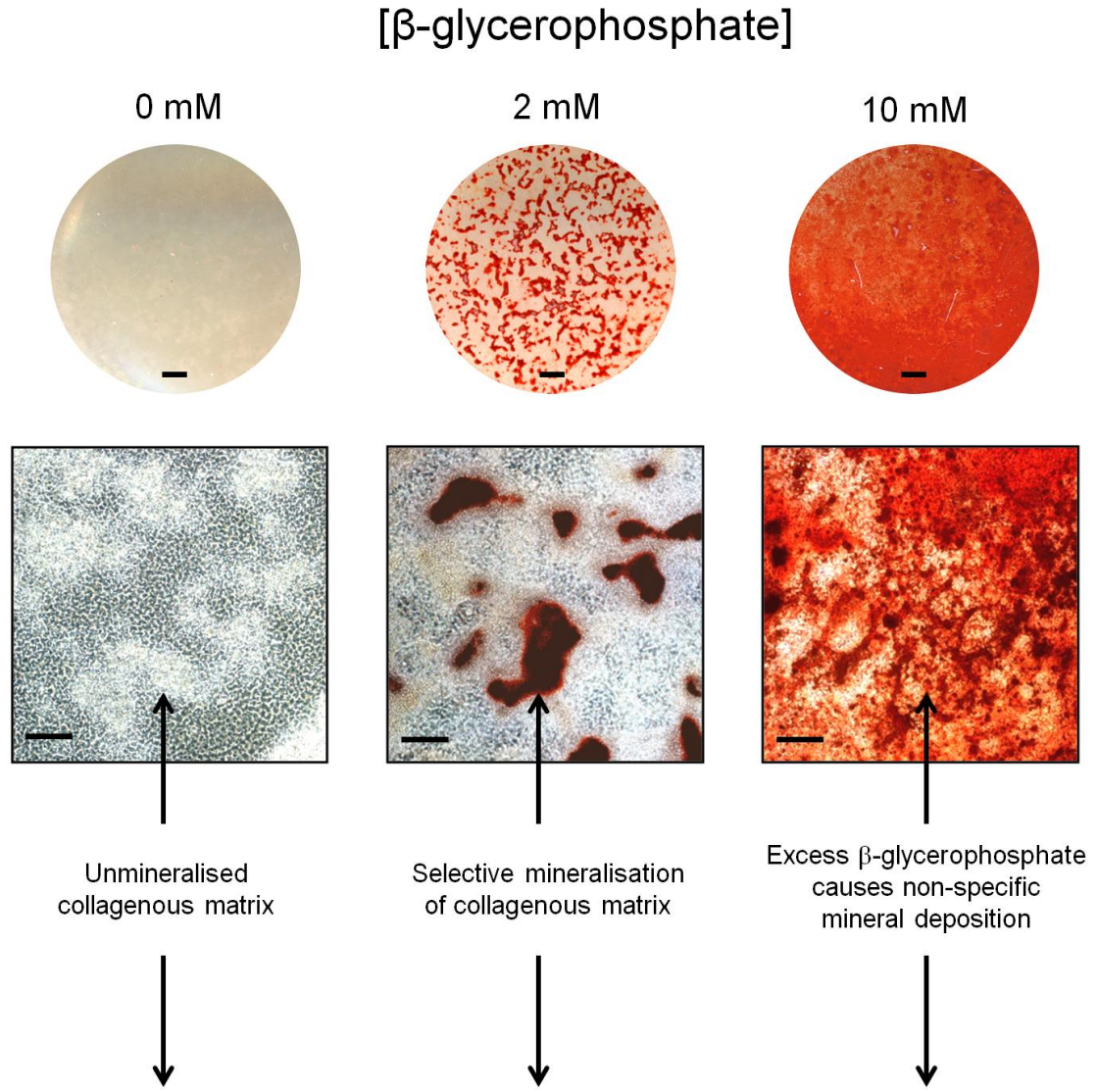
Figure 4.6.a.   *The effect of β-glycerophosphate concentration on bone mineralisation.*

Osteoblasts were cultured for 14 days then stained with alizarin red to demonstrate mineral deposition and cultured for 28 days before visualisation by transmission electron microscopy (TEM).   In the absence of β-glycerophosphate (β-GP), bone formation failed to occur; widespread, unmineralised collagenous matrix is evident in the phase contrast and TEM images (Figure 4.6.b.   overleaf).   Bony structures formed in the presence of 2mM β-GP show specific mineralisation of the collagenous matrix.   In the presence of 10mM β-GP non-specific deposition of bone mineral occurs.   Intracellular mineral deposition causing damage to membranes and internal organelles is also evident in the TEM images.
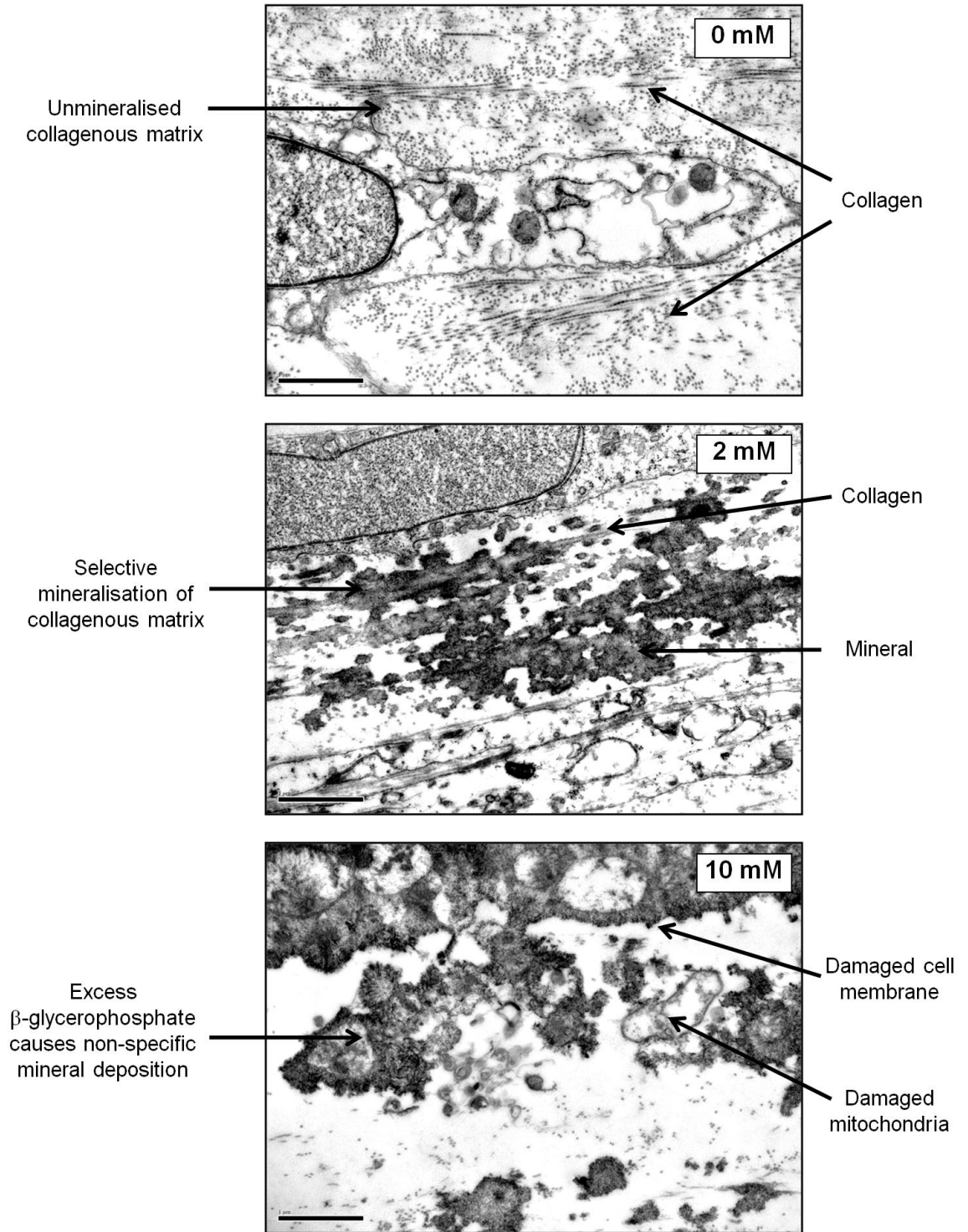
Figure 4.6.b. *TEMs showing the effect of β-glycerophosphate concentration on bone mineralisation.*

Scale bar = 1μm.

Collagen formation

Calvarial and long bone osteoblasts were cultured for 7 or 14 days in supplemented DMEM. Cell monolayers were stained with Masson's trichrome. Clear increases in collagen fibre formation and deposition by osteoblasts in both culture types was observed over the culture period (Figure 4.7.). Measurement of soluble collagen using the Sirius red assay, revealed increases in the amount produced by the cells and released into the media followed the same trend for both osteoblast type, steadily increasing in parallel over the course of the culture (Figure 4.8.).
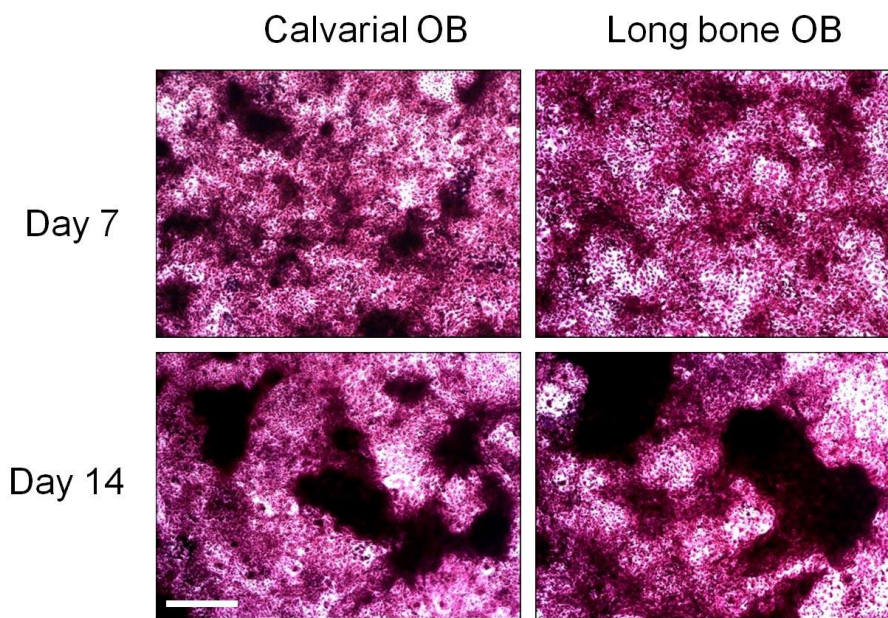
Figure 4.7. *Collagen deposition by calvarial and long bone osteoblasts.*

Cells were grown for 7 and 14 days in supplemented DMEM before monolayers were stained with Masson's trichrome. Dense collagen deposits can be seen as dark purple structures. Scale bar = 0.5cm.
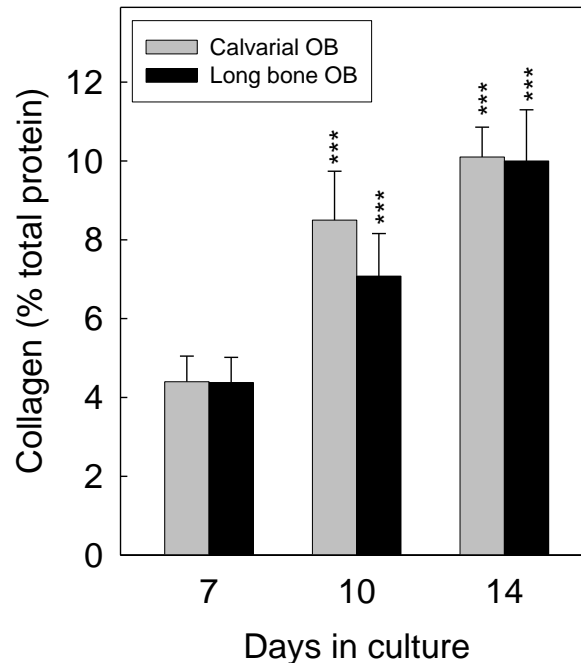
Figure 4.8. *Soluble collagen production by osteoblasts.*

Production of soluble collagen (as a percentage of total protein) measured at days 7, 10 and 14 of culture showed comparable increases for calvarial and long bone osteoblasts. Significance levels were assessed by comparing the test group to the day 7 control. ***, significantly different from day 7 control, $P<0.001$.

Gene expression profiles

Calvarial and long bone osteoblasts displayed comparable expression of osteogenic marker genes at days 7 and 14. Osteocalcin (OCN), a marker of osteoblast differentiation, was barely detectable at day 7 but was strongly expressed by day 14. Increases in mRNA expression were also seen for other markers of osteoblastic differentiation including; osterix (OSX), RANK ligand (RANKL) and collagen type 1 (Col1α1). Expression of the additional osteoblast maturity markers alkaline phosphatase (ALP), osteopontin (OPN), matrix gla protein (MGP) and osteoprotegrin (OPG) (Figure 4.9.), remained constant over time.
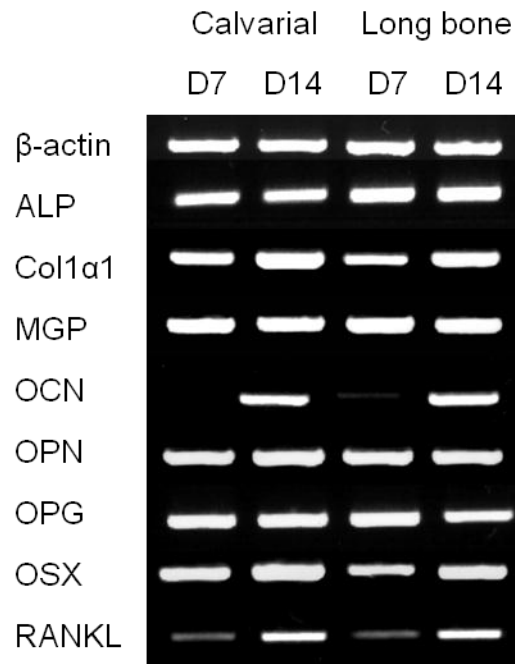
Figure 4.9. *Parallel expression of mRNA for a range of osteogenic markers by calvarial and long bone osteoblasts.*

mRNA assessment using RNA extracted from osteoblasts after 7 and 14 days of culture in supplemented DMEM revealed comparable expression profiles for osteoblastic gene markers for calvarial and long bone osteoblasts at day 7 and day 14.

ALP = alkaline phosphatase; Col1α1 = collagen type I, alpha I; MGP = matrix gla protein; OCN = osteocalcin; OPN = osteopontin; OPG = osteoprotegrin; OSX = osterix; RANKL = RANK ligand.

Osteocalcin mRNA expression was also assessed by qRT-PCR in order to measure the magnitude of the upregulation at day 14 relative to day 7. 36- and 32-fold increases in calvarial and long bone osteoblast OCN mRNA expression, respectively, were observed. Expression of key transcription factors involved in differentiation along other MSC pathways was also quantitatively examined. The 'master regulator' of adipogenesis, PPARγ, showed marginal decreases over the time period of 0.6 and 0.8 fold in calvarial and long bone osteoblasts, and expression of Sox9, a key transcription factor in chondrocyte differentiation, increased by 1.4 and 2.3 fold respectively (Figure 4.10).
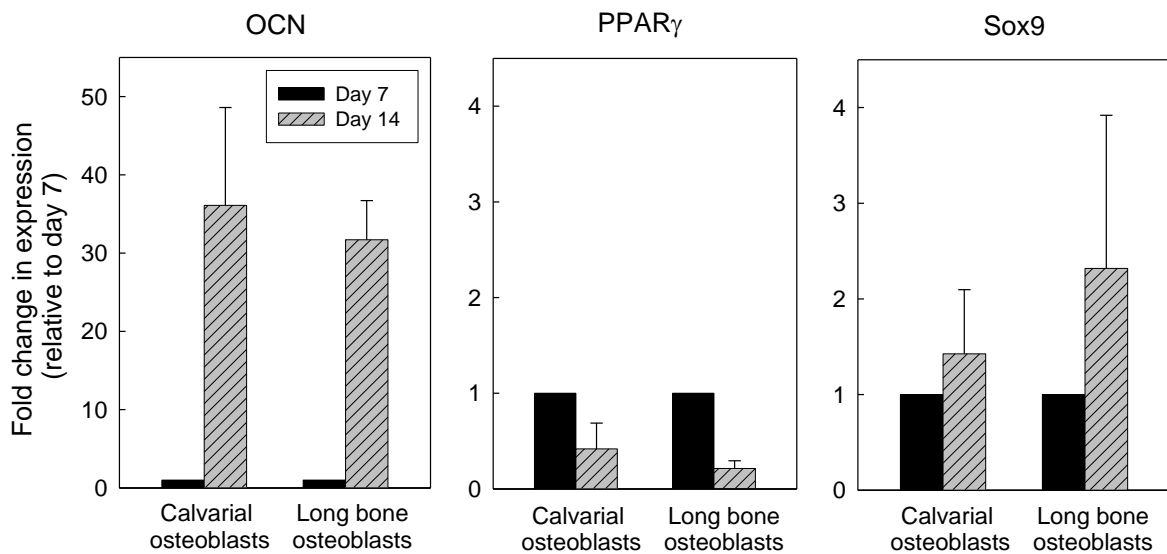
Figure 4.10. *Calvarial and long bone osteoblasts showed dramatic upregulation of osteocalcin but not PPARɣ and Sox9 at day 14 relative to day 7.*

mRNA was extracted from osteoblasts after 7 and 14 days of culture in supplemented DMEM. Quantification of gene expression by qRT-PCR revealed dramatic upregulation of OCN at day 14 relative to day 7 but only marginal changes in the expression of PPARɣ and Sox9.

Response to sex hormones

Both 17-β-oestradiol, the major oestrogen secreted by the pre-menopausal ovary, and 5α-androstan-17β-ol-3-one (dihydrotestosterone (DHT)), the active form of testosterone elicited dose-dependent decreases in bone formation. Total inhibition of bone formation was seen at the highest concentrations (100nm) tested of both hormones (Figure 4.11.).
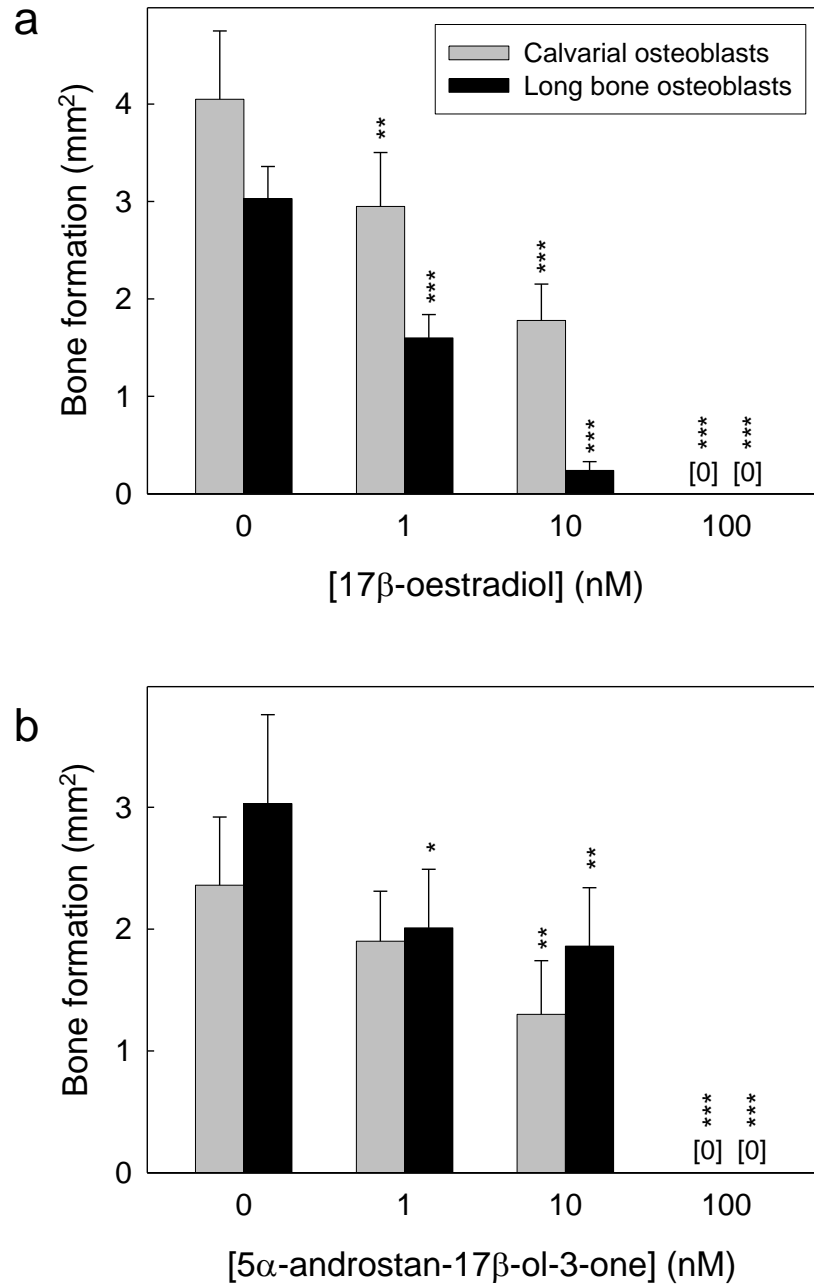
Figure 4.11. *Dose-dependent inhibition of bone formation by gonadal steroids in cultures of calvarial and long bone osteoblasts.*

Osteoblasts were grown in for 14 days supplemented DMEM in the presence of:

a. 17β-oestradiol.

b. 5α-androstan-17β-ol-3-one.

*, significantly different from control, P<0.05. ** = P<0.01, *** = P<0.001.

Discussion

In vitro models for the study of osteoblast behaviour are required for the direct examination of cellular response to exogenous stimuli and thus provide an insight to the *in vivo* behaviour of these cells. It is essential that the cells used in these models behave as closely as possible to the cells in bone tissue, providing robust, reproducible results that can be relied upon.

The experiments described in this chapter indicate that calvarial and long bone osteoblasts exhibit the same behaviour *in vitro*, displaying no clear differences in genotype, phenotype or cellular activity for the parameters tested.

The structures formed by calvarial and long-bone-derived osteoblasts form an extracellular matrix that contains significant amounts of collagen and undergoes selective mineralisation in the presence of a phosphate source. Over the course of the culture trabecular-like structures develop which are approximately the same size and shape as those seen *in vivo*. The spatial arrangement of these structures is similar to that of trabecular bone, with the appearance of spaces that are not dissimilar to those that accommodate vascular channels and bone marrow *in vivo* (Figure 4.12.). These findings indicate that the extracellular matrix formed by these osteoblasts is osseous and are supported by the gene expression studies which revealed dramatic upregulation of osteocalcin in these cells. In addition to this the negligible changes in markers for adipogenesis (PPARγ) and chondrogenesis (Sox9) confirm that the isolated cells are not differentiating along other MSC pathways. Therefore, it can be said with a great deal of confidence that these cells are osteoblasts and the mineralised extracellular structures formed are bone.
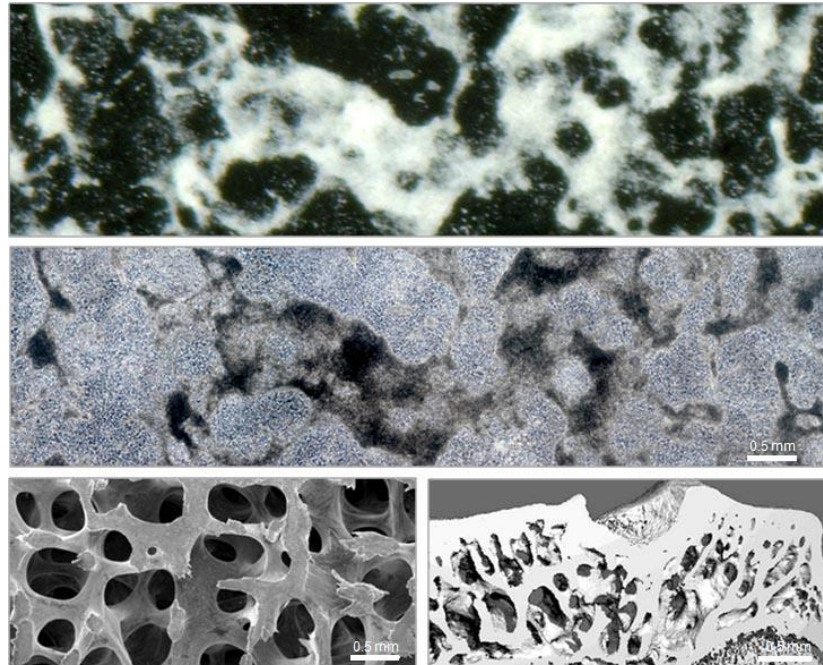
Figure 4.12. *Bone formation by rat long bone osteoblasts and human trabecular bone.*

TOP: Scanned image of bone formed by long bone osteoblasts grown for 28 days in supplemented DMEM. MIDDLE: The same section viewed by phase contrast microscopy. BOTTOM LEFT: Human trabecular bone imaged using scanning electron microscopy (courtesy of Professor Tim Arnett, UCL). BOTTOM RIGHT: Mouse trabecular bone viewed by micro-CT (courtesy of Dr. Isabel Orriss, UCL). Note trabeculae in bottom panels are of approximately similar scale to trabecular features formed *in vitro*. All scale bars = 0.5mm.

The rate of bone formation in these cultures is directly related to the proliferation and maturation rates of the osteoblasts. For both calvarial and long bone osteoblasts, the cellular population approximately doubles between day 4 and day 7, and then again between day 7 and day 10. The images in Figure 4.4. show that the cells are confluent by day 5 and after this time point cell density, and thus cell number, continue to increase. As the culture progresses collagen is deposited by the osteoblasts and mineralisation takes place. The decrease in cell number observed at day 14 (relative to day 10) is a likely consequence of the increased bone formation in the cultures, trapping cells in the mineralised matrix

and rendering them less accessible to the assay reagents. While a decline in replicative capacity of the cells would be predicted to have occurred by day 14 (Rubin, 2002), the total cell number is likely to be higher than that at day 10 if all cells were accessible to the assay reagents and could be counted.

Bone formation by these cells is dependent upon the medium being supplemented with the appropriate additives to facilitate osteogenesis: dexamethasone is required for osteoblastic differentiation from mesenchymal stem cell precursors, ascorbate is an enzyme cofactor for collagen synthesis and β-glycerophosphate provides the phosphate source. While the effect of removing any one of these supplements resulted in almost total abolition of bone formation, the effects on alkaline phosphatase activity were somewhat more varied. Omission of ascorbate from the culture medium caused significant decreases in alkaline phosphatase activity, suggesting that the effects of ascorbate on bone formation go beyond that of being a cofactor for the collagen forming enzymes. It has been suggested that ascorbate has inhibitory effects on kidney alkaline phosphatase which are diminished in the presence of an enzyme substrate (Miggiano *et al.*, 1983). Other reports indicate that ascorbate may be involved in the differentiation and proliferation of osteoblasts from their MSC precursor cells (Franceschi, 1992). Based on the data presented in here, it is not possible to say which role ascorbate is playing in these cultures; further work assessing the differentiation of cells grown in medium lacking ascorbate would required in order for conclusions to be drawn.

The presence of a phosphate source in osteogenic culture medium is essential for mineralisation of the extracellular matrix; β-glycerophosphate is widely used as it provides a stable source of phosphate that is cleaved by alkaline phosphatase when it is required. The concentration of β-glycerophosphate in the medium must be sufficient for selective matrix mineralisation to occur but not in such excess that mineral deposition occurs in

an uncontrolled manner. A review of the available literature on the subject revealed that many studies are performed using 10mM β-glycerophosphate, based on the methodological reports that have been published (Jonsson *et al.*, 1999; Nefussi *et al.*, 1985); one study used β-glycerophosphate as high as 50mM (Buttery *et al.*, 2001). The experiments described here suggest that these concentrations are far too high and would result in non-specific mineralisation of the cell monolayers. As bone formation is used as the end-point for these cultures, this is likely to result in inaccurate quantification of such formation. In addition to this, the electron micrographs shown in this chapter reveal damaged cell membranes and intracellular mineral deposition; it would appear that a concentration of such magnitude leads to an effective 'fossilisation' of cells. Thus, for successful cell growth and selective mineralisation of the collagen structures produced by the osteoblasts, a concentration of β-glycerophosphate of, or around, 2mM should be used.

When β-glycerophosphate was removed from the medium altogether, approximately 1.5-fold increases in alkaline phosphatase activity were observed by both calvarial and long bone osteoblasts. This phenomenon has also been observed in microbes, where increased alkaline phosphatase activity was seen in response to growth in phosphate limited environments (Nakamoto *et al.*, 1979; Raheb *et al.*, 2006). It is likely that upon removal of the phosphate source alkaline phosphatase activity is upregulated as a compensatory mechanism; cleaving phosphate groups off other molecules in an attempt to maintain homeostasis and retain the mineralisation capacity of these cells.

Assessment of the response to both calvarial and long bone osteoblasts to the physiological concentrations of gonadal hormones (Roddam, 2008) revealed dramatic inhibition of bone formation. While previous reports suggest that sex hormones increase osteoblast differentiation and proliferation *in vitro*

(Ernst *et al.*, 1988; Gray *et al.*, 1992; Scheven *et al.*, 1992) the ability of these osteoblasts to form bone was not assessed. *In vivo* the sex hormones, particularly oestrogen, are essential to the maintenance of bone, exemplified by the onset of osteoporosis in post-menopausal women (Turner *et al.*, 1994). The inhibition of bone formation that is observed when osteoblasts are exposed to both oestradiol and DHT indicate that the primary role of these hormones is not anabolic. Consequently, the *in vivo* association between sex steroids and increased BMD (Greendale *et al.*, 1997) is likely to be related to the effect of sex hormones on other cell types. In addition to the protective effect oestradiol has on bone, one possible explanation could be related to the effect of gonadal steroids on endothelial cells. Sex hormones have been associated with vasodilation/vasorelaxation (Orshal *et al.*, 2004; Khalil, 2005) and as such may influence bone formation by increasing its vascular supply.

The data presented in this chapter indicate that although osteoblasts from the calvaria and long bones form bone via different mechanisms *in vivo*, when in culture, bone is formed *de novo* by all cells. A recent report has described differences in expression of the developmental Hox genes between cells derived from the skull and the limb both *in vivo* and in cell culture (Rawlinson *et al.*, 2009). However, given that no clear differences are observed in bone formation by calvarial and long bone osteoblasts described in this chapter, it would appear that osteoblasts have an intrinsic program for the *in vitro* formation of bone which is independent of their anatomical site of origin. The absence of cartilaginous differentiation factors in the *in vitro* environment and the presence of dexamethasone, which rapidly induces osteoblastic differentiation (Rickard *et al.*, 1994), is likely to be the reason for the direct formation of bone in these cultures. Therefore, it would appear difficult to recapitulate endochondral ossification *in vitro* as in essence one would have to recreate the exact timings and sequence of chondrogenic and osteogenic stimuli. Recently, Scotti *et al*, claim to have

achieved this feat. By culturing human MSCs *in vitro* with the appropriate chondrogenic stimuli they argue that it is possible to form endochondral bone *in vitro* and then use bone formed in this manner for successful bone grafts (Scotti *et al.*, 2010).

The novel method for the isolation of long bone osteoblasts that has been described here can be used in addition to the pre-existing calvarial osteoblast assay to obtain osteoblasts, allowing more cells to be yielded from a single animal and providing an alternative method for the study of osteoblast behaviour.

Chapter 5

PTH inhibits bone formation by osteoblasts *in vitro*

Introduction

The use of 'intermittent' parathyroid hormone (rhPTH 1-34) to induce bone growth is becoming increasingly widespread (Tashjian *et al.*, 2006). Intermittent PTH treatment was the first anabolic bone therapy approved in the UK by the National Institute of Clinical Excellence (NICE) and by the US Food and Drug Administration (FDA) for the treatment of low bone mass (Mauck *et al.*, 2006). It is prescribed to women with post-menopausal osteoporosis, men with primary or hypogonadal osteoporosis or men and women with glucocorticoid induced osteoporosis (Kurland *et al.*, 2000; Saag *et al.*, 2007). For all these groups the recommended dosage is 20µg by daily subcutaneous injection. Peak serum concentrations of 4.86µM (Satterwhite *et al.*, 2010) are reached approximately 30 minutes after administration and decline to almost undetectable levels within 3 hours, due the relatively short half life of the drug. The 20µg dose causes transient increases in the serum calcium concentration, beginning after approximately 2 hours and peaking between 4 and 6 hours post administration. Serum calcium levels return to baseline between 16 to 24 hours after each dose (Eli Lilly - Forteo prescription information, 2002; Hodsman *et al.*, 2005). As such, once daily injections provide fluctuations in the blood serum concentration of PTH resulting in an 'intermittent' dosing pattern, reflecting the natural cyclical variations of the amount of PTH circulating in healthy, hormone balanced individuals.

Despite much investigation over the past few decades the precise biochemical mechanisms mediating the anabolic effects of PTH and the reasons

for the phenotypic differences arising due to variations in dosage and exposure patterns remain elusive (Jilka, 2007). Several suggestions as to how intermittent PTH treatment results in bone formation have been proposed. As described in Chapter 1 and reviewed by (Hock, 2001), treatment with PTH is thought to increase bone turnover resulting in a net gain in trabecular bone volume. The increased bone mineral density (BMD) is a result of improved structure in both cortical and cancellous bone (Jiang *et al.*, 2003) and it has been suggested that the number and thickness of trabeculae are also increased (Seeman *et al.*, 2001). Intermittent PTH treatment has also been reported to increase osteoblast differentiation (Ishizuya *et al.*, 1997) and have anti-apoptotic effect on osteoblasts, increasing cell survival and thus bone forming potential (Jilka *et al.*, 1999).

More recent findings have implicated the Wnt signalling pathway in the osteoblastic response to PTH; key molecular components of the pathway have been shown to be regulated by PTH (Kulkarni *et al.*, 2005; Kakar *et al.*, 2007; Chen *et al.*, 2009). Of particular interest is sclerostin (SOST), the osteocyte secreted inhibitor of bone formation (Winkler *et al.*, 2003); it has been suggested that SOST is a target gene for PTH, whereby PTH inhibits the secretion of SOST from osteocytes (Keller *et al.*, 2005; Bellido *et al.*, 2005). These findings have been furthered by studies using transgenic animals where SOST gain-of-function and loss-of-function mutants were seen to display attenuated responses to intermittent PTH treatment, implying that SOST contributes to the PTH-induced bone gain *in vivo* (Kramer *et al.*, 2010). These studies have provided important insights into the mechanism of PTH action and the interaction between different cells in the bone environment, however, it would seem unlikely that relief of the inhibitory action that SOST has on osteoblasts would be sufficient to induce the anabolic effect observed in response to intermittent PTH treatment.

In addition to its effects on bone formation PTH has also been implicated in angiogenesis. Both VEGF and its receptors are expressed by osteoblasts (Tombran-Tink *et al.*, 2004). Upregulation of VEGF (as occurs in hypoxia) results in increased blood vessel formation and thus greater vascularisation of the environment. PTH (1-34) has been shown to stimulate VEGF expression up to 5-fold when administered in combination with vitamin D in osteoblast-like cells (Schlaeppi *et al.*, 1997). Furthermore, the C-terminal PTHrP peptide (PTHrP (107-139)) has been seen to stimulate rapid and transient increases in VEGF expression at both the transcriptional and protein level in human osteoblastic cells (Esbrit *et al.*, 2000). Taken together, these findings suggest PTH may cause an upregulation in VEGF production by osteoblasts and as such, the anabolic effects may be mediated by neovascularisation.

To fully comprehend how PTH mediates bone anabolism an understanding of its direct effects on osteoblast function is vital; however, this area has been neglected. Some *in vitro* studies have been performed; among the most notable findings are that: PTH stimulated the proliferation of human trabecular bone cells (Macdonald *et al.*, 1986); PTH decreased osteocalcin production by human bone cells (Beresford *et al.*, 1984) and that PTH had an inhibitory effect on the differentiation and bone formation of cells derived from rat calvaria (Bellows *et al.*, 1990). These reports suggest that PTH exerts an inhibitory effect on formation and function, despite promoting early proliferation. However, all of these data were published before the true anabolic effects of PTH were widely recognised and, as such were not considered in the same context as they may be today.

More recently is has been suggested that the anabolic affect that is seen *in vivo* can be replicated *in vitro* (Kondo *et al.*, 2009); the authors propose PTH acts primarily on immature osteoblasts, stimulating their differentiation and thus

leading to increased bone formation. However, the dosing pattern used was not reflective of PTH treatment that results in anabolism *in vivo* and as such it is difficult to conclude that this effect alone accounts for the increased bone formation.

Given the importance of PTH as a bone anabolic therapy it is essential that the controversy surrounding its possible mechanism of action be resolved. In this chapter I explore the direct effects of PTH on bone formation using the long bone osteoblast culture system developed and described in Chapter 4. Osteoblasts exposed to PTH in continuous and intermittent doses are examined and the effects on the activity and bone forming potential of these cells are assessed. The direct effect of sclerostin on osteoblasts is also examined. The potential upregulation of angiogenesis is explored and the role of the vasculature in mediating the anabolic response to intermittent PTH treatment is discussed.

Methods

Long bone osteoblasts were isolated and cultured as described in Chapter 4. All other materials and methods used in this chapter are described in Chapter 2.

To examine the response of osteoblasts to PTH (Parathyroid hormone fragment 1-34 rat, CAS number 98614-76-7, Sigma Aldrich, Poole, Dorset, UK), cells were cultured with continuous or intermittent doses of PTH. In continuously treated cultures, 0, 1, 10 or 100nM PTH (diluted in DMEM) was present for the duration of the experiment. Intermittently treated cultures were exposed to PTH in a dosing pattern in line with previously published reports where anabolic effects were seen in response to treatment (Ishizuya *et al.*, 1997; Locklin *et al.*, 2003), and as such, osteoblasts were subject to 100nM PTH for 6 hours every 3 days. Cells were cultured for an average of 14-17 days.

Results

PTH is a potent inhibitor of osteoblastic bone formation

Continuous exposure to PTH inhibited bone formation by both calvarial and long bone osteoblasts in a dose-dependent manner, with complete abolition of bone formation at the highest concentration tested (Figure 5.1). These findings were the stimulus for the rest of the work carried out in this chapter as they raised questions relating to the mechanism of action of PTH *in vivo*. The direct effects of PTH on the number and size of trabeculae formed by long bone osteoblasts are shown in Figure 5.2.
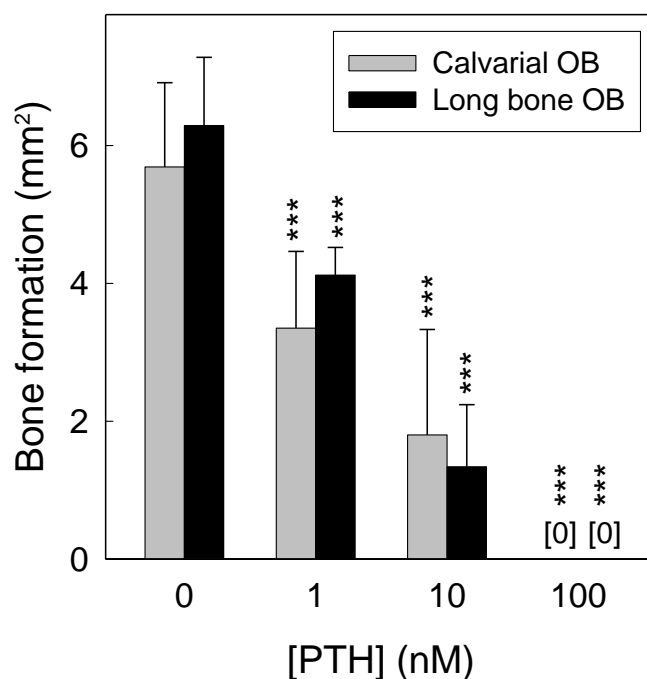
Figure 5.1. *PTH potently inhibits bone formation by both calvarial and long bone osteoblasts.*

Osteoblasts were grown for 14 days in media supplemented with 2mM β-glycerophosphate, 50μg/ml ascorbate, 10nM dexamethasone and continuous exposure to the relevant concentration of PTH. To analyse the amount of bone formed, cell monolayers were stained with alizarin red and bone formation quantified by image analysis. ***, significantly different to control, P<0.001.

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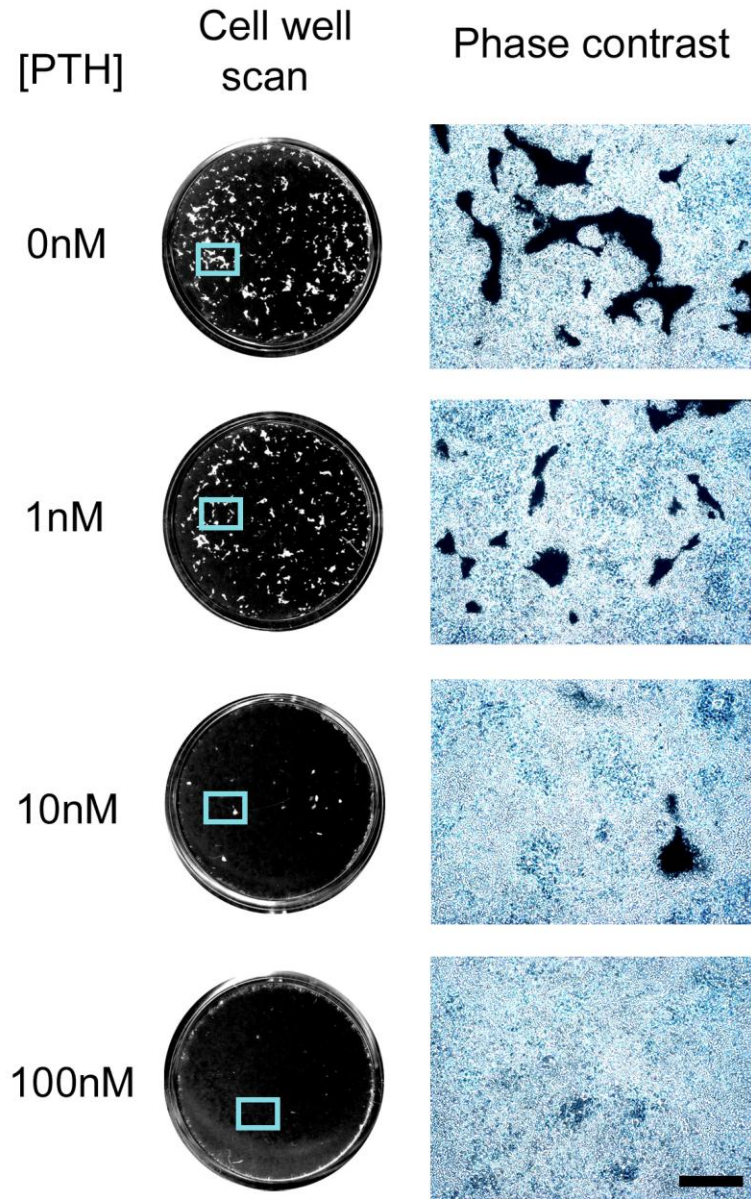
Figure 5.2. *Exposure to PTH caused dose-dependent reductions in both the number and size of trabeculae formed by long bone osteoblasts.*

Cell wells and phase contrast images of structures formed after osteoblasts were grown for 14 days in supplemented DMEM with the relevant concentration of PTH.

Well width = 1.6cm. Scale bar = 0.5 cm.

Intermittent PTH treatment causes dose-dependent decreases in bone formation

When treated with intermittent doses of PTH, bone formation by osteoblasts was inhibited in the same manner as when cells were treated continuously. The inhibition was less marked in the intermittently treated group when compared to the continuously treated group but the observed effect was directly proportional to the duration of PTH exposure, highlighting the direct inhibitory action of PTH on osteoblasts (Figure 5.3).
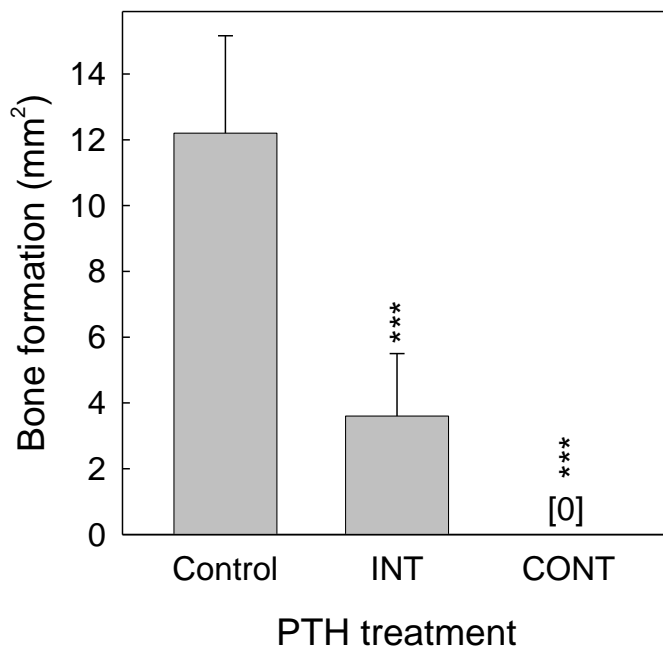
Figure 5.3. *Intermittent PTH treatment inhibits long bone osteoblast activity.*

Osteoblasts were grown for 14 days in supplemented DMEM with PTH treatment as described below. After this time period cell monolayers were stained with alizarin red and bone formation quantified by image analysis. ***, significantly different to control, P<0.001.

INT = Intermittent - 100nM PTH for 6 hours every 3 days. CONT = Continuous - 100nM PTH throughout.

PTH causes decreases in cell number

Continuous exposure to PTH caused dose-dependent decreases in osteoblast number. Apoptosis, estimated by measurement of the specific mono- and oligonucleosomal enrichment of the cytoplasm also decreased, however, the general trend would suggest that this decrease probably reflects the decrease in cell number (Figure 5.4). The dramatic decrease in apoptosis at 10nM PTH suggests that PTH may be having an anti-apoptotic effect at this concentration.
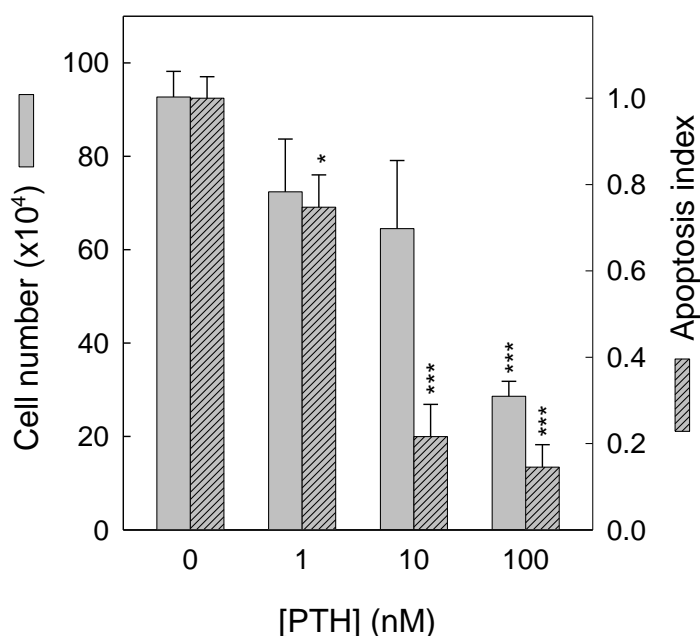
Figure 5.4. *PTH treatment decreases osteoblast cell number and apoptosis.*

Osteoblasts were grown for 14 days in supplemented DMEM and in the presence of PTH. Cell number was assessed using the lactate dehydrogenase assay. Apoptosis was estimated using the cell death detection ELISAPLUS (Roche Diagnostics). The apoptosis index is a measure of the mono- and oligonucleosomes released into the cytoplasm from the nucleus upon initiation of the apoptotic cell death. *, significantly different from control, P<0.05. *** = P<0.001.

PTH decreases osteoblastic alkaline phosphatase activity

Decreases in alkaline phosphatase activity were observed upon treatment with PTH at 10 and 100nM; a 29% reduction in enzyme activity occurred as a result of treatment with 10nM PTH and a significant, 60% decrease when osteoblasts were treated with 100nM PTH. The effect on cells treated with 1nM PTH was negligible. This indicates that PTH reduces the ability of osteoblasts to generate free phosphate and as such, the quantities of phosphate present are not likely to be sufficient to allow mineralisation of the collagenous matrix (Figure 5.5).
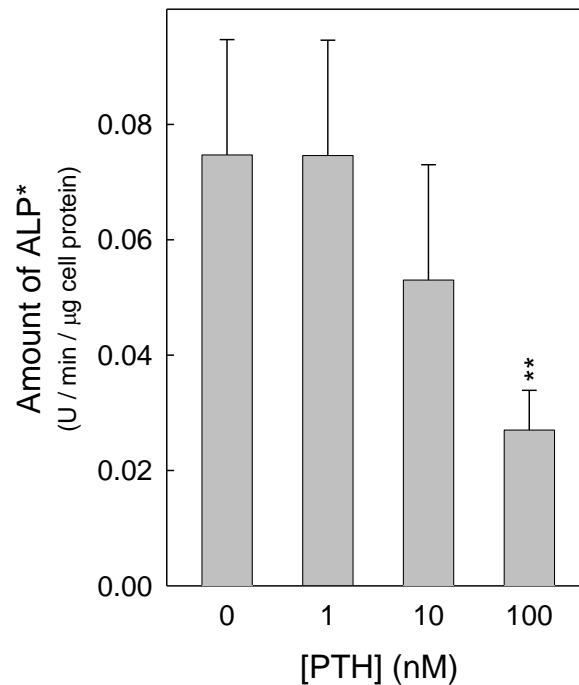
Figure 5.5. *PTH inhibits alkaline phosphatase activity.*

Osteoblasts cultured for 14 days in supplemented DMEM and the indicated concentration of PTH were assayed for alkaline phosphatase (ALP) activity, which was measured relative to the total cell protein present. Amount of ALP* = Amount of alkaline phosphatase measured as enzyme activity (U / min / µg cell protein).

**, significantly different from control, $P < 0.01$.

PTH does not affect extracellular ATP levels

As nucleotides inhibit osteoblast mineralisation (Orriss *et al.*, 2007), cellular ATP release was measured to ascertain if the inhibition of bone formation observed in response to PTH treatment was a consequence of increased release of ATP from osteoblasts. At the higher concentrations of PTH tested, slight increases in ATP release were observed at day 7, but these were much less pronounced by day 14. However, all observed increases in ATP release were accompanied by losses in cellular viability. Dead or dying cells release ATP and as such it is likely that this is the cause of the increases seen, rather than a controlled release in response to PTH treatment (Figure 5.6.).

PTH decreases expression of mRNA for osteogenic markers

RT-PCR revealed marginal decreases in mRNA expression for type I collagen (Col1α1) at the highest concentration of PTH tested. However, strong downregulation of osteocalcin (OCN) in the presence of 10nM and 100nM PTH was observed; additionally osterix (OSX) expression was notably decreased in cells cultured with 100nM PTH. Expression of the PTH receptor (PTHR1) was also dose-dependently decreased. The expression of sclerostin (SOST), a known inhibitor of bone formation, was examined and showed dramatic decreases across the test concentration range but its receptor, Lrp5, was relatively unaffected (Figure 5.7).
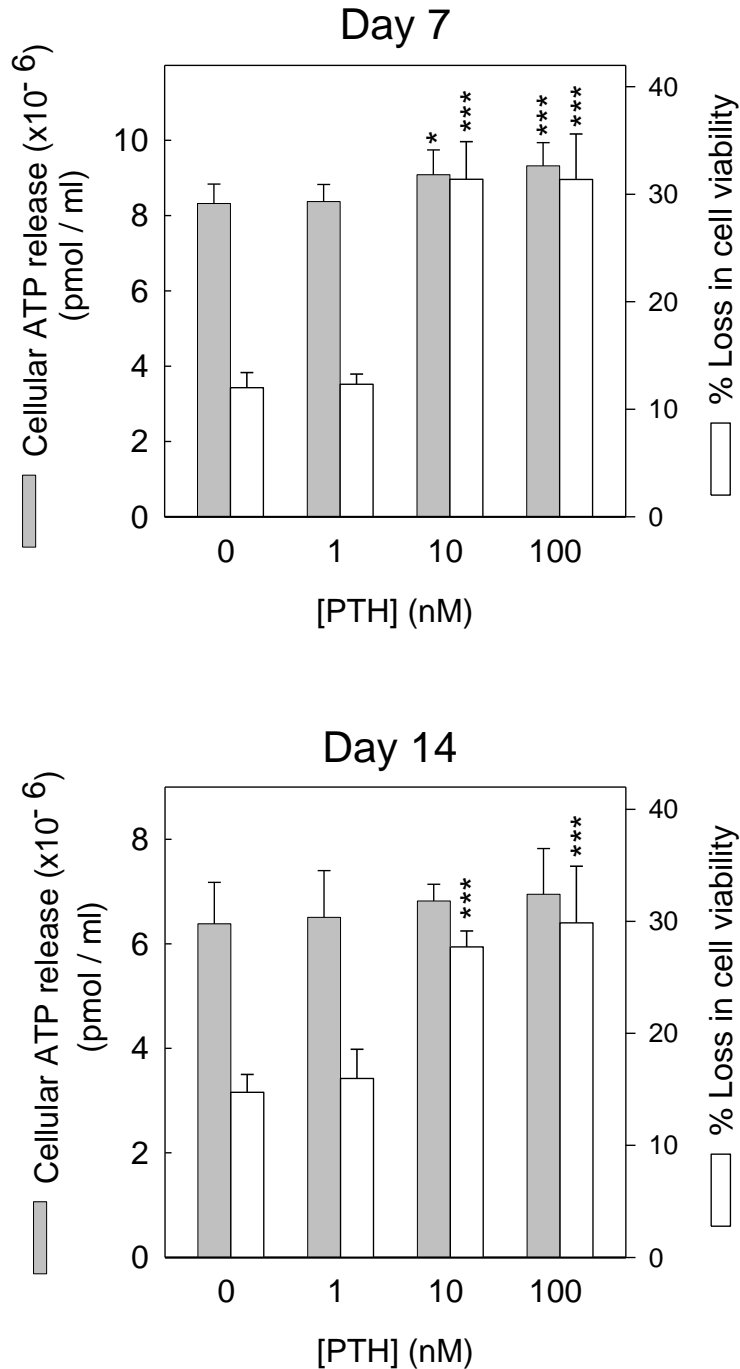
Figure 5.6. *PTH does not cause increases in ATP release by osteoblasts.*

Osteoblasts were grown in supplemented DMEM with PTH. ATP release measurements taken at day 7 and day 14 were normalised for cell number. The percentage loss in cell viability was assessed alongside to ascertain whether ATP released was due to PTH treatment or increased cell death; losses in cell viability suggest increases in ATP release are a result of increased cell death. *, significant difference to control, $P<0.05$. *** = $P<0.001$.
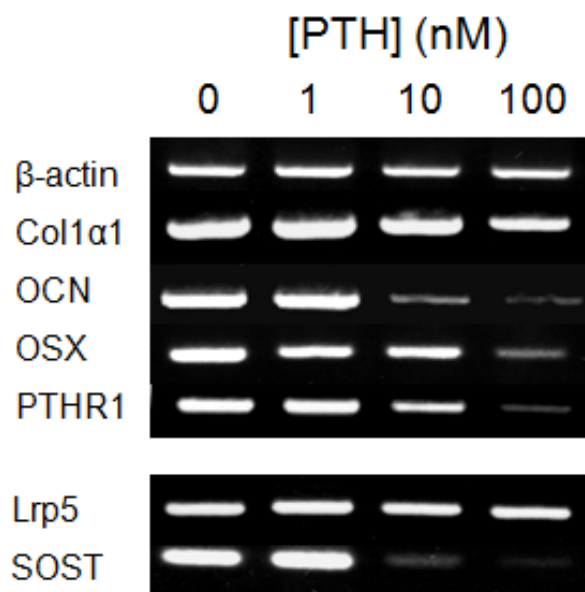
Figure 5.7. *mRNA expression by long bone osteoblasts treated continuously with PTH.*

RNA was extracted from osteoblasts grown for 14 days in supplemented DMEM and PTH as indicated. mRNA expression for all osteogenic gene markers examined was decreased at the highest concentration of PTH tested. Sclerostin (SOST) also showed strong downregulation but the expression of the sclerostin co-receptor Lrp5, appeared unaffected.

Col1α1 = Collagen type 1 alpha 1; OCN = Osteocalcin; OSX = Osterix; PTHR1 = PTH Receptor 1; Lrp5 = Low-density lipoprotein receptor-related protein 5; SOST = Sclerostin.

The effect of sclerostin on osteoblasts

To investigate the effects of sclerostin on osteoblastic bone forming potential a baseline characterisation was performed using a sample of the sclerostin protein (kindly donated by Novartis). Over the concentration range tested (0, 0.1, 1, 10 µg/ml), only small inhibitory effects were seen on bone formation, alkaline phosphatase activity and production of soluble collagen (Figure 5.8.); the only statistically significant inhibition observed was on soluble collagen production at the highest dose of sclerostin tested.
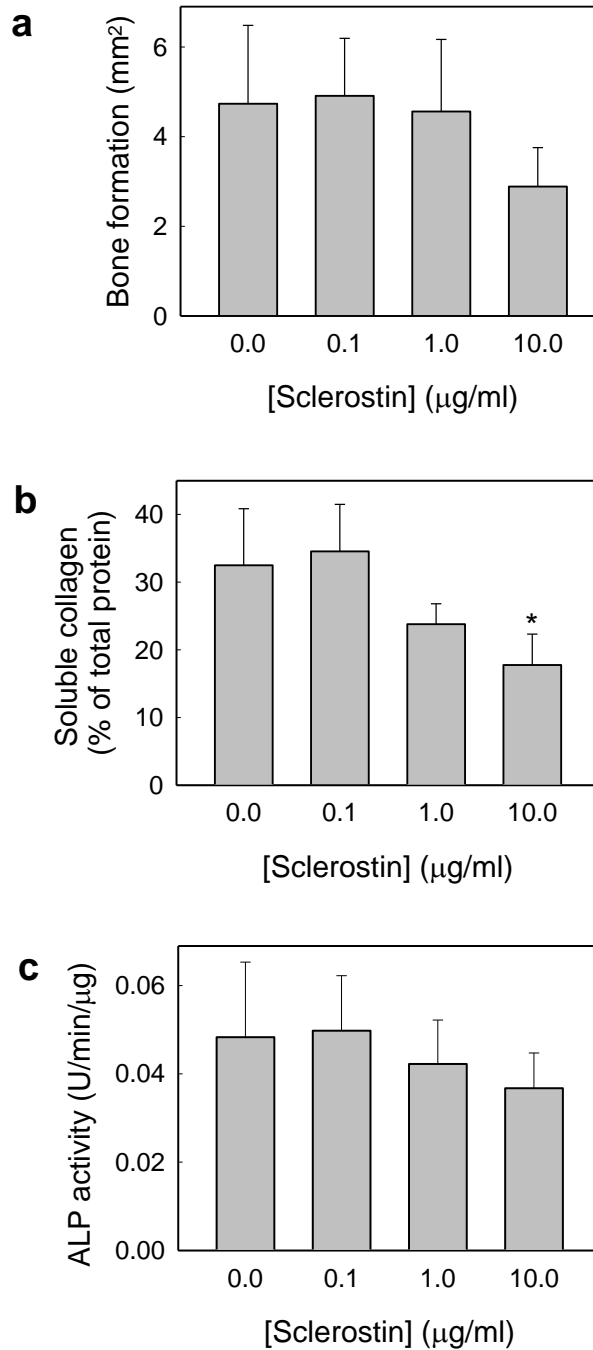
Figure 5.8. *Mild inhibition of osteoblast function by exogenous sclerostin.*

Calvarial osteoblasts were cultured for 14 days in supplemented DMEM and in the presence of increasing concentrations of sclerostin. Modest inhibition of osteoblast activity was observed at the highest concentration tested.

a. Bone formation. b. Soluble collagen production. *, significantly different to control, P<0.05.
c. Alkaline phosphatase (ALP) activity.

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The effect of PTH on VEGF expression

As discussed in the introduction to this chapter, PTH has been reported to have effects on aspects of the vascular supply, causing vasodilation of vascular beds and PTHrP has been implicated in increased angiogenesis via upregulation of VEGF. Thus, the expression of VEGF mRNA by PTH treated osteoblasts was examined. No significant effects on the expression of VEGF mRNA were observed over the concentration range tested. Decreases in osteocalcin expression reinforce the findings of the RT-PCR studies and further indicate PTH may be inhibiting the differentiation of these cells into mature osteoblasts.

Examination of the amount of VEGF protein secreted by long bone osteoblasts indicated that treatment with PTH caused this to be decreased in a dose-dependent manner (Figure 5.9.).
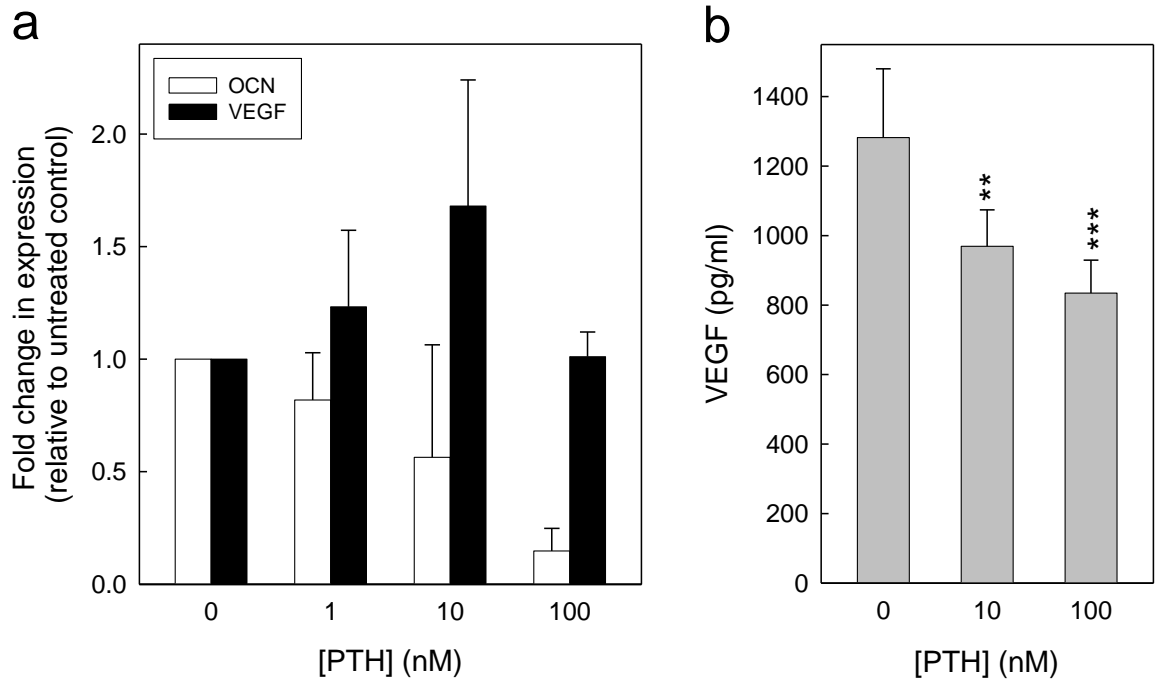
Figure 5.9. *VEGF expression by PTH treated osteoblasts*

Osteoblasts were grown for 14 days in supplemented DMEM and PTH as indicated.

a. RNA was extracted from osteoblasts and qPCR performed to examine mRNA expression of VEGF and OCN in PTH treated osteoblasts. OCN expression decreased over the test range but the fluctuations in VEGF expression suggest PTH is having little effect on its expression.

b. The supernatant from PTH treated cells was removed and analysed for the presence of VEGF protein using a rat VEGF ELISA kit from Invitrogen. Decreases across the test range suggest VEGF is downregulated upon exposure to PTH.   **, significantly different from control, $P<0.01$. *** = $P<0.001$.

Discussion

The results presented in this chapter provide strong evidence that the direct effect of PTH on osteoblasts is to potently inhibit their activity. The data presented demonstrate clear inhibition of bone formation when osteoblasts are subject to either treatment pattern in a time- or dose-dependent manner, with increased exposure leading to increased inhibition of bone formation. These findings suggest that the *in vivo* anabolic response to PTH must be mediated via indirect mechanisms acting on cells other than the osteoblast.

Osteoblast cell number was decreased across the concentration range of PTH tested, this suggests PTH is inhibiting the ability of the isolated pre-osteoblasts to differentiate and then proliferate into fully functional cells. The reduced cell number appeared to be accompanied by decreases in apoptosis, however, apoptosis was measured by examining the oligonucleosome content of cytoplasmic proteins that were released upon cell lysis. The assay used did not account for the reduction in cell number, thus the observed decrease is likely to be a consequence of the decreased cell number. However, at 10nM PTH the decrease in apoptosis was not in line with decreasing cell number; it is possible that at this concentration PTH is suppressing apoptosis, as has been previously described (Schnoke *et al.*, 2009).

The activity of the cells was also reduced by PTH treatment. ALP activity was decreased by approximately one third upon treatment with 10nM PTH and two thirds when treated with 100nM PTH. This suggests that higher concentrations of PTH may be inhibiting the ability of cells to mineralise newly formed collagenous structures. These findings are supported by those from mRNA expression studies; reductions in osteocalcin and osterix expression indicate an inhibition of differentiation by cells exposed to increasing concentrations of PTH. Interestingly, there is little effect on the expression of

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mRNA for type I collagen, supporting the idea that the inhibitory effects of PTH may be more related to the inhibition of mineralisation, rather that the inhibition of collagen formation. The dose-dependent downregulation of the PTH receptor, PTHR1, is also consistent with decreased differentiation. PTHR1 is expressed by lineage committed, maturing osteoblasts (Datta *et al.*, 2009); if PTH inhibits the early differentiation of these cells, the receptor expression would be decreased. It is possible that PTH could be specifically downregulating its own receptor via negative feedback mechanisms, however, given the downregulation of the other markers of osteoblastic differentiation this would seem a more plausible explanation.

Expression of sclerostin mRNA was also subject to strong downregulation by PTH treatment, however, expression of Lrp5, a protein that forms of part of the sclerostin receptor complex, appeared to be relatively unaffected by the treatment. These data support previous findings that suggest PTH may relieve the inhibitory actions of sclerostin on bone formation (Keller *et al.*, 2005; Kramer *et al.*, 2010). To further this work, and examine the extent of the inhibition of sclerostin on osteoblast function, a sample of the sclerostin protein (donated by Novartis) was tested. Osteocyte secreted sclerostin is believed to act on osteoblasts by binding to the Lrp5/Lrp6 receptor complex and inhibiting Wnt/β-catenin signalling (reviewed by (Kneissel, 2010)). As this signalling pathway is implicated in bone mass regulation, inhibition by sclerostin has an inhibitory effect on bone formation *in vivo*. As such, similar effects were expected in *in vitro* studies. To examine this hypothesis, osteoblasts treated with increasing concentrations of sclerostin were cultured and their bone forming ability, soluble collagen production and alkaline phosphatase activity were assessed. For all parameters examined, the inhibitory effects of sclerostin were limited. A possible explanation for these modest inhibitory effects observed could be explained by the differences that may exist between the *in vivo* concentration of sclerostin and

those tested here. As the sclerostin producing osteocytes are buried deep within the bone matrix it is difficult to know the local, *in vivo* concentration of sclerostin and as such there are no detailed reports which discuss it. However, diffusion along canaliculi has been reported to be somewhat restricted (Wang *et al.*, 2005) and as a result it is possible that the local concentration of sclerostin could reach relatively high levels. Consequently, the concentration of sclerostin that osteoblasts are exposed to *in vivo* could be much higher than has been tested and the inhibitory effects much more potent than those seen in the *in vitro* experiments performed in this study.

The strong direct inhibitory action of PTH on bone formation taken with the weak direct inhibitory effect of sclerostin suggests that the *in vivo* anabolic response to PTH is mediated by the interaction of the osteoblast with other local or systemic factors. The requirement of osteogenesis for an adequate vascular supply is becoming increasingly recognised; the findings presented in Chapter 3 show the necessity for oxygen in order for collagen and bone formation to take place and it follows that an increase in local blood flow or supply is essential for significant new bone formation. Thus, it would seem likely that the anabolic effect that PTH treatment has on bone could be linked to an increase in vascular supply. As VEGF is a key factor mediating angiogenesis, its expression by PTH treated osteoblasts was examined. However, the results presented here do not support the idea that PTH stimulates angiogenesis; qPCR investigations revealed no significant change in VEGF mRNA expression with increasing exposure to PTH and analysis of the VEGF protein showed dose-dependent decreases in production indicating that the anabolic response to PTH is unlikely to be a result of neoangiogenesis. It has been suggested that PTH could cause increases in blood flow to vascular beds by increasing vasodilation (Musso *et al.*, 1989; Wang *et al.*, 1984) and cardiac output (Crass *et al.*, 1987). The extent of vasodilation in different vascular bed varies (Wang *et al.*, 1984), but when the

effects were examined on rat kidney, both PTH and PTHrP induced similar concentration-related increases in glomerular blood flow and diameters of preglomerular vessels (Endlich *et al.*, 1995). One report has suggested that PTH induced vasodilation does not affect the vascular beds of osseous tissue (Rambausek *et al.*, 1982) but this has not been clarified; more recently published reports indicate that the vasodilatory effects of PTH are widespread (Laroche, 2002; Rashid *et al.*, 2007). Although this has not been examined directly, it would seem a distinct possibility.

The direct effect of PTH on osteoblasts is potent inhibition of differentiation and bone forming ability. However, given the profound inhibition of bone formation by osteoblasts in culture, these results strongly suggest that the *in vivo* anabolic action of PTH on bone must involve other systemic pathways. The previously published work on the effects of PTH on vasodilation, taken with the findings presented in this chapter, suggest that *in vivo* the anabolic effects seen may be a result of PTH increasing the blood flow to bone and consequently increasing bone formation. PTH works quickly in the body and so this is a preferable suggestion to that of angiogenesis, which with the requirement for new blood vessel formation, would take place over a much longer time period. In addition, the short *in vivo* bioavailability of PTH results in the inhibitory effects on osteoblasts being limited and are overcome by the positive effects of local vasodilation on bone. Consequently, anabolism results. The differential effect of continuous PTH treatment can also be explained by this theory. Chronic exposure to PTH is inhibitory to osteoblasts, as is demonstrated by the experiments performed in this chapter and although continuous PTH may also cause vasodilation, this is not sufficient to overcome the direct inhibitory effect of PTH on osteoblasts. Consequently, bone formation is inhibited. This hypothesis will be further discussed in Chapter 6.

Chapter 6

General discussion

The relationship between cells that produce type I collagen and oxygen has been examined in this thesis. *In vivo*, oxygen is supplied to these cells by the vasculature and as such the influence of vascular supply on cellular function has been considered. Further, a potential role of the vasculature in mediating the response of bone to exogenous stimuli has been proposed.

Hypoxia is one of the most profound effects of reduced vascular perfusion as all cells have an absolute requirement for oxygen for the aerobic generation of ATP. In **Chapter 3** the effect of chronic hypoxia on collagen producing cells derived from the mesenchymal stem cell lineage was investigated. Exposure to hypoxia was found to cause a significant, 80% decrease in collagen deposition by fibroblasts and 88% inhibition of bone formation by osteoblasts. The scale of the inhibitory effect of hypoxia is similar for these closely related cell types and when considered with the other experiments performed suggests that the same mechanisms are likely to be mediating the response to low oxygen in both osteoblasts and fibroblasts. These results indicate that successful wound healing in soft tissues and fracture repair are both dependent on an adequate vascular oxygen supply.

There are a number of possible mechanisms by which hypoxia may cause inhibition of the function of these cells. The data presented in Chapter 3 indicates that hypoxia slows the growth and differentiation of these cells, with reduced proliferation of both cell types seen after culture in hypoxia for 96 hours and delayed expression of markers of osteoblast maturity revealed by the mRNA expression studies. It is possible that the reduced cell number seen in hypoxia

may be a result of apoptosis. Previous work carried out by this research group has suggested that hypoxia does not result in osteoblast apoptosis *in vitro* (Utting *et al.*, 2006) and as such apoptosis of calvarial osteoblasts and dermal fibroblasts cultured in hypoxia were not examined in this study. However, others have reported that hypoxia does induce apoptosis when mesenchymal stem cells are grown in serum deprived media (Zhu *et al.*, 2006), indicating that while hypoxia may not directly induce apoptosis, it may be a consequence of *in vivo* ischemia.

Hypoxia also causes significant inhibition of collagen formation by these cells. As discussed in Chapter 3 collagen production is dramatically inhibited in low oxygen conditions, a likely consequence of the requirement of the prolyl hydroxylase (PHD) enzymes for oxygen. The striking oxygen dependence of the PHD enzymes makes them ideal candidates for cellular oxygen sensing, as such the PHDs involved in the hydroxylation of HIF have been implicated in the cellular response to changing oxygen tensions (Schofield *et al.*, 2004). Reduced activity of the PHDs has been observed in oxygen tensions as high as 10%, with progressive reduction in enzyme activity as the oxygen tension was reduced further (Epstein *et al.*, 2001). As the collagen modifying PHD enzymes share the requirement for oxygen as a cofactor, their activity is also significantly reduced in low oxygen conditions and as such collagen production is inhibited. The findings presented indicate that low oxygen would significantly slow the wound healing process, decreasing cellular proliferation and inhibiting collagen biosynthesis.

The upregulation of VEGF mRNA has been seen previously by osteoblasts exposed to long-term hypoxia (Utting, 2006) and the mRNA study in Chapter 3 demonstrates that VEGF is also upregulated by fibroblasts grown in hypoxia. This indicates that the cellular adaptation to low oxygen is to improve the vascular supply via the induction of VEGF signalling. When considered with

the inhibitory effects of hypoxia on collagen formation by these cells this indicates that increased vascularisation in response to hypoxia is a fundamental adaptation to low oxygen for these cells.

Vascularisation is essential for bone formation, exemplified by the knockout studies that showed HIF1α mediated VEGF expression is required for the proper formation of rodent long bones (Wang *et al.*, 2007). As such, it is a commonly held view that osteogenesis and angiogenesis are coupled (Riddle *et al.*, 2009). In addition, the restoration of an adequate oxygen supply to the site of soft tissue injury is essential in the final stages of wound healing (Braiman-Wiksman *et al.*, 2007). When considered together, the findings presented in this thesis and the published literature on the subject indicate that an oxygen supply sufficient for the proper function and collagen production by these cells is essential to the formation, function and repair of skin and bone. This is exemplified by the successful use of hyperbaric oxygen treatment to decrease healing time in bone (Ueng *et al.*, 1998) and non-diabetic leg ulcers (Hammarlund *et al.*, 1994).

The adhesion studies performed on osteoblasts and fibroblasts showed limited effects. As hypoxia reduces the activity of the majority of cellular functions by osteoblasts and fibroblasts, reduction in the ability to adhere in hypoxia was expected. However, the experiments performed in Chapter 3 revealed that growth in hypoxia resulted in only marginal decreases in the adhesion of these cells to plastic. A likely explanation for this behaviour can be proposed when the *in vivo* situations in which adhesion is likely to take place are considered. In the early stages of wound healing MSCs home to the site of injury in response to the release of inflammatory cytokines (Sordi, 2009; Fong *et al.*, 2011). The first phase of wound healing involves vasoconstriction, slowing the blood flow to the tissue in order to minimise blood loss and is followed by

vasodilation (Irvin, 1985).   If truly unaffected by hypoxia, the findings of the adhesions studies would suggest that both fibroblasts and osteoblasts can adhere to the injured soft tissue or bone surface in preparation for proliferation and regeneration of the tissue once the vascular supply has been restored.

A novel method for the isolation and culture of long bone osteoblasts derived from neonatal rats is described in **Chapter 4**.   The characterisation of these bones alongside their analogous calvarial counterparts showed no clear differences between the cells derived from these anatomical locations when cultured *in vitro*.   Recently published findings by Rawlinson *et al.* indicate there is differential expression of the Hox genes between these two different sources of osteoblasts (Rawlinson *et al.*, 2009).   This was to be expected given the different *in vivo* locations and thus different developmental patterning which would occur.   However, the data presented in this thesis suggest that this does not affect the *in vitro* behaviour of osteoblasts.   Consequently, once removed from their native environments and cultured in osteogenic medium both cell types form bone *de novo*, with no evidence of endochondral bone formation occurring in long bone osteoblast cultures.

As has already been discussed in Chapter 4, Scotti *et al.* (2010) have proposed that it is possible to recapitulate endochondral ossification *in vitro* using marrow derived human adult MSCs.   By engineering human hypertrophic cartilaginous tissue, the authors show it is possible to engineer tissue that has the potential to undergo developmental changes similar to those that take place during limb formation.   If these findings hold true, it holds huge promise for therapeutic treatments.

The bone formation assay developed and described in Chapter 4 provides an additional model for the study of osteoblasts that overcomes some of the shortcomings associated with other *in vitro* models, however, it is not without its

limitations. As is the case with all *in vitro* models, the study of one cell type in isolation allows the exact effects of the test substances to be examined, but this is not reflective of the *in vivo* environment where cells are in contact and interacting with each other and the extracellular matrix (Abbott, 2002). This is exemplified by the work performed examining the osteoblastic response to PTH (Chapter 5). Further, the assay developed has not provided a model for the study of endochondral bone formation, but it has expanded the possibilities for the *in vitro* culture of osteoblasts. By adopting this method the whole animal can be used in a reliable, robust bone formation assay and thus the number of animals required for experiments will be reduced. This is beneficial on both ethical and economical grounds.

Using this method for osteoblast isolation the effects of PTH on osteoblast function and bone formation were examined in **Chapter 5**. The *in vitro* results presented and discussed indicate that PTH had a direct, inhibitory effect on osteoblasts when cells were treated with the hormone. The same inhibitory effects were observed when cells were treated with PTH in intermittent or continuous doses, with the level of inhibition observed being directly related to the duration of exposure to PTH. This indicates that the differences between the anabolic and catabolic effects of intermittent and continuous PTH treatment seen *in vivo* (Uzawa *et al.*, 1995) do not occur as a result of the direct action of PTH on osteoblasts and must involve other local or systemic factors.

It has been proposed that cell types other than the osteoblast and osteoclast could be involved in mediating the anabolic effects of PTH *in vivo*. Pacifici *et al.* have reported that T cells play an essential role in the anabolic response to PTH. Experiments performed using T cell deficient mice revealed that continuous infusion with PTH failed to induce osteoclast formation, bone resorption and bone loss (Gao *et al.*, 2008). In addition, when these mice were

treated with intermittent PTH a blunted increase in bone formation and trabecular bone volume was observed. The normal anabolic response to intermittent PTH treatment was restored upon adoptive transfer of T cells into deficient mice (Pacifici, 2010).   As the response to intermittent PTH is blunted but not completely abolished in T cell deficient mice, this cannot be the only mechanism involved in the anabolic response but it is certainly playing a key role.

Other findings have shown that PTH has dramatic effects on vascular tone, resulting in vasodilation (the key references are discussed in Chapter 5). It is plausible that the vasodilation and concomitant increased blood supply to bone could be playing a role in mediating the anabolic response of intermittent PTH treatment. Thus, it is hypothesised that when administered intermittently the level of PTH in the blood is sufficient to cause vasodilation of arteries, increasing the blood supply to bone and resulting in bone formation. Experiments performed on the rat mesenteric artery have shown that the vasodilation is stimulated by concentrations of PTH in the low nanomolar range (maximal effects were observed in the presence of 30nM PTH and half maximal effects at 4nM PTH) (Nickols *et al.*, 1985). These concentrations are in line with the reported concentration of PTH present in blood serum after subcutaneous injection with teriparatide (Satterwhite *et al.*, 2010). Elevated PTH levels in response to teriparatide administration are only present for a limited time period (approximately 2-3 hours post injection) (Frolik *et al.*, 2003) and as such minute to minute changes in vascular tone result. The lack of angiogenic involvement, as suggested by the reduction in VEGF protein described in Chapter 5, supports this hypothesis. The formation of new blood vessels would take place over a much longer time period; if PTH was having a positive effect on angiogenesis, it would have to be bioavailable for much longer than the 2-3 hours after administration. However, when PTH is present for longer time periods, i.e. when it is administered continuously, the effect of PTH is no longer anabolic.

Continuous exposure to PTH causes hypertension in normal subjects (Hulter *et al.*, 1986) suggesting that the vasodilatory effect of PTH are diminished. Further, it has been reported that prolonged exposure to PTH causes desensitisation of vascular smooth muscle cells (Nyby *et al.*, 1995). Therefore, it would seem reasonable to hypothesise that the anabolic effects of intermittent PTH may be mediated, at least in part, by the vasodilation of the blood vessels in and around bone, resulting in increased blood flow and concomitant osteogenesis. In addition to this PTH also acts directly on osteoclasts, stimulating their activity (Dempster *et al.*, 2005) and as such, bone resorption occurs. The relative amount of osteogenesis associated with intermittent PTH treatment is much greater than the osteoclastic resorption taking place and consequently is anabolic. As osteogenesis is not stimulated by continuous treatment, the relative osteoclastic activity is much greater and bone loss results.

Nitric oxide (NO), is another known signalling molecule with vasodilatory actions (Ignarro *et al.*, 1987). While high levels of NO (as are seen in cases of severe inflammation) inhibit bone formation, normal physiological levels are required for bone formation (Van't Hof *et al.*, 2001). NO has been linked to the anabolic effects of oestrogen *in vivo*, with inhibitors of nitric oxide synthase (NOS) causing decreases in oestrogen-induced bone formation (Samuels *et al.*, 2001). In addition, oestrogen has been seen to increase the synthesis and release of NO by endothelial cells (Hayashi *et al.*, 1995). Thus it is possible that in addition to its direct anabolic effects on bone, NO mediated vasodilation may also contribute to the *in vivo* anabolic effect of oestrogen.

This is not the only situation in which vasodilators are also seen to have bone anabolic effects. The prostaglandins, particularly prostaglandin 2 (PGE_2), is a known vasodilator of vascular beds at multiple sites (Lumley *et al.*, 1982).

PGE$_2$ is also implicated in increased bone formation (Chyun *et al.*, 1984). PGE$_2$ cannot be used as a bone anabolic therapy due to the associated side effects but PGE$_2$ receptor selective agonists have been seen to induce bone healing (Paralkar *et al.*, 2003).

A similar mechanism may also be mediating the effects on bone of other factors. Glucocorticoids are known to inhibit vasodilation, therefore preventing increased blood flow to vascular beds (Perretti *et al.*, 2000). Restriction of bone perfusion is known to impair bone metabolism and consequently may be associated with a reduction in bone mass (Vogt *et al.*, 1997). The association of glucocorticoid use with bone loss and resulting osteoporosis and fracture is well known (Reid, 1999) and is a likely consequence, at least in part, of the reduction in vascular supply induced by steroid use. Therefore, it is possible that glucocorticoids act in a converse manner to PTH, restricting blood flow to bone and consequently resulting in bone loss.

Even though the relationship between osteogenesis and angiogenesis is well documented, the role of the vasculature in mediating the response to bone anabolic therapies has not been widely considered by the literature. The mechanism proposed here suggests that PTH increases bone formation by increasing vasodilation of the vascular network, increasing blood flow to bone.

The relationship between vasodilation and bone formation is not limited to PTH, with other bone anabolic agents also known to increase blood flow. Further, the reciprocal effects that result from glucocorticoid treatment indicate that this hypothesis may provide a link between the actions of these factors on bone and its vascular supply. While the vasodilation hypothesis proposed here is unlikely to be the sole mechanism mediating the anabolic response to PTH, the evidence presented strongly suggests that it is likely to be a contributing factor.

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Future work

The results presented in this thesis and suggestions made in the general discussion point towards a clearer, more descriptive explanation of why intermittent and continuously administered PTH elicit different effects on bone *in vivo*. Whilst this thesis has provided the initial proposition, to achieve a better understanding, the suggested hypothesis needs to be tested so that it can be definitely confirmed if the proposed mechanism for the action of PTH is true.

Although upregulation of VEGF is not involved in the anabolic response, it is possible that other vascular stimuli may be upregulated in osteoblasts in response to intermittent PTH treatment, and these factors may play a role in mediating the changes in vascular tone. Platelet derived growth factor (PDGF) is a stimulator of angiogenesis but also has effects on vasoconstriction and vasodilation (Sakagami *et al.*, 2001). PDGF-A, an isoform of PDGF, is expressed by osteoblasts (Zhang *et al.*, 1991; Yu *et al.*, 1997), but its expression in PTH treated cells has not been investigated. mRNA and protein expression studies could easily shed light on this and indicate whether or not PDGF plays a role in mediating the vascular response to PTH treatment.

Using a basic organ culture of slices of rat artery and subjecting them to intermittent and continuous PTH treatment, the effects on vascular tone could be assessed. However, *in vivo* studies would provide the most useful data, but this would be difficult to measure. As PTHR1 null mice display chondroplaysia and die before birth (Clemens *et al.*, 2001), knockouts cannot be used but targeted knock down or deletion of the receptor in VSMCs could provide a system in which the effects of PTH on bone could be assessed in the absence of the influence from the vasculature.

Conclusion

An adequate vascular supply is essential for the proper function of collagen producing cells. The oxygen requirement for collagen synthesis is absolute: failure to establish or maintain a good vascular network has severe effects on the function of cells producing type I collagen and their ability to produce an extracellular matrix.

This thesis has consolidated and furthered previous findings on the response of osteoblasts and fibroblasts to hypoxia. Furthermore, it has provided a novel method for the study of osteoblast *in vitro* and proposed explanations involving the vasculature in the varied responses to intermittent and continuous PTH treatment. Put in a wider context, the suggested hypothesis could hold true for other factors that affect both bone mass and vascular tone.

The relationship between the vasculature and collagen producing cells is tightly coupled and offers wide ranging possibilities for intervention and thus potential therapeutic targets.

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Appendix I

Primer sequences

Table 1. Primer pairs used for RT cDNA amplification

Gene product	Primer sequence (5'-3')	bp	ta (°C)
Alkaline phosphatase	S - CTCATTTGTGCCAGAGAA AS - GTTGTACGYCTTGGAGAC	238	50
Beta actin	S - GTTCGCCATGGATGACGAT AS - TCTGGGTCATCTTTTCACGG	332	53
Collagen 1α1	S – GGCTATGATGAGAAATCAGC AS - ACCACTGAAACCTCTGTGTC	318	53
DMP1	S - GAAAACAGTGCCCAAGATAC AS - CTGTCGTCTTCCTCAGAGAC	371	53
Lrp5	S- GTACCCGGAAGATCATTGTA AS - GAATGTCCATGGGTGAATAC	303	53
Matrix gla protein	S – CCCTGTGCTATGAATCTC AS - GACTCCGTAACAAAGCGA	297	50
Osteocalcin	S – GCAGACACCATGAGGACCCT AS - GCAGCTGTGCCGTCCATAC	418	59.8
Osteoprotegrin	S – GAGTGTTCTGGTGGACAGTT AS - ACAAGCTCACTAGCTTCAGG	335	53

Gene product	Primer sequence (5'-3')	bp	ta (°C)
Osteopontin	S – CGGTGAAAGTGGCTGAGT AS - GACTCGGGATACTGTTCA	333	50
Osterix	S – GAGGAAGAAGCTCACTATGG AS - GTAGACACTAGGCAGGCAGT	351	55
PTHR1	S - TGGGATACTCCATGTCTCTC AS - TTGGTAGCCAGGAAGTAGAG	307	53
RANK ligand	S – CGAGCGCAGATGGATCCTAAC AS – GACTTTATGGGAACCCGATGG	330	57
Sclerostin	S - CACCATGCAGCTCTCACTA AS - ACGTCTTTGGTGTCATAAGG	228	53
VEGF	S - TCCAGAGAGAAGTCAAGGAA AS - AGAGAGAAGAGCCCAGAAGT	359	65

Table 2. Primer pairs used for cDNA amplification by qRT-PCR

Gene product	Primer sequence (5'-3')
Beta actin	S – GCCTTCCTTCCTGGGTATGG AS – GAGGTGTTTACGGATGTCAACG
Osteocalcin	S – CCAAGCCCAGCGACTCTGAG AS – TCCAAGTCCATTGTTGAGGTAGC
PPARγ	S – TGCCTATGAGCACTTCACAA AS - ATCCATCACAGAGAGGTCCA
Sox9	S – CAGAGAACGCACATCAAGAC AS – GGGCTGTAGGAGATCTGTTG

Appendix II

Abbreviations

ABAM   Antibiotic-antimycotic

ALP   Alkaline phosphatase

ANOVA   One way analysis of variance

ASC   Ascorbic acid

ATP   Adenosine triphosphate

BMD   Bone mineral density

BMP   Bone morphogenic protein

BMU   Basic multicellular unit

BSA   Bovine serum albumin

C/EBP   CCAAT/ enhancer binding protein

CASR   Calcium sensing receptor

CB   Calvarial bone osteoblasts

CBP   CREB-binding protein

cDNA   Complementary deoxyribonucleic acid

CO_2 Carbon dioxide

Col1α1   Collagen type 1, alpha 1

CTGF   Connective tissue growth factor

Dexa   Dexamethasone

dH$_2$O	Distilled water
DHT	Dihydrotestosterone
DMEM	Dulbecco's modified eagle medium
DMP1	Dentine matrix protein 1
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
HBSS	Hanks balanced salt solution
HIF-1α	Hypoxia inducible factor-1α
HRE	Hormone response element
Ihh	Indian hedgehog
iPTH	Intermittent PTH
LB	Long bone osteoblasts
LDH	Lactate dehydrogenase
L-glut	L-glutamine
Lrp5	Low-density lipoprotein receptor-related protein 5
Lrp6	Low-density lipoprotein receptor-related protein 6
mA	Milliamp
M-CSF	Macrophage colony stimulating factor
MgCl$_2$	Magnesium chloride

MGP	Matrix gla protein
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell
N_2	Nitrogen
NADH	Nicotinamide adenine dinucleotide (reduced form)
NFATc1	Nuclear factor of activated T cells 1
O_2	Oxygen
OCN	Osteocalcin
Oligo-dT	Oligo deoxythymidylic acid
OPG	Osteoprotegrin
OPN	Osteopontin
OSX	Osterix
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PHD	Prolyl hydroxylase
pO_2	Partial pressure of oxygen
PO_4^{3-}	Phosphate ion
PPARγ	Peroxisome proliferator-activated receptor gamma
PTH	Parathyroid hormone
PTHR1	Parathyroid hormone receptor 1
PTHrP	Parathyroid hormone related protein

pVHL	Von Hippel Lindau protein
qRT-PCR	Quantitative real-time polymerase chain reaction
RANK	Receptor activator for nuclear factor κ B
RANKL	Receptor activator for nuclear factor κ B ligand
rh-PTH	Recombinant human parathyroid hormone
RNA	Ribonucleic acid
RNase	Ribonuclease
RT-PCR	Reverse transcriptase polymerase chain reaction
Runx2	Runt-related transcription factor 2
Scl-Ab	Sclerostin antibody
SEM	Standard error of the mean
SOST	Sclerostin
Sox5/6	SRY (sex determining region Y)-box 5/6
Sox9	SRY (sex determining region Y)-box 9
TEM	Transmission electron microscopy
TGFβ	Transforming growth factor-β
TNFα	Tumour necrosis factor-α
Ub	Ubiquitin
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
w/v	Weight / volume
Wnt	Wingless-type MMTV integration site

β-actin   Beta actin

βAPN   Beta aminoproprionitrile

β-GP   Beta glycerophosphate

Appendix III

Publications

Taylor SEB, Orriss IR, Lander M, Turmaine M, Key ML, Patel JJ and Arnett TR. 2011. Parathyroid hormone is a potent, direct inhibitor of bone formation by rat osteoblasts *(manuscript in preparation).*

Orriss IR, Taylor SEB and Arnett TR. 2011. Rat osteoblast cultures. *Methods in Molecular Medicine (in press).*

Taylor SEB, Orriss IR, Patel JJ, Brandao-Burch A and Arnett TR. 2010. The anabolic response of bone to PTH: a vascular effect? *Bone*, 47: S133 (abstract).

Patel JJ, Talbot D, Orriss IR, Key ML, Taylor SEB, Karnik K, Arnett TR. 2010. Osteoblast differentiation and bone formation are retarded in hypothermia. *Bone,* 47: S129-S130 (abstract).

Taylor SEB, Orriss IR, Key ML, Patel JJ and Arnett TR. 2010. Formation of bony structures by cultured long bone osteoblasts: potent inhibition by parathyroid hormone. *Bone*, 46: S56-S57 (abstract). *Oral presentation at the International Bone and Mineral Society (IBMS) annual meeting, Davos, Switzerland.*

Orriss IR, Knight GE, Utting JC, Taylor SEB, Burnstock G and Arnett TR. 2009. Hypoxia stimulates vesicular ATP release from rat osteoblasts. *Journal of Cellular Physiology*, 220:155-162.

Taylor SEB, Key ML, Lander M, Orriss IR, Patel JJ and Arnett TR. 2009. A novel method for the isolation and culture of rat long bone osteoblasts. *Bone*, 44: S318-S319 (abstract).

Patel JJ, Orriss IR, Key ML, Taylor SEB, Karnik K and Arnett TR. 2009. Mild hypothermia promotes osteoclastogenesis whilst retarding osteoblast differentiation and bone formation. *Calcified Tissue International,* 85: 180 (abstract).

Taylor SEB, Lander M, Key ML, Orriss IR, Patel JJ and Arnett TR. 2009. Osteoblasts isolated from different anatomical locations display similar characteristics *in vitro*. *Calcified Tissue International,* 85: 172-173 (abstract).

Rat osteoblast cultures

Running title: Osteoblast assay

Authors: Isabel R Orriss, Sarah EB Taylor and Timothy R Arnett

Department of Cell & Developmental Biology

University College London

U.K.

Full Address: Department of Cell & Developmental Biology

University College London

Gower Street

London WC1E 6BT

UK

Phone: 0044 20 76793309

Fax: 0044 20 76797349

Emails: t.arnett@ucl.ac.uk

i.orriss@ucl.ac.uk

1. Introduction

Osteoblasts are derived from mesenchymal stem cells and are responsible for bone formation. A number of different approaches have been developed to study osteoblasts *in vitro*, including bone organ cultures, primary cell cultures and immortalized osteoblast-like cell lines. Combined these methods have provided abundant information about the regulation of osteoblast proliferation, differentiation and function. Osteoblastic cell lines are widely used as a convenient source of large numbers of cells with a relatively stable phenotype. However, many of these osteoblast-like cells do not express the whole range of bone-specific genes and/or form bone *in vitro*. Furthermore, repeated passaging has been shown to cause the loss of the osteoblastic phenotype in some cell lines (*1*).

Primary bone cell cultures were first described in 1964 by Peck and colleagues (*2*), who isolated cells from the parietal and frontal bones of fetal and neonatal rat calvariae using collagenase digestion. The isolated cells proliferated *in vitro* and exhibited high alkaline phosphatase (ALP) activity, although the cultures were contaminated with other cell types such as fibroblasts. In 1974, Wong and Cohn (*3*) used sequential collagenase digestion to obtain a more homogenous population of osteoblastic cells. The first description of the formation of bone nodules by differentiating osteoblasts released enzymically from calvarial bones and cultured with β-glycerophosphate ascorbate and dexamethasone was by Bellows and colleagues in 1986 (*4*).

This chapter describes the isolation, culture and staining of primary osteoblasts from the calvaria and long bones of neonatal rats (*see* **note 1**). The osteoblast bone formation assay has a number of advantages. Firstly, it allows the key function of osteoblasts, namely bone formation, to be studied quantitatively (*5*). Bone formation in this essentially 2-dimensional culture system appears to recapitulate the intramembranous (woven) bone formation seen in histological sections from developing animals – *i.e.* large-scale structures resembling trabeculae appear. Secondly, it offers the opportunity to study separately the processes of bone matrix deposition and mineralisation (*6*). Thirdly, it allows the extracellular environment to be tightly regulated (*e.g.* pH, pO_2) in a manner which is not possible using bone organ cultures or *in vivo* (*7,8*). Fourthly, osteoblast activity can be studied in an environment that is relatively free from the influence of other cell types normally found in bone such as endothelial cells, nerves and haematopoietic cells. Lastly, osteoblasts can be studied at clearly identified stages of differentiation from the immature, proliferating cells present early in the cultures through to the mature bone-forming osteoblasts in late stage cultures.

Two important pitfalls should be highlighted here. The first concerns the use of inappropriately high concentrations of the alkaline phosphatase substrate, β-glycerophosphate in many published descriptions of osteoblast cultures. At high concentrations, β-glycerophosphate causes generalised mineral deposition and impaired cell viability (the osteoblasts effectively fossilise), with the result that the cultures fail to progress beyond the formation of small nodules. The second, related pitfall concerns the frequent confusion between mineral deposition (which also occurs on teeth, in caves and in kettles) and true bone formation, which involves selective mineralisation of a collagenous matrix deposited in characteristic patterns. Osteoblast cell lines (and many other cell types) express alkaline phosphatase and can mineralise in the presence of sufficient β-glycerophosphate – but this is not bone formation.

2. Materials

All solutions, instruments and tissue culture plastics should be sterile.

1. **Animals**: The cells are obtained from the calvariae and long bones of neonatal (2-3 day old) rats. The number of animals to use depends on the experiments to be performed; typically one animal will yield 10^7 and 5×10^6 cells from the calvaria and long bones, respectively (*see* **notes 1 and 2**).

2. **Phosphate buffered saline (PBS)**: For storing tissues prior to use and washing cells.

3. **Supplemented Dulbecco's modified essential medium (sDMEM)**: Add 10% foetal calf serum (FCS), 2mM L-glutamine and 100U/ml penicillin, 100µg/ml streptomycin, 0.25µg/ml amphotericin (mixture is known as antibiotic/antimitotic or AB/AM).   This supplemented DMEM (sDMEM) should be stable at 4°C for at least 4 weeks.

4. **Osteogenesis DMEM (osDMEM)**; to the sDMEM described above add 2mM β-glycerophosphate (*see* **note 3**), 10nM dexamethasone and 50µg/ml ascorbate. Always make fresh on day of use.

5. **Sodium hydroxide (NaOH)**: 6M NaOH to alter the pH of the culture medium if required (*see* **note 4**).

6. **Trypsin:** 1% (*w/v*) trypsin solution (free of calcium and magnesium, which inhibit trypsin activity).

7. **Collagenase:** 0.2% (*w/v*) collagenase solution made up in Hank's balanced salt solution (HBSS); filter sterilise. Note that HBSS contains calcium, which is required for collagenase activity.

8. **Fixative**: 2.5% glutaraldehyde in dH_2O or 70% ethanol; prepare fresh before use.

9. **Alizarin red stain for mineralised bone nodules:** 1% (w/v) alizarin red in dH_2O; prepare fresh before use.

10. **Alkaline phosphatase (ALP) staining**: alkaline phosphatase kit (Sigma kit 86C-1KT).

11. **Sirius red stain for deposited collagen**: Sircol™ dye reagent (Biocolor kit S1005)

12. **Tissue culture plastics:** Large petri dishes (100mm), 5ml flat bottomed tubes, 15ml and 50ml centrifuge tubes, $75cm^2$ tissue culture flasks and 24 well tissue culture plates.

13. **Dissection** tools: Scalpels and blades (no.20), tweezers and scissors

3. Methods

To keep the cell cultures sterile, normal techniques for working under sterile conditions should be employed (working in a flow cabinet, use of sterile media and instruments, etc).

3.1. *Isolation and culture of primary osteoblasts from neonatal rat calvaria*

1. Sacrifice 2 neonatal (2-3 days old) rats and sterilise with 70% ethanol. Place each cadaver in a large petri dish.

2. Remove the head using large scissors; transfer the body to a separate petri dish for long bone isolation (*see* **section 3.2.**)

3. Grasp the head at the nape of the neck and make a small incision along the base of the skull (small scissors will make the cleanest cut). Carefully remove the skin and the brain tissue from the skull using a scapel and tweezers.

4. Cut away the jaw and scrape off any excess tissue and cartilage from around the edge of the calvaria.

5. Cut the calvaria in half and place in a flat- bottomed 5ml tube; wash with PBS.

6. Repeat steps 2-5 for the second animal.

7. Incubate the calvariae in 1% trypsin (1ml/calvarial bone) for 10 minutes at 37°C. Remove and discard the trypsin solution; wash in sDMEM (serum and calcium will inactivate any residual trypsin).

8. Incubate in 0.2% collagenase solution (800 µl / calvarial bone) for 30 minutes at 37°C.

9. Remove the collagenase digest, discard and replace with fresh solution for a further 60 minutes at 37°C.

10. Keep the final digest and transfer to a 15ml conical base centrifuge tube. Wash calvaria with sDMEM (5ml), transfer the solution to the 15ml tube containing the final digest.

11. Spin the cell solution at 1,500 g for 5 minutes at room temperature. Discard the supernatant and resuspend the cell pellet in sDMEM (1ml/calvarial bone). Pool the cell suspensions.

12. Add 20ml of sDMEM to 2 x 75cm^2 flasks; add 1ml of cell suspension to each flask.

13. Incubate the flask at 37°C/5% CO_2 until the cells reach confluency (~3 days).

3.2. Isolation and culture of primary osteoblasts from the long bones of neonatal rats

1. Take the bodies of the animals used for the calvarial osteoblast isolation (*see* **section 3.1**).

2. Remove the limbs from the body by cutting with sharp scissors at the point closest to the body, preserving as much of the limb as possible.

3. Using a scalpel cut off the paws and cut the limb in half (at the joint).

4. Remove the skin and scrape away the soft tissue from the limbs.

5. Cut off the epiphyses and place the bone fragments into a flat- bottomed 5ml tube. Wash and vortex in PBS.

6. Incubate the bone fragments in 1% trypsin (1ml / animal) for 10 minutes at 37°C. Remove and discard the trypsin solution; wash in sDMEM.

7. Incubate in 0.2% collagenase solution (1ml) for 30 minutes at 37°C.

8. Remove the collagenase digest, discard and replace with fresh solution for a further 60 minutes at 37°C.

9. Keep the final digest and transfer to a 15ml conical base centrifuge tube. Wash remaining bone fragments with sDMEM (5ml), transfer the solution to the 15ml tube containing the final digest.

10. Spin the cell solution at 1,500 g for 5 minutes at room temperature. Discard the supernatant and resuspend the cell pellet in sDMEM (1ml).

11. Add 20ml of sDMEM to a 75cm^2 flasks; add cell suspension.

12. Incubate the flask at 37°C/5% CO_2 until the cells reach confluency (~3 days).

3.3. Bone formation assay

This protocol is used for osteoblasts from both calvarial and long bone isolations.

1. Once the cells in the 75cm^2 have reached confluency (~3 days), remove the sDMEM and wash with PBS. Add 1% trypsin (2ml/flask) and incubate for 10 minutes at 37°C.

2. Inactivate the trypsin by adding 10ml of sDMEM; transfer the cell suspension to a 15ml centrifuge tube.

3. Spin the cell solution at 1,500 *g* for 5 minutes at room temperature. Discard the supernatant and resuspend the cell pellet in sDMEM (1ml/flask). Pool the cell suspensions.

4. Perform a cell count using a haemocytometer; seed the cells in 24 well tissue culture trays at a density of 2.5 x 10^4 cells/well in osDMEM.

5. Half the medium should be exchanged every 2-3 days. Cultures will typically be fully confluent by day 4; they will begin to form discrete bone nodules from about day 10, with extensive networks of mineralising 'trabeculae' developing from day 14 (**Figs. 1 & 2**).

6. The pH should be monitored at every medium change and maintained at ~pH 7.4 by the addition of 6M NaOH if required (*see* **notes 4 and 5**).

7. ALP activity (ALP assay kit, Biotron) and soluble collagen production (Sircol Red assay, Biocolor) can be assayed throughout the 14 day culture period using commercially available kits.

3.4. Fixation and staining

1. On termination of the experiment, carefully wash the cell layers with PBS.

2. Transfer to fixative: 2.5% glutaraldehyde for 5 minutes for alizarin red and ALP staining, 70% ethanol for one hour for Sirius red staining.

3. Wash twice with PBS and leave to air dry (Sirius red staining only).

4. For cell layers to be stained with alizarin red or for ALP, wash three times with 70% ethanol and leave to air dry.

5. Stain as required (**Fig. 2**):
   - Alizarin red for 5 minutes followed by three washes with 50% ethanol; allow to air dry.
   - ALP for ~30 minutes in the dark; wash with dH$_2$O and allow to air dry.
   - Sirius red for 1 hour; wash with dH$_2$O and allow to air dry.

3.5. Quantification of mineralised bone formation

1. Once fully dry, scan plates at 2000 dots/cm^2 on a high-resolution flat-bed scanner. We use an Epson Perfection 4990 photo/slide scanner. Transmitted light scanning of alizarin red-stained plates is commonly used but unstained cell layers also yield excellent high-contrast images in both transmitted and reflected light modes (the latter showing the white bone trabeculae against a dark background) (**Fig. 2**).

2. Apply a circular mask to the image of each cell well and convert to a binary image (*e.g.* using 'Adobe Photoshop').

3. Use an image analysis program (e.g. 'Image J' – available free from http://rsbweb.nih.gov/ij/) to determine the number and surface area of mineralised bone nodules in the binary images of each individual well, using constant threshold and minimum particle size levels.

3.6. Statistics

We routinely use one-way analysis of variance (ANOVA) to analyse experiments. Although often neglected, adjustments for multiple comparisons between treatment groups (*eg*, the Bonferroni correction) are frequently needed (*see* **note 6**).

4. Notes

1. ***Calvarial cells vs. long bone cells:*** Although the flat bones of the skull and the limb bones are formed by different developmental processes, osteoblasts derived from either source appear to behave similarly in culture.

2. ***Young cells vs. old cells:*** An important reason why the rat osteoblast culture system described here is able to form bone is that the initial cell population is obtained from neonates – and thus has considerable growth potential. Cells derived from adult animals (including human donors), may have progressed too far towards their 'Hayflick limits' to be capable of the rapid expansion to high cell density required for true osteogenic differentiation.

3. ***Concentration of β-glycerophosphate****:* Bone mineralisation requires a supply of calcium and orthophosphate (Pi), both of which are required for hydroxyapatite crystal formation. A key source of phosphate in bone is alkaline phosphatase, which hydrolyzes a range of phosphate-containing substrates including adenosine triphosphate (ATP) and inorganic pyrophosphate (**6**). An additional important mechanism by which ALP promotes mineralisation, however, is by hydrolysing pyrophosphate, a ubiquitous physicochemical inhibitor of calcium phosphate deposition. To form mineralised bone nodules *in vitro*, an additional source of phosphate is required. The most widely used phosphate source is β-glycerophosphate (β-GP), although phosphate itself can also be used. **Fig. 3** illustrates the critical importance of β-GP concentration in this culture system (see also Orriss *et al*, 2007) (**6**). In osteoblast cultures lacking β-GP, organic matrix is deposited but mineralisation fails to occur. Osteoblasts cultured with 2mM β-GP reproducibly form abundant bony structures with characteristic "trabecular" morphology; alizarin red staining shows that mineralisation is confined to these matrix structures. In contrast, culture with 5-10mM β-GP causes a widespread, dystrophic deposition of mineral as soon as the differentiating osteoblasts begin to express significant amounts of ALP, leading to mineralisation of the cells themselves (**Fig. 3A**). This impairs cell viability (**Fig. 3B**), with the result that only small mineralised nodules are able to form (rather than the striking trabecular structures evident with lower concentrations of β-GP). Thus, it is critical that a low concentration of β-glycerophosphate is used in this assay.

4. ***The importance of pH***: Extracellular pH is an important factor in the regulation of bone mineralisation (**7**). Acidosis causes a selective, inhibitory action on matrix mineralisation by inhibiting alkaline phosphatase expression and activity, whilst increasing mineral solubility (**7**). For example, even a reduction in pH from pH 7.43 to pH 7.32 will reduce bone nodule formation *in vitro* by ~60% (**Fig. 4**). Due to its high bicarbonate concentration, DMEM is more alkaline than some media (such as minimum essential medium, MEM); in equilibrium with 5% CO_2, it is buffered to pH ~7.4. The metabolic activity of osteoblasts will cause progressive acidification, particularly in mature, bone forming cultures when cells numbers are high. To ensure that pH does not affect bone mineralisation in this assay it may sometimes be necessary to add a small volume of OH^- ions (as 6M NaOH) to the culture medium to ensure an average running pH of 7.4.

5. ***Measuring the medium pH***: Accurate measurement of the operating pH of mammalian osteoblast cultures is important for meaningful comparison of results from different laboratories. For HCO_3^-/CO_2 buffered media, accurate pH measurement can only be achieved by the use of a standardized blood gas analyser. We use a Radiometer ABL 705 blood gas analyser (Radiometer, Crawley, UK), which features a multi-electrode system to measure pH, pCO_2 and pO_2 in a 200µl injected sample (cyclic time ~ 2 minutes). The first medium measurement taken immediately after removing the culture plates from the incubator is assumed to provide a pCO_2 value that is identical for all wells and that reflects the actual pCO_2 during incubation. It should be noted that opening the incubator door during experiments, may cause perturbations in CO_2 levels that affects the measured pH and pCO_2 values.

6. ***Statistics***: Because of interassay variability, statistical comparisons should be performed only within one assay, and not between different assays.
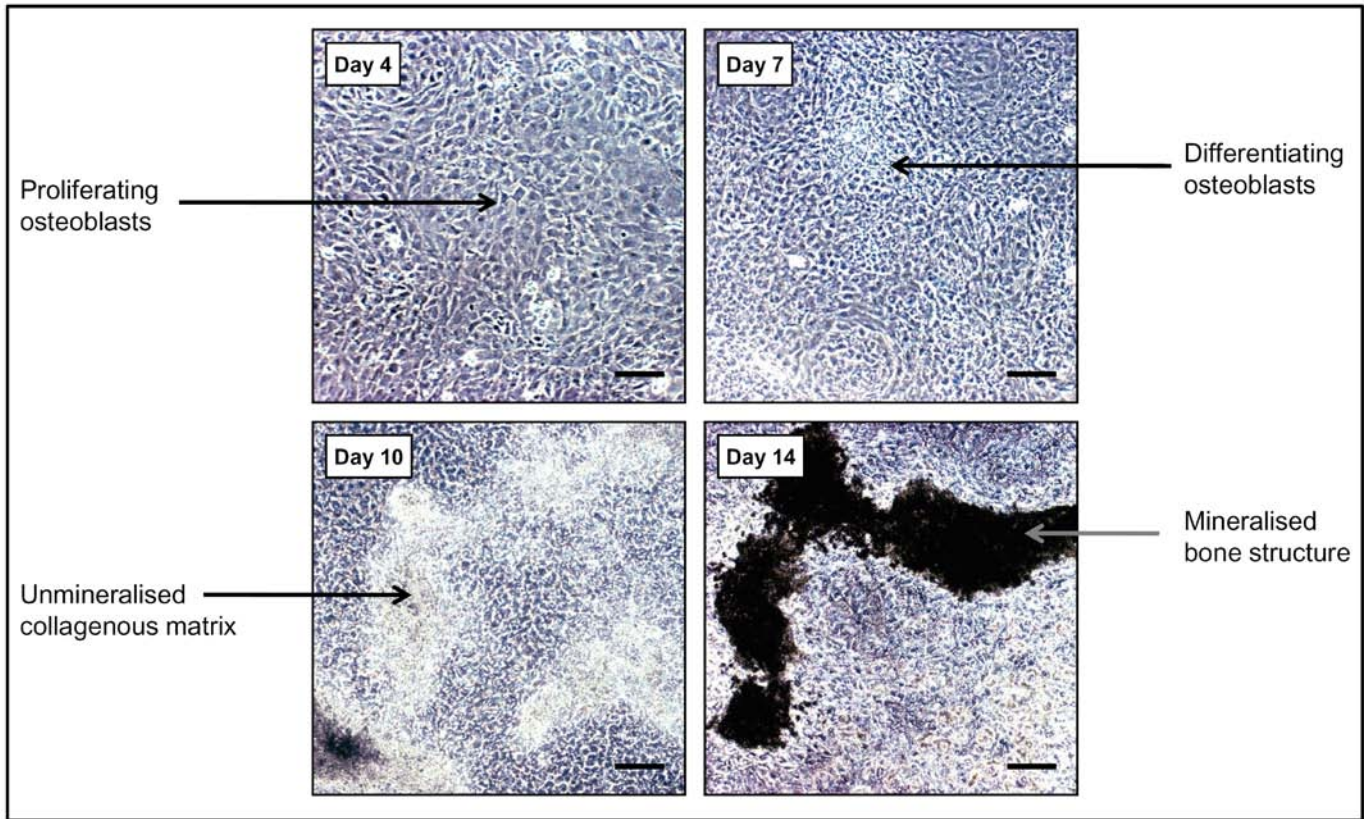
Figure 1. Orriss *et al, 2010*

Figure 1. *Phase contrast microscopy of primary rat osteoblast cultures.* Representative images are of unstained cell layers. By day 4 of culture, a confluent monolayer of cells is evident. At day 7, the cells are more compacted and organic matrix is beginning to be deposited (as shown by the arrow). By day 10, there is abundant deposition of unmineralised collagenous matrix and mineralisation (dark area at lower left of image) is commencing. After 14 days of culture, there is widespread formation of mineralised matrix networks. Scale bar = 25µm.
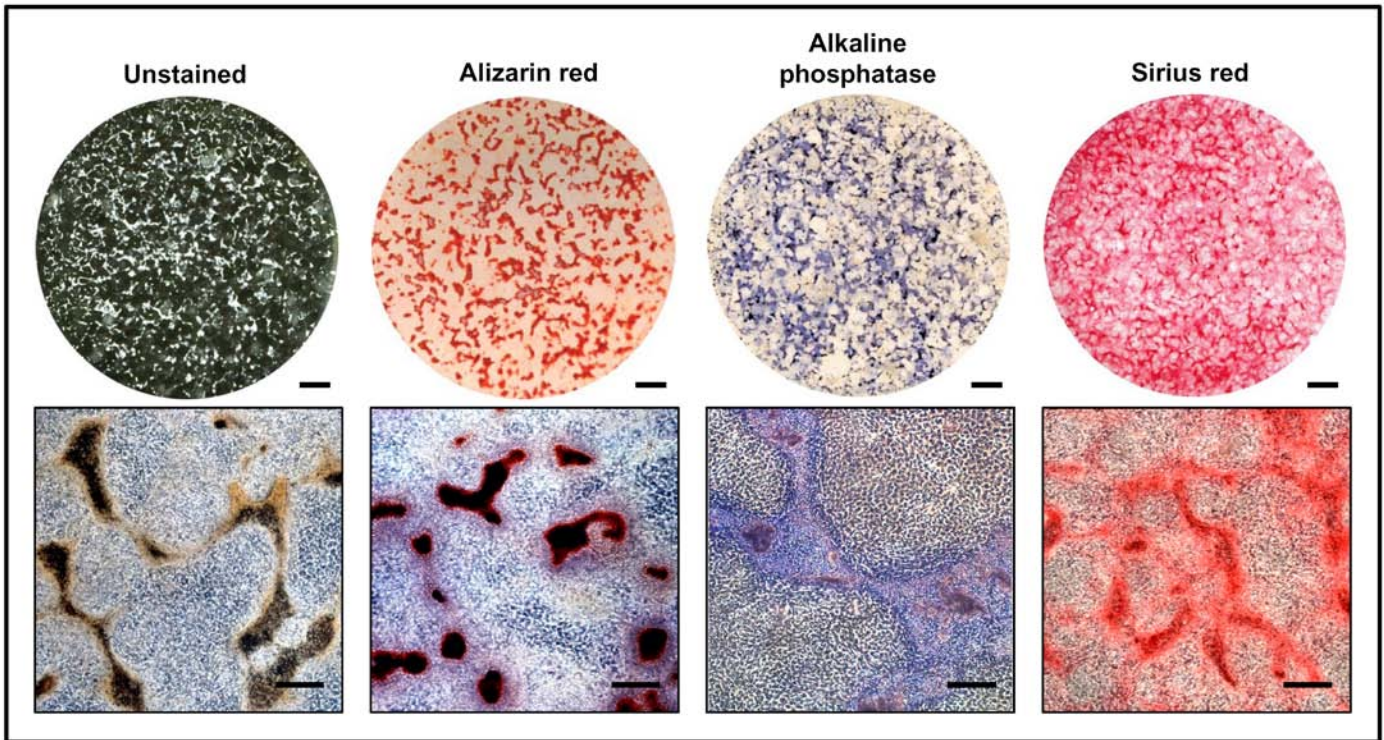
Figure 2. Orriss *et al, 2010*

Figure 2. *Different staining methods in primary rat osteoblast cultures.* Images show 14 day osteoblast cultures either left unstained, or stained with alizarin red (to demonstrate calcium), alkaline phosphatase (ALP) or sirius red (to demonstrate collagen). Bone formed in these cultures exhibits a typical "trabecular" morphology, with discrete mineralisation confined to matrix nodules, as shown in the unstained and alizarin red-stained images. In the unstained image at lower left, unmineralised matrix appears brown and mineralised matrix black. Intense ALP staining (also 'trabecular') is evident in mature, bone forming osteoblasts. Sirius red staining shows the presence of collagen fibres in these cultures. Scale bars = 0.1cm (cell well scans, upper row) and 25μm (phase contrast images, lower row).
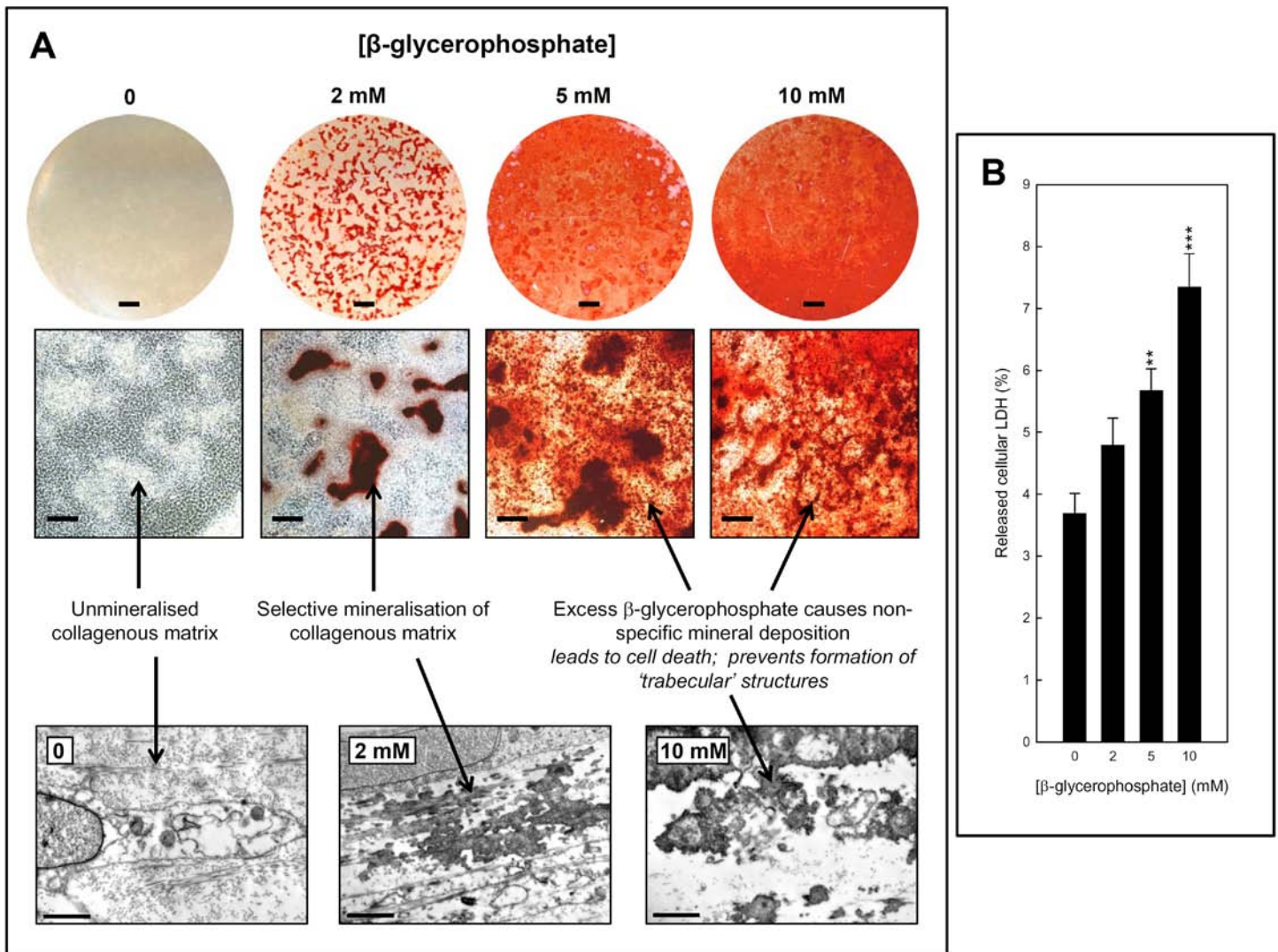
Figure 3. Orriss *et al, 2010*

Figure 3. *The effect of the β-glycerophsophate concentration on bone mineralisation and osteoblast viability in vitro.* **(A)** Rat calvarial osteoblasts were cultured for 14 days before staining with alizarin red to demonstrate mineral deposition. Rat long bone osteoblasts were cultured for 28 days before fixation and transmission electron microscopy (TEM). In the absence of β-glycerophosphate (β-GP), bone mineralisation fails to occur due to insufficient inorganic phosphate; widespread, unmineralised collagenous matrix is evident in the phase contrast and TEM images. Bone formed in the presence of 2mM β-GP exhibits a typical "trabecular" morphology; mineralisation is confined to these structures. In the presence of 5-10mM β-GP, widespread, non-specific (dystrophic) deposition of bone mineral occurs across the cell monolayer, with inhibition of normal matrix deposition. Intracellular deposition of mineral that causes damage to cell membranes and organelles is also evident. **(B)** Increased β-GP concentration is associated with decreased osteoblast viability, assessed as lactate dehydrogenase (LDH) release after 14 days in culture. Values are means ± standard error of the mean (SEM) (n = 6); significantly different from control *** = $p < 0.001$, ** = $p < 0.01$. Scale bars = 0.1cm (cell well scans), 25µm (phase contrast images) and 1µm (TEM).
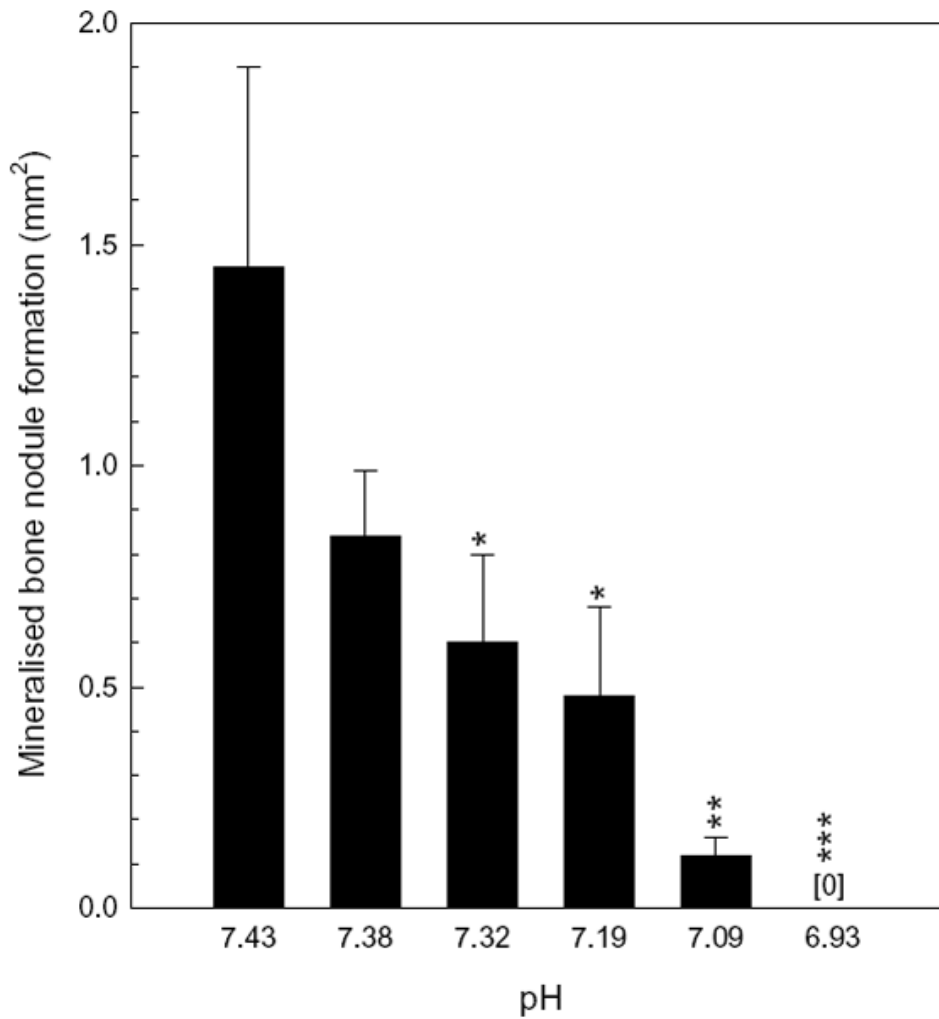
Figure 4. Orriss *et al, 2010*

Figure 4. *Inhibitory effect of acidosis of bone mineralisation by cultured rat osteoblasts.* In 14 day cultures, decreasing pH caused progressive reduction in the formation of mineralised nodules stained with alizarin red, with complete inhibition at pH 6.93. Values are means ± standard error of the mean (SEM) (n = 6); significantly different from control (pH 7.43) value, ** = p <0.001, * = p < 0.05.

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