An Investigation into the Process of Early Osteoblastic Differentiation

A Thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medicine

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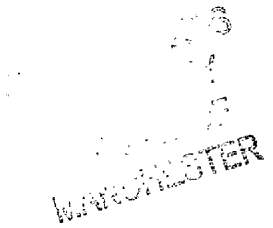
(DXKUT)



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Abstract

level to excess osteoclasis and/or reduced osteoblastosis. In order to study the processes leading to the latter a histomorphometric investigation of osteoblastic function was carried out in 153 cases with established osteoporosis. This demonstrated a reduction in the number of osteoblasts in up to 62% of cases, indicating a failure of osteoblast recruitment and/or maturation from the stem cell pool. The involvement of different bone compartments in osteoporosis was studied by histomorphometry, in 159 cases, demonstrating reduction in bone formation rate in the trabecular and cortical compartments in up to 82% of cases, compared to only 43 to 44% of cases for the periosteal and sub-cortical compartments. Reduction in the trabecular compartment was mirrored by reduction in the cortical bone in 85% of cases, whilst the same was true for the periosteal and sub-cortical compartments in 66% of cases, indicating coupling of bone formation within, but not between, the two sets. The existence of the postulated switch in differentiation of marrow stromal cells from the osteoblastic to the adipocytic lineage in osteoporosis was investigated by measurement of the ratio of adipocytic to haematopoietic tissue in osteoporosis, which was elevated (43% vs 22% in controls). The increase in the amount of adipose tissue was most significantly associated with the trabecular apposition rate, supporting the postulated switch. As a first step to identifying the cellular processes responsible for the alteration in osteoblastic differentiation in osteoporosis indicated by these studies primary human bone marrow stromal cell culture was used to isolate differentiation stage-specific genotypic markers of early osteoblastic differentiation, in both adherent and non-adherent models of osteoblastic differentiation. Adherent stromal cells were isolated from human marrow and their capacity for osteoblastic differentiation established by stimulation with dexamethasone. Reseeding of initially non-adherent marrow stromal cells yielded further adherent cells, which showed the same capacity for osteoblastic differentiation. The non-adherent cells were negative for the antibody STRO-1, whilst both the initially and subsequently adherent cells were positive, indicating that the non-adherent cells represented an earlier stage in the osteoblastic lineage. For both systems, poly (A) reverse transcription polymerase chain reaction was used to produce cDNA libraries representative of all the mRNA species in stromal cells undergoing osteoblastic differentiation. In order to minimise genetic heterogeneity between the cells used to produce the cDNA, clonal populations of STRO-1 positive adherent cells in the adherent model, and of adherent cells subsequent upon reseeding of glycophorin A negative non-adherent cells in the non-adherent model, were used. Subtractive hybridisation was then used to isolate genes upregulated during early osteoblastic differentiation of adherent stromal cells, and during adherence of non-adherent marrow stromal cells, the identity of which was established by library screening and gene sequencing. Four genes upregulated in the former were identified, i.e. 14-3-3 zeta, KIAA0081, non-skeletal tropomyosin and alpha enolase, the involvement in osteogenesis of the first 3 of which was confirmed by *in-situ* hybridisation studies in normal and abnormal human adult and fetal bone. Each of the 3 were upregulated in healing fracture callus, and in the growth plate, the osteoblasts and the periosteal mesenchyme in fetal bone, whilst 14-3-3 zeta showed variable expression in a variety of fetal skeletal dysplasias; initial transfections of primary marrow stromal cells with 14-3-3 zeta antisense oligomer abrogated osteoblastic differentiation. A more comprehensive tissue distribution than previously reported was obtained for KIAA0081. Two genes upregulated during adherence of non-adherent stromal cells were identified, aldolase A and CGI-120 protein, the involvement of both of which in early osteogenesis was confirmed by *in-situ* studies. These genotypic markers will facilitate study of the process of early osteoblastic differentiation, and of its perturbation in osteoporosis.

Declaration

No portion of the work referred to in this thesis has been submitted in support of an application or qualification of this or any other university or institute of learning.

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To Ann and our children, James, John, Michael and Tom

"And now my story is told. Like my journeys, it has not always followed the directions I thought it might, and sometimes the outcomes have remained obscure until the very end. It has been a voyage of discovery for me, as all journeys should be. It has become an act of love and trust. I have tried to be guided by the truth and have discovered that, as in my running, honesty, courage and a determination to succeed bring rewards and joys quite unlooked for....

And as I run, I shall wonder: is this the beginning of the end or is it just the end of the beginning?"

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Publications arising from this work

Published Articles

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Byers, R. J., Denton, J., Hoyland, J. A., Freemont, A.J., (1999). Differential patterns of altered bone formation in different bone compartments in established osteoporosis. *J Clin Path* **52**: 23-28.

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Prizes

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Chapter 1

General introduction

1.1 Normal Bone Structure

Bone is a specialised form of connective tissue providing support, protection and attachment. It also plays an important role in homeostasis, principally through modulation of calcium fluxes. It is a dynamic tissue and is best considered in terms of its component cells, and the matrix which they synthesise, as well as the bone marrow, consisting of haematopoietic cells and stromal cells.

1.1.2 Organisation of bone structure

1.1.2.1 Macroscopic organisation

The adult skeleton can be divided into the axial and the appendicular skeleton, and is composed of long, flat and short bones. The axial skeleton comprises the vertebral column, the pelvis and the skull, whilst the appendicular skeleton includes the upper limb girdle, the rib cage and the bones of both upper and lower limbs. The shape of each bone is related to its function (Revell, 1986). The long bones give maximal mechanical advantage, the short bones facilitate balance and weight bearing, and the flat bones provide protection for vital organs.

1.1.2.2 Microscopic organisation

In addition, bone can be divided into two main types: woven and lamellar (Eriksen *et al.*, 1994). Woven bone is the first type of bone to develop in embryonic life and is present in fetuses and young children. It is composed of large, irregular osteocytic pieces of bone, and has irregular lamellae. It is not strong and as a consequence has greater flexibility than mature, lamellar bone. Lamellar bone forms the majority of the normal adult skeleton and is composed of collagen fibres arranged in parallel sheets. It is present in both cortical and cancellous, or trabecular, bone, and provides tensile strength at the expense of flexibility.

Cortical bone comprises approximately 80% of the adult skeleton (Dempster, 1992) and consists of columns of concentric lamellae, which together with canaliculi present between the lamellae, comprise a Haversian system (Carleton & Short, 1954). Each of these systems directly abuts its neighbours and runs roughly parallel to the long axis of the one, along lines of stress. The majority of the canaliculi also run parallel to the long axis of the bone and are connected to a central Haversian canal, which runs the length of the Haversian system, by smaller canaliculi which radiate out from the central canal, and connect each lamellae to the next layer. The canaliculi contain fluid and osteocytes. It is thought that mechanical stress induces fluid flows within the canaliculi, which in turn exert

shear stress on the osteocytes, producing a cellular response to mechanical stress (Mosley & Lanyon, 1998; Skerry, 1997).

Trabecular bone consists of orientated lamellae present in the marrow space within bone. The lamellae are orientated in parallel sheets in trabeculae, which form a lattice across the marrow space. As in the compact bone the trabeculae lie along lines of stress, and provide mechanical strength disproportionate to their volume. The strength of the bone is due to both the mineral density (Turner, 1989) and the geometric arrangement of the trabecular struts (Biewener *et al.,* 1996; Majumdar *et al.,* 1998).

Attempts to identify biochemical markers of bone turnover have concentrated on the cortical and trabecular compartments. Whilst such markers generally give a picture of overall activity in the entire skeleton, they have also indicated that different remodelling rates are present in the different bone compartments, both in normal and disease states (Delmas, 1990).

Between the cortical and trabecular compartments lies a small zone of subcortical or endosteal bone, which has a high level of remodelling (Parfitt, 1983). The outer surface of the metaphysis is covered with periosteum, immediately beneath which lies a thin layer of periosteal bone. This consists of growing bone, composed of osteoblasts and associated osteoid and calcified matrix. It is responsible for concentric transverse growth of the diaphysis and merges into the cortical bone. The osteoblasts covering the periosteal bone are thought to arise from the stromal cells located within the periosteum (Bruder *et al.,* 1997 & 1998).

1.2 Bone modelling and growth

1.2.1 Bone modelling and remodelling

Modelling and remodelling are distinct processes that need to be distinguished from each other (Dempster, 1992). Modelling is the process whereby the bone alters its shape throughout the growth phase, whilst remodelling is a continuous process whereby old, mature bone is replaced by new bone. Therefore, whilst modelling results in formation of a new structure, remodelling causes little or no change in bone shape. It is a dynamic process and occurs in focal and discrete packets of bone. The remodelling of each packet takes about 3 to 4 months and is separate, both spatially and temporally, from the remodelling of other packets (Mundy, 1995a). The packets of bone have been called the bone remodelling

units. The process of remodelling takes place in an organised manner and has four stages, namely activation, resorption, reversal and formation.

1.2.2 Activation and resorption

Osteoclastic activation is the first step and involves adhesion of osteoclasts to bone via integrins on the surface of the osteoclast membrane and RGD (arginine-glycine-asparagine) motifs in proteins at the bone surface, such as osteopontin (Miyauchi A *et al.*, 1991). The osteoclast becomes polarised, with development of a ruffled border adjacent to the bone surface. Lysosomal enzymes are released into the space between the osteoclast and the bone surface, and hydrogen ions generated by carbonic anhydrase II are pumped across the ruffled border, providing an optimal environment for proteolytic degradation of bone (Blair *et al.*, 1989). Following resorption the osteoclast, which is a motile cell, moves on and osteoblasts are recruited to the site of resorption, where they proliferate, laying down osteoid which is then mineralised to form new bone (reviewed in Mundy, 1995a).

1.2.3 Formation

Bone resorption and subsequent formation are closely coupled in normal bone (Hayden *et al.*, 1995). Several factors are involved in this process. Resorption releases transforming growth factor beta (TGFβ) and bone morphogenetic proteins (BMPs) from the bone matrix (Mundy *et al.*, 1995). Transforming growth factor beta is chemotactic for osteoblasts, as is platelet derived growth factor (PDGF) (Zheng *et al.*, 1992) which is also released. Both also stimulate osteoblastic proliferation and increase the number of committed osteoblasts. This phase of osteoblast proliferation is followed by differentiation, stimulated by BMPs (Urist, 1997; Sakou, 1998; Kaplan and Shore, 1996), especially BMP-2 and BMP-4 (Li *et al.*, 1996; Chen *et al.*, 1997; Amedee *et al.*, 1994; Fromigue *et al.*, 1998; Reddi and Cunningham, 1993). The insulin-like growth factor system is also important in mediating coupling (Hayden *et al.*, 1995). Insulin-like growth factors (IGFs) promote osteoblast proliferation and are released from the bone by resorption, and synthesised by both osteoclasts and osteoblasts, acting in both a paracrine and an autocrine manner (Linkhart *et al.*, 1996).

1.2.4 Fetal bone development

The fetal skeleton is formed by two processes: intramembranous ossification and endochondral ossification. Intramembranous ossification is responsible for the formation of the flat bones, such as the calvarial and facial bones, and the small bones, such as the

vertebral bodies, which are initially formed by intramembranous ossification, whilst the long bones of the limbs are formed by endochondral ossification.

1.2.5 Intramembranous ossification

During intramembranous ossification bone arises directly from a mesenchyme (Cruess, 1982), which condenses to form a membranous cast of the bone being formed. As the mesenchymal cells condense collagen is deposited, forming a fibrous area. Osteoblasts differentiate from the stromal mesenchymal cells and produce osteoid, which is then mineralised. As more mineralised matrix is produced some osteoblasts become entrapped within it where they persist as osteocytes. The bone gradually replaces the fibrous tissue, growing within the membranous anlagen as bone spicules. Meanwhile, vascular mesenchyme condenses on the outside of the membrane to form periosteum, the inner layer of which differentiates into osteoblasts. Consequently, two types of bone are produced, an inner vascular zone of cancellous bone from the membrane, which is surrounded by an outer relatively avascular plate of cortical bone (Cruess, 1982).

1.2.6 Endochondral ossification

Initially the long bones are represented by a model composed of condensed mesenchyme, which is replaced by hyaline cartilage (Snell, 1975). Ossification begins, at about the eight week of gestation, in the middle of the cartilaginous shaft (Ogden & Rosenberg, 1988). The chondrocytes undergo hypertrophy, then apoptosis, and the matrix becomes mineralised. The outer layer of the mesenchyme undergoes osteoblastic differentiation in the same manner as for intramembranous ossification, and begins to lay down an outer layer of periosteal bone (Cruess, 1982). This outer layer of bone maintains the integrity of the developing long bone as the central part of the cartilaginous shaft is broken down and replaced by a bud of vascular tissue. Chondroclasts present in the bud break down the cartilaginous core of the shaft, which is then replaced by cancellous bone by osteoblasts, which also arise from the vascular bud. This represents the primary ossification centre and forms the diaphysis. At either end of the developing bone lie the epiphyses, composed of cartilage, and separated from the diaphysis by the fetal growth plate.

1.2.7 Fetal growth plate

Longitudinal growth of fetal long bones is determined largely by growth at the growth plate (Ogden & Rosenberg, 1988; Price *et al.,* 1994). However, the anatomy of the growth

plate in the fetus is relatively poorly characterised, though the anatomy of the post-natal growth plate is well defined and will therefore be reviewed initially to provide orientation for discussion of the fetal growth plate.

In post-natal long bones the growth plate consists of resting, proliferative and hypertrophic zones (reviewed in Brighton, 1978 & 1984). The resting zone lies next to the epiphysis and is composed of spherical chondrocytes, lying either singly or in pairs, separated by cartilaginous matrix. In the proliferative zone there is a transition from spherical to flattened proliferative chondrocytes, which are aligned in columns parallel to the long axis of the bone (Brighton, 1984). The cells in this zone are separated by less matrix than elsewhere in the growth plate, and the zone provides most of the longitudinal growth of the bone. The hypertrophic zone contains large spherical chondrocytes, again aligned in columns, but becoming progressively vacuolated and less ordered as they undergo terminal hypertrophic differentiation, followed by apoptosis (Hillarby *et al.*, 1996).

Calcification of the matrix starts in the hypertrophic zone, and initially occurs on or within matrix vesicles, present in the longitudinal septa of the matrix (Poole *et al.*, 1984, 1989). The C propeptide of type II collagen becomes concentrated in the mineralising sites and there is an associated replacement of the amorphous calcium phosphate that is initially deposited by crystalline hydroxyapatite. Consequently, the longitudinal septa become calcified by crystal growth and coalesce. This process occurs in the lower half of the hypertrophic zone, which is therefore referred to as the zone of provisional calcification. It has been suggested that some of the molecules secreted by chondrocytes in this zone stimulate capillary invasion (Cowell *et al.*, 1987).

Distal to the growth plate lies the metaphysis, which is generally defined as that area immediately distal to the last intact transverse septum at the base of each cell column of the growth plate, and proximal to the narrower diaphysis (Brighton, 1984). From this area capillary sprouts invade the cartilage of the growth plate. The capillaries are involved in degredation and removal of the non-mineralised transverse septa and associated cells. In this area osteoblasts are observed lining calcified cartilage bars, known as primary spongiosa, whilst the secondary spongiosa begins a little further down the septa, where osteoblasts begin to lay down bone (Brighton, 1984).

Though there have been many radiographic (Martin & Higginbottom, 1971), ultrasonographic (Elejalde & Elejalde, 1986; Gentili *et al.*, 1984) and gross anatomical

studies of the development of the fetal growth plate, few studies have investigated the dynamic changes occurring in the growth plate during gestation at the microscopic level in humans. Rodrigeuz *et al* (1992) studied the growth plate in the fetal and neonatal period. In the first half of pregnancy the fetal growth plate was highly cellular, with little of the cellular organisation and matrix seen in post-natal life. However, from the 20th to the 40th week of gestation the thickness of the growth plate decreased due to reduction of both matrix and cellular components, especially the latter. Consequently the relative volume occupied by matrix, and the amount of matrix per cell, increased. Cellular organisation was more ordered, and, interestingly, the thickness of the growth plate increased post-natally, but these changes had no effect on longitudinal growth rate. Measurement of bone apposition in 19 week fetuses, by tetracycline labelling prior to therapeutic termination, demonstrated maximal mineralisation at the periosteal surface, the rate for the cancellous and endocortical compartments both being roughly half that for the former (Glorieux *et al.,* 1991). In addition, trabecular osteoid thickness was greatest close to the growth plate, in the region of the primary spongiosa, and fell from the epiphysis, as did the periosteal mineral apposition rate. This study highlights the clear compartmentalisation and organised mineralisation present in fetal bone.

1.3 Bone cells

1.3.1 Osteoclasts

Osteoclasts are the cells responsible for bone resorption. They are thought to arise from a haematopoietic stem cell, sharing a common precursor with the macrophage/monocytic lineage (Parfitt, 1998). They break down bone by a combination of enzymic digestion and acid hydolysis and, coupled to osteoblastic activity, act to maintain dynamic turnover and renewal of bone integrity (Vaananen, 1996).

Osteoclasts are giant multinucleated cells responsible for bone resorption and arise from haematopoietic tissue, sharing a common precursor with cells of the monocytic/ macrophage lineage. They are found on endosteal bone surfaces, in Haversian systems, and occasionally on periosteal surfaces (Mundy, 1995a). Whilst not common on normal bone surfaces, they are frequent at sites of active remodelling, such as in growing bones, or in hyperparathyroid bone disease. They are large multinuclated cells, containing up to 100 nuclei, though usually less than 10. The nuclei are centrally placed, containing one or two nucleoli. The cytoplasm contains primary lysosomes, numerous mitochondria and is

polarised, the cell membrane adjacent to the bone being folded (the ruffled border). This border attaches to the bone forming a tight seal, beneath which resorption occurs. The cytoplasm next to the ruffled border contains no organelles (the clear zone), though is rich in actin filaments which act to anchor the osteoclast to the bone surface. Osteoclasts can be identified by presence of the calcitonin receptor (Takahashi *et al.,* 1988), TRAP (tartrate-resistant acid phosphatase) expression, and by positivity for the monoclonal antibody 23c6, which recognises the alphaV/beta3 subunit of the vitronectin receptor (Horton *et al.,* 1991). Both the calcitonin receptor and 23c6 however are found on other cell types (Mundy, 1995a).

Osteoclasts attach to bone via adhesion receptors, specifically the alphaV/beta3 vitronectin receptor, alpha2/beta1 collagen receptor, and alphaV/beta1 receptor (Nesbitt *et al.,* 1993), of which the vitronectin receptor is thought to be most important (Horton *et al.,* 1991), mediating not only attachment but also movement across the bone surface. The sealing membrane zone is thought to be attached to the bone via a specific type of cell-matrix interaction (Vaananen & Horton, 1995). Following attachment the osteoclast becomes polarised into three distinct domains, the ruffled border, the sealing zone and the basal membrane, opposite to the ruffled border. The ruffled border forms the 'resorbing organ' and it has been suggested that it is a specialised form of lysosomal membrane since it contains a lysosomal proton pump and the mannose-6-phoshate receptor (Baron *et al.,* 1988). The cytoskeleton undergoes intense reorganisation during polarisation, with accumulation of podosomes and actin-integrin complexes, that anchor extracellular proteins via their integrin subunit, specifically the vitronectin receptor. Following this actin becomes arranged as a ring, a pattern seen only in resorbing cells (Vaananen, 1996). During resorption the actin ring is rapidly broken down under the action of calcitonin. Bone mineral and matrix are broken down by the production of an acidic pH at the bone surface, by the proton pump (Baron *et al.,* 1988), with consequent dissolution of the hydroxyapatite crystals (Vaananen, 1996), together with the secretion of lysosomal cysteine proteinases (Goto *et al.,* 1994) and matrix metalloproteinasas (Chambers *et al.,* 1985), which together degrade bone matrix proteins.

1.3.1.1 Osteoclast lineage

Osteoclasts are derived from the haematopoietic system (Gothlin & Ericsson, 1976; Walker, 1973 & 1975; Kahn & Simmons, 1975). Co-culture of mouse bone marrow and spleen cells contain multinucleated cells, which express an osteoclastic phenotype (TRAP

and calcitonin receptor positivity) upon stimulation with 1,25-dihydoxy vitamin D3 (1,25-vitamin D3), parathyroid hormone (PTH) or prostaglandin E2 (PGE2). These markers were simultaneously expressed in a population of mononuclear cells, and subsequently these mononuclear cells, which are part of the monocyte-macrophage system, have been shown to be osteoclast precursors (Suda *et al.,* 1996b). Differentiation of monocytes, and of some tissue macrophages, to osteoclasts requires the presence of marrow stromal cells and osteoblasts (Suda *et al.,* 1992b), and a number of cell lines that support osteoclast differentiation are now avaliable (Suda *et al.,* 1996b). Intriguingly, direct cell-to-cell contact is required in co-culture for osteoclast formation (Suda *et al.,* 1992b). The process of osteoclast differentiation from the monocyte-macrophage system is outlined in figure 1.1. For each stage in the lineage mutations have been reported in different types of osteopetrotic mice, all of which possess the same resultant phenotype, namely marked increase in skeletal density and corresponding narrowing of the marrow cavities (Marks, 1989). Specifically, the knockouts generated include the grey-lethal (*gl/gl*), micropthalmic (*mi/mi*), osteosclerotic (*oc/oc*), and osteopetrotic (*op/op*) mice. The first two are cured by bone marrow transplantation, indicating the presence of defects in the maturation of osteoclast progenitors, whilst the latter two are not, indicating that they have defects of the bone microenvironment. Two other artificial models of osteopetrosis have been generated in mice, by disruption of the proto-oncogenes c-*fos* (Wang *et al.,* 1992) and c-*src* (Soriano *et al.,* 1991). The *op/op* mice do not generate M-CSF, the defect appearing to lie in the osteoblasts, since co-culture of *op/op* osteoblasts and normal spleen cells did not support osteoclastosis except in the presence of the recombinant growth factor M-CSF (macrophage/monocyte-colony stimulating factor), whilst co-culture of *op/op* spleen cells with normal osteoblasts resulted in generation of many osteoclast-like cells (Marks, 1989). The nature of the gene defect in *oc/oc* mice is unknown, but is thought to involve disruption of ruffled border formation by the osteoclasts (Marks, 1989). In the *mi/mi* mice osteoclasts also fail to develop ruffled borders, though the mechanism whereby the protein encoded by *mi*, which is a member of the basic-loop-helix-leucine zipper family, effects this normally is not known. The nature of the defect in grey-lethal mice is presently unknown.

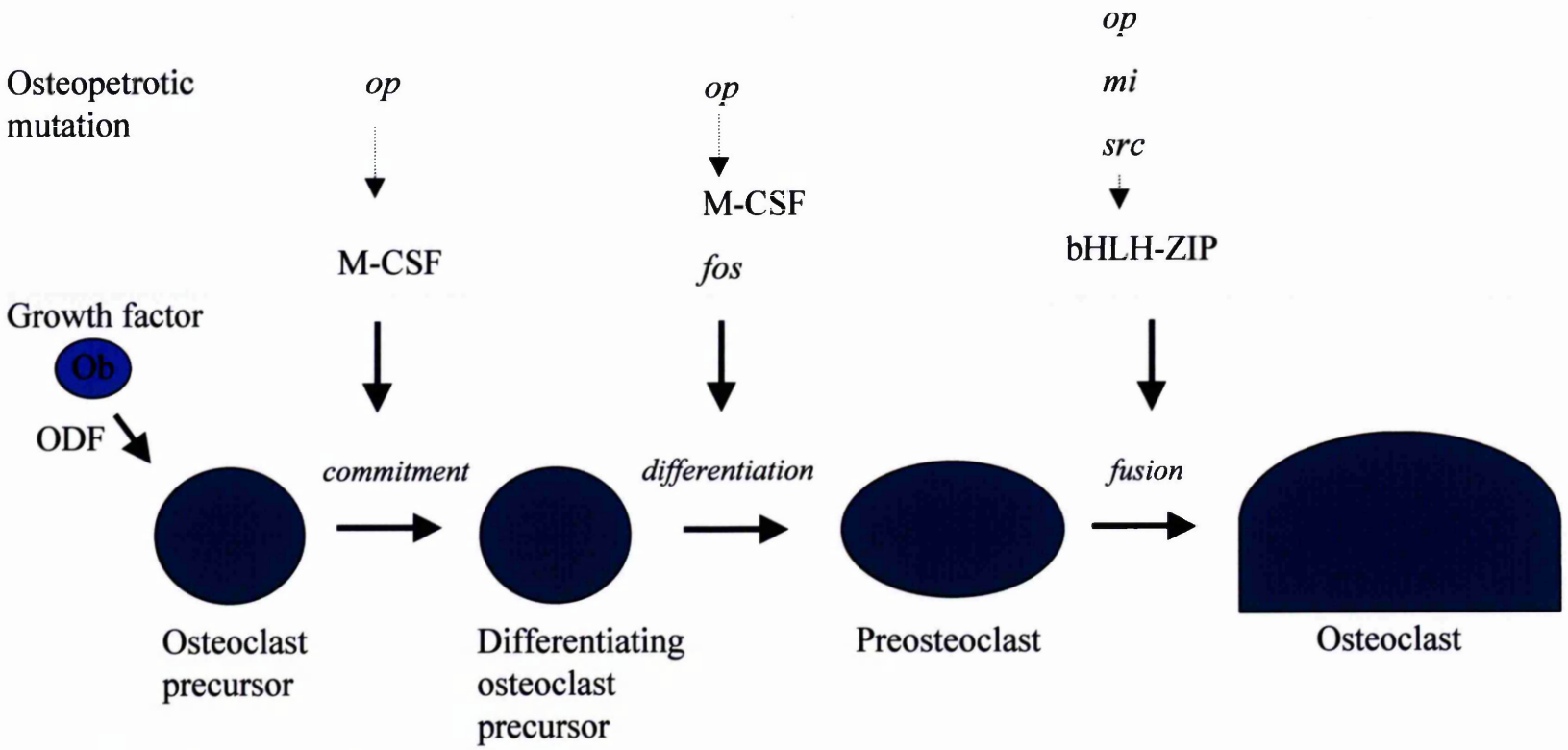


Figure 1.1 Osteoclast differentiation. Growth factors involved at each stage, and the murine osteopetrotic mutants resulting from defects at each stage are shown (see text for details of mutations). Ob - osteoblastic cells, ODF - "osteoclast differentiation factor", bHLH-ZIP - basic helix-loop-helix-leucine zipper protein (adapted from Suda *et al.*, 1996).

Osteoclast differentiation is regulated by 1,25-dihydroxy vitamin D3 (Kurihara *et al.,* 1990) and PTH (Uy *et al.,* 1995). Until recently receptors for both these hormones had been detected only on osteoblasts (Mee *et al.*, 1996), and both are thought to act on osteoclast precursors indirectly by stimulating the production of an osteoclast differentiation factor by osteoblasts (Suda *et al.,* 1992b; Martin *et al.,* 1998). Stimulation by 1,25-dihydroxy vitamin D3 is mediated via the vitamin D receptor, whilst two further signal transduction pathways are responsible for mediating the effects of other stimulants, namely the gp130 pathway for the interleukins IL-6 and IL-11, oncostatin M (OSM) and leukaemia inhibitory factor (LIF), and cyclic AMP (cAMP) for PTH, PGE2 and IL-1 (Martin *et al.,* 1998). Each of these factors induces osteoclast differentiation. In addition, M-CSF plays an important role in osteoclast development (Sarma & Flanagan, 1996), acting to augment proliferation, differentiation, migration and survival, whilst cultures lacking M-CSF fail to generate osteoclasts (Tanaka *et al.,* 1993), and it has been proposed that M-CSF acts to effect a switch in the differentiation of monocytes from the macrophage to the osteoclast pathway (Mbalaviele *et al.,* 1995). Interestingly, with the exception of M-CSF, the remainder of the reported osteoclastogenic factors, including IL-1, TGBβ, tumour necrosis factor alpha (TNFα), IL-6, IL-11, PGE2 and PTH, have been shown to be not essential for osteoclast development by genetic ablation experiments; mutation of only csf-1, which encodes for M-CSF leads to arrest at the pre-osteoclast stage (Suda *et al.,* 1996b).

IL-6 acts as a paradigm for the gp130 signalling pathway. It exerts is activity via a cell surface receptor that consists of two components; a ligand-binding protein, gp80 (IL-6 receptor), and the non ligand-binding protein gp130 (Taga *et al.,* 1989). The soluble IL-6R (sIL-6R), which lacks transmembrane and cytoplasmic domains, interacts with IL-6 to stimulate osteoclastic differentiation, whilst either of them alone fail to do so (Tamura *et al.,* 1993). Interestingly, sIL-6R has been detected in the urine and serum of healthy subjects, and its level is increased in patients with multiple myeloma (Gaillard *et al.,* 1993), HIV infection (Honda *et al.,* 1992) and rheumatoid arthritis (Kotake *et al.,* 1996), in whom excess IL-6 is also present (Hirano *et al.,* 1988), suggesting that the two form a complex in these patients which induces bone destruction. IL-6 has also been implicated in the recruitment of osteoclasts caused by oestrogen deficiency (Jilka *et al.,* 1992).

Most recently, the role of the new TNF-family molecule osteoprotegerin ligand (OPGL) (Kong *et al.,* 1999), and of osteoprotegerin (OPG) (Lacey *et al.,* 1998), in osteoclastogenesis has been defined. OPGL (also known as TRANCE, RANKL and ODF) is expressed by osteoblast/stromal cells, is induced by 1,25-vitamin D3, IL-11, PGE2 and

PTH, and acts to both mediate osteoclastogenesis, in the presence of M-CSF, and to activate mature osteoclasts. It effects both of these actions without the need for either 1,25-vitamin D3 or co-culture with stromal cells, indicating that it corresponds to the previously uncharacterised osteoclast differentiation factor (ODF) (Martin *et al.,* 1998). By contrast, osteoprotegerin (OPG), also known as osteoclastogenesis-inhibitory factor, inhibits osteoclast differentiation (Simonet *et al.,* 1997). OPG is a soluble cytokine produced by osteoblasts, under stimulation by 1,25-vitamin D3, BMP-2, IL-1 and TNFα, and is a member of the TNF receptor family (Hofbauer *et al.,* 1998). It appears to act at the level of the TRAP positive, β3-integrin positive osteoclast precursors (Tan *et al.,* 1997), and may act as a neutralising factor for a TNF-related protein that normally acts as an osteoclast maturation factor (Lacey *et al.,* 1998). OPGL, either on the surface of osteoblasts, or in a soluble form, binds to OPG to block its inhibitory effects on osteoclastogenesis (Kong *et al.,* 1999).

Finally, very recent evidence has been reported in support of a role for the transcription factor nuclear factor kappa beta (NFkB) in maturation of TRAP negative to TRAP positive precursors, and for survival of mature osteoclasts by inhibition of apoptosis (Jimi *et al.,* 1998). IL-1 induces activation of NFkB in osteoclasts, with associated degradation of it inhibitor, IKBA, and translocation of the p65 subunit of NFkB from the cytoplasm to the nucleus (Jimi *et al.,* 1998). In contrast however, Hing *et al* (1998) found that NFkB did not protect against apoptosis, but that it was important for cell maturation, by interaction with RANKL/OPGL, and proposed that RANKL activated NFkB, in turn upregulating osteoclast expression of IL-1, which in turn promotes maturation. Since NFkB is increased in inflammation this model may explain the increase in osteoclastogenesis that occurs during chronic inflammatory arthropathies. Furthermore, since oestrogen can bind to NFkB, and block the upregulation of IL-6 that it induces, this may be a possible explanation for the decrease in osteoclastic apoptosis that occurs in post-menopausal osteoporosis.

1.3.2 Osteoblasts

Osteoblasts are the cells responsible for bone formation. They are thought to arise from the stromal system of bone (Owen, 1985), maturing through a series of progenitor stages to become mature osteoblasts (Aubin *et al.,* 1995). They are responsible for synthesis of new bone and, coupled to osteoclastic activity, act to maintain bone integrity.

They are cuboidal cells possessing a single eccentric nucleus and abundant cytoplasm (Hughes & Aubin, 1997). They are located as a monolayer closely apposed to the bone and osteoid surface, whilst the preosteoblasts are more spindle shaped and lie in loosely packed layers between the mature osteoblasts and the bone marrow. Mature osteoblasts have a well-developed endoplasmic reticulum and Golgi apparatus, reflecting their synthetic function (Aubin & Liu, 1996). They produce and mineralise new bone matrix, secrete growth factors, which become stored in the bone matrix, and regulate bone resorption by acting as accessory cells for osteoclastic differentiation and function. They produce and secrete a wide array of bone matrix proteins, including membrane-associated alkaline phosphatase, collagen type I, osteocalcin (Ocn), bone sialoprotein (BSP), osteopontin (Opn) and proteoglycans (Liu *et al.*, 1994; Rickard *et al.*, 1996; Hughes & Aubin, 1997). The importance of each for bone integrity are given below, but it is also important to note their use in defining the stages of osteoblast differentiation (Hughes & Aubin, 1997; Aubin & Liu, 1996; Liu *et al.*, 1994; Aubin *et al.*, 1995). The expression of alkaline phosphatase increases with differentiation (Aubin *et al.*, 1995; Liu *et al.*, 1994), later falling with the onset of mineralisation. It is thought to be important for mineralisation, and inhibition of its activity with levamisole blocks mineralisation *in vitro* (Bellows *et al.*, 1991). However, alkaline phosphatase is also present in preosteoblasts, and is reported to appear before osteocalcin (reviewed in Aubin & Liu, 1996), limiting its utility in defining the stage of osteoblast differentiation. Collagen type-I is also characteristically expressed by both mature osteoblasts and preosteoblasts (Rickard *et al.*, 1996), as is osteopontin (Shalhoub *et al.*, 1992). Furthermore, neither of these two proteins are bone specific. Conversely, bone sialoprotein is restricted to skeletal associated cells and found on osteoblasts, but not preosteoblasts (Liu *et al.*, 1994). Finally, osteocalcin is also highly restricted to bone, and is only found in mature osteoblasts (Ducy & Karsenty, 1995; Liu *et al.*, 1994). A number of antibodies have also been generated against cells at different maturational stages in the osteoblast lineage (Walsh *et al.*, 1994; Haynesworth *et al.*, 1992), but with the exception of HOP-26 (Joyner *et al* 1997), STRO-1 (Simmons *et al.*, 1991) and SB-10 (Bruder *et al* 1997), these are all directed against more mature cells, frustrating study of cells early in the osteoblastic lineage (Aubin *et al.*, 1995).

1.3.2.1 Osteoblast lineage

Osteoblasts arise from preosteoblasts (figure 1.2), which lie directly subjacent to the osteoblasts *in vivo*, and undergo differentiation first to bone lining cells and finally to osteocytes. The details of the morphology and function of osteocytes are detailed in 1.3.3, but their ontogeny will be considered together with osteoblast differentiation.

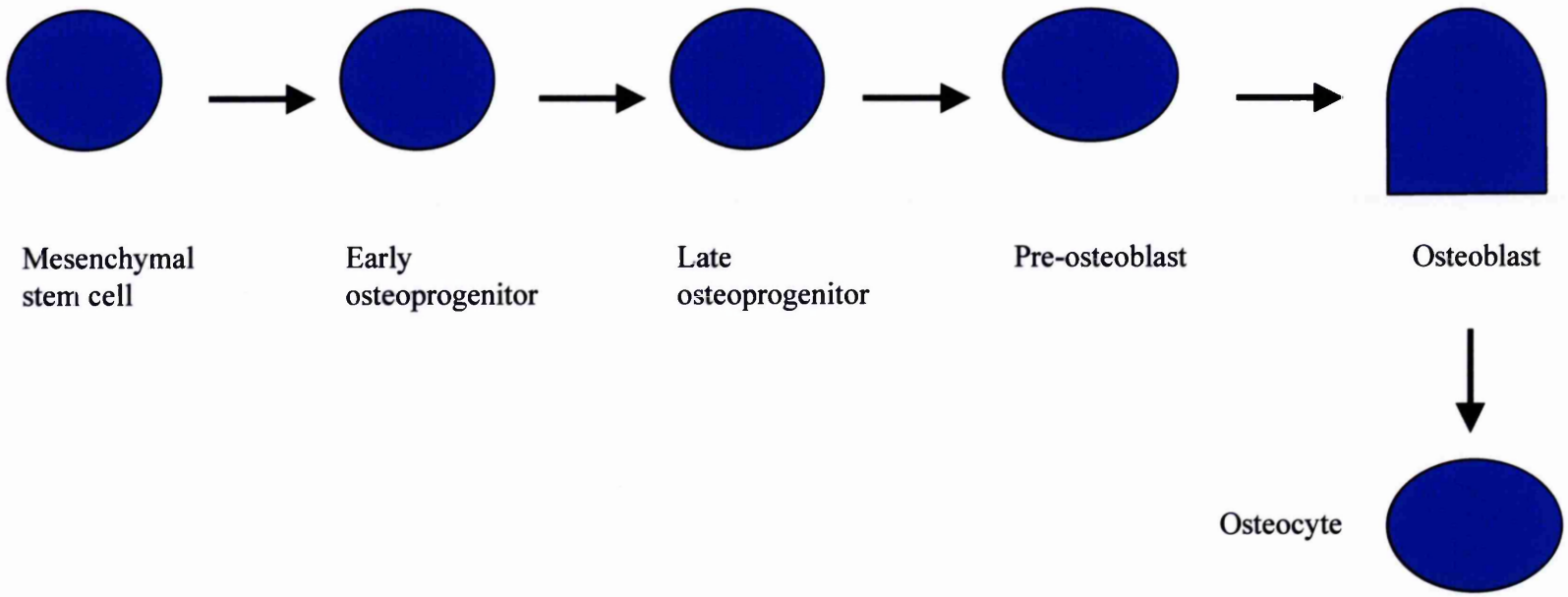


Figure 1.2 Postulated steps in the osteoblast lineage, implying recognisable stages of differentiation. (Adapted from Aubin *et al.,* 1995). The number of stages between the mesenchymal stem cell and the late osteoprogenitor stages is presently unknown.

Understanding of osteoblastic differentiation has been gained from a variety of culture models (reviewed in Hughes & Aubin, 1997). Kinetic studies of bone cell production indicate retention of proliferative potential by preosteoblasts which is lost in osteoblasts, lining cells and osteocytes (Owen, 1967). About 20% of mature osteoblasts become osteocytes, whilst the rest are presumed to be eliminated by apoptosis (Owen, 1970). Consequently osteoblasts must be continually replaced by differentiation of preosteoblasts, which in turn develop form stromal osteoprogenitors (Caplan, 1991; Simmons, 1996; Beresford, 1989). The nature of the stem cell pool from which these are derived is one of the most vexed questions in bone biology and will be discussed in detail in section 3.1.1.3. Apart from the antibodies STRO-1, SB-10 and HOP-26 (Joyner *et al.,* 1997; Simmons *et al.,* 1991; Bruder *et al.,* 1997) no markers exist for the earliest cells in the lineage (Aubin *et al.,* 1995). Cells showing a restricted commitment to the osteoblast lineage, osteoprogenitor cells, may exist in two different forms (reviewed in Burwell, 1994). One population, determined osteogenic precursor cells, (DOPC), is capable of constitutive differentiation *in vitro*, whilst the other, inducible osteogenic precursor cells, (IOPC), requires stimulation for induction of the osteoblast phenotype. The progression of these precursor cells to a more mature phenotype can be monitored by their differential expression of bone related proteins (reviewed by Hughes & Aubin, 1997; Aubin *et al.,* 1995; Liu *et al.,* 1994). Collagen type-I is the first marker expressed. It is present on osteoprogenitors, preosteoblasts, and on mature and mineralising osteoblasts. Osteopontin is weakly expressed in osteoprogenitors and then more strongly in all latter stages. Alkaline phosphatase is not present in osteoprogenitors and increases with progression from preosteoblast to mature osteoblast. It is still present, but in lower amounts, in mineralising osteoblasts. Osteocalcin is present in mature osteoblasts only, persisting during mineralisation. Bone sialoprotein is expressed first on preosteoblasts, falling upon mineralisation. PTH receptor expression is seen in mature osteoblasts, and to a lesser extent mineralising cells, whilst parathyroid hormone-related peptide (PTHrP) is first expressed on preosteoblasts, maximally on mature osteoblasts, and less so with onset of mineralisation. Whilst these patterns appear to provide a clear differentiation map upon which to locate any cell, most of the data has been collated from a variety of cell culture systems, which may influence cell behaviour, and gene expression, in different ways (Hughes & Aubin, 1997).

In view of this attempts have been made to refine characterisation of the osteoblast lineage by generation of antibodies against different stages (Aubin *et al.,* 1995; Bruder *et al.,* 1997; Haynesworth *et al.,* 1992; Joyner *et al.,* 1997; Simmons *et al.,* 1991). The antibodies SH-2

(Haynesworth *et al.,* 1992) and SB-10 (Bruder *et al.,* 1997) both recognise stromal stem cells, but whilst SH-2 is restricted, SB-10 also reacts against osteoprogenitor cells. STRO-1 is present on osteoprogenitor cells, and is thought to be present at the earliest stage of the osteoblast lineage (Simmons *et al.,* 1991; Gronthos *et al.,* 1999). It overlaps with expression of alkaline phosphatase, appearing before it and disappearing prior to the loss of alkaline phosphatase expression. The expression profile of HOP-26 (Joyner *et al.,* 1997) has not been as clearly defined, but it is present on osteoprogenitor cells, absent on osteoblasts, and is thought to be specific for stromal cells. Antibodies reactive against latter stages, SB-3, SB-2 and SB-5 (Haynesworth *et al.,* 1992), have also been described, but are less useful for study of early osteoblast ontogeny.

From the above it can be seen that few markers exist to identify cells early in the osteoblast lineage, either *in vitro* or *in vivo*. This has frustrated efforts to define the nature and biology of the osteogenic stem cell (Aubin *et al.,* 1995), a detailed overview of which is given in section 3.1.1.

1.3.3 Osteocytes

The osteocyte is the most abundant cell type in bone and there are approximately 10 times as many osteocytes as osteoblasts in adult human bone. They reside in lacunae in the bone matrix and are thought to arise from mature osteoblasts, though the process by which they do so is poorly understood (Nijweide *et al.,* 1996). During bone formation, some of the osteoblasts are left behind and become embedded in the newly laid-down osteoid matrix (Nefussi *et al.,* 1991). Concurrent with this they lose some osteoblastic characteristics and acquire new ones, including their typical morphology. Osteocytes are stellate shaped cells and are enclosed within the lacuno-canalicular network of bone. The cell body lies within the lacuna from which long, slender cytoplasmic processes extend into the canalicular network (Nijweide *et al.,* 1996), thereby effecting connections between osteocytes via gap junctions at the end of the processes (Doty, 1981). Each osteon therefore contains a syncytium of gap junction-coupled cells, which is also connected to the cells at the bone surface, including osteoblasts.

Osteocytes have limited capacity for matrix synthesis and their major role is to act as the mechanoreceptors of bone (Cowin *et al.,* 1991). *In vivo* loading studies of the turkey ulna demonstrated increased osteocytic expression of glucose-6-phosphate dehydrogenase (G6PD) in response to intermittent loading (Skerry *et al.,* 1989). Similar studies have demonstrated increased tritiated uridine uptake and increased IGF-I expression in

osteocytes in response to loading (El-Haj *et al.,* 1990). Stress causes interstitial fluid flow in the canalicular network and it is thought that the osteocytes respond to this rather than to direct deformation of the bone (Skerry, 1997). *In vitro* investigations of the effects of fluid flow on osteocytes support this hypothesis (Nijweide *et al.,* 1996). Shear stress produced by fluid flow over cultured osteocytes increases intracellular cAMP levels (Reich *et al.,* 1990), via PGE2 (Reich & Frangos, 1991), which is also increased. They appear to be more sensitive to fluid shear stress than to hydrostatic pressure and are more sensitive than osteoblasts, and have therefore been termed the professional mechanosensory cells of bone (Nijweide *et al.,* 1996). Modulation of the activity of osteoblasts and osteoclasts in response to stress is thought to be via the release by osteocytes of prostaglandins (Reich & Frangos, 1991) and nitric oxide (Turner *et al.,* 1998; Kawata & Mikuni-Takagaki, 1998; Rawlinson *et al.,* 1996).

1.4 Bone matrix and bone-specific proteins

Bone consists of bone cells which exist in a microenvironment contiguous with the haematopoietic and marrow stomal system, and which owes its structural integrity to the matrix proteins and other bone-specfic proteins secreted by osteoblasts. Each of these can be considered as markers of the osteoblast lineage and their biology is reviewed below.

1.4.3 Alkaline phosphatase

In man, four alkaline phospahatse isoenzymes exist, each of which is encoded by a different gene (Henthorn, 1996). These include the placental, placental-like, intestinal and tissue non-specific alkaline phosphatase (TNSALP) isoforms. The latter is expressed in liver, bone and kidney and is also known as L/B/K alkaline phosphatase. Complementary deoxyribonucleic acid (cDNA) clones for tissue-nonspecific alkaline phophatase have been isolated from a number of mammals (Terao *et al.,* 1990; Henthorn *et al* 1988) and all encode a 524 amino acid polypepetide which contains a 17-amino-acid signal peptide (Henthorn, 1996). Apart from the mouse and rat, which are 97% identical, the other cDNAs are 83-89% homologous, whilst the amino acid sequences are 90% the same. The messenger ribonucleic acids (mRNAs) are approximately 2.5 kilobases (kb) long and don't hybridise with the tissue-specific isoforms, but show divergent nucleotide sequence at the 5 prime (5') end, due to alternate promoter usage.

TNSALP comprises 13 exons, of which two at the 5'end are noncoding, 'leader', exons, designated 1a and 1b (Henthorn *et al.,* 1988). Since each of these exons has its own

promoter sequence they are incorporated into the mRNA in a mutually exclusive manner, resulting in two types of mRNA, each encoding an identical polypeptide, but with a different 5' untranslated sequence (Henthorn, 1996). Use of the exons/promoters is tissue specific, and varies between osteoblastic cell lines, though most studies of the expression of TNSALP have not attempted to discriminate between the two forms.

Expression *in vitro* can be stimulated by treatment with dexamethasone, retinoic acid or dibutyryl cAMP (Leboy *et al.,* 1991; Cheng *et al* 1994). TNSALP enzyme activity and mRNA accumulation are increased by up to 2000-fold by treatment with dibutyric acid cyclic AMP (db-cAMP) in mouse fibroblasts (Favier *et al.,* 1981), though no cAMP responsive element has been found in the promotor (Struder *et al.,* 1991). Similarly, the mechanisms involved in increased transcription by dexamethasone and retinoic acid are unknown (reviewed in Henthorn, 1996).

Alkaline phosphase is a membrane bound protein and acts in mineralisation (Whyte, 1994). Consequently most of what is known about its function has come from observations of defective mineralisation, and in particular from hypophosphatasia, in which the alkaline phosphatase gene is mutated (Whyte, 1994). Since its discovery in the 1920's many physiological roles have been proposed for alkaline phosphatase, including hydrolysis of phosphate esters to provide nonphosphate esters, transferase action for the synthesis of phosphate esters, regulation of inorganic phosphate (Pi) metabolism and maintenance of levels of phosphoryl-metabolites (Whyte, 1996). The diversity of roles ascribed to it reflects its wide tissue distribution, and, in the placenta, it binds to the Fc receptor of IgG, suggesting that it acts in the transcytosis of this immunoglobulin (Makiya *et al.,* 1992). However, more specifically, its roles in mineralisation include local increase of Pi levels, inactivation of inhibitors of hydroxyapatite crystal growth, transport of Pi, and action as a calcium binding protein or a calcium/magnesium-ATPase, or as a phosphoprotein phosphatase that prepares the skeleton for ossification (Whyte, 1994). However, its physiological role is still poorly understood, as evidenced by the fact that its bioassay still relies on the hydrolysis of artificial substrates (Whyte, 1996).

Hypophosphatasia may present across a wide age range, and the specific skeletal defect seen is dependent upon this, but essentially all cases, regardless of age, exhibit hypomineralisation of bones, together with loss of dentition (Rathbun, 1948; Fraser, 1957). The clinical complications range from hypoplastic lungs in perinatal cases, to repeated stress fractures in adults. Serum alkaline phosphatase levels are low, whilst both calcium

and Pi levels are normal, and may even be increased (Whyte, 1996). Missense mutations occur in the TNSALP gene (Henthorn & Whyte, 1992). Ten different such mutations have now been reported, each of which results in alteration of an amino acid residue that is conserved in mammalian TNSALPs (Henthorn & Whyte, 1992). Three-dimensional crystallography indicates that these changes appear to disturb the catalytic pocket or structurally important metal ligand binding sites in the molecule (Henthorn & Whyte, 1992). These observations, together with biochemical and clinical data, from patients with hypophosphatasia have been formulated by Whyte into an overview of how TNSALP may act physiologically in man (Whyte, 1996). Increased endogenous levels of phosphoethanolamine (PEA), inorganic pyrophosphate (Ppi), and pyridoxal 5'-phosphate (PLP) indicate that TNSALP is catalytically active toward a variety of substrates with a fairly variable chemical structure, and, since these putative natural substrates are normally present in extracellular fluid at nano-molar or micro-molar concentrations, TNSALP acts at substrate levels that are much lower than those used in routine clinical assays. TNSALP functions primarily as an ectoenzyme. Consequently, PEA, PPi and PLP acumulate extracellularly in hypophophatasia. The source of PEA accumulation is unclear, but might reflect degradation of the phosphatidylinositol-glycan moiety that anchors proteins to the cell surface, whilst PPi is formed by the action of nucleoside triphosphate pyrophosphatase (NTP-PPi-ase). PLP originates in the liver. The extracellular accumulation of PPi, which at low concentrations promotes calcium phosphate deposition and at high concentrations acts as an inhibitor of hydroxyapatite crystal growth, results in deposition of calcium phosphate and calcium pyrophosphate crystal deposition, reflecting a regional effect at low concentrations of PPi. The high extracellular concentrations of PPi at sites of mineralisation meanwhile result in inhibition of calcification, resulting in osteomalacia.

1.4.4 Type I collagen

Type I collagen is the most abundant extracellular protein in bone, and is essential for strength of bones (Rossert & de Crombrugghe, 1996). It exists as a triple helix of three polypepetide chains, each of which contain Gly-X-Y repeats, by virtue of which each chain is coiled in a left-handed helix, whilst the chains assemble in a right-handed triple helix (Prockop *et al.,* 1979). At least 19 different collagens have now been reported, of which types I, II, III, V and XI are fibrillar, with high tensile strength. Type I is a major constituent of bone, whilst type III is rarely found in mature bone, though it is present in healing fracture callus. Type X is the major type present in cartilage and is secreted by hypertrophic chondrocytes (Rossert & de Crombrugghe, 1996).

Each molecule of type I collagen is composed of two α1 chains and one α2 chain, both of which consist of a long helical domain which is preceded by a short N terminal peptide, and followed by a short C terminal peptide. It is secreted as a propeptide from which the N and C termini are cleaved by proteases, leaving the mature molecules which then assemble into fibrils (Prockup *et al.,* 1979). In man the gene encoding the α1 chain is located on the long arm of chromosome 17 (17q21.3-q22) and that for α2 the long arm of chromosome 7 (7q21.3-q22) (Retief *et al.,* 1985). The genes consist of 41 and 42 exons respectively (all of which are conserved between the two, since two of them are joined in the α1 gene), each between 45 to 162 basepairs (bp) long, and the difference in gene size for the two chains (18kb for α1, and 38kb for α2) is due to differences in the size of the introns (Rossert & de Crombrugghe, 1996). Following post-transcriptional exon splicing and 3' end polyadenylation the mature mRNA is translated. The resultant propeptide α1 and α2 chains undergo hydroxylation and glycosylation, following which the three associate at the C terminal end and the triple helix is formed. The helix is then transported to the extracellular space, in which proteases cleave the C and N terminal peptides, dramatically reducing the solubility of the collagen molecules, which then spontaneously assemble into fibrils (Prockop *et al.,* 1979; Prockop 1990). Mutations in the coding sequence of either the pro-α1 (I) or pro-α2 (I) genes gives rise to osteogenesis imperfecta (Kuivaniemi *et al.,* 1991). In most cases this leads to the production of normal amounts of an abnormal chain, which either inhibits complete folding of the triple helix or prevents correct fibril assembly (Kadler *et al.,* 1991).

Collagen type I synthesis is under the regulation of a number of cytokines and growth factors, and hormones. Transforming growth factor β induces production of type I collagen, both *in vivo* and *in vitro* (reviewed in Rossert & Crombrugghe, 1996), whilst TNFα reduces production in most models (Nannes *et al.,* 1989; Diaz *et al.,* 1993), both effects occurring at the level of transcription. IL-1, interferon gamma (IFNγ), and IL-10 all inhibit production of collagen type I, whilst IL-4 increases production. IGF-I induces production of collagen I *in vivo* and *in vitro* (Thiebaud *et al.,* 1994; Schmid *et al.,* 1989), as does IGF-II (Thiebaud *et al.,* 1994), whilst basic fibroblastic growth factor (bFGF) inhibits it's production (Hurley *et al.,* 1992 & 1993). PGE2 has a biphasic effect, inducing production of collagen type I at low concentrations, and inhibiting it at high concentrations (Raisz & Fall, 1990). Corticosteroids (Canalis, 1983) and parathormone (Kream *et al.,* 1980) both inhibit production of collagen type I *in vitro*, whilst the effect of 1,25-vitamin

D varies with the stage of differentiation of the cells used, being stimulatory in immature cells and inhibitory in more mature cells (Rossert & Crombrugghe, 1996).

The transcriptional regulation of the type I collagen genes has been extensively studied (reviewed in Rossert & Crombrugghe, 1996). Several functional cis-acting elements are present in the proximal promotor of the pro-α2 (I) gene, one of which is a binding site for the CCAAT-binding protein CBF, whilst the others bind Sp1 and NF1/CTF (Rossert & Crombrugghe, 1996).

1.4.5 Osteopontin

Osteopontin (Opn) is a phosphorylated glycoprotein present in bone matrix and in a variety of other tissues, including kidney, brain, hypertrophic chondrocytes and odontoblasts, and it has been implicated in control of a number of normal and pathological processes (Butler, 1989). In particular however, in bone it plays an important role in cell attachment and migration (Oldberg *et al.,* 1986).

It is an acidic glycoprotein of between 44 to 75 kilo-Daltons (kDa) comprising 264 - 301 amino acids depending on species, amongst which it shares approximately 40% homology, except for the chicken sequence which shows only 19% identity with the other species (human, rat, pig, rabbit, mouse, bovine) (Bulter *et al.,* 1996). A hydrophobic leader sequence of 16 amino acids, which is well conserved, is present at the start in all species, and a further 3 sequences are highly conserved, all of which are involved in cell attachment and spreading. In humans it is encoded by a single copy 9kb gene, which maps to the locus 4q13, and which can undergo differential mRNA splicing to produce variant forms (Young *et al.,* 1990). Osteopontin is synthesised by preosteoblasts, osteoblasts (Liu *et al.,* 1994), and osteocytes (Arden *et al.,* 1996), and is regulated, at the transcriptional level, by TGFβ1 and TGFβ2 (Alsina *et al.,* 1996), both of which upregulate its expression in mouse osteoblast-like cell lines. It's expression is also upregulated by retinoic acid, 1,25 vitamin D3, endothelin, and bone morphogenetic proteins, whilst calcitonin and parathormone are inhibitory (Butler *et al.,* 1996). Vitamin D response elements (VDREs) are present in the promotor (Noda *et al.,* 1990), and the presence of other transcription factor binding sites, for AP-1 and Sp-1, has been reported (Denhardt & Guo, 1993).

Osteopontin is localised to osteoblasts and osteocytes, to the fibroblast-like cells associated with the periosteum (Butler *et al.,* 1996), and in rat embryonic tissue, to hypertrophic chondrocytes (Mark *et al.,* 1988). In the latter model it is expressed prior to mineralisation,

whilst in a neonatal rat model its expression was most intense at the mineralisation front; it was also present in the adjacent osteoid (Pinero *et al.,* 1995). Ultrastructural studies have localised it uniquely amongst the bone matrix proteins to the cement lines which lie between areas of bone laid down at different times, suggesting a role in maintaining structural connection between such areas of bone (Butler *et al.,* 1996). The existence of the RGD sequence, which interacts with αvβ3 integrins, in Opn suggested a role in cell attachment (Oldberg *et al.,* 1986), which has subsequently been confirmed by RGD peptide blocking experiments. Opn induces attachment and spreading of fibroblasts (Somerman *et al.,* 1987), via αvβ3 (Ross *et al.,* 1993) and possibly also αvβ5 and αvβ1 (D'Errico *et al.,* 1995), but its role in attachment of osteoclasts is less certain. *In vitro* osteopontin inhibits both *de novo* apatite formation and apatite growth (Boskey *et al.,* 1993), a function dependent on the presence of phosphate groups, suggesting that *in vivo* it acts to regulate mineralisation (Butler *et al.,* 1996).

1.4.4 Osteocalcin

Osteocalcin (Ocn), also called bone gla protein (BGP), is a member of a large family of mineral-binding extracellular proteins called the gla proteins (reviewed in Ducy & Karsenty, 1996). The gla proteins are synthesised by osteoblasts and chondrocytes (Hale *et al.,* 1988; Hauschka *et al.,* 1989) and share modified glutamic acid residues (gla residues) which confer a high affinity for Ca^{2+} and hydroxyapatite. Osteocalcin is a small protein of 46-50 amino acids and accounts for approximately 10% of the non-collagenous proteins of bone (Gallop *et al.,* 1980). Until recently it was thought to be exclusive to bone, but is now known to be present at low levels in other tissues (Knoblauch *et al.,* 1998). The human gene is less than 1kb long and encodes four exons, of which exons 1 to 3 are those of prepro-osteocalcin, whilst exon four codes for the mature protein (Ducy & Karsenty, 1996). Three nearly identical Ocn genes have been identified in the mouse osteocalcin locus, two of which, OG1 and OG2, are expressed only in bone, whilst the third, osteocalcin-related gene (ORG) is found only in the kidney in postnatal life (Desbois *et al.,* 1994). Osteocalcin synthesis is controlled by 1,25-dihydroxy vitamin D3 (Price & Baukol, 1980), via a vitamin D responsive element in the Ocn promotor (Morrison *et al.,* 1993). The promotor also contains a TATA box (Ducy & Karsenty, 1996), two glucocorticoid responsive elements (Morrison *et al.,* 1993; Stromstedt *et al.,* 1991), and a TNFα responsive element (Li & Stashenko, 1993). In addition, two osteoblast-specific elements, OSE1 and OSE2, have been identified in the osteocalcin promotor. By definition these are expressed only in osteoblasts, and were identified and verified by their ability to confer osteoblast-specific expression to reporter genes *in vivo* (Ducy & Karsenty, 1996). OSE1

binds a factor prior to the onset of mineralisation, suggesting that it is differentiation stage specific, whilst OSE2 binds irrespective of the stage of osteoblastic differentiation. Recently OSE2 has been shown to be pivotal in the induction of early osteogenesis (Otto *et al.,* 1997) (see section 1.5.1 below).

1.5 Control of bone formation

1.5.1 Genetic control of skeletal differentiation

Genetic control of skeletal growth and maintenance involves direction of both cellular differentiation and patterning. In the past decade skeletal developmental biology has focussed on the latter (Kaplan & Shore 1996), in particular the role of the helix-loop-helix transcription factors paraxis and scleraxis (Burgess *et al.,* 1995), the Hox (Fromental-Ramain *et al.,* 1996) and Pax (Lacombe, 1995) genes, and the hedgehog / patched signalling pathway (Ingham, 1998a & 1998b). More recently however, several studies have begun to unravel the genetic factors controlling cellular differentiation in the skeleton, and in particular those responsible for osteoblastic differentiation (Otto *et al.,* 1997). Stein and Lian (1993) have reviewed the molecular control of osteoblast differentiation and proliferation. In a fetal rat calvarial osteoblast model the first 10 to 12 days post harvest is characterised by active proliferation, with expression of cell cycle (histone) and cell growth (c-myc, c-fos and c-jun) regulated genes; c-myc and c-fos support proliferation by encoding transactivation factors (Stein & Lian, 1993). This phase is associated with expression of type I collagen and TGFβ, and is followed by a phase of differentiation, during which alkaline phosphatase is upregulated and proliferation decreased (Stein & Lian, 1993). Further differentiation to a mineralising phenotype is reflected in expression of other bone-related genes, namely bone sialoprotein, osteopontin and osteocalcin. Of these the bone-specific gene for osteocalcin shows regulation by Fos-Jun protein complexes, which are able to occupy the VDRE and osteocalcin box regulatory elements, specifically at AP-1 sites, in the osteocalcin gene, blocking transcription; this explains the absence of osteocalcin expression in proliferating osteoblasts, and its upregulation upon mineralisation (Stein & Lian, 1993; Liu *et al.,* 1994; Ducy *et al.,* 1996). It is postulated that release of the Fos-Jun complex from the AP-1 sites permits their occupancy by the vitamin D receptor complex and other transcription factors (Stein & Lian 1993).

The pivotal role of osteocalcin in osteoblast development has been underscored by a number of recent studies into the action of the transcription factor Cbfa1 (Otto *et al.,* 1997;

Komori *et al.,* 1997; Mundlos *et al.,* 1997; Ducy *et al.,* 1997; Dickman 1997). Osteocalcin was until recently thought to be expressed exclusively in osteoblasts and in no other matrix-producing cell, though it has been detected in other tissues by polymerase chain reaction (PCR) (Knoblauch *et al.,* 1998). It is a skeletal Gla protein, whose function is to control mineralisation (Otto *et al.,* 1997; Ducy *et al.,* 1996). Two osteoblast-specific cis-acting elements, OSE1 and OSE2, have been localised to the promotor region of the mouse osteocalcin gene (Ducy & Karsenty, 1996). Of these Osfa2, the protein that binds to OSF2, is related to the Runt/core binding factorA (Cbfa) family of transcription factors. Cbfa proteins are the mammalian homologs of the Drosophila runt protein which is involved in neurogenesis and sexual differentiation and bind to DNA through a 128 amino acid domain called the runt domain (Ducy *et al.,* 1997). Three Cbfa proteins, encoded by different genes have been identified (Otto *et al.,* 1997). Of these Cbfa1 was originally thought to be T-cell specific (Ogawa *et al.,* 1993), but has recently been shown to encode for Osfa2, which is a full length transcript of Cbfa1 (Ducy *et al.,* 1997).

During early mouse embryogenesis Osf2/Cbfa1 is initially expressed in all areas of mesenchymal condensation, upto 12.5 days post-conception (dpc), but thereafter its expression is restricted to cells of the osteoblast lineage (Ducy *et al.,* 1997). Those cells positive at 12.5 dpc also expressed type II collagen, indicating the capacity for differentiation along either the osteoblast or chondrocyte lineage. Interestingly, the Meckel's cartilage was negative at 12.5 dpc; these cells are fully differentiated chondrocytes at 12.5 dpc and express alpha 1(II) collagen. At 16.0 dpc Osf2/Cbfa1 was present only in areas of ossification, whilst matrix gla protein, a gene expressed in chondrocytes but not osteoblasts, was absent in these areas (Ducy *et al.,* 1997). DNA-binding studies showed that Osf2/Cbfa1 bound to the OSE2-like elements in the promotor regions of osteocalcin, alpha 1(I) collagen, BSP and osteopontin, and that it enhanced their expression. Furthermore it was able to induce their expression in non-skeletal cells, including the skin fibroblast cell line C3H10T1/2 (Ducy *et al.,* 1997). In Cbfa1-deficient mice there is normal skeletal patterning, but the skeleton is made of cartilage rather than bone, demonstrating that the role of Osf2/Cbfa1 as a regulator of osteoblast differentiation is non-redundant (Komori *et al.,* 1997; Otto *et al* 1997). In heterozygotes there was delayed closure of the sutures of the fontanelles and hypoplastic clavicles, a phenotype identical to that seen in the human disease cleidocranial dysplasia (CCD) (Otto *et al.,* 1997). Subsequently deletions, stop codon mutations and insertion mutations have been identified in the Cbfa1 gene in patients with CCD (Mundlos *et al.,* 1997). Osf2/Cbfa1 is induced by stimulation with BMP7 (OP1), suggesting that it is part of the BMP signalling

cascade (Ducy *et al.,* 1997). Appropriately, BMP7 is known to act at an earlier stage of osteoblast differentiation than the other BMPs, notably BMP2 and BMP4 (Li *et al.,* 1996). By contrast, 1,25-vitamin D3 down-regulates Osf2/Cbfa1, and is also known to prevent terminal osteoblast differentiation clinically, causing aplastic bone disease (Goodman *et al.,* 1994). However, whilst Osf2/Cbfa1 is clearly pivotal in osteogenesis, it may act in combination with other nuclear factors, in particular other OSFs (Ducy & Karsenty, 1995). In this regard another cis-acting element, OSE1, binds to a different OSF, and a further sequence (Ducy & Karsenty, 1995), this time controlling expression of the alpha1(I) collagen gene has recently been identified (Rossert *et al.,* 1996). Interestingly, *in vitro* osteoclacin expression can be induced in cells from Cbfa1-/- mice by stimulation with recombinant human bone morphogenetic protein 2 (rhBMP-2), suggesting the importance of other transcription factors in BMP-induced osteocalcin synthesis (Komori *et al.,* 1997). Indeed, down-regulation of osteocalcin does not explain the complete lack of osteogenesis in Cbfa1-/- mice since osteocalcin knockout mice show increased bone formation (Ducy *et al.,* 1996).

1.5.2 Bone morphogenetic proteins

Purification studies to establish what factors were responsible for the ability of demineralised bone to induce mineralisation isolated the bone morphogenetic proteins (BMPs), which form part of the TGF beta superfamily (Urist, 1994; Hogan, 1996). More than 40 BMP proteins have been isolated (Sakou, 1998), including BMP-2 to BMP-6, osteogenic protein-1 (OP-1/BMP-7) and OP-2 (BMP-8), and growth/differentiation factor-5 (GDF-5) and GDF-6, also called cartilage-derived morphogenetic protein (CDMP)-1 and –2 (Erlacher *et al.,* 1998; Sakou, 1998; Reddi & Cunningham, 1993). Along with other members of TGF beta superfamily they encode secreted polypeptides which share a conserved pattern of 7 cysteines, and, uniquely, amino acid homology at the carboxy-terminus. In particular, high degrees of amino acid sequence homology exist between BMP-2 and -4, between BMP-5, -6 and –7, and between GDF-5 and –6 (Sakou, 1998).

In vitro BMP-2 induces osteoblast differentiation in a variety of cell types (Chen *et al.,* 1997; 1998; Sakou, 1998), though in one study this effect only occurred at high doses whilst lower doses resulted in adipocytic differentiation (Wang *et al.,* 1990). Such differentiation occurs at the expense of proliferation (Fromigue *et al.,* 1998). BMP-7 (OP-1) induces osteoblastic and chondroblastic differentiation of osteoprogenitor cells and appears to act at an earlier stage of differentiation than BMP-2 and –4 (Li *et al.,* 1996a). In

SaOS-2 cells rhBMP-7 upregulated expression of BMP-6, but depressed levels of BMP-2 and –4 (Honda *et al.,* 1997). Further evidence of the autocrine/paracrine nature of BMP regulation was provided by Chen *et al* (1997) who showed enhancement of BMP-3 and BMP-4 by BMP-2 during the mineralisation phase in fetal rat calvarial osteoblasts. The osteogenic effect of BMP-3 (osteogenin) on human osteoprogenitor cells was demonstrated by Amedee *et al* (1994). Proliferation was reduced whilst osteogenic markers (osteocalcin and type I collagen) were increased. However, their effects are not restricted to the osteoblastic lineage. They can also induce chondrocytic differentiation (Hoshi *et al.,* 1997), and have important effects in early post-implantation development and patterning (Hogan, 1996). Specifically BMP7 is required for kidney, eye and limb development in the mouse. In a chick model ectopic Sonic hedgehog (Shh) expression leads to upregulation of BMP-2 and –4, though the generality of this observation remains to be tested. Indeed, many of the studies into expression, and control thereof, of BMPs outside bone have served to demonstrate that the effects of, and upstream controls on, the expression of BMPs differ between tissues. For example, whilst in bone and epithelia BMPs depress proliferation, in the ectoderm, kidney and eye, BMPs are needed for cell proliferation (Hogan, 1996).

In vivo BMPs, particularly BMP-2, BMP-4, BMP-5 and BMP-7, induce formation of bone and cartilage, with mutations resulting in a number of skeletal mutations, such as *Short ear* mice due to defects in BMP-5 (Kingsley *et al.,* 1992) and *brachypodism* mice due to mutations in GDF-5 (Storm *et al.,* 1994). In humans GDF-1/CMP-1 gene mutations result in Hunter-Thompson chondrodysplasia (Thomas *et al.,* 1996) which is phenotypically similar to brachypodism. In keeping with their in role in controlling differentiation outside the skeleton disruption of BMP-4 results in severe mesodermal defects with consequent early embryonic death (Winner *et al.,* 1995). Similarly, (OP)-1/BMP-7 knockout mice show defective renal and ocular development (Hogan, 1996).

The upregulation of BMPs during fracture repair has stimulated interest in their clinical use to facilitate repair of large bony defects (Wang, 1993; Riley *et al.,* 1996). BMP-4 is upregulated in osteoprogenitor cells during the early stages of fracture repair in mice, specifically in cells negative for Gla protein, a marker of a more mature osteoblastic phenotype (Nakase *et al.,* 1994). Others have shown increased expression of BMP-2/-4 in the cambium layer of the periosteum adjacent to the fracture shortly after injury, with reduction later on (Li *et al.,* 1998). BMP-7/OP-1 is also upregulated in the early stages of fracture repair and appears to act at an earlier stage than BMP-2/-4 (Onishi *et al.,* 1998). Congruent with these findings, both rhBMP-2 and rhBMP-7/OP-1 have induced repair of

large segmental defects in animal studies, though clinical results have not been as good (Sakou, 1998).

1.5.3 Hormonal regulation of osteoblast function

Investigations of the hormonal control of bone formation have largely been driven by the need to understand the abnormalities underlying altered function in a variety of diseases, including hyperparathyroidism, post-menopausal osteoporosis and osteomalacia. Consequently most studies have focused on the action of parathyroid hormone, calcitriol and oestrogen.

1.5.3.1 Parathyroid hormone

Parathyroid hormone (PTH) is a peptide produced by the parathyroid glands in response to falling serum calcium concentration, acting to maintain normal calcium homeostasis by stimulating osteoclastic bone resorption, renal tubular reabsorption of calcium and production of 1α-hydroxylase in the kidney (Alsina *et al.,* 1996). When used pharmacologically, PTH has complex effects on bone depending on whether it is administered continuously, resulting in increased resorption, or intermittently, stimulating formation (Dempster *et al.,* 1993).

The primary target cell is the osteoblast, whilst osteoclasts only resorb bone in response to PTH when in the presence of osteoblasts, and consequently PTH is thought to increase osteoclastic resorption indirectly via osteoblasts (Rodan and Martin, 1981). Functional PTH receptors have been found on both mature and immature osteoblastic cells (Rouleau *et al.*, 1988 & 1990; Moore *et al.,* 1997), though Teti *et al* (1991) also found receptors on osteoclasts and their precursors. Intriguingly, PTH has been shown to inhibit bone resorption in isolated osteoclasts, and it may therefore have dual actions on osteoclastosis, an indirect stimulatory one via osteoblasts and a direct inhibitory one on osteoclasts (Dempster *et al.,* 1993).

In vitro PTH acts to inhibit collagen synthesis, alkaline phosphatase expression and osteocalcin secretion by osteoblasts, and suppresses appositional bone formation in cultured rat osteoblasts (Gray & Jones, 1998). It stimulates proliferation of osteosarcoma derived osteoblasts at low doses, but inhibits proliferation at high doses (Martin *et al.,* 1989). The anabolic effect is mediated by an increase in proliferation of precursors

(Watson *et al.,* 1999), but it must be remembered that its effects on proliferation will vary dependent on the culture system used (Martin *et al.,* 1989; Van der Plas *et al.,* 1985).

The stimulation of bone formation observed at low doses of PTH (Dempster *et al.,* 1993) is probably mediated by the growth factors IGF-I (Canalis, 1996), released from stromal cells, and TGF-beta (McCarthy *et al.,* 1989), released from the bone matrix by osteoclasts. *In vitro* it increases the expression of differentiation associated osteoblast genes, including type I collagen (Thiebuad *et al.,* 1994), bone sialoprotein (Yang & Gerstenfeld, 1996) and osteocalcin (Yu & Chandrasekhar, 1997). However, direct evidence for PTH-induced proliferation of mature osteoblasts in the postnatal skeleton is lacking, leading to the suggestion that the source of the increased osteoblast number in the PTH-treated skeleton is primarily the osteoprogenitor pool (Watson *et al.,* 1999). In addition, PTH continues to stimulate bone formation over treatment periods of several months, supporting the notion that it stimulates recruitment of new osteoblasts from the stem cell pool. Kostenuik *et al* (1999) investigated the role of PTH and IGF-I in the recruitment of osteoblasts in unloaded bone and found resistance to proliferation of osteoprogenitor cells when stimulated with PTH, compared to that observed in normally loaded bone. The proliferative effect of PTH on the cells was mediated by IGF-I.

1.5.3.2 Calcitriol

Calcitriol, or 1,25-dihyroxyvitamin D3, is produced in the proximal tubules of the kidney and is a potent bone-resorbing agent (Alsina *et al.,* 1996). It increases intestinal absorption of calcium and phosphate and *in vivo* is necessary for normal mineralisation. Receptors for calcitriol are present on osteoblast and their precursors (Chen *et al.,* 1983; Narbaitz *et al.,* 1983; Mee *et al.,* 1996 & 1997) but there are wide discrepancies between *in vitro* and *in vivo* studies of its action and, similar to PTH, this suggests that its main target is the osteoblast precursor pool. In short-term bone organ cultures, calcitriol inhibits bone collagen synthesis (Raisz *et al.,* 1980), and, when administered to cultured osteoblast-like cells, it increases expression of osteocalcin and alkaline phosphatase (Price & Baukol, 1980; Manolagas *et al.,* 1981). In prolonged culture of fetal rat calvarial cells however, it inhibited the formation of mineralised bone nodules, and, in rodent osteoblasts and osteosarcoma cell lines it inhibited osteoblast proliferation in a dose-dependent manner (Chen *et al.,* 1983). Its effects therefore, like those of PTH, are closely linked to the cell system, and in particular the stage of differentiation, used.

1.5.3.3 Oestrogen

Oestrogen receptors have been identified in both osteoblasts (Komm *et al.,* 1988; Hoyland *et al.,* 1997) and osteoclasts (Oursler *et al.,* 1991; Hoyland *et al.,* 1997), but the reported effects on osteoblast function are conflicting (Turner *et al.,* 1994), probably due to the different culture systems used in each study. Whilst most studies have focused on the effect of oestrogen on osteoclastosis (Manolagas, 1998), oestrogen has been reported to increase, decrease, or have no effect on proliferation of cells of the osteoblast lineage, and, similarly to have a stimulatory (Qu *et al.,* 1998), inhibitory, or no effect on bone matrix protein production (Alsina *et al.,* 1996). These discrepant reports reflect the use of different culture systems, whilst *in vivo* however it clearly enhances bone formation. This may be an indirect effect through its modulation of the production of cytokines (Oursler *et al.,* 1991). It stimulates TGF-beta production by osteoblasts (Oursler *et al.,* 1991; Qu *et al.,* 1998), a process mediated by the nuclear protooncogenes c-fos and c-jun (Turner *et al.,* 1994), and also alters production of IL-6, IGF-I, IGF-II and TNF-α (Turner *et al.,* 1994). In particular, loss of oestrogen following the menopause triggers an increase in IL-1, IL-6 and TNF due to loss of suppression of IL-6 and TNF gene transcription by oestrogen (Manolagas, 1998).

1.5.4 Cytokine regulation of bone formation

Osteoblasts are capable of producing and responding to a wide variety of cytokines and other locally acting growth factors. Bilbe *et al* (1996) performed PCR phenotyping of primary human osteoblasts, in conjunction with the human osteosarcoma derived cell lines TE-85, MG-63 and SaOS-2. There was strong concordance between the expression profiles for each of the cultures, and, specifically, IL-6, IL-8, IL-11, TNF alpha and beta, and LIF were expressed in all four, together with the receptors for IL-4, IL-7, fibroblast growth factor-I (FGF-I), IGF-I, and PTH. In addition, the growth factors PDGF-alpha and beta, PTHrP, TGF-beta, and BMPs 1, 2 and 3, together with the bone matrix proteins matrix gla protein (MGP), osteocalcin and osteonectin, were also expressed by all four cell types. As for any cellular system, the effects of these on osteoblast function are complicated by interaction between cytokines (MacDonald and Gowen, 1992) and by combinations of autocrine and paracrine effects, making transfer of *in vitro* results to *in vivo* difficult.

The TGF-betas are members of a large family of genes including activins, BMPs and growth and differentiation factors (GDFs) (Centrella *et al.,* 1994). They are produced as precursor molecules, undergoing post-translational processing prior to secretion as an

inactive complex, latent TGF-beta (Bonewald & Dallas, 1994). The latent form is secreted by osteoblasts and incorporated into the bone matrix, which constitutes the most abundant source in the body (Hauschka *et al.,* 1986), from which it is thought to be activated during osteoclastosis by acidic pH (Centrella *et al.,* 1994) and proteases. It has an autocrine action since osteoblasts not only synthesise it, but also possess receptors for it (Robey *et al.,* 1987), and TGF-beta mRNA expression in human osteoblasts is upregulated by stimulation with TGF-beta (Subramaniam *et al.,* 1995). In most studies TGF-beta stimulated collagen synthesis and expression of markers of osteoblastic differentiation, including osteonectin, alkaline phosphatase, fibronectin, osteopontin and osteocalcin (Noda and Rodan, 1987; Noda *et al.,* 1988; Noda, 1989), and, *in vivo*, subcutaneous injection causes increased periosteal bone formation (Noda and Camilliere, 1989). *In vivo*, TGF beta is upregulated in the early stages of fracture repair (Bolander, 1992; Andrew *et al.,* 1993a). Its main action *in vivo* is thought to be recruitment and differentiation of osteoblast precursors (Mundy *et al.,* 1995), which may explain why osteoblast-specific overexpression of TGF-beta in a murine model led to an osteoporotic phenotype (Erlebacher and Derynck, 1996). The association of a splice variant of the TGF beta 1 gene with osteoporosis confirms its importance in maintenance of normal bone strength (Langdahl *et al.,* 1997).

The insulin-like growth factors IGF-I and IGF-II are weakly mitogenic for osteoblasts, but stimulate activity of differentiated osteoblasts (Linkhart *et al.,* 1996), increasing osteocalcin and collagen type I synthesis, and resulting in increased bone formation (Spencer *et al.,* 1993). IGF expression is high in the growth plate and in healing fracture callus (Andrew *et al.,* 1993b; Linkhart *et al.,* 1996), whilst mice with a null mutation of the type I IGF receptor have delayed skeletal development and ossification (Liu *et al.,* 1993). Similarly, serum IGF I is low in osteoporosis (Reed *et al.,* 1995), whilst treatment of ovariectomized mice with IGF-I increased bone size (Ammann *et al.,* 1996). IGF is produced by osteoblasts and stromal cells (Zhang *et al.,* 1991 & 1995) and is enhanced by PTH (Alsina *et al.,* 1996), only modestly stimulated by growth hormone (Canalis, 1994), and decreased by glucocorticoids. Oestrogen and prostaglandin E2 both increase IGF-I synthesis, the latter possibly via PGE2-responsive elements in the IGF-I promotor (Pash *et al.,* 1995). Levels of IGF mRNA are reduced by rat tail suspension (Zhang *et al.,* 1995). The insulin growth factors are involved in coupling bone resorption and formation. They are stored in the bone matrix and their release during resorption leads to stimulation of osteoblastic bone formation (Hayden *et al.,* 1995). The bone concentration of IGF I is site and age dependent (Linkhart *et al.,* 1996), and is less in osteoporotic bone. The local regulation of IGF is modulated via the IGF binding proteins (IGFBPs), which form large

complexes with IGFs *in vivo* (Linkhart *et al.*, 1996). The six known IGFBPs are produced by osteoblasts and can either increase (IGFBP-5), or decrease (IFGBP-1) bone formation (Alsina *et al.,* 1996), making interpretation of results complex.

Apart from the bone morphogenetic proteins, the other cytokines with prominent effects on osteoblast formation are platelet-derived growth factor, the prostaglandins, the interleukins 1, 6, 4 and 11, TNF alpha and PTHrP (reviewed in Alsina *et al.,* 1996). Platelet-derived growth factor stimulates proliferation of osteoblasts (Canalis, 1994), and is involved in fracture repair (Andrew *et al.,* 1995; Bolander *et al.,* 1992). *In vivo* prostaglandins are anabolic (Alsina *et al.,* 1996), though the target cell is undetermined. Non-adherent cells have been suggested (Scutt & Bertram, 1995) and recent work from the same group supports this hypothesis (Miao & Scutt, 1998). Tumour necrosis factor alpha is produced by macrophages and monocytes. It inhibits bone formation (Alsina *et al.,* 1996), stimulating resorption *in vitro* and has been implicated in the pathogenesis of osteoporosis. Additionally it is thought to be responsible for the uncoupling of formation and resorption that occurs in malignancy (Alsina *et al.,* 1996). Parathyroid hormone-related peptide has an important regulatory role in development of the fetal growth plate. It is produced by cells in the perichondrium (Lanske *et al.,* 1996) in response to Indian hedgehog (Ihh) secreted by differentiating chondrocytes (Vortkamp *et al.,* 1996) and acts on the same chondrocytes in a feedback loop to prevent more chondrocytes from differentiating. Consequently patients with mutation of the receptor for PTH/PTHrP develop metaphyeal chondrodysplasia (Schipani *et al.,* 1996). Its role in osteoblast development however is less clear. It is expressed in a range of rat cell lines and in rat calvarial cultures (Suda *et al.,* 1996a), in which it was present in greater amounts in less mature cells. Furthermore, the PTH/PTHrP receptor is temporally expressed during osteoblast differentiation (McCauley *et al.,* 1996), suggesting an autocrine action (Suda *et al.,* 1996a).

1.5.5 Glucocorticoids

Glucocorticoids are physiological regulators of growth and development in many systems in which, in general, they inhibit cell proliferation (Shalhoub *et al.,* 1992). *In vivo* they enhance bone resorption and decrease formation, with resultant osteopenia (Baylink, 1983), whilst *in vitro* the synthetic glucocorticoid dexamethasone is widely used to induce primarily osteoblastic (Cheng *et al.,* 1994; Beresford *et al.*, 1993; Leboy *et al.,* 1991), but also adipocytic differentiation (Dorheim *et al.*, 1993; Beresford *et al.,* 1992 & 1993; Locklin *et al.*, 1995). Dexamethasone stimulated osteoblastic differentiaiton of marrow

stromal cells, in both human (Cheng *et al.,* 1994) and rodent models (Leboy *et al.*, 1991), is accompanied with reduced proliferation and recruitment of preosteoblasts (Delany *et al.,* 1992). This effect is augmented by co-stimulation with PTH and PGE2 (Cheng *et al.*, 1994), the former possibly via the ability of glucocorticoids to increase PTH binding and receptor expression (Urena *et al.,* 1994). The molecular processes underlying these changes are not clearly understood. Dexamethasone causes a transient increase in Fos (Shalhoub *et al.,* 1992) and Jun mRNA, two components of the AP-1 complex (Subramaniam *et al.,* 1992). It has been suggested that interaction between the AP-1 complex and an orientation-specific cis-acting element in the human alpha1(I) collagen gene leads to down regulation of the latter in response to dexamethasone (Delany *et al.,* 1994). AP-1 has also been implicated in the down-regulation of osteocalcin reported with dexamethasone (Beresford *et al.,* 1984; Morrison & Eisman, 1993), since the osteocalcin gene has several AP-1 sites, though others have reported increased levels of osteocalcin in response to corticosteroids (Shalhoub *et al.,* 1992). Glucocorticoid receptor binding sites have also been found in the TATA box and the VDREs of the gene, and receptor binding to these sites may result in steric hindrance of gene transcription (Morrison & Eisman, 1993).

Glucocorticoids also modulate osteoblast activity by an effect on the growth factors secreted by bone cells. TGF-beta activity, which promotes bone growth, is upregulated by glucocorticoids (Shalhoub *et al.,* 1992), possibly by increased release of the latent molecule from the bone matrix by increased lysosyme activity (Oursler *et al.,* 1993). IGF-I expression is inhibited by glucocorticoids (Delany *et al.*, 1994; Alsina *et al.,* 1996). Since IGF-I enhances osteoblastic collagen synthesis and increases proliferation of preosteoblasts (Canalis, 1996), this leads to reduced bone formation. Additionally, reduction in IGFBP levels by glucocorticoids is likely to alter IGF activity (Canalis, 1996).

In a fetal rat calvarial model Shalhoub *et al* (1992) found upregulation of osteocalcin and osteopontin in response to dexamethasone, and down regulation of fibronectin and collagen; alkaline phosphatase levels rose after 20 days of treatment following an initial fall. These trends were maintained with further treatment. Dexamethasone co-stimulation with 1,25-vitamin D3 exaggerated the effect of 1,25-vitamin D3 on gene expression. The same trends in mRNA expression were found by Leboy *et al* (1991), who also found that expression of osteocalcin required additional stimulation with 1,25-vitamin D3, though this was not necessary in Shalhoub's work (Shalhoub *et al.,* 1992). Subsequently dexamethasone has routinely been used to induce osteoblastic differentiation in both

human and rodent models of stromal cell differentiation (Hughes & Aubin, 1997). Cheng *et al* (1994), Rickard et al (1994) and Beresford *et al* (1994, 1992) confirmed this utility of dexamethsosone, though, interestingly, early studies of the effect of glucocorticoids used hydrocortisone (Bellows *et al.,* 1987; Wong *et al.*, 1990). However, contradictory results have been reported for the effects of, and need for, dexamethasone in differentiation of bone derived cells of the osteoblast lineage reflecting the discrepancies present between different culture systems (Bellows & Aubin, 1989; Maniatopoulos *et al.,* 1988). These can be explained by differences in the stage of differentiation of the cells present (Beresford *et al.,* 1993). In particular, fetal calvarial cultures contain mature osteoblasts and do not require dexamethasone for formation of nodules, whilst marrow stromal cultures, containing earlier progenitor cells, do (Beresford *et al.,* 1993).

Recent data from fetal rat calvaria have shown that the effects of Dex *in vitro* are modulated by BMP-6, which in the same model was sufficient for induction of osteoblastic differentiation, though augmented by co-stimulation with dexamethasone (Boden *et al.,* 1997). This report provides a possible molecular pathway for the action of dexamethasone, and, since the expression of BMP-6 is closely related to that of Indian hedgehog during skeletal morphogenesis (Iwasaki *et al.,* 1997), may explain its differential effects dependent upon stage of differentiation. Interestingly, BMP-6 is upregulated by oestrogen (Rickard *et al.,* 1998), providing a link between the two hormones. This link however, is at variance with the observed differences in the effects on bone mass of the two on bone mass, since oestrogen is considered osteoinductive (Qu *et al.,* 1998), whilst most *in vivo* studies report loss of bone mass with dexamethasone (Baylink, 1983). The latter is thought to be due partly to decreased formation and partly to increased resorption (Conaway *et al.,* 1996; Dempster,1989). Li *et al* (1996b) carried out a dose-response study to corticosteroid deficiency or excess in growing rats and showed that glucocorticoid excess inhibited bone growth and turnover, but did not cause osteopenia. Since the animals in the study were maintained on calcium supplements, a possible explanation for the previous findings of reduced bone mass (Goulding & Gold, 1988) may be the result of uncontrolled low dietary calcium (Li *et al.,* 1996b). Shen *et al* (1997), in a follow up to the work of Li *et al* (1996b) investigated the effect of prednisolone on bone mass, either with or without supplementary calcium and found that with supplementary calcium prednisolone not only did not result in bone loss, but may have had a protective effect on the skeleton through the inhibition of bone resorption. This contradicts the common finding of osteopenia in glucocorticoid-treated patients (Baylink, 1983), but one must also take into consideration the demonstrated species differences in the effects of glucocorticoids and the fact that patients

receiving glucocorticoids often have other risk factors for bone loss, such as immobilisation or oestrogen deficiency. Notably, in rats who were either immobilised or ovariectomised concurrently with prednisolone administration, bone loss was observed; the same was also seen in those rats fed a low calcium diet. This study points the way to a more rational consideration of the effects of glucocorticoids *in vivo*, and indicates that the discrepancies observed between *in vivo* and *in vitro* studies may largely be due to artefact (Shen *et al.*, 1997).

1.6 Osteoporosis

Osteoporosis is characterised by a reduction in bone mass and alteration of the trabecular microarchitecture, with resultant increased susceptiibity to fracture (Burckhardt *et al.*, 1991). Consequently, it causes fractures predominantly in those bones with a high proportion of trabecular bone, since this is the compartment most severely affected by the process in most cases (Peel & Eastell, 1995). It is usually asymptomatic prior to the development of fractures, at which point significant loss of bone mass has already occurred. The lifetime risk of a fracture in Caucasian women is nearly 40%, and in the United Kingdom it has been estimated to cause 60,000 hip fractures, 50,000 radial fractures and 40,000 vertebral fractures annually (Melton, 1995; Melton *et al.*, 1993), with significant resultant morbidity, mortality and cost to the National Health Service (Cooper, 1993). Considering that by the time fractures occur bone mass has already been markedly reduced, the key to its treatment in the future will be prevention and early detection (Kannus, 1999). To this end its natural history, clinical genetics and molecular pathobiology are presently the focus of intense research.

1.6.1 Epidemiology and natural history of osteoporosis

Normally bone formation follows, and is coupled to, resorption. Coupling of formation and resorption ensures that bone mass is maintained during remodelling, and is thought to be mediated through the action of both local and systemic factors (Mundy, 1995a & 1995b). Osteoporosis is an example of a metabolic bone disease in which the normal coupling of bone formation and resorption has been lost, with the result that bone resorption exceeds formation, with consequent net loss of bone (Manolagas, 1998; Rehman *et al.*, 1995). As can be seen from figure 1.3 this may occur in states of reduced bone formation, increased resorption, or in high turnover when both are increased, but where the rise in resorption exceeds that of formation. Similarly, bone mass will also fall despite normal levels of resorption if formation is reduced. Other metabolic diseases in which there is failure of

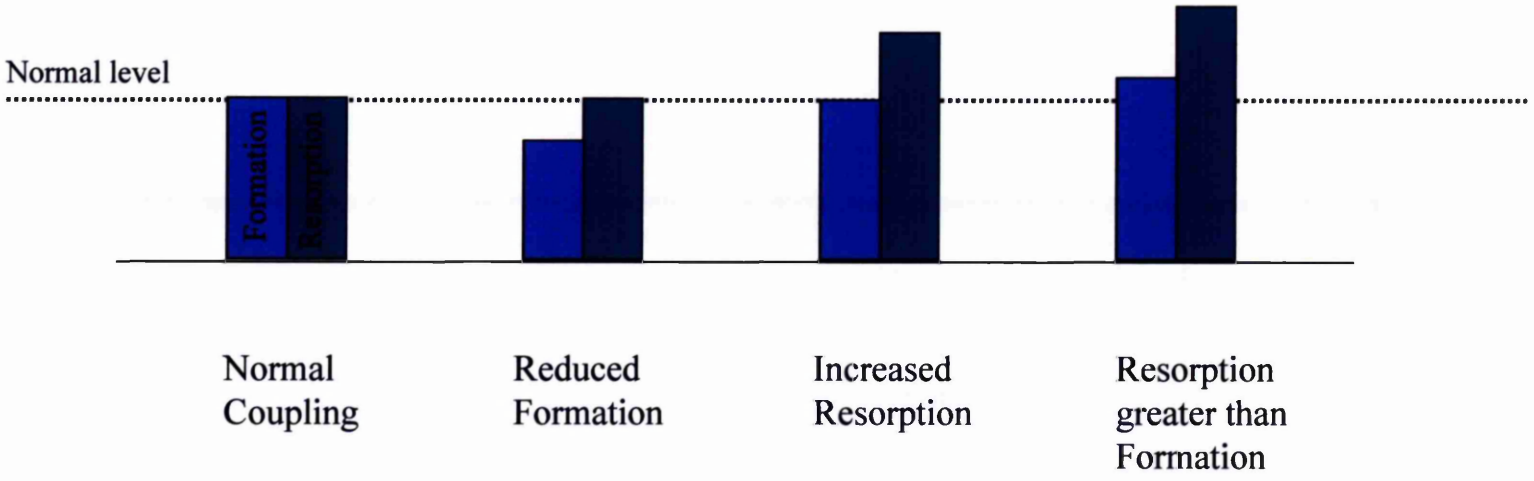


Figure 1.3 Un-coupling of bone formation and resorption in osteoporosis, resulting in net excess of resorption compared to formation. In normal state coupling of formation and resorption maintains bone mass, whilst net loss results from increased resorption, or decreased formation alone, or increase of resorption greater than that of formation in high turnover bone disease.

normal coupling include renal bone disease, aluminium bone disease and osteopetrosis. Interestingly, coupling of formation and resorption is largely maintained in Paget's disease of bone, but, since osteoclasis is markedly increased in this disease, bone formation is also upregulated, resulting, paradoxically since the primary lesion is osteoclastic, in the formation of large bones. The bone laid down however, is disorganised and consequently weak.

Factors involved in the mediation of coupling include prostaglandin E2 (Jee & Ma, 1997), parathyroid hormone (Kostenuik *et al.,* 1999; Gray & Jones, 1998; Li *et al.,* 1999; Weinreb *et al.,* 1997) and IL-1 (Alsina *et al.,* 1996), each of which can stimulate, apparently independently, both bone resorption and formation. Unequal effects of these factors on formation and resorption would therefore bring about the failure of coupling seen in osteoporosis. It has been reported, for example, that oestrogen increases skeletal resistance to parathyroid hormone-induced resorption (Cosman *et al.,* 1993). Local factors, released from the bone matrix during resorption, also mediate coupling, including IGF (Canalis, 1997; Hayden *et al.,* 1995) and TGF-beta (Mundy *et al.,* 1995). Since the production of these factors by osteoblasts is stimulated by oestrogen (Oursler *et al.,* 1991; Turner *et al.,* 1994) their abundance in bone would decrease after the menopause. Finally, it has been proposed that, since bone structure and mass are regulated by mechanical load (Mullenderm *et al.,* 1998; Mason *et al.,* 1997), by a system that is also modulated by oestrogen, the mechanical loads themselves may act as a coupling factor (Kannus *et al.,* 1996; Carter *et al.,* 1996). The strain resulting from a given load will be greater in thinner trabeculae, causing an increase in bone formation and reduced resorption, whilst thick trabeculae would experience the reverse (Kimmel, 1993). By extension, perforated trabeculae, which are by definition non-load bearing since strain can no longer be transmitted along them, would show high levels of resorption, a situation which is observed *in vivo*. The ways in which coupling is altered specifically in osteoporosis are detailed below.

Osteoporosis is characterised by reduced bone mass, for which there are a number of causes, including the endocrinopathies, malignant disease, drugs, including alcohol, glucocorticoids and heparin, malabsorption and chronic renal or liver disease, though the most common forms are post-menopausal and senile osteoporosis. It has been defined on the basis of bone mass measurement by the World Health Organisation (Kanis *et al.,* 1994). Individuals with a bone mineral density (BMD) of 2.5 standard deviations (SD) or more below the mean for young normal subjects are considered to have osteoporosis,

whilst measurements from 1-2.5 SD below the mean are defined as low bone mass (osteopenia). Frost (1997) however has proposed an alternative definition and classification based on the biomechanical pathogenesis rather than on the severity of the accompanying medical conditions. In the classification proposed 'true osteoporosis' would be that such that the patient's usual physical activities cause bone pain, and/or spontaneous fractures, and 'physiologic osteopenia' such that normal activity would not lead to bone pain, or fractures, which would only result from a fall. Combinations of both types, and transient osteopenia, such as following a severe traumatic fracture, are the other divisions in the proposed classification. As such the classification focuses attention on the underlying cause and attempts to match pathobiology to clinical scenarios and outcomes.

In practise most cases of osteoporosis are associated with sex steroid deficiency, specifically loss of oestrogen at the menopause, are 'senile', or are due to administration of glucocorticoids, of which the first group is largest (Manolagas, 1998). Consequently osteoporosis is more common in women, in whom its incidence increases with age. In women it's incidence rises after the age of 45 years and is mainly responsible for fractures of the forearm up to the age of 65 years, after which the incidence of hip fractures rises sharply. By contrast, in men the incidence of fractures does not start to rise until after 75 years of age (Melton *et al.,* 1993; Cooper 1993).

In both men and women bone mass increases during the first two decades, reaching a peak in the early twenties. Thereafter bone mass gradually declines, until it falls below a fracture threshold (Johnston & Slemenda, 1995). Both the peak bone mass and the rate of bone loss following its attainment are crucial in determining the time to fracture, and, therefore, the overall risk, or prevalence, of fracture in the population. Consequently, even small population-level increases in peak bone mass, and small reductions in age-related bone loss rates could lead to a significant reduction in the rate of osteoporotic fractures (Kannus, 1999). Peak bone mass is affected by genetic factors, as detailed below, and by both diet and exercise.

There is very convincing evidence to link dietary calcium deficiency with osteoporosis (Lindsay & Nieves, 1994). Calcium deficiency in animals causes osteoporosis (Heaney *et al.,* 1982), whilst osteoporotic patients show negative calcium balance. Calcium absorption from the gut declines in all individuals with ageing, partly due to a reduction in the capacity for increasing absorption of calcium to cope with reduced levels in the diet that is present in younger individuals (Slovik *et al.,* 1981). Children receiving small calcium

supplements attain higher peak bone mass than those with unsupplemented diets (Johnston *et al.,* 1992). Furthermore, dietary calcium intake is less in females than males at all ages, particularly in adolescent females in whom calcium intake is often insufficient to maintain normal calcium balance. However, just how dietary calcium deficiency could cause bone loss is unclear. One possibility is the induction of secondary hyperparathyroidism, which is supported by evidence that calcium and vitamin D supplements decrease hip fracture rates in the elderly and suppress PTH levels (Chapuy *et al.,* 1992). Alternatively, since oestrogen may stimulate vitamin D3 production (Mundy, 1995b), increased calcium requirements following the menopause could be due to oestrogen deficiency, leading to decreased production of 1,25 vitamin D3, which would cause decreased gut absorption of calcium (Mundy, 1995b).

Human and animal studies have shown that physical activity is important in increasing bone mass, density and strength (Kannus, 1999). The starting age is crucial, the benefit being doubled if the activity is started before or at puberty rather than after it. Bone tissue also responds to exercise in adulthood, although at this stage exercise is better at preserving bone rather than adding new bone (Kannus *et al.,* 1996). Nevertheless, the bone preserving action of exercise in adulthood may still be important in maintaining bone strength and preventing osteoporotic fractures since only small percentage gains in preservation of bone mass and density will result in significant reductions in risk of fracture. However, the type, frequency, intensity and duration of exercise that best produce the desired bone changes are not yet well determined, but current evidence suggests that impact type exercise that creates versatile strain distributions through the bone structure can best improve bone strength (Kannus *et al.*, 1996; Skerry, 1997; Frost, 1997; Bennell *et al.,* 1997). Sports such as squash, tennis, aerobics, gymnastics, or weight training may best fulfill these criteria, whilst in older adults brisk walking, stair climbing, dancing and calisthenics are suitable. Despite the clear effect of physical activity, and mechanical loading, on bone mass and density, which may amount to up to a 50% increase in both parameters, the exact mechanisms by which this occurs are unknown (Kannus *et al.,* 1996). Animal experiments indicate that the training effect is most likely transmitted by stimulation of osteoblastic activity (Yeh *et al.,* 1993), and less so by reduction in osteoclasis. Prostaglandins, growth factors and oestrogen have all been implicated in mediation of the process (Kannus *et al.,* 1996), and details of their role in the development of osteoporosis are given below.

1.6.2 Clinical genetics of osteoporosis

The pathogenesis of osteoporosis, like that of all other multifactorial diseases, is the result of a complex interplay of genetic susceptibility (Smith *et al.,* 1973; Christian *et al.,* 1989; Seeman *et al.,* 1989) and environment (Kannus, 1999). In order to address the former of these variables several groups have identified genetic polymorphisms associated with osteoporosis, using linkage and association studies (Lander & Schork, 1994). Principal amongst these polymorphisms are those of the vitamin D receptor gene (VDR) allele. Polymorphisms of the receptor were first reported by Morrison *et al* (1992), who identified common polymorphisms in the VDR gene which were correlated with osteocalcin levels (Morrison *et al.,* 1992), and with bone density in both twins and unrelated individuals (Morrison *et al.,* 1994), and suggested that common allelic variations in the gene accounted for up to 75% of the genetic basis of differences in bone mineral density (BMD). The VDR gene (Baker *et al.,* 1988) is located on chromosome 12 (Labuda *et al.,* 1992) and consists of nine exons, spanning 60-70kb of genomic DNA. The primary locus for polymorphisms is the intronic sequence (approximately 1320bp long) between exons 8 and 9, in which restriction sites for the endonucleases BsmI, TaqI and Apa I are present. By convention the presence of a BsmI restriction site in the VDR allele is designated by a lower case letter 'b', whilst in it's absence the upper case 'B' is used. Individuals are therefore homozygous for the presence (bb) or absence of the restriction site (BB), or heterozygous (Bb). Similarly, the presence or absence of the ApaI and TaqI restriction sites are designated 'a' or 'A', and 't' or 'T' respectively. Morrison *et al* (1992) found that BB predicted higher osteocalcin levels, and was associated with low BMD in an Australian Caucasian population, whilst Spector *et al* (1995) reported similar findings for polymorphism at the TaqI locus, which was in strong linkage disequilibrium with the BsmI site, in a UK population. Other studies supporting the association of polymorphisms in the VDR gene with osteoporosis include those of Fleet *et al* (1995), who studied the BsmI site, and Riggs *et al* (1995) who studied both the BsmI and ApaI sites. Whilst all the above studies were carried out on Caucasian women, similar findings have been reported from a Japanese study, looking at the BsmI and ApaI sites (Gross *et al.,* 1996).

Other workers however have failed to confirm these reports. A twin study by Hurstmeyer *et al* (1994) found no relationship between VDR genotype and BMD at any of the four skeletal sites measured, whilst Looney *et al* (1995) found no increase in the prevalence of the BB genotype in osteoporotic women. Other studies which have failed to find an association between VDR genotype and BMD include those of Garnero *et al* (1995), Scholler *et al* (1995), Melhus (1994), Keen *et al* (1995) and Kroger *et al* (1995). It remains

to be answered as to how polymorphisms in the VDR gene could affect BMD, but determination of such a mechanism would help resolve the disputed association of VDR gene polymorphism with osteoporosis.

Presently, other candidate genes for the heritable component of osteoporosis are being studied, principal amongst which are the COL1A1 and COL1A2 genes, which encode for the collagen α1 (I) and collagen α2 (I) chains of collagen type I respectively (Grant *et al.*, 1996). Protein coding mutations of both of these genes give rise to a severe osteoporotic phenotype in the form of osteogenesis imperfecta (Rowe, 1991). The protein coding regions of both COL1A1 and COL1A2 are normal in osteoporotic patients (Spotila *et al.*, 1994), but Ralston and colleagues have described a G-T polymorphism that affects a binding site for the transcription factor Sp1 in the first intron of the COL1A1 gene which is over represented in patients with osteoporotic fractures (Grant *et al.*, 1996; Garnero *et al.*, 1998). Gel shift assays demonstrate that the T substitution ('s' allele) increases affinity of the Sp1 binding site for DNA, and assays of allele specific transcripts in heterozygotes show more 's' derived transcripts than those derived from the normal, 'S', allele (*Dean et al.*, 1998a). Increased transcription of the 's' allele is accompanied by an increase in alpha 1 chain production, possibly resulting in the formation of collagen alpha 1(I) homotrimers (Dean *et al.*, 1998b). Such homotrimers would be weaker than normal α1 (I) / α2 (I) heterotrimers because of altered inter-molecular crosslinking (Dean *et al.*, 1998b). This hypothesis is of particular relevance to osteoporosis since α1 (I) homotrimer formation causes increased bone fragility in osteogenesis imperfecta, which is due to a mutation in the COLIA2 gene (Chipman *et al.*, 1993). In keeping with the hypothesis, biomechanical studies have shown reduced bone strength in cores of bone obtained from patients carrying the 's' allele (Dean *et al.*, 1998b).

Finally, allelic variation at the interleukin-1 receptor anatagonist (IL-1ra) gene has been associated with early postmenopausal bone loss at the spine (Keen *et al.*, 1998). IL-1ra binds to IL-1 receptors, competing with both IL-1α and IL-1β without IL-1 agonist activity (Dinarello, 1991). IL-1 is a powerful stimulant of bone resorption (Dinarello, 1991), and is present at high levels in the monocytes of patients with 'high turnover' osteoporosis (Pacifici *et al.*, 1993). Conversely, in animal studies administration of IL-1ra blocks the increase in osteoclastosis seen after ovariectomy (Kitazawa *et al.*, 1994). The human IL-1ra gene maps to chromosome 2q and consists of four exons (Lennard *et al.*, 1992). The second intron contains a polymorphic site associated with bone loss, and may be of

functional significance since the region also contains three potential protein-binding sites (Keen *et al.,* 1998).

1.6.3 Molecular pathobiology and cellular mechanisms of osteoporosis

The epidemiology of osteoporosis clearly indicates the presence of different groups of patients under the generalised definition of osteoporosis as a BMD of greater than 2.5 SD below the mean bone mass (Rehman *et al.*, 1995). Whilst the classification of Frost (Frost, 1997) is not yet in current use, it is useful in directing attention away from the clinical consequences i.e. low bone mass, to those of causation. Manolagas (1998) has reviewed the cellular mechanisms of osteoporosis, and in particular the processes in those cases due to either sex-steroid withdrawal, administration of glucocorticoids, or 'old age', so called senile osteoporosis.

Maintenance of bone mass and trabecular microarchitecture is obviously dependent on the balance between osteoblastic formation and osteoclastic resorption, and alteration in either side of the equation may lead to loss of bone mass. The level of peak bone mass attained in early adult life is an important factor in an individual's risk of developing osteoporosis (Kannus, 1999). However, from a therapeutic and medical perspective the pathogenesis of bone loss is probably more important, though parenthetically, twin studies (Eisman *et al.,* 1993; Kelly *et al.,* 1993) indicate that the rate of bone loss is less strongly influenced by genetics than peak bone mass.

The rate and extent of bone resorption is determined by the rate of osteoclast recruitment and activity. Consequently agents that increase bone resorption *in vivo* and osteoclast formation *in vitro* have been implicated, including parathyroid hormone, prostaglandin E (PGE), IL-1, TNFα, and IL-6 (Reddy & Roodman, 1998; Manolagas, 1988; Martin *et al.,* 1998). These cytokines are particularly important in the pathogenesis of post-menopausal osteoporosis, due to the withdrawal of oestrogen (Jilka, 1998; Manolagas, 1998). *In vitro* oestrogen inhibits the production of IL-6 (Jilka, 1998) and PGE2 (Kawaguchi *et al.,* 1995) by macrophages, and of IL-6 by marrow stromal cells (Jilka *et al.,* 1992). Inhibition of IL-6 production, which also occurs with administration of selective oestrogen receptor modulators (SERMS) such as raloxifene, is due to interactions of the oestrogen receptor with transcription factors, especially NF-KB and RANKL (Jilka, 1998). Loss of oestrogen following the menopause therefore results in increased IL-6, which in turns leads to activation of NF-KB (Galien *et al.,* 1996), resulting in increased osteoclast survival.

Hughes *et al* reported induction of apoptosis in osteoclasts by oestrogen (Hughes *et al.*, 1996). IL-6 therefore leads directly to increased resorption, whilst injection of an IL-6 neutralising antibody to gonadectomised female mice prevented an increase in osteoclastogenesis (Poli *et al.*, 1994). Conversely, IL-6 is seemingly unimportant for osteoclastogenesis under normal conditions, and administration of an IL-6 antibody to oestrogen sufficient mice has no effect on osteoclastogenesis (Poli *et al.*, 1994). IL-1 and TNF are also strong stimulators of osteoclast formation (Kimble *et al.*, 1995), but are produced at very low levels by marrow stromal cells and instead their main source is bone marrow macrophages and monocytes. They stimulate IL-6 production by stromal cells (Devlin *et al.*, 1998), and recently have been shown to be regulated by oestrogen (Kimble *et al.*, 1997; Sunyer *et al.*, 1997), but whilst TNF is reduced by oestrogen, IL-1 is increased. It is likely therefore that their role in the pathophysiology of osteoporosis is an indirect one, since they also act to inhibit osteoblast differentiation and stimulate production of M-CSF, which is osteoclastogenic (Reddy & Roodman, 1998). The role of TGF-beta in the development of osteoporosis is tenuous (Manolagas, 1998), whilst serum IGF I levels decline in parallel with bone mass with age. Conversely, mice with high levels of IGF I have high bone mineral density (Rosen *et al.*, 1997). IGFs stimulate bone matrix formation, but why their levels should fall with age is unclear, though alteration in the levels of their binding proteins IGFBP-4 and IGFBP-5 could be responsible (Canalis 1997).

Senile osteoporosis is characterised by slow bone loss and occurs in elderly eugonadal men, in whom, in contrast to the acute drop in oestrogen occurring in women during the menopause, there is slow and progressive decline in androgen levels (Manolagas, 1998). The reduction in bone mass in this group of patients is thought to be due principally to a reduction in the amount of bone formed, and specifically to reduced numbers and activity of osteoblasts. The reasons for this are presently obscure, but a mouse model exists, the so-called senescence accelerated mouse-P6 (SAMP6), in which there is decreased osteoblastogensis, a low rate of bone formation, reduced bone mineral density, and increased marrow adipocity (Jilka *et al.*, 1996). This is in agreement with the hypothesis that there exists a reciprocal relationship between osteoblastogenesis and adipogenesis, with each lineage arising from the uncommitted stem cell of the CFU-F(see section 2.1.3). Alteration of the balance of differentiation in favour of adipogenesis is thought to be responsible for osteoporosis under this model (see section 2.1.3).

Finally, some cases of osteoporosis occur following exogenous administration of glucocorticoids. Glucocorticoid-induced osteoporosis is characterised by diffuse bone loss, of both cortical and cancellous bone, consequent upon reduced bone formation. This is thought to be due to both defective osteoblastogenesis and an increase in osteoblast apoptosis (Manolagas, 1998). The latter factor has been studied in depth by Manolagas and colleagues, who found induction of osteoblastic apoptosis *in vitro* by glucocorticoids (Jilka *et al.,* 1998). Osteoblast apoptosis appears to be mediated by IL-6 type cytokines and TGF-β (Jilka *et al.,* 1998), via the expression of the cyclin dependent kinase inhibitor p21 (Bellido *et al.,* 1997). In support of these observations, both mice receiving glucocorticoids and patients with glucocorticoid-induced osteoporosis exhibit increased osteoblast apoptosis (Weinstein *et al.,* 1998). The effects of glucocorticoids on osteoblast gene expression, differentiation and proliferation are reviewed in section 1.5.5, but the precise mechanisms whereby they reduce osteoblastogenesis *in vivo* remain unclear (Delany *et al.,* 1994). Recently, Scutt *et al* (1996), using an assay of rodent bone marrow stromal cell recruitment, reported different rates of colony formation and osteoblastic differentiation depending upon the dose of dexamethasone given. In particular, the optimum concentrations for differentiation of stromal cells to the osteoblastic lineage were 10^{-7} M and 10^{-8} M, whilst concentrations either less than or in excess of these resulted in reduced colony formation rates. Since the doses of glucocorticoids used often give peak serum concentrations of 10^{-5} M, this provides an explanation for the apparent contradiction between the role of glucocorticoids *in vitro* and *in vivo*, which appears to be a reflection of the dose at which the steroid is added.

1.7 Overall objectives of investigation

The importance of osteoblast differentiation, and its alteration, in osteoporosis is disputed, though most attention to now has been focused on the upregulation of osteoclastogenesis. Since osteoporosis may also result from decreased bone formation it is important to characterise the extent and nature of this factor in the pathogenesis of osteoporosis. Furthermore, the postulated switch in differentiation of osteoblastic precursors from the osteoblastic to the adipocytic lineage in osteoporosis requires examination.

It can be seen from the above that whilst the later stages in the osteoblastic lineage are well characterised, information regarding the earlier stages is relatively scanty. Furthermore, whereas many studies have investigated the role of growth factors in osteoblastic differentiation, and the possible molecular mechanisms underpinning their effects, the

majority of these have been carried out using either animal or immortalised cell culture systems, of doubtful relevance to normal human osteogenesis. The use of such varied systems also accounts for the wide variation in the results reported by different groups. The use of primary human cells is therefore preferable.

In order to address these themes, the investigation was carried out in three parts. Firstly, a detailed histomorphometric analysis of osteoblastic activity, of bone formation in the different bone compartments, and of the relationship between bone formation and the proportion of marrow adipose tissue, was performed in established cases of osteoporosis. Secondly, primary human bone marrow culture was used to isolate and identify genes differentially expressed during the very early stages of osteoblastic differentiation, both in adherent and non-adherent models of osteogenesis, and the importance of the genes identified for normal and abnormal bone formation assessed by hybridisation studies. Thirdly, the functional role of the genes identified was investigated by use of anti-sense transfection experiments. The details of the various components of the investigation are outlined in the flow chart shown in figure 1.4, which should be referred to in the following chapters.

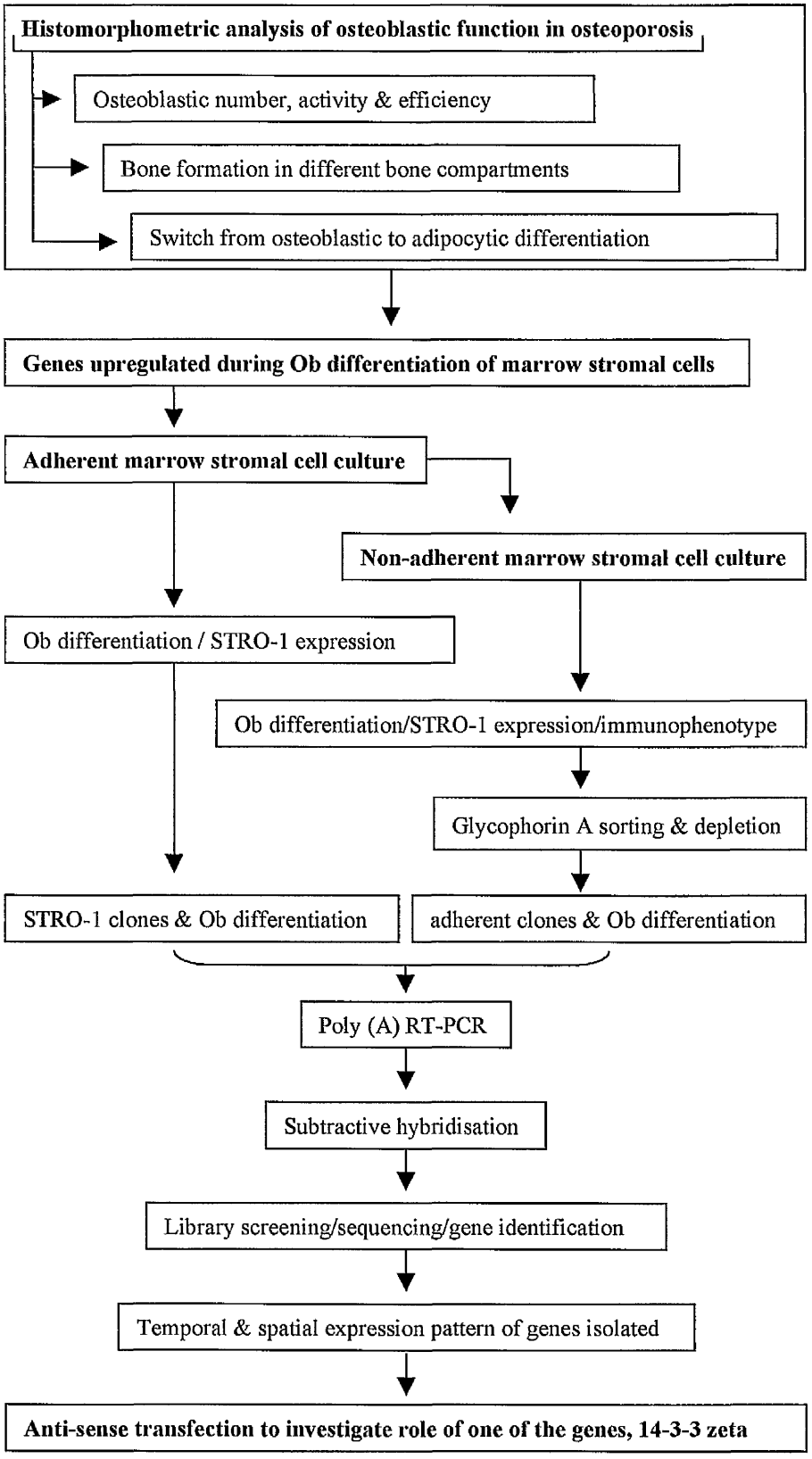


Figure 1.4 Flow chart of investigations undertaken. Ob=osteoblastic differentiation

Chapter 2

Histomorphometric analysis of osteoblast function and differentiation in established osteoporosis

2.1 Introduction

Osteoporosis is a clinical syndrome characterised by a reduction in bone mass (osteopenia), usually in the trabecular compartment, and resultant low trauma fractures (Peel & Eastell, 1995). It is an important cause of morbidity and mortality in the elderly, especially in women, and the economic cost of osteoporotic femoral fractures alone is substantial. At the cellular level it has been shown to be due to an imbalance in osteoblast bone formation and osteoclast bone resorption (Manolagas, 1998), i.e. uncoupling, different pathogenic mechanisms being responsible for alteration in each side of the equation (reviewed in section 1.6), though the contribution to bone loss made by each of the equation *in vivo* is unclear. Histomorphometric studies have been carried out to define these factors (Rehman *et al.,* 1995) and are reviewed below. In addition, as alluded to in the introduction (section 1.3.2.1), osteoblasts arise from a mutipotential stem cell, which is also capable of differentiation along the adipocytic lineage, and there is evidence indicating that a switch from the osteogenic to the adipocytic lineage occurs in osteoporosis (Hirano & Iwasaki, 1992). Finally, since osteoporosis is due in part to reduced mechanical loading of the bone (Shen *et al.,* 1997), as demonstrated most graphically during space flight (Vico *et al.,* 1998), the contribution made by the different bone compartments to the bone loss seen may vary. The principles utilised in bone histomorphometry, the changes reported in osteoporosis, and the basis of the osteoblastic / adipocytic switch will be reviewed.

2.1.1 Bone histomorphometry

Bone histomorphometry allows quantitative assessment of bone remodelling and structure (Compston, 1997). By convention bone biopsies from the iliac crest are used, and most use transverse biopsies, in which two cortices together with the intervening cancellous bone are present. The biopsies are processed to resin, avoiding the need for demineralisation, and usually stained with von Kossa or a van Geison stain to differentiate osteoid from mineralised bone, whilst 1% toluidine blue is used for definition of bone cell morphology and to facilitate polarised light microscopy. Examination of tetracycline fluorescence is achieved in unstained sections by visualisation under blue (365 nm) light (Compston, 1997).

The histological sections used for bone histomorphometry are two-dimensional fractions of a three-dimensional structure. The data obtained can be extrapolated to three-dimensional

quantities, but the stereological formulae used to do this assume that the data is isotropic (Parfitt *et al.,* 1983). However, since this is not the case for bone many prefer to express the raw two-dimensional data only. The data obtained by histomorphometry can be divided into static measurements, such as osteoblast number, or dynamic measurements, such as bone formation rate; a list of the parameters commonly used is given in table 2.1. The nomenclature and the units used were defined in 1987 by the American Society of Bone and Mineral Research Histomorphometry Nomenclature Committee (Parfitt *et al.,* 1987). According to the guidelines all data are expressed in the format of the source (the structure on which the measurement is made; for example, bone tissue or surface), the measurement (primary or derived) and the referent (area or perimeter in two-dimensional, and volume or surface in three-dimensional terminology). The most commonly used source is cancellous bone, though other possible sources include the endocortical and periosteal surfaces, whilst cortical bone has largely been ignored by most histomorphometrists, despite its predominance in the skeleton and its importance as a determinant of fracture risk (Compston, 1997).

Histomorphometric data is usually obtained by point-counting utilising a series of grids or lines that are inserted into the eyepiece of the microscope (Compston, 1997), though recently a number of semi-automated and automated computerised systems have been developed (Compston *et al.,* 1993). These have not only greatly simplified the collection of two-dimensional data, but have also allowed the use of more sophisticated methods of assessment of bone structure. Since the structural determinants of cancellous bone strength are three-dimensional, their assessment on conventional sections provides only indirect information about them (Odgaard, 1997). However, there is evidence that the measurements from two-dimensional sections are representative of three-dimensional structure (Odgaard & Gundersen, 1993). Methods used to assess bone structure include conventional measurement of trabecular width, separation and number, either with an eye-piece graticule, or by computerised image analysis, whilst less commonly used techniques include strut analysis (Garrahan *et al.,* 1986), star volume (Vesterby, 1990), and fractal analysis (Weinstein *et al.,* 1992). Strut analysis is based on a topological classification of trabeculae, or struts, and on the definition of free ends (termini) and nodes (junctions). The number of termini and nodes are counted, a node/terminus ratio derived, and the different strut types counted, with node-to-node and node-to-loop types being correlated with connectivity, whilst struts with termini are not (Garrahan *et al.,* 1986). Star volume is the mean volume of the solid material (trabecular star volume) or

Variable Type	Variables	Definition (Units)
Static Trabecular Bone Parameters	Trabecular Bone Volume	BV/TVt (%)
	Wall Thickness	(um)
	Osteoid Surface	OS/B (%)
	Osteoid Volume	OV/BV (%)
	Osteoid Thickness	(um)
	Osteoblast Surface	ObS/BS (%)
	Mineralising Surface 1	MS/BS or dLS+1/2sLS (%)
	Mineralising Surface 2	MS/OS or sLS+dLS (%)
	Mineralising Surface	dLS/sLS (%)
	Eroded Surface	ES/BS (%)
	Osteoclast Surface	OcS/BS (%)
	Osteoclast Number	Noc/TVt (/mm^2 x10^{-2})
	Trabecular Thickness	(um)
	Trabecular Number	(/mm^3)
	Trabecular Separation	(um)
Static Cortical Bone Parameters	Cortical Thickness	(um)
	Cortical Volume	CV/TVc (%)
	Cortical Wall Thickness	(um)
	Osteoclast Number	Noc/TVc (/mm^2 x10^{-2})
	Sub-cortical Osteoclasts	Noc/BSs (/mm x10^{-2})
Dynamic Parameters	Trabecular Apposition Rate	(um/day)
	Cortical Apposition Rate	(um/day)
	Bone Formation Rate	BFR/BVt [dLS+1/2sLS] (%/year)

Table 2.1 Standard histomorphometric variables measured. Each variable was measured according to the guidelines described, and using the standard units defined by, Parfitt *et al* (1987).

empty space (marrow star volume) that can be seen unobscured from a randomly chosen point of measurement (Vesterby, 1990).

Recently a number of techniques have been developed specifically to generate three-dimensional images of bone, including reconstruction of serial sections (Odgaard, 1997), scanning and stereo microscopy (Genant *et al.,* 1996), and high resolution and microcomputed tomography (Muller *et al.,* 1996; Muller *et al.,* 1998) and magnetic resonance imaging (Majumdar *et al.,* 1998). Comparison of the results from high-resolution computed tomography of, and histological sections from, the same pieces of bone indicate a high degree of concordance between the two methods *vis a vis* trabecular microstructure (Muller *et al.,* 1996), though the fidelity of the data obtained from three-dimensional imaging is dependent upon the spatial resolution (Kothari *et al.*, 1998). This is particularly true for measures such as trabecular thickness, whilst traditional morphometric measures, such as trabecular spacing and number showed weak resolution dependency (Kothari *et al.,* 1998).

2.1.2 Histomorphometric analysis of osteoporosis

However, whilst studies of postmenopausal osteoporosis have supported such a concept of heterogeneity (Rehman *et al.,* 1995), there is debate as to the principle cause. Some studies have shown that 'uncoupling of bone' is caused by increased bone resorption (Heaney *et al.,* 1978). This leads to perforation of the bone trabeculae, which results in a greater increase in bone fragility than would be expected from the loss of bone mass alone (Parfitt, 1981). Steiniche (1995) found three patterns of bone loss in osteoporosis, namely: 1) reversible bone loss depending on the magnitude of the remodelling space, which is the amount of bone resorbed and not yet reformed during the remodelling sequence, 2) irreversible thinning of the trabeculae due to a negative balance at the remodelling site, and 3) irreversible loss of whole trabecular elements caused by deep resorption lacunae perforating the trabecular plates. Other studies suggest that the predominant abnormality is the failure of osteoblasts to fill resorbed spaces (Vesterby *et al.,* 1989; Kleerekoper *et al.,* 1985), either after increased or normal levels of resorption. Both these observations provide an explanation for the fact that although the bone mass is significantly reduced, by up to 20-30% in postmenopausal osteoporosis, a substantial overlap exists between the bone mass in patients with vertebral fractures and normal controls (Steiniche, 1995). Consequently, beside the slight reduction in trabecular bone volume seen in osteoporosis, significant differences in microstructure exist between osteoporotic patients and normal

controls. These changes in structure are probably a consequence of trabecular plate perforations (Steiniche, 1995).

Notwithstanding the nature of the defect produced however, understanding of the pathobiology of osteoporosis relies on a precise definition of osteoblast and osteoclast activity. In an attempt to provide this Rehman *et al* (1995) carried out a histomorphometric analysis of osteoblast and osteoclastic parameters in trephine biopsies from a large group of patients with established postmenopausal osteoporosis. They found five distinct groups on the basis of histomorphometric changes in cell function, namely: group 1, decreased osteoblastic and osteoclastic activity; group 2, decreased osteoblastic and increased osteoclastic activity; group 3, increased osteoblastic and osteoclastic activity; group 4, no bone surface cell activity; group 5, apparently normal osteoblastic and osteoclastic activity. In particular, the data pointed to a wide variation in the number and activity of bone surface osteoblasts, and to a lack of correlation between osteoblastic and osteoclastic activity. Jackson *et al* (1987) found reduced numbers and activity of osteoblasts in idiopathic male osteoporosis, whilst Arlot *et al* (1984), in a histomorphometric study of 27 patients with idiopathic osteoporosis found poor double labelling of trabecular osteoid, suggesting impaired formation of bone at these surfaces. This phenomenon was not accompanied by increased width of osteoid seams (as seen in osteomalacia), indicating that formation and mineralisation of the matrix were in equilibrium. Thus some patients with osteoporosis who are rapidly losing bone have low rates of formation of trabecular bone both by individual osteoblasts and in relation to available bone surfaces (Arlot *et al.,* 1984). Given the promising treatments that can increase trabecular bone volume in osteoporosis, such as parathyroid hormone-related peptide (Stewart, 1996) and sodium fluoride (Mundy, 1995c), there is now therefore clearly a need to examine in more detail the nature and extent of altered osteoblast activity in osteoporosis. Furthermore since, as reviewed in Manalogas (1998), perturbation of normal osteoblastic differentiation is likely to be important in idiopathic osteoporosis, there is also a need to examine osteoblast recruitment.

Finally, about 20 % of human bone is trabecular and 80% cortical, though the trabecular bone is metabolically more active and osteoporotic fractures tend to occur at sites composed of more than 50% trabecular bone, such as the lumbar vertebrae, and femoral neck (Peel & Eastell, 1995). As a result most studies have concentrated on the reduction in trabecular bone mass (Courpron *et al.,* 1976; Avioli, 1997; Ellis & Peart, 1972), with little attention to the cortical, sub-cortical or periosteal compartments, though, for example, sub-

cortical bone thinning is known to cause decreased cortical bone width (Hernandez *et al.*, 1997).

2.1.3 Adipocytic / osteoblastic switch

The reduction in trabecular volume that occurs in osteoporosis is associated with a decrease in bone marrow cellularity, and a parallel increase in marrow adiposity (Hirano & Iwasaki, 1992). The cellular component of the bone marrow consists of haematopoietic and stromal cells (Abboud & Lichtman, 1995), and it has been hypothesised that there is a reduction in differentiation of osteoblasts from the stromal cell precursor pool in favour of adipocytic differentiation (Kajkenova *et al.*, 1997). The details of the stromal system of bone are reviewed in section 3.1.1.3, whilst those of the latter stages of osteoblast differentiation have been covered in section 1.3.2, and so in this section I will concentrate on the evidence for the switch mentioned above.

Owen and colleagues, in a series of papers (Owen, 1988; Owen *et al.*, 1987; Beresford *et al.*, 1992; Bennett *et al.*, 1991), established the clonogenic capacity of bone marrow stromal cells, describing first of all heterogeneity of the CFU-F in terms of osteogenic differentiation as assessed by alkaline phosphatase expression *in vitro* (Owen *et al.*, 1987). The differentiation of the CFU-F was amenable to alteration by stimulation with either epidermal growth factor, which reduced alkaline phosphatase expression, and with hydrocortisone, which increased its expression (Owen *et al.*, 1987). Further evidence for the ability of the CFU-F to undergo a switch in differentiation was provided by the same group in 1991, when they reported osteogenic differentiation, both *in vitro* and *in vivo* using diffusion chambers, of rabbit adipocytic colonies generated *in vitro* from marrow stromal cells (Bennett *et al.*, 1991). The yield of osteogenic tisssue *in vivo* was greater from implantation of fibroblastic colonies, though three of the eight adipocytic colonies implanted went on to form bone. *In vitro* adipocytic colonies derived from primary culture of marrow stromal cells underwent differentiation to an osteoblastic phenotype when the serum used to supplement the culture medium was changed from rabbit to fetal calf serum. This indicates that the cells that had initially differentiated in an adipocytic direction were able to revert to a more proliferative stage and differentiate along the osteogenic pathway (Bennett *et al.*, 1991). Subsequently Beresford *et al* (1992) demonstrated the presence of an inverse relationship between differentiation of adipocytic and osteogenic cells in rat marrow stromal cell culture. Adipocytic differentiation was assessed morphologically and by expression of collagen type IV and of aP2, whilst osteogenic differentiation was

assessed on the basis of alkaline phosphatase expression and by induction of bone sialoprotein and osteocalcin. For primary cultures maintained in the presence of fetal calf serum, the presence or absence of dexamethasone at different stages induced a switch to either the osteogenic or adipocytic lineage. Specifically, addition of dexamethasone after an initial period of culture induced adipogenesis at the expense of osteoblastic differentiation, whilst its presence from the outset, followed by addition of 1,25-vitamin D3, caused the reverse. Conversely, when the cells were stimulated with dexamethasone, in the presence of fetal calf serum, from the outset then both osteoblastic and adipocytic differentiation was observed. The same conditions however, are now routinely used to induce osteogenic differentiation of stromal cells, whilst the substitution of rabbit for fetal calf serum, as initially reported by Bennett *et al* (1991) is used to stimulate adipocytic differentiation.

In addition to the *in vitro* studies referred to above, several *in vivo* models supporting the hypothesis have recently been reported. The immortalised human bone marrow stromal cell line HCC1 is capable of differentiation along either the osteoblastic or adipocytic lineage dependent upon culture conditions (Brown *et al.,* 1997). Hicok *et al* (1998) reported six similar cell lines, derived from human bone marrow stromal cells, immortalised with the SV40 antigen and capable of differentiation along either lineage, whilst another similar cell line hOP 7 was reported by Houghton *et al* (1998). Interestingly hOP 7, which exhibited an osteoprogenitor phenotype *ab initio* and underwent further differentiation to a more mature osteoblastic phenotype when stimulated with dexamethasone, showed acquisition of an adipocytic phenotype when cultured in the presence of rabbit serum. The pre-incubation of the rabbit serum with activated charcoal abrogated its adipogenic action, indicating that factors present within the serum are responsible for the switch (Houghton *et al.,* 1998). The nature of the factor or factors involved is unknown, though specific fatty acids present in rabbit but not fetal calf serum are candidates (Schmidt *et al.,* 1996). In particular, fatty acids, which are altered after the menopause, are involved in induction of the transcription factors that co-ordinate adipogenesis, namely the CAAT/enhancer binding proteins (C/EBP α,β,δ) and the perioxisome proliferator-activated receptors (PPAR $\alpha,\gamma 2,\delta$) (Gimble *et al.,* 1996). The role of the latter group of transcription factors in adipogenesis is underlined by the finding that the thiazolidinediones, a new class of synthetic anti-diabetic compound, bind to peroxisome proliferator-activated receptors and reduce bone mineral density, with an associated increase in the number of marrow adipocytes (Jennermann *et al.,* 1995). Finally, Thompson *et al* (1998) reported the development of a series of marrow stromal cell lines

derived from $p53^{-/-}$ mice. The lines were grouped into three categories. Those in the first were heterogeneous, comprising stem cells capable of spontaneous generation of foci containing either adipocytes or mineralising osteoblasts, and those in the second corresponded to mature osteoblasts. Those in the third however, whilst also appearing to correspond to mature osteoblasts, were capable of marked adipocytic differentiation when stimulated with, in this case, dexamethasone (0.1μM / 10^{-7} M), indomethacin (50μM) and ascorbic acid (50μg/ml). This report not only supports the hypothesis that stromal cells can switch from the osteoblastic to the adipocytic lineage, but also indicates, as suggested by Dorheim *et al* (1993), that in at least some situations adipocytes may be derived from osteoblast-like cells, suggesting the presence of phenotypic plasticity.

Previous studies have shown a correlation between the increase of adipose tissue and the decrease in cancellous bone in bone marrow (Meunier *et al.,* 1971), and in some this has been linked to a decrease in bone formation (Hirano & Iwasaki, 1992). However, these studies have largely been conducted using postmenopausal samples, and may therefore be confounded by the effect of age on the ratio of adipose to haematopoietic tissue. The cellularity of bone marrow varies from between 60 to 90% in the first decade to between 10 to 50% in the seventh decade (Hartsock *et al.,* 1965). This has been shown by point counting methods (Kerndrup *et al.,* 1980) as well as by more sophisticated image analysis methods, notably by Hirano & Iwasaki (1992) who reported a decrease in the haematopoietic tissue, with a parallel increase in adipose tissue, in the bone marrow of postmenopausal osteoporotic women. The increase in adipose tissue was proportionate to the decrease in cancellous bone, and to the ratio of osteoid perimeter / bone perimeter, regardless of age. On the other hand, the ratio of eroded perimeter / bone remained almost constant, until the amount of adipocytic tissue increased significantly, indicating that an increase in adipose tissue causes an imbalance in bone formation and resorption, with resultant bone loss. However, this study (Hirano & Iwasaki, 1992) was conducted in a group of postmenopausal women, all over the age of 50 years, without age/sex matched controls. It is therefore necessary to establish whether or not the same pertains to both males and females, from a wider age range, with osteoporosis.

2.1.4 Objectives of histomorphometric studies

On the basis of the above, histomorphometric analysis of bicortical iliac crest biopsies taken from patients with established osteoporosis was used to investigate three areas: 1) the differential pattern of osteoblast dysfunction, 2) the differential pattern of altered bone

formation in different bone compartments, and 3) the alteration of the haematopoietic to adipocytic ratio, in patients with established osteoporosis.

2.2 Methods

2.2.1 Bone histomorphometry

Histomorphometry was carried out according to standard protocols using bicortical iliac crest biopsies. Bone histomorphometry quantitatively assesses bone remodelling, modelling and structure by a range of static and dynamic parameters, providing precise characterisation and monitoring of disease states. The iliac crest is the usual site for the biopsy, which ideally consists of both cortices together with the intervening trabecular bone. The procedure is carried out under local anaesthetic using an 8mm diameter trephine needle, within which a central trocar is present and which is used to core out a cylindrical piece of bone between 1.5 to 3cm long and 2mm in diameter.

2.2.2 Case selection

Histomorphometric data from iliac crest biopsies was abstracted from the files of 159 patients (45 male, 114 female: mean age 55 years, range 26 to 75 years) referred over a 5 year period (1990-1995) to either Hope Hospital, Salford, or the Manchester Royal Infirmary, Manchester with established osteoporosis. In each case the diagnosis of established osteoporosis was determined radiologically by the presence of at least one non-traumatic vertebral crush fracture, or a lumbar spine (L2-L4) bone mineral density less than 2.5 standard deviations below peak bone mass (assessed by dual energy absorptiometry). Biopsies with no measurable histomorphometric abnormalities were selected as normal controls. However, since such biopsies were very uncommon it was necessary to include some uni-cortical iliac crest biopsies. Each patient had received two doses of oral dimethyl-chlor-tetracycline (10-15 mg/kg body weight), the first 15-18 days before the biopsy and the second 10 days later. In each case biopsy was performed within four to seven days of the last tetracycline dose.

2.2.3 Biopsy processing and preparation of sections

Upon receipt the biopsies were fixed in absolute alcohol and processed three times in LR white resin monomer (London Resin Co., London), the last two taking place under reduced

pressure. Resin polymerisation was carried out overnight at 60^{O} C. Twenty-seven 5 μm step serial sections were cut through each block with a tungsten tipped knife. Groups of three sections were stained with toluidine blue (pH 4.2), or using the modified Giemsa or Von Kossa techniques. For fluorescence microscopy, 20 μm unstained sections were cut at different levels throughout the block. Routine histomorphometric analysis was carried out manually by an independent observer (Professor A J Freemont, University of Manchester) unconnected with the subsequent analysis and image analysis was carried out semi-automatically using a Magiscan 600 image analyser. Only those biopsies in which an adequate core of bone was present were included. Normal ranges were derived from a group of locally recruited normal control subjects (Rehman *et al.*, 1994).

2.2.4 Histomorphometric analysis

Parameters were measured using standard techniques (Parfitt *et al.,* 1983; Compston *et al.,* 1989) and defined according to the terminology proposed by Parfitt *et al* (1987). For each of the biopsies a full range of dynamic and static parameters (detailed in table 2.1) were measured at the time of initial diagnostic analysis. When tetracycline labelled sections were analysed, the total length of mineralising surface (tLS) was divided into two components - those with two (double labelled surface, dLS) and those with only one label (single labelled surface, sLS). The latter represented regions of the bone surface that had either stopped or started mineralisation between the two doses of label. Osteoid surface (OS) was identified in toluidine blue stained sections and defined as an unmineralised surface at least 3 μm thick.

2.2.4.1 Analysis of differential pattern of osteoblast dysfunction

Counting osteoblasts is often impractical because of their number and indistinct cell boundaries. A surrogate for osteoblast number was assessed by measurement of the ratio osteoblast surface / bone surface (ObS/BS). Two measures of mineralising surface were used. Firstly the proportion of osteoid surface bearing either single or double label (sLS+dLS/OS) was used to assess the percentage of osteoblasts actively mineralising at any one time. Secondly the ratio of double to total labelled surface (dLS/tLS) was used to indicate the degree to which those active at any one time remained active thereafter i.e. the efficiency. If dLS was equal to tLS i.e. dLS /tLS = 100%, then all those active when the first label was applied would also have been active when the second was and could therefore be said to be functioning at a high degree of efficiency.

2.2.4.2 Analysis of the differential pattern of altered bone formation in different bone compartments

Bone formation was measured in each of the four main compartments (figure 2.1). Trabecular and cortical bone formation rates were assessed by measurement of the trabecular and cortical apposition rates, which were calculated by measurement of the distance between the labelled surfaces divided by the time interval between the two label doses. The periosteal and sub-cortical bone formation rates were similarly assessed by measurement of the distance between the labelled surfaces. Each of the four parameters (periosteal, cortical, sub-cortical and trabecular bone apposition rates) were measured in each patient.

2.2.4.3 Analysis of the alteration of the haematopoietic to adipocytic ratio

The ratio of adipocytic to haematopoietic tissue in von Kossa stained sections in each biopsy was measured by semi-automated image analysis using a Quantimet 600S. Since the ratio of haematopoietic to adipose tissue varies normally between para- and intra-cortical sites the ratio was measured in three fields in each biopsy, one adjacent to each cortical surface and one in the middle of the biopsy. The average of these was taken as the ratio for the biopsy. For each of the 3 fields the live image (figure 2.2a) was captured digitally, within which the amount of haematopoietic tissue was measured automatically by selection of tissue above a pre-set grey scale cut-off (figure 2.2b). This represented stained nuclear and cytoplasmic tissue, whilst the optically clear adipocytic tissue was measured by subtraction of the haematopoietic from the total area. The optimum grey scale cut-off was established by carrying out replicate measurements on the same fields in 10 randomly selected biopsies across a range of grey scale cut-offs. The results were plotted and the optimum cut-off selected. The area for measurement was selected with a cursor, omitting trabecular struts and artefacts (figure 2.2c), and the ratio of adipocytic to haematopoietic tissue, as defined by the grey scale cut-off as noted above, measured within it (figure 2.2d); the marrow directly adjacent to the endosteal surface was largely excluded from analysis due to the frequent artefactual separation of the marrow from this surface, with resultant possible error in estimation of true adipocytic area.

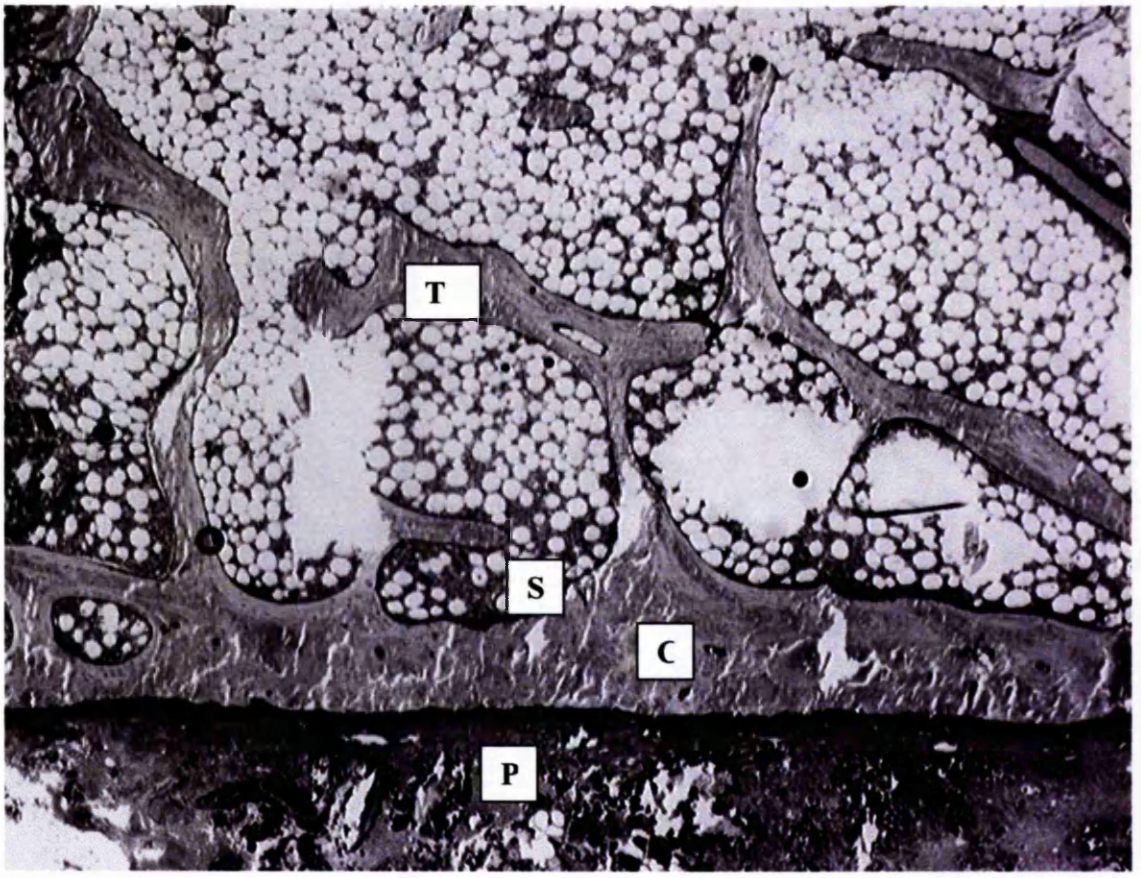


Figure 2.1 Iliac crest biopsy showing location of compartments measured (C-cortical, T-trabecular, P-periosteal, S-sub-cortical); haematoxylin and eosin stain, magnification x100.

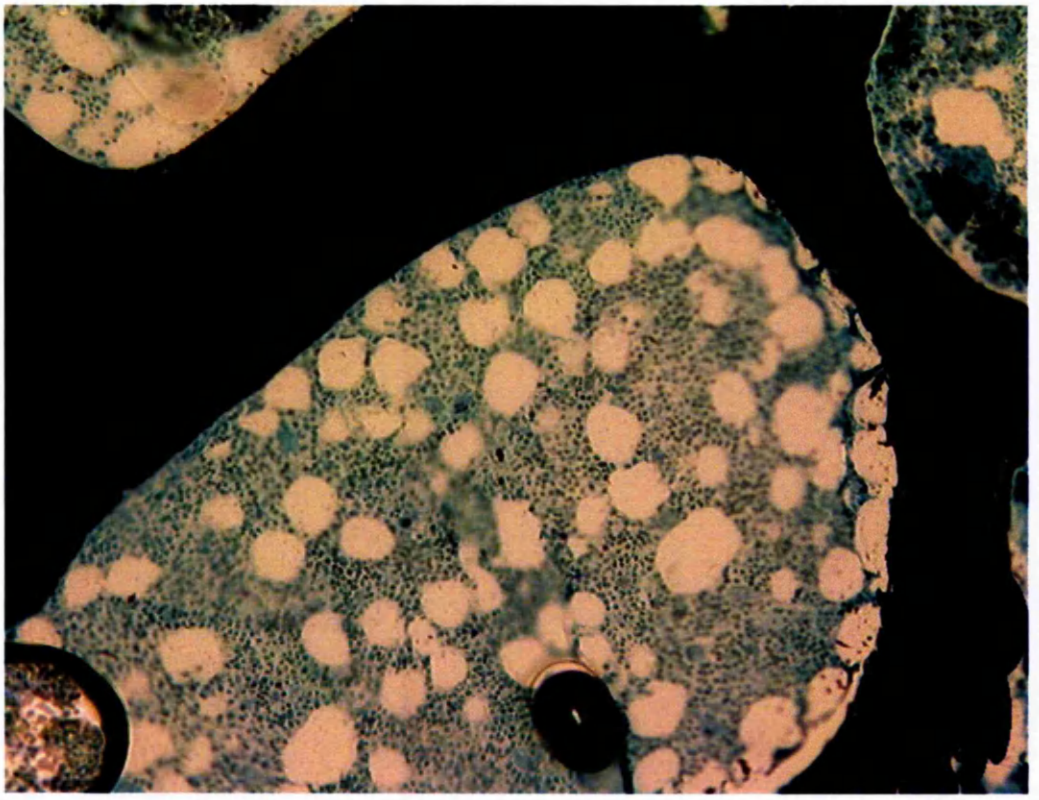


Figure 2.2a. Live image of representative field of bone marrow captured digitally; von Kossa stain, magnification x 40.

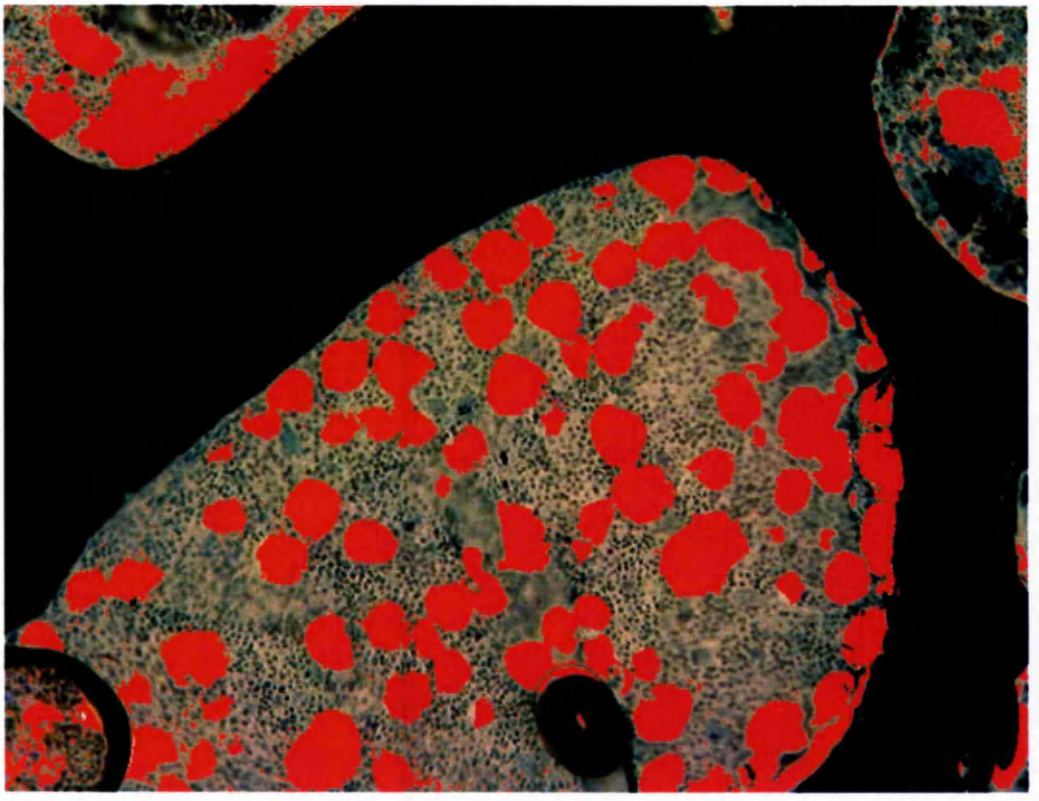


Figure 2.2b. Adipocytic tissue (red) highlighted by semi-automated selection of area above grey scale cut off; von Kossa stain, magnification x 40.

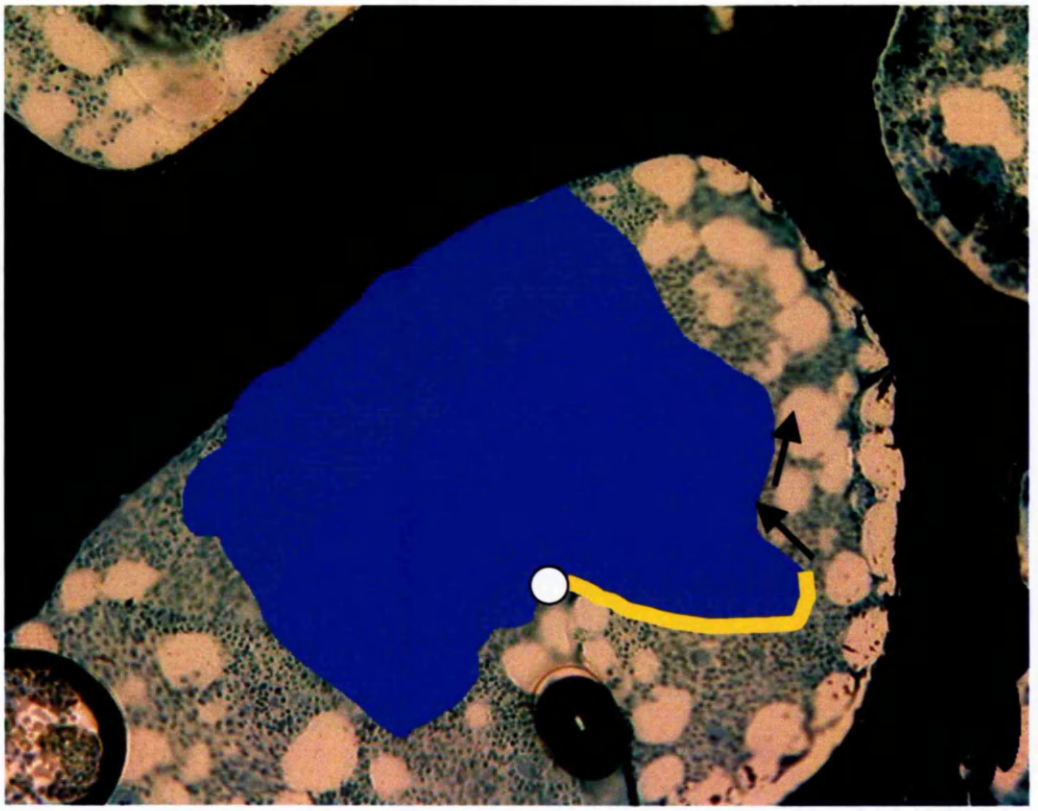


Figure 2.2c. Area for calculation of adipocytic ratio (blue) selected with cursor, omitting trabecular struts and artefacts; von Kossa stain, magnification x 40.

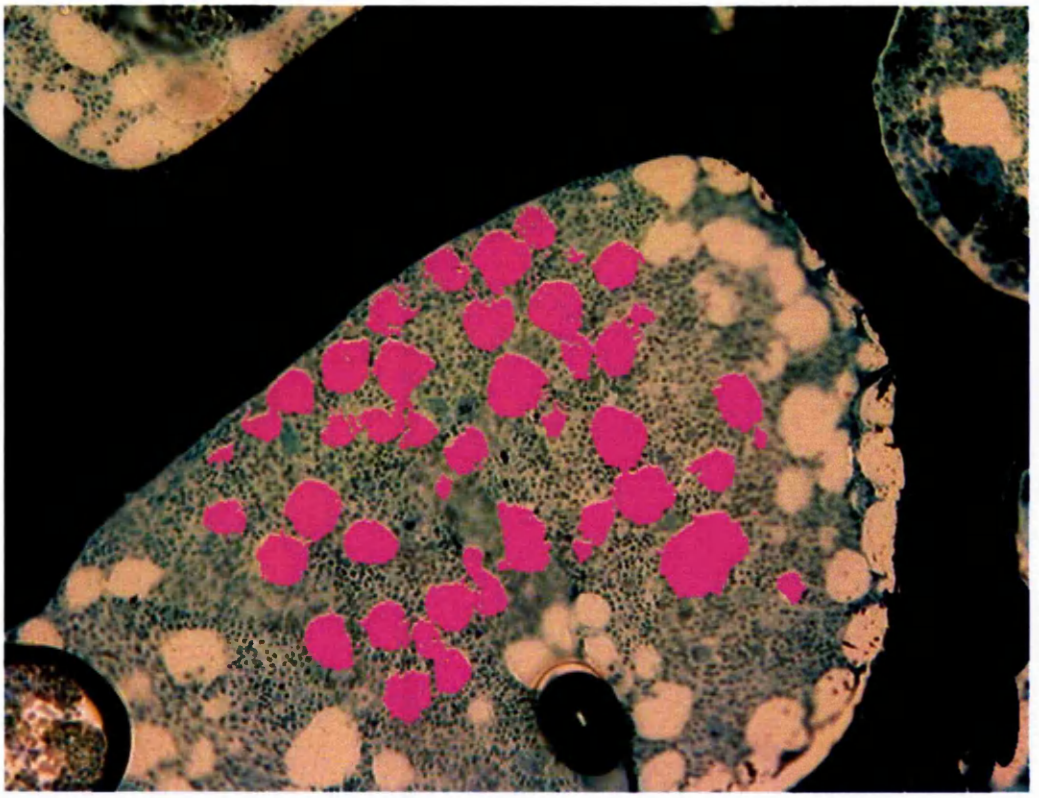


Figure 2.2d. Adipocytic tissue (purple) highlighted by subtraction from grey haematopoietic tissue within area selected and ratio of clear/adipocytic to grey/haematopoietic-stromal tissue calculated; von Kossa stain, magnification x 40.

The investigator's intra-observer error was tested by measurement of the ratio in 6 randomly selected biopsies twice with an interval of 2 weeks, and the inter-observer error by independent measurement of the ratio in 6 randomly selected biopsies by the investigator and two validators (John Denton & John Ratjaratnam, University of Manchester).

2.2.5 Statistical analysis

Z-scores were calculated, according to published guidelines (Rehman *et al.*, 1995), to facilitate analysis of the parameters of osteoblastic function and of bone formation rate in the different compartments. Statistical comparison of the haematopoietic / adipocytic ratios was carried out using a two sample Student's t-test (for parametric data) and standard multiple regression analysis, using Minitab, used to identify the factors contributing to the variation of the sample ratio. Each of the histomorphometric variables that had been recorded at the time of initial diagnostic analysis were used as factors. A stepwise approach was adopted and multiple regression equations, accounting for the variability of the sample ratios, generated.

2.3 Results

2.3.1 Analysis of differential pattern of osteoblast dysfunction

Fully informative biopsies were available for 153 of the 159 cases initially selected. Each of the three parameters in each patient were expressed as raw values and as z-scores. The mean and the spread of the raw values for each of the three parameters are shown in table 2.2, together with the mean and spread of the population means against which the raw values were compared to obtain z-scores.

The population mean and standard deviation varied with age and sex within each parameter and so, whilst the appropriate age/sex matched means were used to produce the z-scores for each parameter in each patient, the mean and spread of the means is shown to give an overall impression of the deviation of the raw data from the norm. Similarly, the spread of the standard deviations of the normal values for each parameter is also given in table 2.2.

The results demonstrate a marked reduction in the mean raw value compared to the mean normal population value for each parameter. The raw values and the corresponding

Table 2.2 - Mean values for both raw data and normal population values for each parameter, together with spread of normal means and standard deviations.

	Osteoblast No. (ObS/BS)	% Active (sLs + dLs/OS)	Efficiency (dLs/tLs)
Mean normal mean value	5.0	71.2	55.1
Mean raw value	3.0	49.9	26.7
Spread of normal mean	4.2 - 6	48.3 - 81	43.3 - 58
Spread of normal SD	0.5 - 1. 05	5.4 - 7.55	3.7 - 5.55

Table 2.3 - Three dimensional matrix of parametrical changes based on Z scores. Each cell is given in the format:- osteoblast number (ObS/BS) : percentage osteoblasts active (sLS + dLS/OS) : efficiency of osteoblasts active (dLS/tLS), each parameter being designated either normal(N), high(H), or low(L). The number of cases with each combination of changes is indicated.

NNN 9 NNH 3 NNL 14	HNN 1 HNH 2 HNL 2	LNN 12 LNH 1 LNL 23
NHN NHH 1 NHL 2	HHN HHH HHL	LHN LHH 1 LHL 1
NLN 2 NLH NLL 14	HLN HLH 1 HLL 1	LLN 3 LLH LLL 60

Figure 2.3 - Graph of raw value (shaded square) and corresponding age/sex matched mean (black diamond) for each patient in parameter: a) osteoblast number (ObS/BS); b) percentage osteoblasts active (sLS + dLS/OS); & c) efficiency of osteoblasts active (dLS/tLS); for each the parameter value is given on the y-axis and the patient on the x-axis

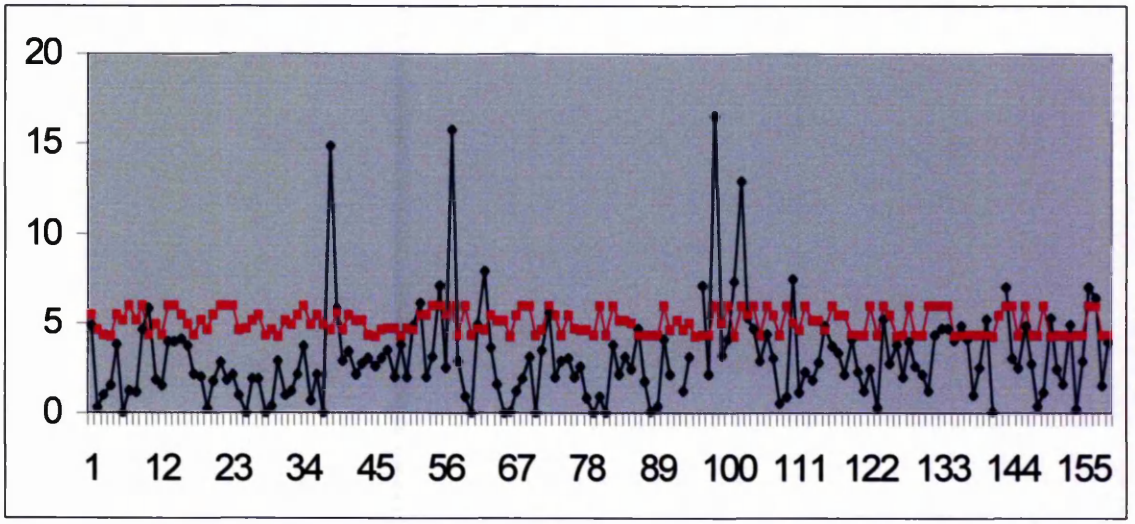


Figure 2.3a

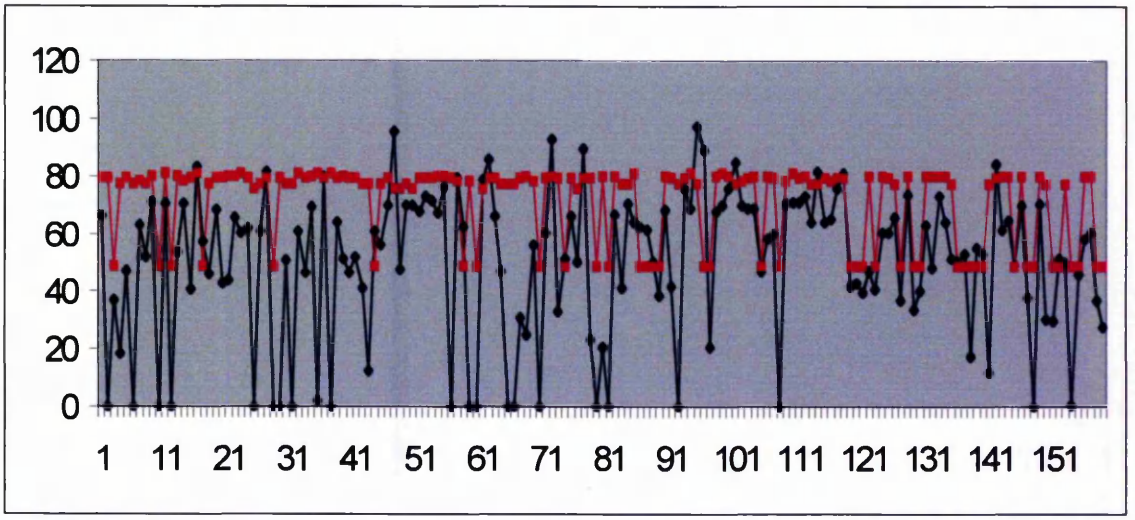


Figure 2.3b

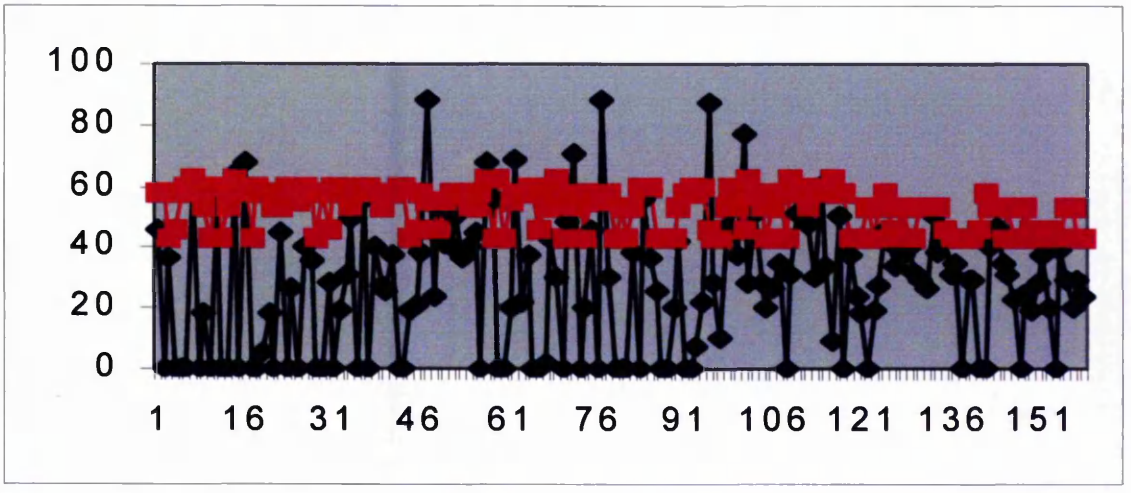
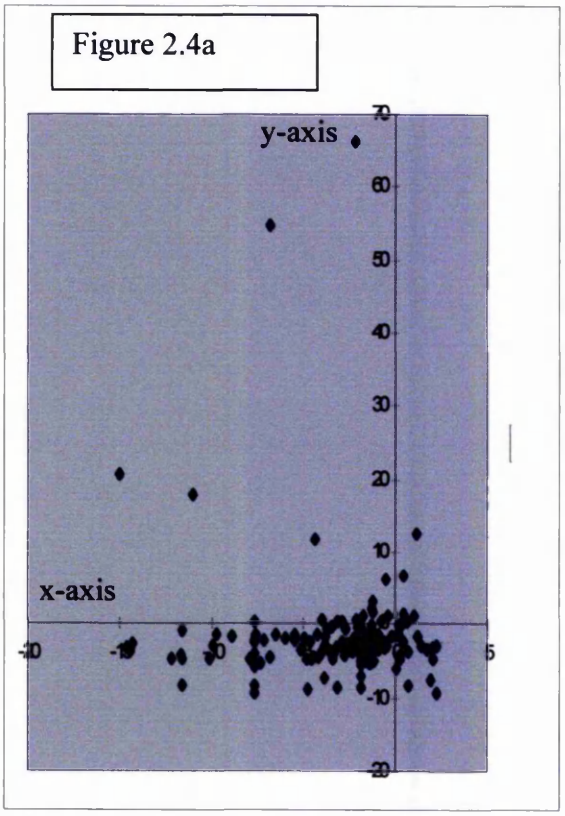
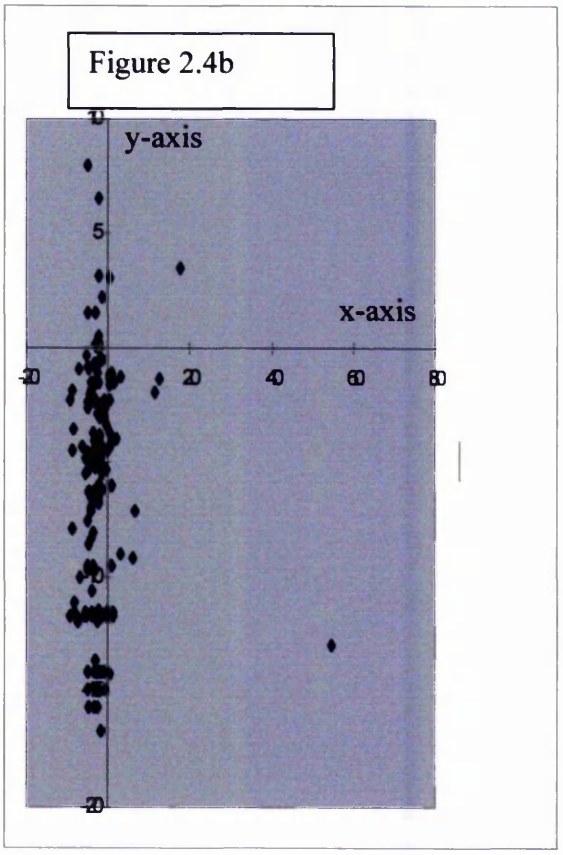


Figure 2.3c

Figure 2.4 - Scatter plots of z-scores of: a) osteoblast number (ObS/BS) vs percentage osteoblasts active (sLS + dLS/OS); b) osteoblast number (ObS/BS) vs efficiency of osteoblasts active (dLS/tLS); and c) percentage osteoblasts active (sLS + dLS/OS) vs efficiency of osteoblasts active (dLS/tLS). For each plot the variable on the x-axis is given before that on the y-axis.





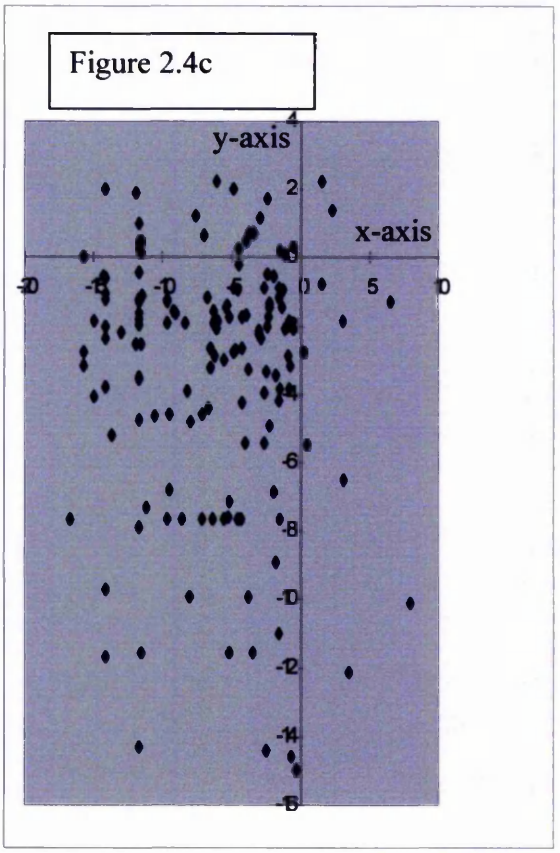


Table 2.4 - Groups of patients with each combination of parametrical change (combinations represented in less than 4 patients (21 cases in total) were not considered large enough for independent analysis)

	No.	Osteoblast No. (ObS/BS)	% Active (sLs + dLs/OS)	Efficiency (dLs/tLs)
Group 1	9	normal	normal	normal
Group 2	14	normal	normal	low
Group 3	14	normal	low	low
Group 4	23	low	normal	low
Group 5	12	low	normal	normal
Group 6	60	low	low	low
Others	21	aggregate of smaller groups and not analysed further		

Table 2.5 Demographic details of patients in the different histomorphometric groups

	No. Males : Females	Mean Age (SD) in years
Group 1	1 : 8	59.5 (10.5)
Group 2	2 : 12	60.1 (8.1)
Group 3	2 : 12	56.1 (7.2)
Group 4	5 : 18	56.5 (8.6)
Group 5	4 : 8	53.4 (11.0)
Group 6	26 : 34	52.7 (12.0)

Table 2.6 Z scores of histomorphometric parameters in the different groups

	Osteoblast No. (ObS/BS)	% Active (sLs + dLs/OS)	Efficiency of Osteoblasts (dLs/tLs)
Group 1	-0.5	-1.0	-0.9
Group 2	-0.8	-0.5	-4.8
Group 3	-0.2	-3.9	-6.1
Group 4	-3.5	-0.9	-5.5
Group 5	-3.7	-1.0	-1.0
Group 6	-4.4	-6.5	-10.4

age/sex matched normal population means, which therefore vary from patient to patient, for the entire data set of each parameter are shown in figure 2.3. In order to analyse the nature of these changes further, z-scores were calculated for each parameter in each patient, in order to allow comparisons to be made between values from patients of differing age and sex. A z-score for an individual value is the number of standard deviations by which the value differs from the mean of normal age and sex matched controls for the local population (Rehman *et al.,* 1994). Z-scores allow the standardisation of data and a z-score of either greater than 2 or -2 was taken as abnormal. Z-scores for each parameter were plotted against each other (figure 2.4). These scatter plots showed no clustering of patients, though there was a wide distribution of values for each parameter. In order to identify subgroups within this distribution each of the three parameters in each patient was designated on the basis of its z-score as either high ($Z>2$), normal ($2>Z>-2$) or low ($Z<-2$). A 3 dimensional matrix was then used to determine the number of patients presenting with any given combination of parametrical change on the basis of these designations (table 2.3), and groups of patients with similar changes identified (table 2.4). Demographic details for each group are given in table 2.5 and the mean z-scores for each of these groups are shown in table 2.6.

Group 1 comprised 9 patients with normal osteoblast numbers, percentage activity, and efficiency, and were osteoporotic due to excess osteoclastic activity in 6 cases, whilst the remaining three showed a reduction in osteoblast number which did not fall below 2 standard deviations from the population mean. Group 2 contained 14 patients who showed a reduction in efficiency of osteoblast activity, but a normal number of osteoblasts and percentage active. Group 3 also contained 14 patients, who had normal osteoblast numbers, but showed reduction in both the percentage and efficiency of osteoblasts active. Group 4 held 23 patients and showed a normal percentage of osteoblasts active, but a reduction in absolute osteoblast number and in the efficiency of those active. Group 5 contained 12 patients who had low numbers of osteoblasts, but a normal percentage and efficiency of osteoblast activity. Group 6 contained 60 patients who showed a reduction in all three parameters. In addition 21 patients showed a variety of other combinations of parametrical change, but none of these smaller groupings contained more than 3 patients and they were not considered large enough for meaningful conclusions to be drawn from them. Though differences in the proportions of male and female cases, and mean ages of cases were present between groups these were not statistically significant.

2.3.2 Analysis of the differential pattern of altered bone formation in different bone compartments

Fully informative biopsies were available for 157 (113 female, 44 male) of the 159 cases initially selected. The patients ranged in age from 26 to 75 years, with a mean age of 54.9 years (SD 11.3 years). Each of the four parameters in each patient were expressed as raw values and as z-scores. The mean and the spread of the raw values for each of the four parameters are shown in table 2.7, together with the mean and spread of the population means against which the raw values were compared to obtain z-scores. The population mean and standard deviation varied with age and sex for the trabecular and cortical apposition rates and so, whilst the appropriate age/sex matched means were used to produce the z-scores for each parameter in each patient, the mean and spread of the means is shown to give an overall impression of the deviation of the raw data from the norm. Similarly, the spread of the standard deviations of the normal values for each parameter is given in table 2.7. The population mean and standard deviation were calculated across all ages for the periosteal and sub-cortical apposition rates.

The results demonstrate a reduction in the mean raw value compared to the mean normal population value for each parameter (table 2.7). The mean reduction was greatest in the cortical and trabecular apposition rates (4.4 and 4.1 standard deviations below the mean respectively), just over 2 standard deviations below the mean for the sub-cortical apposition rate (2.3), and less than 1 SD below the mean for periosteal formation (0.9) (table 2.8). The raw values and the corresponding age/sex matched normal population means, which therefore vary from patient to patient, for the entire data set of each parameter are shown in figure 2.5. In order to analyse the nature of these changes further, z-scores were calculated for each parameter in each patient, to allow comparisons to be made between values from patients of differing age and sex (table 2.8). A z-score of either greater than 2 or -2 was taken as abnormal. On this basis, the trabecular apposition rate was normal in 26 cases (16.5%), increased in 3 (2.0%), and reduced in 128 cases (81.5%), whilst the cortical apposition rate was normal in 30 cases (19.1%), increased in 4 (2.6%), and reduced in 123 cases (78.3%). Conversely, the periosteal apposition rate was normal in 76 cases (48.4%), increased in 13 (8.3%), and reduced in just 68 cases (43.3%), whilst the sub-cortical apposition rate was normal in 77 cases (49%), increased in 11 (7%), and reduced in just 69 cases (44%).

Z-scores for each parameter were plotted against each other (figure 2.6). These scatter plots showed no clustering of patients, and, except between the periosteal and subcortical

Table 2.7 - Mean values for both raw data and normal population values for bone formation in each compartment, together with spread of normal means and standard deviations.

	Trabecular	Cortical	Periosteal	Sub-cortical
Mean normal mean value	0.59	0.72	1.9	3.7
Mean raw value	0.20	0.27	1.25	2.68
Spread of normal mean	0.4 - 0.69	0.6 – 0.79	NA	NA
Spread of normal SD	0.05 – 0.17	0.05 – 0.215	0.75	0.45

Table 2.8 - Mean Z scores for bone formation in each compartment

	Trabecular	Cortical	Periosteal	Sub-cortical
Mean Z score	-4.10	-4.43	-0.87	-2.28
SD of Z scores	3.19	3.66	1.79	3.87

Table 2.9 Correlation coefficients between Z scores of bone formation rates in each compartment

	Trabecular	Cortical	Periosteal
Trabecular			
Cortical	0.302		
Periosteal	0.267	0.177	
Sub-Cortical	0.283	0.231	0.588

Figure 2.5 - Graph of raw value (black diamond) and corresponding age/sex matched mean (shaded square) of bone formation for each patient in each compartment: a) trabecular; b) cortical; c) periosteal; and d) sub-cortical. For each plot the value is plotted on the y-axis for each individual patient, on the x-axis.

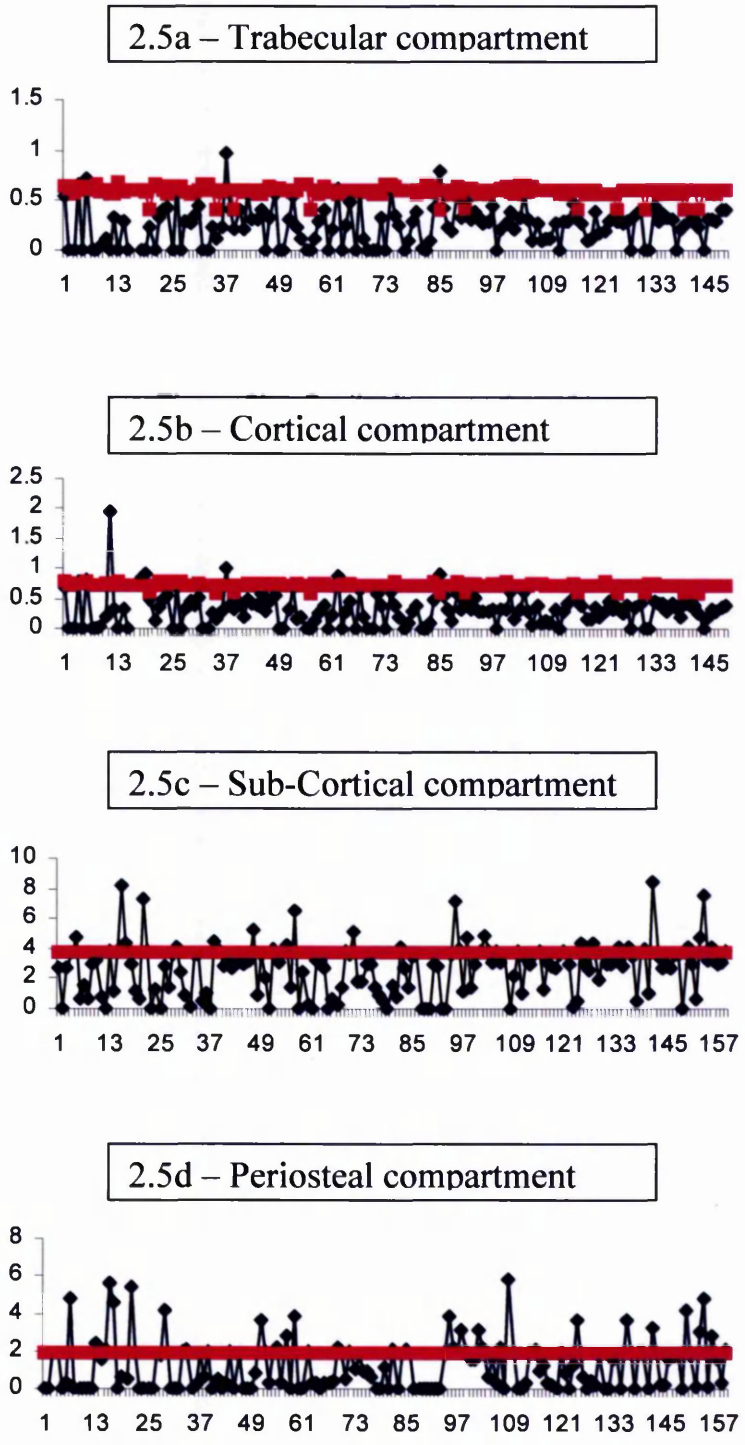


Figure 2.6 - Scatter plots of z-scores of bone formation in: a) trabecular vs cortical compartments; b) trabecular vs periosteal compartments; c) trabecular vs sub-cortical compartments; d) cortical vs periosteal compartments; e) cortical vs sub-cortical compartments; and f) periosteal vs sub-cortical compartments

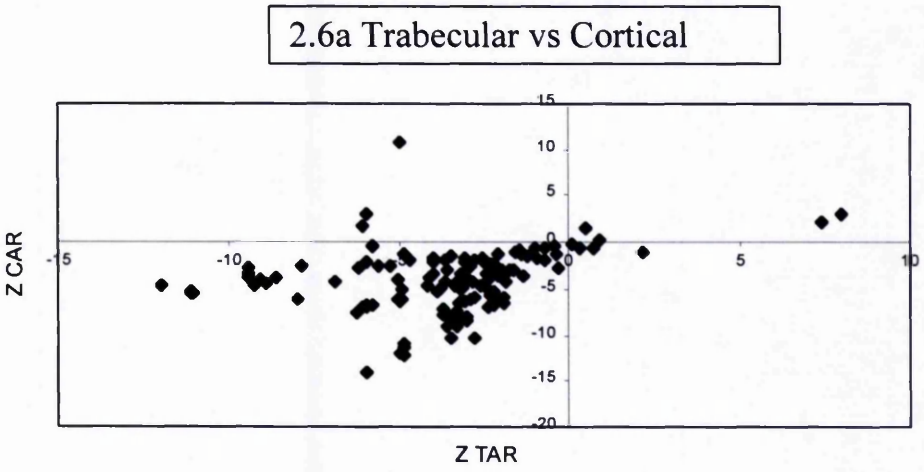


Figure 3b - Z PAR vs Z TAR

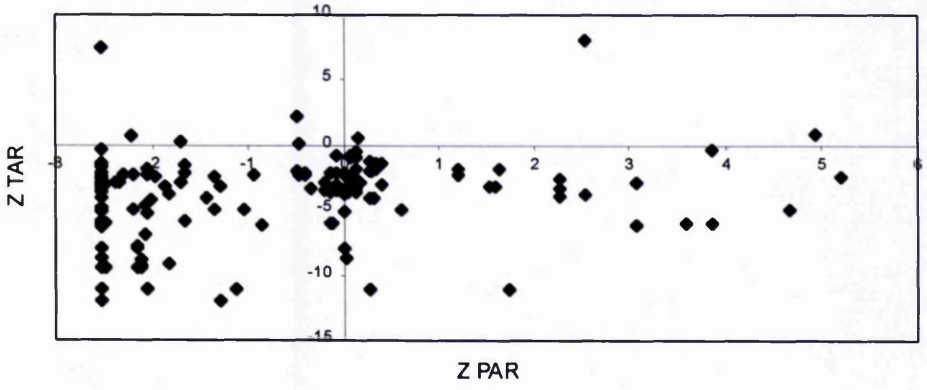
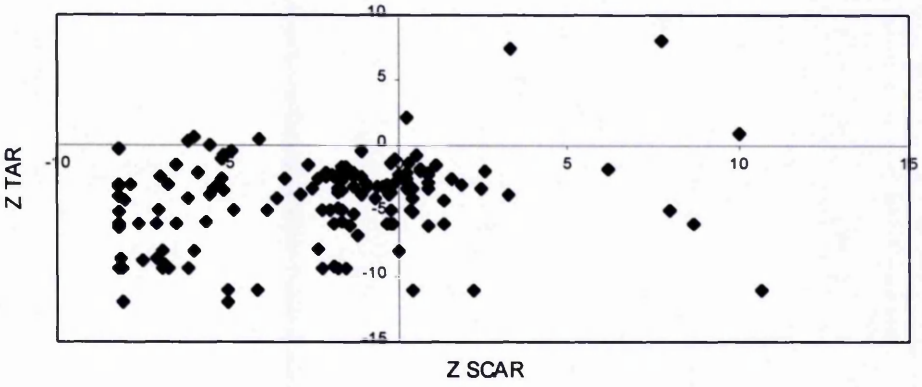
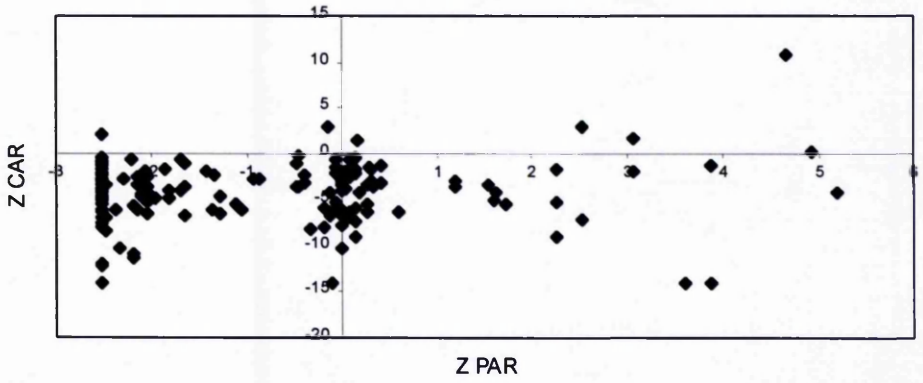


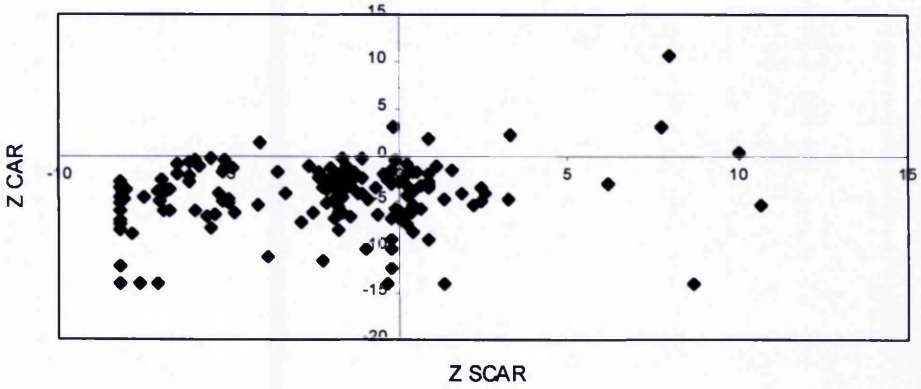
Figure 3c - Z SCAR vs Z TAR



2.6d Cortical vs Periosteal



2.6e Cortical vs Sub-cortical



2.6f Periosteal vs Sub-cortical

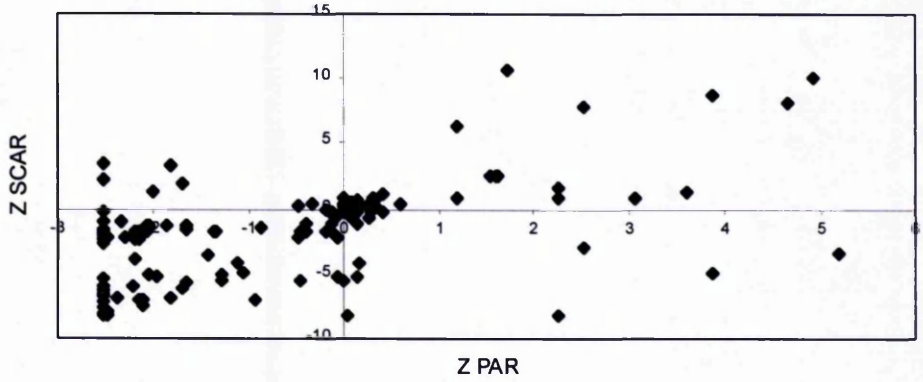


Table 2.10 - Matrix of parametrical changes based on Z scores of bone formation rates in each compartment. Each cell is given in the format:- Periosteal, Sub-cortical, Trabecular, and Cortical compartments, each parameter being designated either normal(N), high(H), or low(L). The number of cases with each combination of changes is indicated.

NNNN 6 NNNH NNNL 3 NNHN 1 NNHH NNHL NNLN 3 NNLH NNLL 37	NHNN 1 NHNH NHNL 2 NHHN NHHH NHHL NHLN NHLH 1 NHLL 3	NLNN 5 NLNH NLNL NLHN NLHH NLHL NLLN 2 NLLH NLLL 12
HNNN HNNH HNNL HNHN HNHH HNHL HNLN 3 HNLH HNLL 2	HHNN 1 HHNH HHNL HHHN HHHH 1 HHHL HHLN HHLH 1 HHLL 1	HLNN 1 HLNH HLNL HLHN HLHH HLHL HLLN HLLH HLLL 3
LNNN LNNH LNNL 2 LNHN LNHH LNHL LNLN 2 LNLH LNLL 17	LHNN LHNH LHNL LHHN LHHH 1 LHHL LHLN LHLH LHLL 1	LLNN 3 LLNH LLNL 1 LLHN LLHH LLHL LLLN 2 LLLH LLLL 39

Table 2.11 Demographic details of patients in the different histomorphometric groups

	No. Males : Females	Mean Age (range / SD) in years
Group 1	17: 22	56.1 (29 – 74 / 11.6)
Group 2	2: 35	56.2 (26 – 75 / 10.1)
Group 3	4: 13	55.8 (42 – 72 / 8.0)
Group 4	3: 9	52.2 (32 – 69 / 11.5)
Group 5	2: 4	57 (48 – 72 / 8.9)
Group 6	2: 3	44 (27 – 53 / 10.8)

Table 2.12 Mean Z scores of histomorphometric parameters in the different groups

	Trabecular	Cortical	Periosteal	Sub-cortical
Group 1	-6.28	-6.29	-2.45	-6.23
Group 2	-3.54	-5.09	-0.15	-0.18
Group 3	-4.94	-4.77	-2.36	-1.42
Group 4	-5.63	-5.10	-0.97	-5.18
Group 5	-1.06	-1.10	-0.12	-0.23
Group 6	-0.14	-0.34	-0.39	-5.23

appositional rates, poor correlation between the parameters (table 2.9). However, in view of the clear difference in the proportion of cases showing reduction in either trabecular / cortical, or sub-cortical / periosteal apposition rates an attempt to identify subgroups within the data set was made. Each of the three parameters in each patient was designated on the basis of its z-score as either high (Z>2), normal (2>Z>-2) or low (Z<-2), as explained above, and a four dimensional matrix used to determine the number of patients presenting with any given combination of parametrical change (table 2.10). Six groups of patients with similar changes were identified; only those parametric combinations represented in more than 3 cases were judged sufficiently frequent to be considered as groups. Demographic details for each group are given in table 2.11 and the mean z-scores for each of these groups are shown in table 2.12.

Group1 was the largest and contained 39 patients with a reduction in all four parameters. Group 2 contained 37 patients with reduced cortical and trabecular formation, but normal periosteal and sub-cortical formation. Group 3 contained 17 patients with reduced formation in the periosteal, cortical and trabecular compartments, and normal sub-cortical bone formation. Group 4 contained 12 patients with reduced formation in the sub-cortical, cortical and trabecular compartments, and normal perisosteal bone formation. Bone formation was reduced, but within normal limits in group 5, comprising 6 patients, whilst the smallest group (5 patients) showed reduced bone formation in the sub-cortical compartment only. The mean age was similar in all of the groups (52.2 to 57 years) except group 6 (44 years) and was also similar to the overall mean age (54.9 years).

The designation of parametrical change (normal i.e. none, increased or reduced) was equal in the trabecular and cortical compartments in 134 cases (85.4%). Out of these 134 cases the parametric designation in both the periosteal and sub-cortical compartments was the same as that of the trabecular and cortical compartments in 47 cases (35.1%), but different in either the periosteal or sub-cortical compartment in 87 cases (64.9%). Out of these latter 87 cases the change in the periosteal and sub-cortical compartments was the same in 41 cases (47.1%). Overall the designation of change was the same in the periosteal and sub-cortical compartments in 88 cases (65.7%).

2.3.3 Analysis of the alteration of the haematopoietic to adipocytic ratio

Suitable bicortical iliac crest biopsies were available from 132 osteoporotic patients (44 male, 88 female) ranging in age from 5 to 80 years, with a mean age of 55 years. The

control group consisted of 9 cases (5 female, 4 male) ranging in age from 21 to 70 years (mean age 55 years).The greatest rate of change in adipocytic area occurred for grey scale cut offs between 200 to 210 (arbitary units of light intensity), indicating that discrimination between clear and stained tissue was most sensitive, and therefore informative, in this range. As a result of this finding the grey scale cut off was set at 205 for all calculations of adipocytic and haematopoietic area.

The overall ratio of adipocytic to haematopoietic tissue in the cases with osteoporosis was significantly greater than in the control group (osteoporotic mean 43.06% vs normal mean 22.4%; $p<0.001$). The difference in ratios between male and female osteoporotic cases was small (male mean 44.42%, std.dev.=12.68 vs female mean 42.29%, std.dev.=13.02). The inter-observer correlation between the values generated by the principal investigator and those of the two validators were 0.90, for validator 1, and 0.78, for validator 2; the principal investigators mean adipocytic / haemaotopoietic ratio for the test cases was 44.63%, whilst those for the validators were 44.87% and 43.15%. The intra-observer correlation between the mean ratios generated by the principal investigator form the same cases on two separate occasions (44.63% and 47.42%) was 0.99.

Multiple regression on the histomorphometry data for the patients showed the following equations that account for the variability of the data set. Each histomorphometric variable was regressed individually with the sample adipocytic / haematopoietic ratios, and the p-values and unadjusted R^2 values tabulated. From table 2.13, according to statistical convention, the variables that had p-values less than 0.05, i.e. less than 5 % chance that it does not contribute to the overall variation of the data set, were selected (table 2.13, *shaded*) and included in linear multiple regression analysis. The following table of p-values was obtained (table 2.14); the regression equation is included for completeness. Again, by convention, the variables with p-values less than 0.05 were retained (table 2.14, *shaded*), resulting in the following regression equation:

Ratio = 77.6 + (1.01 x Age) - (0.396 x Wall Thickness) - (1.02 x Osteoid Volume) - (0.113 x Trabecular Thickness) - (0.00401 x Trabecular Separation) - (0.217 x Cortical Wall Thickness) + (10.8 x Trabecular Apposition Rate) + (0.102 x Bone Formation Rate)

Table 2.13 - Significance of all variables in Multiple Regression Analysis. Variables with a p-value less than 0.05, i.e. those with a less than 5% of association with the variation of the haematopoietic / adipocytic ratio by chance alone, were used in a further round of regression analysis. The R^2 value for each of the variables reflected the degree to which change in each variable was associated with the change in the ratio.

Variable	p-value	R^2 value
Age	*0.002*	6.8
Trabecular Bone Volume	*0.000*	15.1
Wall Thickness	*0.000*	18.4
Osteoid Surface	0.148	1.6
Osteoid Volume	*0.002*	7.0
Osteoid Thickness	*0.002*	7.5
Osteoblast Surface	*0.042*	3.2
Mineralising Surface 1	0.119	1.9
Mineralising Surface 2	0.250	1.0
Mineralising Surface	0.051	2.9
Eroded Surface	0.438	0.5
Osteoclast Surface	0.731	0.1
Osteoclast Number	0.596	0.2
Trabecular Thickness	*0.000*	15.6
Trabecular Number	0.086	2.2
Trabecular Separation	*0.015*	4.5
Cortical Thickness	*0.002*	7.0
Cortical Volume	0.114	1.9
Cortical Wall Thickness	*0.000*	14.4
Osteoclast Number	0.836	0.0
Sub-cortical Osteoclasts	0.904	0.0
Trabecular Apposition Rate	*0.004*	6.3
Cortical Apposition Rate	0.518	0.3
Bone Formation Rate	*0.002*	6.9

Table 2.14 - Variables that only contribute significantly to the regression equation (shaded); only those variables with a p-value less than 0.05 were statistically significantly associated with change in the haematopoietic / adipocytic ratio.

Variable	p-value
Age	0.000
Trabecular Bone Volume	0.137
Wall Thickness	0.000
Osteoid Volume	0.000
Osteoid Thickness	0.476
Osteoblast Surface	0.385
Trabecular Thickness	0.000
Trabecular Separation	0.000
Cortical Thickness	0.059
Cortical Wall Thickness	0.000
Trabecular Apposition Rate	0.000
Bone Formation Rate	0.000

An R^2 value of 98.5 % was obtained with all the variables having p-values of less than 0.001, indicating that 98.5% of the variability of the adipocytic / haematopoietic ratio was associated, within the set of parameters initially used, with these factors, of which the greatest contribution to the variability of the ratio was made by the trabecular apposition rate, followed in descending order by osteoid volume, age, wall thickness, cortical wall thickness, trabecular thickness, bone formation rate and trabecular separation.

2.4 Discussion

The results for each of the three analyses performed are discussed below separately, after which a concluding section is appended.

2.4.1 Analysis of differential pattern of osteoblast dysfunction

Osteoporosis is a clinical syndrome characterised by the end-point of osteopenia and resultant low trauma fractures (Peel & Eastell, 1995). It encompasses a widely heterogeneous population and previous studies have indicated that a better understanding of the mechanisms underlying osteoporosis and a firmer basis for therapeutic intervention will be achieved by sub-classification to produce more homogeneous patient groups (Steiniche, 1995). Most such attempts have utilised clinical parameters, but recently a histomorphometric approach has been used which demonstrates different groups with altered osteoblast and osteoclast function (Rehman *et al.,* 1995). This emphasises the need to know and target the cellular mechanisms leading to reduction in bone mass (Ericksen *et al.,* 1990; Manolagas *et al.,* 1995; Garabedian, 1995; Ziegler *et al.,* 1995) and directs attention to the different patterns of cell dysfunction that can occur in the disease. Demonstrating reduced osteoblast activity, and/or number, would therefore be of considerable pertinence, especially in view of the present intense work into the processes modulating osteoblast proliferation and activation (Bruder *et al.,* 1994; Lian & Stein, 1994; Marie, 1994; Aubin *et al.,* 1995; Rickard *et al.,* 1996; Beresford 1989). Histomorphometric data was analysed from 153 patients with established osteoporosis in order to investigate and characterise the existence of different patterns of osteoblast dysfunction.

A small number of patients (group 1) showed either no significant change in any of the osteoblast parameters, or an increase in osteoclastic activity. The largest single group (group 6) showed a decrease in all 3 parameters, i.e. reduced osteoblast number, percentage active and the efficiency of active cells indicating poor recruitment of osteoblasts from the precursor pool and poor activity once recruited. The majority of

patients however belonged to neither group but showed various deficiencies in recruitment and/or activity. Both osteoblast number and function were reduced in 83 patients. These could be subdivided into 2 subgroups (groups 4 and 6), the majority (group 6) showing reduction in both functional measures (percentage activity, and efficiency of activity), whilst those in group 4 showed a fall in efficiency only, a fall in the efficiency of active cells occurring more frequently than a fall in the percentage of cells active. This suggests that the more common alteration in function is a relative one, reflected in a fall in the level of efficiency of active osteoblasts, rather than an absolute one of a reduction in the number of osteoblasts active. By contrast, there were no large subgroups with normal efficiency but reduced percentage of osteoblasts active. This emphasises that a relative reduction in osteoblast activity is more common than an absolute one and suggests a sequential change with an initial relative reduction in level of activity which eventually leads to total lack of function, and therefore to a reduction in the percentage active. In support of this reduced efficiency was observed in a total of 101 cases and was the most frequent abnormality detected, and though the number of cases showing reduced osteoblast number was similar (95 cases), the group showing reduction in osteoblast number only was the smallest (12 cases), further indicating that reduced activity is more frequent than absolute number. The mechanisms involved in control of osteoblast number and function do seem to be separate in some patients, with groups 2 and 3 showing normal number of osteoblasts but reduction in one or both of the functional parameters. Similarly, group 5 showed a reduction in osteoblast number alone. In 21 patients the groupings involved very small numbers of patients (up to 3), and though this reflects further the heterogeneous nature of osteoporosis and therefore supports the approach used, these groups were omitted from further analysis since whilst they may represent less frequent patterns of dysfunction, the possibility that they represent outliers cannot be excluded.

In further interpretation of the above results however the following caveats must be noted. Firstly, the osteoblast number was assessed using the ratio of osteoblast surface to bone surface. Whilst this is an acceptable proxy it relies on similarity of osteoblast size across cases, and care must therefore be taken in extending the results directly to consideration of actual numbers, rather than proportionate quantities, of osteoblasts.

Previous studies have used cluster analysis to identify discrete groups (Rehman *et al.*, 1995). Although broad groups were identified scatter plots of the data showed that no formal clusters, representing discrete entities, were present, but rather that the data was normally distributed over a wide area. However, though discrete clusters or entities do not

exist in a data set there is still a clinical and categorical utility in splitting or subdividing the data using set limits, such as the upper and lower limits of the normal distribution, as in this case. This allows data sets, which necessarily lie along a spectrum, such as those for many diseases, to be subdivided and simplified in a meaningful manner. Histomorphometric data was analysed from a large cohort of osteoporotic patients to determine the nature of changes in osteoblastic function and has demonstrated that there are different patterns of osteoblastic function. In particular the results suggest that the number and function of osteoblasts in osteoporosis may be altered in different ways although in every case the end point is a reduction in bone mass (Manolagas, 1998). Our data could be interpreted as showing that a reduction in cellular function is initially due to a reduction in level of activity, which eventually leads to cessation of activity and consequent exit from the active pool. These results emphasise that at the time of presentation the cellular dysfunction is heterogeneous (Rehman *et al.,* 1995). To reverse the dysfunction to improve bone mass necessitates recognising the specific defect in a given patient at the time of presentation and emphasises the utility of histomorphometry in this analytical process. In addition, they indicate the nature of the possible cellular mechanisms operating, and demonstrate that more detailed understanding of the cellular pathology of osteoblasts in osteoporosis will allow more focused and effective treatment. In particular our data indicate that a significant proportion of patients have decreased numbers of osteoblasts. To target this defect would require a greater understanding of the site and differentiation pathways of pre-osteoblasts than is currently available (Aubin *et al.,* 1995).

2.4.2 Analysis of the differential pattern of altered bone formation in different bone compartments

The results confirm the high frequency of reduced bone formation in the trabecular compartment in osteoporosis (81.5%), but also indicate a similarly high frequency of reduced bone formation in the cortical compartment (78.3%). The correlation between these two parameters was low (0.3), though when the cases with a similar designation of change were analysed according to their z-score the level of bone was equal in the two compartments in 85.4% of cases. Likewise, the distribution of level of bone formation was similar in the periosteal and sub-cortical compartments, with low formation in 48.4% and 49%, and normal formation in 43.3% and 44% of respectively. Additionally, the designation of change in the periosteal and sub-cortical compartments was the same in

65.7% of cases. Notably, the designation of change was different in the periosteal / sub-cortical compartments from that in the cortical / trabecular compartments in 64.9% of the cases in which the level of formation in the latter were equal, and the same in just 35.1%. Furthermore, of the cases in which the level of formation differed between the two sets of compartments (cortical / trabecular, periosteal / sub-cortical), the designation of the level of bone formation was the same in the periosteal and sub-cortical compartments in nearly half (47.1%).

These results show grouping of the four compartments into two separate sets, cortical / trabecular and periosteal / sub-cortical, with different patterns of altered bone formation in osteoporosis. Bone formation was reduced more frequently in the former group than in the latter (78.3% / 81.5% vs 43.3% / 44%). The degree of this reduction was similar in the cortical and trabecular compartments (mean z –scores of -4.43 and -4.1 respectively), though the degree of reduced bone formation was greater in the sub-cortical than the periosteal compartment (mean z –scores of –2.28 and –0.87 respectively). This was reflected in the wider range of z-scores for the sub-cortical compartment, as well as a lower minimum z-score (-8.22 vs –2.53). Conversely, both the range and maximum and minimum z-scores were similar in the cortical and trabecular compartments. However, notwithstanding this difference in the degree of reduced bone formation between the periosteal and sub-cortical compartments, they demonstrate close linkage of level of bone formation, whilst the correlation between their z-score was also higher than for any other combination (0.59). This indicates close linkage of the level of bone formation in the periosteal and sub-cortical compartments, which are often spared from the reduction in bone formation common in the cortical and trabecular compartments, between which there also appears to be linkage of bone formation. This suggests that fundamentally different processes are involved in normal control, and consequent perturbation by osteoporosis, of bone formation in the two sets of compartments. The nature of such differences is speculative. Possible candidates from animal and human studies include differences in the response to hormones within different compartments (Jee & Ma, 1997; Benz *et al.,* 1991), and the different modulation of strain that occurs through structures as oppose to at their surfaces (Mosley *et al.,* 1997). Obviously, in order to reduce the effects of osteoporosis on bone strength it will be most important to concentrate efforts on augmentation of cortical and trabecular bone formation, should different therapeutic modalities be shown to be more or less specific for one or other of the compartments studied. It is interesting that the compartments contributing most to bone strength were also those most frequently affected.

No formal clusters were identified after initial study of the scatter plots, which showed that the data were normally distributed over a wide area. However, as has previously been demonstrated, even when discrete clusters do not exist in a data set there is still a clinical and categorical utility in splitting or subdividing the data using set limits, as in this case. Using z-scores of –2 or +2 to divide the data set into low, normal or high bone formation, and grouping those with a particular pattern of parametrical change in the four compartments, six groups were identified. The largest group showed reduced formation in all four compartments, though the number showing reduction in the trabecular and cortical compartments only was nearly as large. These two groups accounted for 24.8% and 23.6% of the cases respectively, constituting the majority of cases of osteoporosis. This provides an explanation for the observation that the periosteal and sub-cortical compartments are often affected differently to the trabecular and cortical compartments, but also indicates that there are large numbers of patients in whom the periosteal and sub-cortical compartments are normal. These patients may have less severly deranged biochemical indices and may have a more impressive improvement in their biochemical indices of bone formation, due to the contribution made by the these two compartments, but without corresponding increase in overall bone density and resultant mechanical strength due to reduction in the cortical and trabecular compartments. Additionally, they indicate the presence of a profound biological difference in the response of these compartments to the variety of disease factors responsible for osteoporosis, as well as further emphasising the clinical heterogeneity of the condition. That the distinction between the two groups is not artificial is demonstrated by the large difference in the mean z-scores for the periosteal and sub-cortical compartments between them (-2.45 vs –0.15 and –6.23 vs –0.18 respectively). The two groups had a similar age profile, but females were much more common in the latter group, suggesting that the latter possibility is true, though more precise determination of the cause or causes will require further work.

Both groups three and four showed reduced cortical and trabecular bone formation, with similar mean z-scores for both of these compartments, together with reduction in one or other of the periosteal or sub-cortical compartments. Group three had reduced periosteal bone formation, whilst in group four sub-cortical formation was reduced. The reason for this difference between the groups is unclear, and it is unlikely to be artefactual in group four, since the z-scores for the periosteal and sub-cortical compartments in both groups lay far from the –2 cutoff, though the mean scores were closer to the cutoff in group three, and the possibility of artefact cannot be excluded for this group. The low mean z-score for the sub-cortical compartment in group four is mirrored in group six, in which there is reduced

formation in the sub-cortical compartment alone. Furthermore the reduction in this compartment is of similar magnitude in the two groups. The number of cases with reduced sub-cortical and normal periosteal formation (17 in groups four and six) is equal to that for the converse, and there is no evidence for a biological preference for alteration in either of these two compartments. Interestingly, group six was the youngest and the only one with more males than females, but the low numbers involved reduce the value of these particular observations. Group five showed normal bone formation in all four compartments; osteoporosis was diagnosed clinically in each of these, but though bone formation was reduced in each case, the degree of reduction (between 1 to 2 standard deviations below the mean) was insufficient for designation as 'low' using a z-score of –2 as the cutoff.

The results demonstrate close linkage of the level of bone formation in the periosteal and sub-cortical compartments, which are often spared from the reduction in bone formation common in the cortical and trabecular compartments, between which there also appears to be linkage of bone formation. The data indicate that different processes are likely to be involved in normal control, and consequent perturbation by osteoporosis, of bone formation in the two sets of compartments. The nature of these differences remains speculative, but the results emphasise the importance of defining more precisely the different microenvironments in bone. Such studies will not only further understanding of normal bone formation, but will allow elucidation of the reasons for differential involvement by osteoporosis, with possible consequences for therapy.

2.4.3 Analysis of the alteration of the haematopoietic to adipocytic ratio

Previous studies have shown a correlation between the increase of adipose tissue and the decrease in cancellous bone in bone marrow (Meunier *et al.,* 1971), and in some this has been linked to a decrease in bone formation (Hirano & Iwasaki, 1992). However, these studies have largely been conducted using postmenopausal samples (Hirano & Iwasaki, 1992), and may therefore be confounded by the effect of age on the ratio of adipose to haematopoeitic tissue. The cellularity of bone marrow varies from between 60 to 90% in the first decade to between 10 to 50% in the seventh decade (Hartsock *et al.,* 1965). This has been shown by point counting methods (Kerndrup *et al.,* 1980) as well as by more sophisticated image analysis methods, which also showed that the cellularity in individual biopsies varied from field to field. This explains the not infrequent clinical finding of discordance between the cellularity reported in bone marrow aspirate and trephine samples

taken at the same time. In order to overcome this the adipocytic / haematopoeitic ratio was measured in three fields in each patient. In doing so the assumption was made that adipocytes were of similar size across cases. Though counting the actual numbers of adipocytes would be a more precise measure adipocytic area, as used by the workers note above, is an acceptable proxy.

The present study was conducted over a wide age range and importantly the control group, albeit small, was composed of patients with a similar range of ages, and the same mean age, overcoming the limitations of previous studies. The increase in adipocytic tissue found by others was confirmed, with consequent parallel decrease in cellularity. When analysed on a longitudinal basis this was related to a minor degree to age, but, since the control group was matched for age the increase was present independent of age when all the data from the test and control groups was used. This reflects the fact that within the group as a whole there remained the effect of age present in the normal population, but that over and above this the amount of adipose tissue was greater in osteoporotic patients than controls. However, the only factor contributing more to the variability in the adipocytic / haematopoeitic ratio than age was the trabecular apposition rate, which is a direct correlate of osteoblast activity. Interestingly, an association between the amount of adipose tissue and bone formation has previously been demonstrated (Hirano & Iwasaki, 1992), but in a smaller sample that contained postmenopausal women only. Measures of bone resorption, including osteoclast number and surface, were not associated with the variability in the A/H ratio by multivariate analysis, in keeping with other reports of a lack of association with resorption (Hirano & Isawaki, 1992). Furthermore, in the present study, the variability was associated less strongly with measures reflecting the balance between formation and resorption, namely wall thickness, and bone formation rate. Similarly, the variability was strongly associated with osteoid volume, reflecting new bone formation. Paradoxically the variability was not strongly associated with osteoblast surface, though this may reflect the fact that in many cases of osteoporosis, it is the function rather than absolute number of osteoblasts that is reduced (Rehman *et al.,* 1995). Interestingly, the variability was only weakly associated with trabecular separation, which is thought to reflect resorption, with reduction in trabecular strut connectivity, a parameter not routinely measured.

Osteoblasts are thought to arise from pluripotent marrow stromal cells, which are also capable of differentiation along the adipocytic, chondrocytic or myoblastic lineages (Aubin *et al.,* 1995), and there is accumulating evidence that an inverse relationship exists between osteoblastic and adipocytic differentiation of marrow stromal cells *in vitro* (Gimble *et al.,*

1996; Beresford *et al.,* 1992). However, whilst the association between an increase in marrow fat and loss of trabecular bone is well documented, few studies have investigated the role of the bone marrow in bone formation using dynamic *in vivo* measurements. The adipocytic / haemtopoietic ratio was used as a proxy for assessment of the amount of stromal tissue present, and has shown strong association between variability in this parameter and trabecular apposition, demonstrating a close functional link between the balance between adipocytic and haematopoietic/stromal tissue and osteoblastic activity. This supports the hypothesis that a clonal switch between the adipocytic and osteoblastic lineages occurs in osteoporosis (Jilka *et al.,* 1996) and strengthens the relevance of *in vitro* studies of altered stromal cell differentiation in osteoporosis (Manolagas, 1998).

2.4.4 Conclusions

The results discussed above indicate that a failure of osteoblast recruitment and maturation is present in a substantial number of cases of osteoporosis, that reduction of bone formation in osteoporosis is more frequent in some bone compartments than others, and support the hypothesis that there is a switch in the differentiation of the CFU-F from the osteoblastic to the adipocytic pathway. The nature and extent of the association between the different groups of osteoblast function and compartmental bone formation was not determined and would be interesting to study in the future. In order to study these events at the cellular level it is first necessary to understand the process of normal early osteogenesis. However, whilst the latter stages in the osteoblastic lineage are well characterised, by a range of phenotypic and genotypic markers, relatively few markers exist for cells early in the lineage. The consequent inability to recognise them, both *in vitro* and *in vivo*, has hampered understanding of this process, thereby confounding attempts to unravel the cellular pathobiology of osteoporosis. Previous attempts to identify genotypic markers of these cells have used animal or immortalised cultures, of uncertain relevance to normal human physiology, or hetrogeneous cultures of cells, the use of which precludes the assignment of genetic changes identified to one population of cells or another. In order to overcome these limitations, primary culture of human bone marrow stromal cells was used, as detailed in chapter 3.

Chapter 3

Identification of genes differentially expressed during early osteoblastic differentiation of human bone marrow stromal cells

3.1 Introduction

The results of the histomormorphometric studies described in chapter 2 indicate that in many cases (62%) of osteoporosis there is a failure of recruitment of osteoblasts from their stem cell pool, and that there may be a switch in maturation of their precursors from the osteoblastic to the adipocytic lineage. In order to understand the reasons for this it is first necessary to understand the process by which osteoblasts normally differentiate.

3.1.1 Early Osteoblastic differentiation

The latter stages of osteoblastic differentiation have been well characterised in animal and human models, and the differential expression of stage specific markers defined (see 1.3.2) (Aubin *et al.,* 1995). In addition, a number of monoclonal antibodies reactive against the cells latter in the osteogenic lineage have been generated (Aubin *et al.,* 1993), but few markers, and in particular molecular markers, exist for cells earlier in the lineage (Simmons, 1996). In order to establish such markers it is necessary to study cells at the earliest stage of the osteoblastic lineage. A variety of cell types have been proposed for this stage, namely pericytes, osteochondrocytic precursors, and bone marrow stromal cells.

3.1.1.1 Pericytes

Vascular pericytes can differentiate into osteoblast-like cells *in vitro*, suggesting that these cells may represent a potential source of osteoprogenitor cells in the adult (Brighton *et al.,* 1992). Bovine capillary and microvessel pericytes were grown in monolayer and synthesised alkaline phosphatase positive, mineralising colonies. The colonies were positive for collagen and for osteocalcin. Doherty *et al* (1998) reported a similar osteoblastic phenotype in cultured bovine pericytes, and, in addition, were able to demonstrate the acquisition of STRO-1 reactivity by the cells. They confirmed the osteoblastic potential of the cells by expression of bone sialoprotein, osteocalcin, osteonectin, and osteopontin, and by formation of bone when the pericytes were transplanted into diffusion chambers in athymic mice. Furthermore, the diffusion chambers also contained cartilage, soft fibrous tissue and adipocytic cells, suggesting that the pericyte population *in situ* serves as a reservoir of primitive precursor cells capable of giving rise to osteoblasts, chondrocytes, adipocytes and fibroblasts (Doherty *et al.,* 1998). Such quadripotentiality of differentiation of the osteoblast precursor has also been reported by Dennis *et al* (1999). Both Brighton *et al* (1992) and Doherty *et al* (1998) found that the phenotype of the pericytes was dependent to a greater or lesser extent on the oxygen

tension in which they were cultured. This aspect was studied in detail by Reilly *et al* (1998), who reported that whilst alkaline phosphatase expression was greatest in those cells cultured at low oxygen tension (3%), the expression of osteocalcin was greatest in those maintained in 21% oxygen (Reilly *et al.,* 1998). The other factors controlling the process are largely unknown, but a role has been suggested for thrombospondin-1 by Canfield *et al* (1996), who reported its expression during nodule formation, and its downregulation following the onset of mineralisation. Mineralisation was promoted by the presence of anti-thrombospondin-1 antibodies, suggesting that thrombospondin-1 acts to inhibit perictye differentiation (Canfield *et al.,* 1996).

3.1.1.2 Osteochondrocytic precusors

The existence of a common osteochondrocytic precursor has been postulated by several groups, but remains disputed. Thesingh *et al* (1991) reported osteoblastic trans-differentiation of fetal murine hypertrophic chondrocytes, in co-culture with cerebrum, and suggested that an unidentified brain-derived growth factor was responsible for the process. Cancedda *et al* (1992) demonstrated osteoblastic differentiation of chick hypertrophic chondrocytes, under the influence of ascorbic acid and, more dramatically, retinoic acid, whilst the same group also reported chondrocytic differentiation of mature osteoblastic cells (Manduca *et al.,* 1992). The transfer of hypertrophic chondrocytes from suspension to anchorage-dependent culture conditions is thought to be important in modulating the trans-differentiation of hypertrophic chondrocytes (Gentili *et al.,* 1993), but the mechanisms involved are unclear. The presence of ascorbic acid is important (Sugimoto *et al.,* 1997). An alternative view to the trans-differentiation of one cell type to another is the existence of lineage plasticity, both at the level of the precursor cells and of the mature cells. Several groups have reported similarities in the phenotype of osteoblastic and chondroblastic cells (Hughes *et al.,* 1995; Galotto *et al.,* 1995; Lian *et al.,* 1993), whilst recently Dennis *et al* (1999) reported a murine cell line, BMC9, capable of chondrocytic, adipocytic, stromal or osteoblastic differentiation dependent on culture conditions. It has been suggested that *in vivo* the hypertrophic chondrocyte undergoes either apoptosis or trans-differentiation to an osteoblastic cell, dependent on its specific location (Bianco *et al.,* 1998; Roach *et al.,* 1995). Hence, those in the growth plate undergo apoptosis, whilst those positioned at the "borderland" between cartilage and osteogenic tissues undergo further differentiation to bone producing cells (Bianco *et al.,* 1998).

3.1.1.3 Stromal system of bone marrow

In order to understand the stromal system of the bone marrow it is first necessary to consider the architecture of the bone marrow, and the process of haematopoiesis (Abboud & Lichtman, 1995), by analogy with which the biology of the stromal system has been described (Owen, 1988; Quesenberry, 1995).

Bone marrow is first identifiable at five months of gestation and maintains granulocytic and megakaryocytic proliferation (Abboud & Lichtman, 1995); fetal erythropoiesis taking place in the liver. Adipocytes first appear in the diaphyses in the fourth year and thereafter progress centripetally so that by 18 years of age haematopoietic elements are only found in the vertebrae, ribs, skull, pelvis and proximal epiphyses of the femora and humeri. The marrow space is supplied with blood by nutrient arteries, which penetrate the cortex and bifurcate into ascending and descending medullary arteries, off which radial branches supply the marrow. Here these vessels anastamose with periosteal capillaries derived from muscular arteries before passing into a sinusoidal network which supports the haematopoietic environment. This network drains into a central venous sinus, from which the blood exits the bone marrow via emissary veins. Whilst the architectural arrangement of the haematopoietic elements is well known, localisation of the stromal cells is poor. The adventitial reticular cells responsible for synthesis of the reticular fibre network on which the haematopoietic cells rest are contiguous with the wall of the vascular sinus, forming part of its coat. From them the reticular fibres extend out along with their cytoplasmic processes. These cells express alkaline phosphatase, CD10 and CD13 and can differentiate along the smooth muscle lineage (Abboud & Lichtman, 1995), but, though some have reported adipocytic differentiation in them (Abboud *et al.,* 1993), they do not give rise to osteoblast-like cells. Osteoblasts and osteoclasts are present at the bone surfaces, and electron microscopy has also demonstrated elongated spindle cells thought to represent osteoprogenitors (Abboud & Lichtman, 1995).

The haematopoietic cells of the marrow arise from pluripotent stem cells capable of differentiation to either lymphopoietic or haematopoietic multipotential stem cells. The common lymphoid stem cell gives rise to T and B progenitor cells, which differentiate to mature T and B cells. The myeloid stem cell undergoes further differentiation to a number of unipotential progenitors, each giving rise to cells of a particular lineage (Quesenberry, 1995). These unipotential cells give rise to relatively primitive, highly proliferative clones, termed colony forming units. Each particular colony forming unit is named according to the mature cell resultant from its further differentiation, i.e. CFU-erythroid (CFU-E), CFU-megakaryocyte (CFU-Meg), CFU-granulocyte-macrophage (CFU-GM), and so on (Quesenberry, 1995).

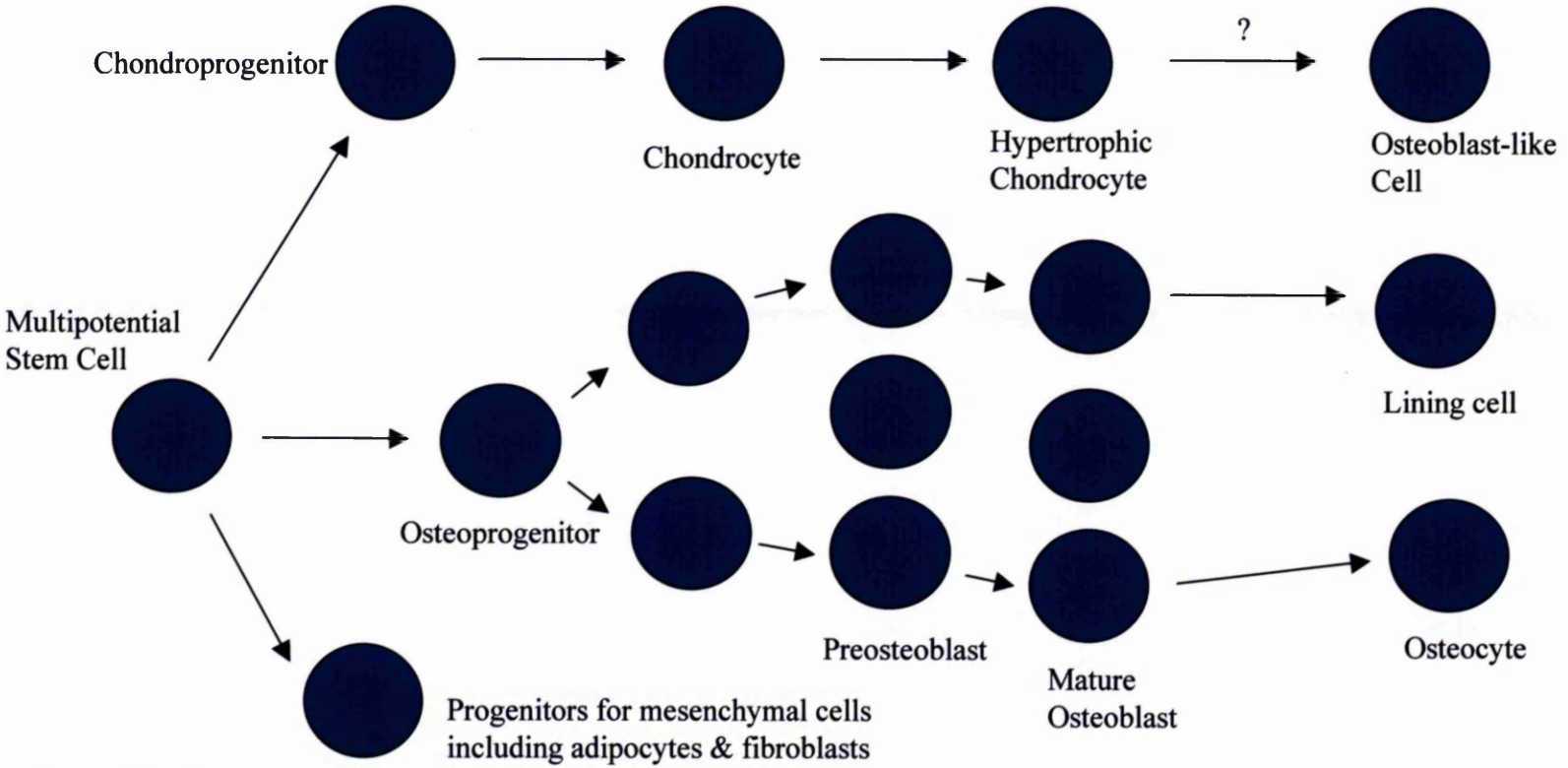


Figure 3.1 Postulated osteoblast lineage scheme showing derivation of the osteoblastic cells from a multipotential mesenchymal progenitor cell and relationship of these cells to other mesenchymal lineages. Whilst the latter stages of osteoblastic differentiation are well characterised, the earlier stages, between the stem cell and the osteoprogenitor stages are unknown. (Adapted from Aubin *et al.*, 1995).

However, whilst the stages present in each of these lineages, and the growth factors responsible for their maturation, are well documented, the same is not true for the proposed CFU-fibroblastic (Aubin *et al.,* 1995; Beresford, 1989) which, by analogy with the haematopoietic system, represents a primitive stem cell capable of differentiaiton along the osteocytic, adipocytic, fibroblastic, myogenic, and possibly chondrocytic, lineages (Aubin *et al.,* 1995; Caplan 1991) (figure 3.1).

Culture of unfractionated marrow cell suspensions results in precipitation of an adherent population that initially comprises a mixture of stromal fibroblasts and haematopoietic cells (Beresford, 1989), though the latter are eliminated by density gradient centrifugation prior to seeding (Cheng *et al.,* 1994). In mixed cultures the haematopoietic cells either die or are removed by media changes, resulting in a pure stromal cell culture (Friedenstein, 1976). Macrophages may be prominent initially, particularly in murine cultures, but disappear with longer culture (Beresford, 1989). Fibroblast monolayers form best when cells are cultured at densities higher than 5×10^5 cells/cm^2, whilst at lower densities fibroblastic colonies are seen (Friedenstein, 1976).

Stem cells are generally considered to be capable of extensive self-renewal, without change in their properties, and of differentiation to several subtypes of mature cell (Lajtha, 1979). Both *in vitro* and *in vivo* CFU-F cells have high proliferative potential (Friedenstein *et al.,* 1982) and are capable of differentiation along the osteoblastic and adipocytic lineages, fulfilling the requirements for a stem cell (Beresford, 1989). Studies using radio-labelled thymidine indicate that the stem cell is noncycling. Consequently, radiothymidine pulse-labelling experiments to track the passage of osteoprogenitor cells and their progeny are likely to under-represent the true picture (Simmons, 1996).

Despite the evidence for a mesenchymal stem cell definite proof for its existence is lacking and until it is possible to identify unequivocally single stem cells and their progeny, and to identify such cells *in vivo*, will remain so (Simmons, 1996). In particular, though stage-specific bone markers are known for cells latter in the osteoblastic lineage, until recently there were no known specific markers for cells early in the lineage. Three markers, STRO-1, HOP-26 and SB10 (Joyner *et al.,* 1997; Simmons *et al.*, 1991; Bruder *et al.,* 1997), have been developed (see 3.1.1.5), but none of them have been used to track cell differentiation at the single cell level. Liu *et al* (1994) used reverse transcription poly (A) polymerase chain reaction to analyse gene expression for bone specific markers in single cells harvested from differentiating cultures of stromal cells, demonstrating differences in gene

expression in cells drawn from fibroblastic, or from either less or more mature osteoblastic colonies, consistent with the pattern observed by others in analyses of whole cultures (Rickard *et al.,* 1996). However, the single cell approach also showed that there is considerable heterogeneity in the repertoire of osteoblast-associated markers present in different cells, suggesting variation in the switch-on of osteoblast phenotype (Liu *et al.,* 1994; Aubin *et al.,* 1995). Secondly, some cells from the colonies classified as fibroblastic morphologically expressed genes consistent with their being osteoprogenitor cells (Liu *et al.,* 1994). Recently the same group has used a similar single cell method to isolate a novel gene expressed during early osteoblast differentiation (Candeliere *et al.,* 1999), and there are now several groups investigating the molecular control of stromal cell differentiation, using a variety of techniques (detailed in section 3.1.3).

During osteoblast differentiation osteopontin appears first, followed by bone sialoprotein and osteocalcin (Liu *et al.,* 1994; Aubin *et al.,* 1995) (figure 3.2). Alkaline phosphatase increases from a low level and decreases with mineralisation. In some studies osteocalcin is detectable only upon mineralisation, whilst in others it appeared shortly following osteopontin (Hughes & Aubin, 1997). Collagen type I is either initially high, thereafter decreasing, or remains at the same level throughout differentiation (Hughes & Aubin, 1997).

3.1.1.4 Transcriptional control of early osteoblastic differentiation

Recently the central role of the transcription factor Cbfa1 in osteoblastic differentiation has been documented in a large number of reports (Ducy *et al.,* 1997; Komori *et al.,* 1997), as reviewed in section 1.5.1. Initial reports of its role in bone were based on observations from cbfa1-/- fetuses, in which its absence was associated with craniofacial abnormalities (Otto *et al.,* 1997; Mundlos *et al.,* 1997). However, these reports restricted its role to osteogenesis, but it is now known to be involved in chondrogenesis also (Kim *et al.,* 1999). Recently it has also been implicated in tooth development (Jiang *et al.,* 1999); interestingly, altered dentition was part of the phenotype of the knockout mice. In addition, it now also appears to exert an effect after birth, when it acts as a transcriptional activator of bone formation, such that its overexpression induces osteopenia due to reduced activity of mature osteoblasts (Ducy *et al.,* 1999). The confusion developing amongst reports of its action is partly due to the existence of different isoforms (Harada *et al.,* 1999). Whilst each of the three isoforms identified are involved in modulation of osteoblast differentiation, they do so to differing extents (Harada *et al.,* 1999).

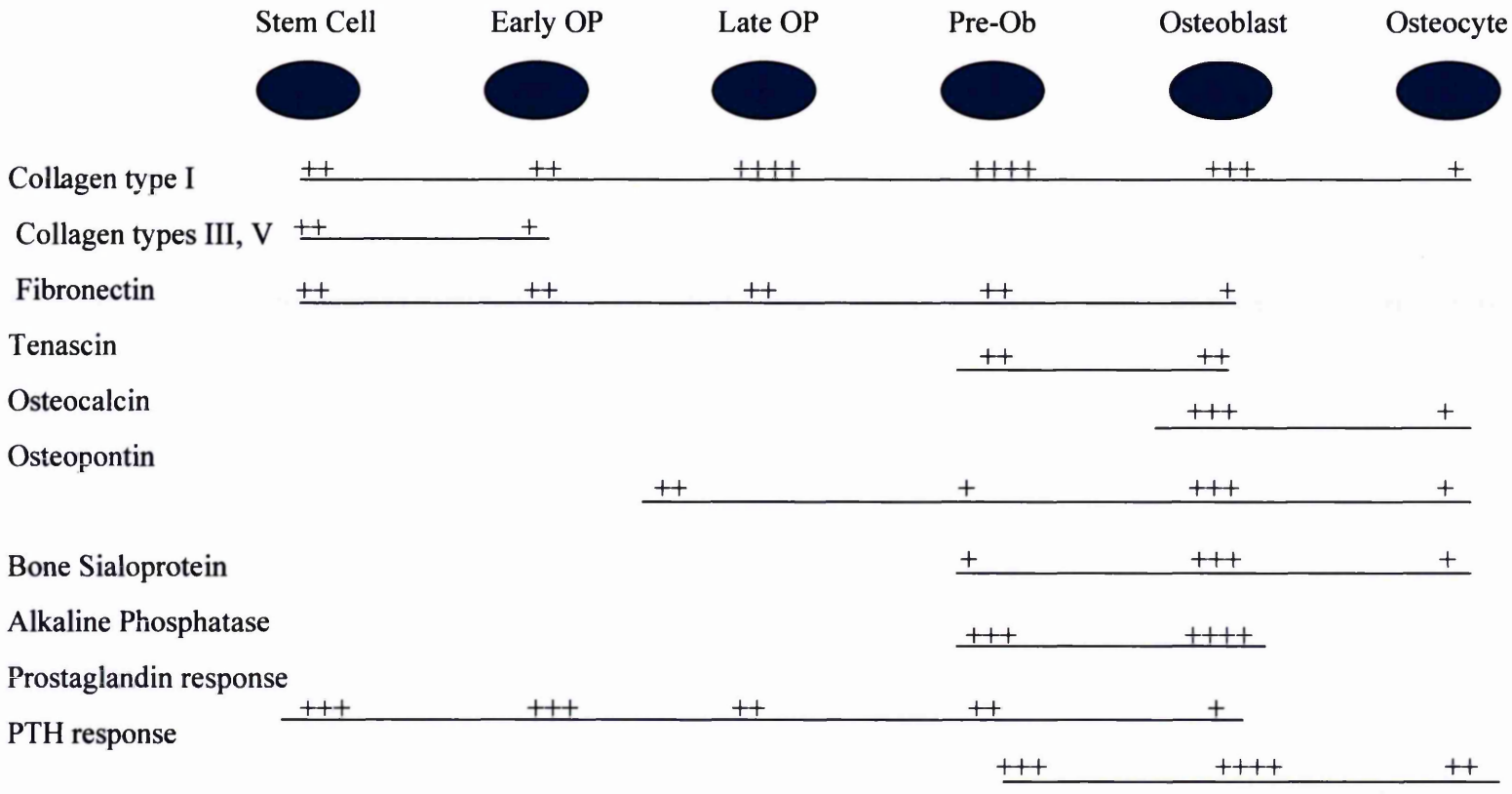


Figure 3.2 Approximate schema of the changes in markers present at the different stages of osteoblastic differentiation. OP - Osteoprogenitor, Ob - Osteoblast. (Adapted from Hughes & Aubin 1997)

Other transcription factors recently reported in osteogenesis include DERMO-1, a member of the helix-loop-helix type transcription factor group, which appears to act as a negative regulator of differentiation in osteoblasts (Tamura & Noda, 1999). Msx2, a homeodomain transcription factor that regulates craniofacial development *in vivo* and osteocalcin (Ocn) promoter activity *in vitro*, also acts as a negative regulator of differentiation, suppressing Ocn expression in the craniofacial skeleton at stages immediately proceeding odontoblast and osteoblast terminal differentiation (Bidder *et al.,* 1998). The AP-1 (activating protein complex-1) fos/jun transcription factor complex binds to AP-1 sites in the vitamin D receptor element and the osteocalcin box within the osteocalcin gene promotor (Stein & Lian, 1993). Expression of c-fos and c-jun occur primarily during the proliferative period of osteoblastic maturation, with associated binding to the AP-1 sites. This prevents binding by stimulatory factors, such as the vitamin D receptor, resulting in suppression of osteocalcin transcription. Release of the AP-1 related proteins i.e. c-fos/c-jun, from the AP-1 binding sites permits occupancy by the vitamin D receptor, with consequent upregulation of osteocalcin expression, reduced proliferation and increased differentiation.

PTH acts to modulate transcriptional activity in osteoblasts in a number of ways (Alvarez *et al.,* 1998; Selvamurugan *et al.,* 1998). Selvamurugan *et al* (1998) reported parathyroid hormone regulation of the rat collagenase-3 promoter in osteoblastic cells through the co-operative interaction of the activator protein-1 site and the runt domain (OSE2/AML1/Cbfa1) binding sequence, demonstrating that both these sites, i.e. the AP-1 and Cbfa1 binding sites, are widespread in osteogenic genes. Alvarez *et al* (1998) found binding of the PTH-responsive osteoblast nuclear matrix architectural transcription factor to the rat type I collagen promoter, providing a possible molecular pathway by which osteoblast structure is coupled to COL1A1 expression. The role of the osteoblast nuclear matrix in modulation of gene expression has received a lot of attention recently and has been reviewed by Bidwell *et al* (1998). The nuclear matrix MAR (matrix attachment region)-binding proteins include topoisomerase II-α and -β, bright and nucleolin, and the osteoblast nuclear matrix proteins NMP1(nuclear matrix protein)/YY1, NMP2/Cbfa1, NMP4 and topoisomerase II-α and -β. They are thought to act by translating a change in cytoskeletal organisation into a bend or twist in the promotor of target genes, since the degree of supercoiling and bending of the promotor DNA can regulate transcriptional activity. In particular, two osteoblast nuclear matrix, DNA-binding proteins, NM3 and NM4, bind close to known regulatory regions in the type I collagen promotor. NM4 binds within a minor groove of the COL1A1 promotor, inducing a bend in the DNA. NM3 binds in a region of the promotor containing a CarG box, an element that has been implicated in

DNA binding and is in proximity to other potential regulatory elements, including a putative vitamin D response element (VDRE) and an AP-1 site.

DERMO-1, noted above, is one of the helix-loop-helix type transcription factors, (others of which include HES-1) which is expressed in osteoblastic cells, suppressed by 1,25(OH)(2) vitamin D-3, and in turn suppresses 1,25(OH)(2) vitamin D-3 enhancement of osteopontin gene expression (Matsue *et al.,* 1997). Similarly, Kazhdan *et al* (1997), in a study of the interaction of nuclear proteins with oligonucleotides corresponding to various basic helix-loop-helix (bHLH) binding sequences (known as E-boxes), found that bHLH gene expression was not required for the early steps of osteogenesis and that moreover, inhibition of bHLH protein binding to a MEF1-type (muscle creatine kinase enhancer) E box might be an integral part of osteogenic commitment.

3.1.1.5 Markers of early osteoblastic differentiation

A range of monoclonal antibodies has been generated against cells in the osteoblast lineage, the stage restriction of which (figure 3.3) has been reviewed in section 1.3.2.1. In particular however, only three are available for identification of cells at the earliest stages of the lineage, namely STRO-1, HOP-26 and SB-10 (Joyner *et al.,* 1997; Simmons *et al.*, 1991; Bruder *et al.,* 1997). At the time at which the initial work detailed in this chapter was carried out STRO-1 was the only one of the three generally available.

STRO-1 (Simmons & Torok-Storb, 1991) is a monoclonal antibody (IgM) that was raised by immunisation of mice with CD34 positive human bone marrow mononuclear cells. It binds to approximately 10% of BM mononuclear cells, more than 95% of which are nucleated erythroid precursors (Simmons & Torok-Storb, 1991), though these can be removed by prior density gradient centrifugation of the marrow. It does not react with committed progenitor cells of the haematopoietic lineage (colony-forming unit granulocyte-macrophage [CFU-GM], erythroid burst [BFU-E], and mixed colonies [CFU-Mix]), whilst fibroblast colony-forming cells (CFU-F) are present exclusively within the STRO-1 positive fraction (Simmons & Torok-Storb, 1991). In long-term bone marrow culture conditions, STRO-1 reactive cells generate adherent cell layers containing many stromal cell types, including adipocytes, smooth muscle cells and fibroblastic elements. These cell layers are able to support the generation of clonogenic cells and mature haematopoietic cells from a population of CD34 positive cells, and retain the capacity to generate further layers of cells with an identical cellular composition to that of the parent

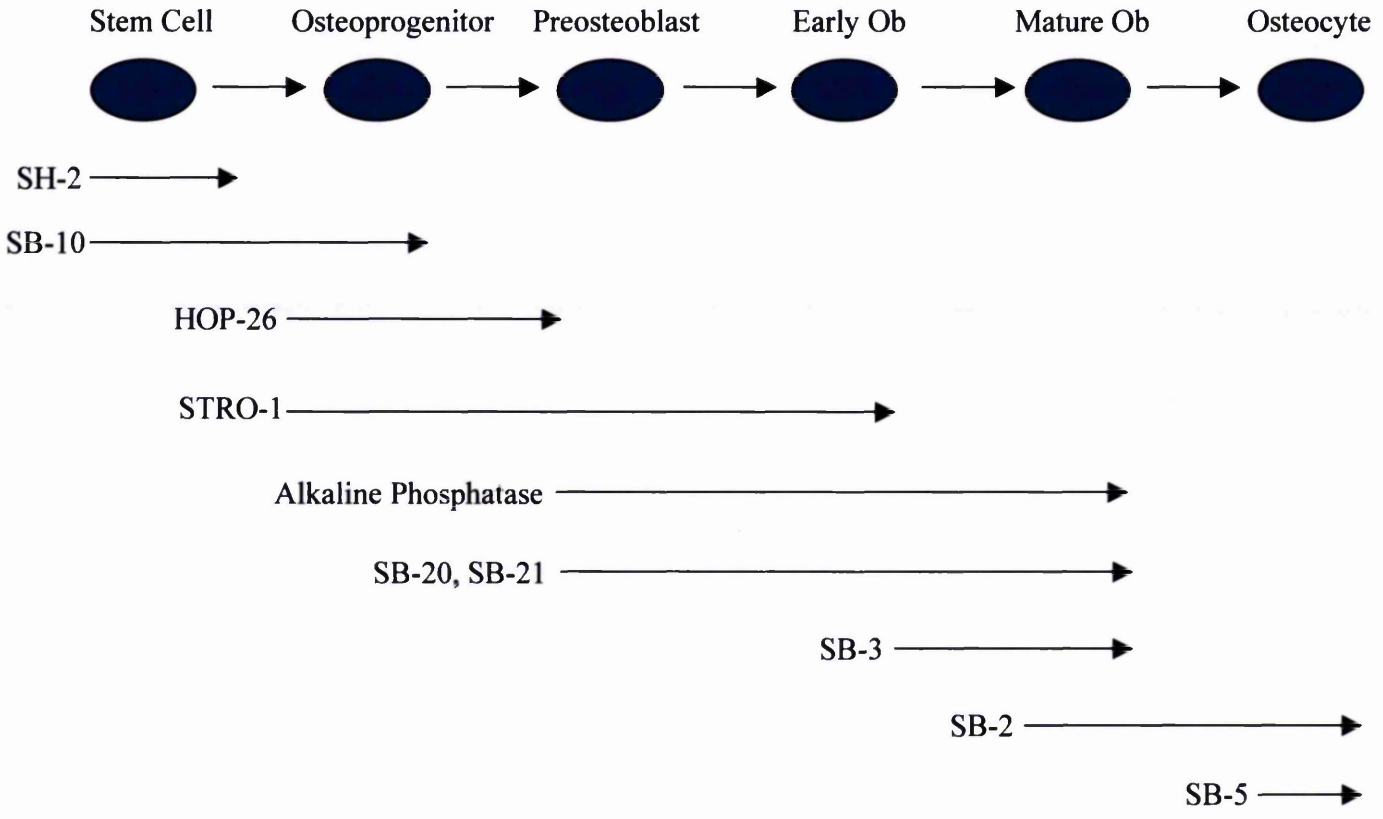


Figure 3.3 Expression of selected cell surface antibodies reactive with cells at different stages in the osteoblastic lineage, compared with the expression of alkaline phosphatase (see text for details of antibodies). Ob - Osteoblast (Adapted from Bruder et al 1997).

culture (Simmons & Torok-Storb, 1991). The osteogenic potential of the CFU-F derived from the STRO-1 positive fraction has been established by culture of the cells, in the presence of dexamethasone (10^{-8}M), ascorbic acid (100μmol/l) and inorganic phosphate, resulting in production of alkaline phosphatase positive colonies after 2 weeks, and in mineralisation after a further 2 weeks in culture (Gronthos *et al.,* 1994). The growth factor requirements of the STRO-1 positive cells under serum-free conditions have been defined (Gronthos & Simmons, 1995). L-ascorbate and dexamethasone were essential for CFU-F colony formation in the absence of serum, whilst platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) demonstrated the greatest ability to support colony growth, both in a dose-dependent manner. Receptors for both PDGF and EGF were detected on more than 90% of the CFU-F, though, whilst receptors were also detected for insulin-like growth factor and nerve growth factor, neither of the two supported colony formation (Gronthos & Simmons, 1995).

Since these initial reports of the utility of STRO-1 for the enrichment and culture expansion of the CFU-F, a number of groups have used it to identify and follow the differentiation of early osteoblast precursors (Stewart *et al.,* 1996; Oyajobi *et al.,* 1999). Oyajobi *et al* (1999) used magnetic activated cell sorting with STRO-1 antibody to isolate STRO-1positive, (STRO-1(+)), cells from non-adherent human fetal BM stromal cells. Immunoselected STRO-1(+) cells were immortalized using SV-40 large T antigen and a clone, F/STRO-1(+) A, with weak alkaline phosphatase (ALP) activity was selected. Preconfluent F/STRO-1(+) A cells showed immunoreactivity for osteopontin, alpha 1(I) procollagen, and parathyroid hormone-related peptide, but not for the late osteoblast differentiation markers, osteocalcin (Ocn), or bone sialoprotein, whilst differentiation of F/STRO-1(+) A cells was induced by dexamethasone and 1,25-dihydroxyvitamin D-3, as shown by increased ALP activity. In addition, osteogenesis occurred in F/STRO-1(+) A cells cultured in three-dimentional aggregates, as assessed morphologically, histologically, and biochemically.

Most recently the differential expression of STRO-1 during osteoblast differentiation has been defined by Gronthos *et al* (1999). Cells expressing STRO-1 alone (STRO-1+ve/AP-ve) exhibited a pre-osteoblastic phenotype, both functionally by their reduced ability to form mineralised bone, and phenotypically by the lack of their expression of bone-related markers, including bone sialoprotein, osteopontin, and the parathyroid receptor. Conversely, the STRO-1-ve/AP+ve and STRO-1-ve/AP-ve fractions appeared to correspond to fully differentiated osteoblasts, whilst the STRO-1+ve/AP+ve fraction

represented an intermediate stage of development. In particular, when re-cultured, the STRO-1+ve/AP-ve fraction alone was able to give rise to all four of the subsets, yielding the same proportions of STRO-1/AP expression as the original primary cultures. Interestingly, although often regarded as restricted to mature osteoblasts, osteocalcin was detected in all four of the subsets, though detection was at the level of mRNA rather than protein. However, it should also be noted that osteocalcin has been detected in pre-osteoblasts *in vivo* (Herbetson & Aubin, 1997), and may not therefore be as tightly regulated as previously thought.

In contrast to STRO-1, which is reactive against unfixed cells only, HOP-26 (Joyner *et al.,* 1997) reacts against fixed cells, rendering it applicable for use in routinely processed sections. Raised against human marrow stromal fibroblast cell cultures, it is an monoclonal IgM antibody, and is strongly reactive with cells in marrow stromal colonies at early stages of differentiation, before expression of alkaline phosphatase, and decreases after the cells reach confluence and express alkaline phosphatase (Joyner *et al.,* 1997). In suspensions of fixed marrow it is immunoreactive with a very small proportion of cells (<1% of the nucleated cells), though it is also reactive with viable cells, indicating a cell surface location. Immunopanning to enrich for HOP-26 positive cells demonstrated that the majority (70%) of the CFU-F present were bound by the antibody. In sections of human fetal limb it was restricted to cells close to the developing bone, in the periosteum and between the developing bone trabeculae, whilst in adult trabecular bone it was reactive with only occasional cells within the marrow space, and unreactive with osteoblasts, adipocytes, and fibrous tissue (Joyner *et al.,* 1997). Intriguingly, positive cells were noted close to blood vessels in the adult bone marrow. Since angiogenesis precedes mineralisation *in vivo*, and osteogenic differentiation has been demonstrated in pericytes (Schor *et al.,* 1995), this may be significant. HOP-26 does not react with other tissues, including skin, muscle, appendix, brain, or the osteoblast cell lines SaOS-2 and MG63, and its restriction to early cells makes it a useful tool for study of early osteogenesis *in vivo*.

Whilst STRO-1 and HOP-26 have been well characterised in terms of their expression profile, the epitope that they react against is unknown in both cases. SB-10 meanwhile (Bruder *et al.,* 1997), also raised by immunisation with human mesenchymal stem cells, has been cloned and corresponds to activated leukocyte adhesion molecule (ALCAM) (Bruder *et al.,* 1998). As with HOP-26, SB10 is reactive with undifferentiated stromal cells and early osteoprogenitors, but no longer reacts once the cells express alkaline phosphatase. The staining pattern of another antibody developed by the same group, SB-

20, is reciprocal to that of SB-10. In particular, in sections of human fetal long bone, SB-10 was positive in the outer, alkaline phosphatase negative, periosteal cells, whilst SB-20, which shows an identical staining pattern to another monoclonal generated along side it SB-21, was positive in the inner, more cuboidal and alkaline phosphatase positive cells (Bruder *et al.,* 1997). SB-10 is not reactive with non-human species, including chick, rat, rabbit, dog and cow, but was reactive with non-skeletal tissues, including brain, intestine and skin, in human fetal samples, though this extra-skeletal reactivity was absent in adult samples. The functional significance of ALCAM / SB-10 was demonstrated by induction of an osteoblastic phenotype in stromal cells cultured in the presence of anti-ALCAM antibody fragments, though this effect was only observed when the osteoinductive agent dexamethasone was also present (Bruder *et al.,* 1998). Other adhesion molecules are also involved in osteogenesis, including NCAM (Lee *et al.,* 1992), OB-Cadherin (Okazaki *et al.,* 1994), whilst mature osteoblasts express ICAM-1 and VCAM-1 (Tanaka *et al.,* 1995b), suggesting that changes in adhesion molecule expression have implications for cellular differentiation beyond those of morphology alone.

3.1.2 *In vitro* osteoblast and stromal cell culture

A wide number of *in vitro* models exist for osteoblast and bone marrow stromal cell culture and can be divided into primary cultures or immortalised cell lines, each of which may be of human or animal, usually rat or murine, origin. In addition, the cells present in trabecular bone (osteoblast-like cells) and bone marrow (stromal cells) cultures differ in terms of their stage of differentiation, and consequently will respond differently to given stimuli (Beresford *et al.,* 1993). It is therefore important to consider the properties of the system chosen in experimental design.

In vivo culture of either osteoblast-like or stromal cells in diffusion chambers has been widely used (Ashton *et al.,* 1980). It is closer to a normal physiological state compared to *in vitro* culture since the cells are exposed to the entire range of nutrients and chemokines that they would normally be in their normal state. However it has the disadvantages of higher cost, difficulty in modulation of the system, and the longer time course needed for experimentation. It is the system of choice for establishing the relevance of an observation *in vivo*, but *in vitro* culture remains superior for testing wide ranges of stimuli and for gene expression studies.

3.1.2.1 Culture medium and supplements

The optimum culture conditions for osteoblastic differentiation of marrow stromal or osteoblast-like cells have been reviewed by Beresford *et al* (1993), using the formation of mineralised nodules as the end-point. Most investigators use a rich culture medium (Hughes & Aubin, 1997), such as alpha modified minimal essential medium, which contains nucleotides, supplemented with 10 – 15% fetal bovine serum and ascorbate; antimicrobial supplements including penicillin G, streptomycin and amphotericin are usually added at 1% final concentration. Ascorbate (ascorbic acid, vitamin C) is added to a final concentration of 0.5-1% (Beresford *et al.,* 1993) and stimulates both cell proliferation (Harada *et al.,* 1991) and expression of differentiation markers such as ALP and Ocn (Malaval *et al.,* 1994). It is an absolute requirement for nodule formation and mineralisation and is thought to act by promoting collagen synthesis, though it does not by itself affect collagen gene expression. Its action can be blocked using RGD-containing peptides, indicating that its effects are mediated via matrix deposition (Harada *et al.,* 1991). This media allows extended culture and continuation of cell proliferation after confluence has been reached. After culture of calvarial derived osteoblast like cells in such medium nodules positive for ALP form. Addition of dexamethasone (10^{-10} - 10^{-6} M), which is added in absolute ethanol, increases expression of the osteoblastic phenotype in such cultures, and in cultures of other osteoblast-like cells, including trabecular explant and digest cultures and the cell lines ROS 17/2.8, MC3T3-E1, and UMR 106.06, reviewed in Hughes & Aubin (1997). The physiological level of natural glucocorticoid is 10^{-8} M, and addition of dexamethasone is needed to induce ALP expression in marrow stromal cultures from rat, rabbit or human sources, whilst addition of beta-glycerophosphate (βGP) is needed for initiation of mineralisation in both osteoblast-like and stromal cell cultures. Beta-glycerophosphate acts as a substrate for ALP, resulting in an increased concentration of inorganic phosphate, resulting in calcium deposition (Bellows *et al.,* 1991 & 1992).

3.1.2.2 Osteoblastic cell lines

A wide variety of clonal cell lines are currently available, including both multipotential and established osteoblastic cell lines. The former are capable of differentiation along a number of lineages and include the mouse embryonic fibroblast line C3H 10T1/2 clone 8, the rat calvarial cell lines RCJ 3.1 and ROB-C26, and the mouse mesodermallly derived cell line C1 (Hughes & Aubin, 1997). Recently two human immortalised marrow stromal cell lines have been reported, hOP 7 (Houghton *et al*, 1998) and HCC1 (detailed in Brown, 1999), both of which were generated by retroviral transduction of human marrow stromal cells

Characteristic	*MC3T3-E1*	*ROS17/2.8*	*UMR106.06*	*HOS/TE-85*	*MG-63*	*SaOS-2*
Bone formation *in vivo*	ND	+	+	ND	ND	+
Mineralised matrix						
Formation *in vitro*	+	-	-	-	-	+
Alkaline phosphatase	+	+	+	-	-	+
cAMP response to PTH	+	+	+	+	+	+
1,25 Vitamin D3 response						
or receptor	+	+	+	+	+	+
Type I collagen synthesis	+	+	+	+	+	ND
Type III collagen synthesis	+	+	-	ND	+	ND
Osteocalcin synthesis	+	-	-	-	-	-
Osteonectin synthesis	ND	+	ND	ND	ND	+
Osteopontin synthesis	+	+	ND	ND	ND	ND

ND = not determined

Table 3.1 Phenotypic characteristics of commonly used established osteoblastic cell lines (Adapted from Hughes & Aubin, 1997)

with the SV40 large T antigen. Both are representative of uncommitted cells and are capable of either osteoblastic or adipocytic differentiation. The HCC1 cell line (Brown, 1999) undergoes osteoblastic differentiation when stimulated with dexamethasone (10^{-6}M) and 1,25-vitamin D3 (10^{-7}M) in the presence of 10% fetal bovine serum, and adipocytic differentiation when cultured in the presence of 15% rabbit serum and dexamethasone (10^{-6}M); the hOP 7 line responds in a similar manner. The cell lines CH3 10T1/2 clone 8, RCJ 3.1, and C1 are additionally capable of myoblastic and chondrocytic differentiation (reviewed in Hughes & Aubin, 1997) following stimulation with 5-azacytadine, BMP-2 and BMP-4 and dexamethasone.

A number of established osteoblastic cell lines are also available, derived from either murine, rat or human tissue. The characteristics of the different lines are shown in table 3.1.

3.1.2.3 Primary osteoblast-like cell culture

Osteoblast-like cells correspondent to the latter stages of the osteoblast lineage are isolated from fragments of trabecular bone, from a variety of sites, including the iliac crest, sternum, knee, or femoral shaft or head, and from either fetal or adult donors. Regardless of the source of the tissue, the osteoblast-like cells are cultured either directly from finely diced bone fragments, or after collagenase digestion to remove the cells from the bone surfaces (Hughes & Aubin, 1997).

Explant culture of mature trabecular bone results in outgrowth of osteoblast-like cells and has been widely used due to its simplicity (Auf'mkolk *et al.*, 1985), though it generates a heterogeneous mix of osteoblastic cells at varying stages of differentiation, together with non-osteoblastic cells such as fibroblasts, and, to a lesser extent, adipocytes. It is therefore appropriate for investigation of the effects of stimuli on overall bone function, but not for identification of differentiation or lineage specific effects. Success of the method depends on fine dicing of the bone fragments prior to explant culture, followed by undisturbed culture for a sufficient period of time to allow outgrowth of cells. Cells isolated in this way cells are pre-osteoblastic and osteoblastic, and express alkaline phosphatase, type I collagen, ostocalcin and osteonectin, show a cAMP response to PTH stimulation and respond to 1,25 dihydroxyvitamin D3 (Auf'mkolk *et al.*, 1985). Cells with a similar phenotype and differentiation profile can be isolated by collagenase digestion of trabecular bone fragments (Robey & Termine, 1985; Wergedal & Baylink, 1984).

Stromal cells are derived from bone marrow, either femoral, iliac or sternal, and comprise a heterogeneous mixture of cell lineages, including multi-potential mesenchymal precursor cells, as well as committed cells (Owen *et al.*, 1987; Matsuyama *et al.*, 1990). The technique relies for success upon adequate disaggregation of the marrow sample, though this is usually accompanied by centrifugation over a density gradient which results in separation of the mononuclear cell fraction (Cheng *et al.*, 1994), which contains the stromal cells, from bone fragments and erythrocytes, including erythroid precursors. The cells are left undisturbed in culture medium for 2 to 7 days for cells to adhere, after which they are culture expanded. The osteogenic capacity of the cells has been verified by culture in diffusion chambers (Haynesworth *et al.*, 1992). The cells are initially fibroblastic and do not express ALP (Liu *et al.*, 1994), which is induced by stimulation with dexamethasone (Cheng *et al.*, 1994).

The age of the donor affects the number, proliferation and differentiation of osteoblast-like or stromal cells present in samples. Cells derived from fetal sources show a greater ability to give rise to a range of differentiated phenotypes than those from older donors, suggesting an increased number of undifferentiated progenitor-type cells in these cultures (Hughes & Aubin, 1997). Studies also show a reduced number of total colony-forming cells and bone-forming cells in bone marrow stromal cell cultures from older donors (Tsuji *et al.*, 1990), though the capacity for differentiation along the osteoblastic lineage of those cells present appears to be similar for cultures generated from old and young donors (Evans *et al.*, 1990).

3.1.3 Analysis of differential gene expression

A myriad of methods has recently been developed for detection of differential gene expression. The majority are based on two different techniques, namely subtractive hybridisation and differential display; as the former was used in the present investigation it will be discussed last. In addition, recently several groups have reported the use of expressed sequence tags (ESTs), whilst the development of cDNA microarray technology has facilitated direct library screening, both to isolate and identify differentially expressed genes.

3.1.3.1 Differential display

Differential display has been more widely used than subtractive hybridisation, but has been reported to produce more false positives (Carulli *et al.,* 1998a). The steps used in the procedure are: 1- reverse transcription of mRNA using oligo-(dT) primers containing one or two additional nucleotides at the 3'end, 2- PCR amplification of the cDNA fragments (tags) using a primer anchor of arbitrary sequence, 3- polyacrylamide gel electrophoresis to resolve and visualise the amplified cDNA fragments. Bands present on the gels from one sample but not the other represent tags present in that sample but not the other. Excision of the band allows further amplification and characterisation of the cDNA. It has been successfully used to isolate genes involved in osteoblast physiology (Mason *et al.,* 1997; Adams *et al.,* 1999; Schuetze *et al.,* 1998), though the glutamate transporter isolated by Mason *et al* (1997) initially proved to be a false positive and its importance was only realised in subsequent studies following from its initial identification.

Compared to subtractive hybridisation, in which each experiment will detect differences in one direction only, differential display allows detection of genes whose expression is up- or down-regulated (Carulli *et al.,* 1998a). Furthermore, more than two mRNA populations can be directly compared with each other at the same time. Unfortunately, it is less sensitive in detecting rare mRNA species, and requires many primer combinations for adequate representation of mRNAs. Since a high number of false positives are associated with it, elimination of which has to be performed by verification of differential expression of excised tags by Northern hybridisation (Mason *et al.,* 1997), the technique is highly labour intensive and time consuming compared to subtractive hybridisation.

3.1.3.2 Expressed sequence tags (ESTs)

Expressed sequence tags (ESTs) are partial cDNA sequences, generated from expressed mRNA, and thus point directly to expressed genes. They are generated by random cloning of the mRNA species expressed by a cell type or tissue, and have been used to identify genes upregulated during osteoblast differentiation (Carulli, *et al.*, 1998a; Carulli, *et al.*, 1998b). The relative abundance of each EST is assessed via the number of clones representative of each sequence. The method produces a large number of cloned sequences rapidly, and at relatively low cost, but lacks specificity and, as a consequence, only a limited number of novel genes can be identified at a time (Carulli *et al.,* 1998a). Carulli *et al* (1998b) isolated 4,000 ESTs, of which approximately 3,000 were homologous to sequences previously reported. Of the remainder the majority were also found to

correspond to known genes, and few novel genes were identified. The technique was used in conjunction with microarray screening, a necessary expedient given the large number of clones generated by the technique (Carulli, *et al.*, 1998a; Carulli, *et al.*, 1998b).

3.1.3.3 Serial analysis of gene expression (SAGE)

Short (9-11bp) cDNA tags derived from mRNA species are concatenated with 4bp spacers, which can then be sequenced. The tag sequence is sufficient to identify the corresponding cDNA as long as its sequence is in the database. Alternatively, an oligonucleotide corresponding to it can be used to screen a cDNA library. The relative abundance of each transcript is assessed from the number of sequence tags representative of it, as with ESTs. The technique is both quantitative and qualitative, and capable of rapid, detailed analysis of thousands of transcripts. It was used by Rantakokko *et al* (1996) to identify genes upregulated during osteoclastic and chondrocytic differentiation, but has generally been used less widely than the other techniques (Carulli *et al.,* 1998a).

3.1.3.4 cDNA microarray hybridisation techniques

Recently much attention has focused on the use of cDNA microarrays for determination of the mRNA fingerprint of a cell type or tissue (Carulli, *et al.*, 1998a; Carulli, *et al.*, 1998b). Oligonucleotides are bound to a microchip, which is then hybridised with mRNA from the sample to be analysed. After post-hybridisation stringency washes the microchip is examined microscopically to determine the pattern of binding of the sample to the oligonucleotides. This is generally visualised by fluorescence labelling of the mRNA prior to hybridisation. The method is expensive, but capable of analysing the expression pattern of literally thousands of different genes in a single day, though the fact that it can only screen for predetermined, and therefore known, oligonucleotides, limits its use for detection of novel, or unexpected, genes (Carulli *et al.*, 1998a). Since these are often the very ones of special interest in the study of differential gene expression occurring during differentiation the technique is of limited use in this context.

3.1.3.5 Subtractive hybridisation

This is a widely used, but technically challenging, technique for isolation of genes upregulated in one cell type or tissue compared to another (Brown *et al.,* 1997; Hillarby *et al.,* 1996; Candeliere *et al.,* 1999). The method of poly (A) reverse transcription polymerase chain reaction (RT-PCR) which is usually used to generate the starting cDNA for the technique is highly sensitive (Brady & Iscove, 1993), being able to detect rare mRNAs present at as low as 0.01% of the total mRNA species. Furthermore, since the

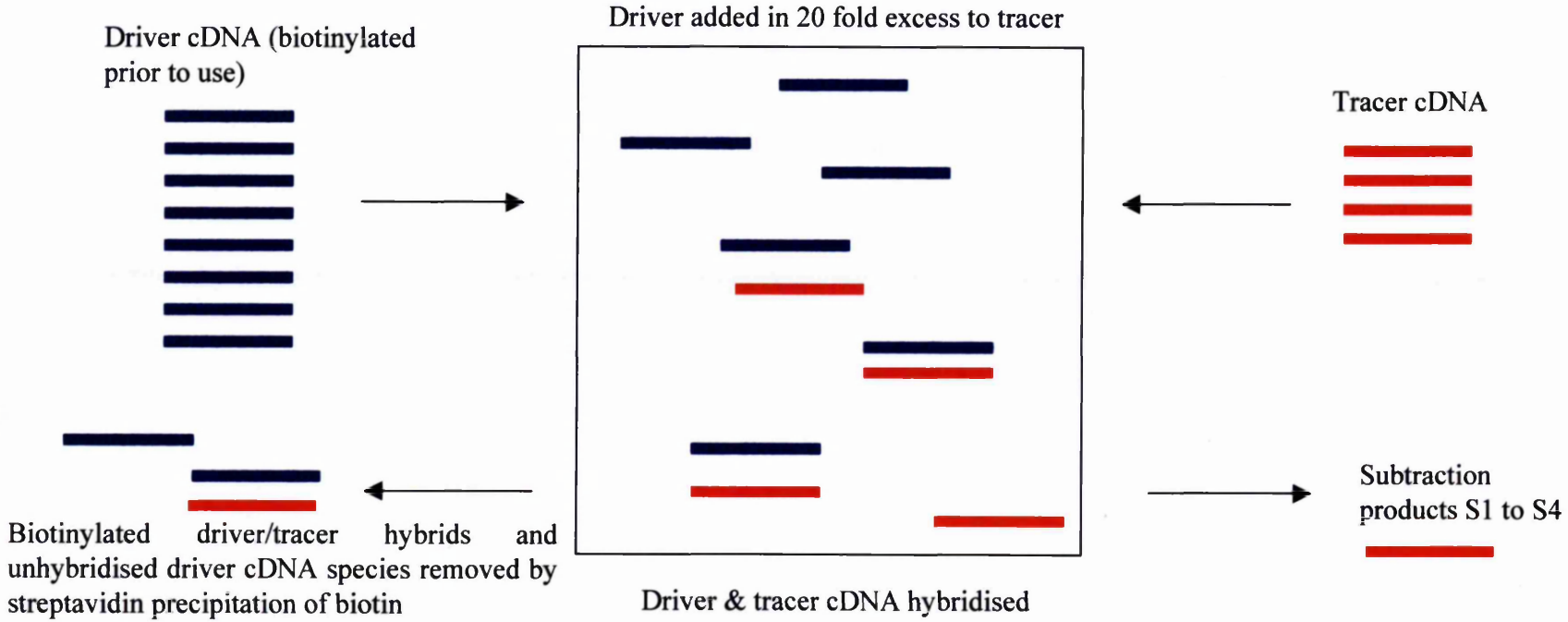


Figure 3.4 Principle of subtractive hybridisation. Driver cDNA is photobiotinylated prior to use and hybridised in a 20 fold excess with tracer cDNA. Biotinylated driver/tracer hybrids and unhybridised driver cDNA species are removed by streptavidin precipitation of biotin, leaving the first subtraction product, S1. This is then substituted for the tracer cDNA in a repeat of the process, yielding a second subtraction product, S2. Two further rounds of subtraction, using S2 and its product S3 in the place of tracer cDNA yields the final subtraction product, S4, which comprises cDNA representative of all the mRNA present in the tracer, but not the driver, cDNA.

product of the method is amenable to poly (A) PCR, the upregulated cDNA pool isolated is capable of indefinite renewal. The use of poly (A) PCR truncates all the cDNAs to between 500 to 700bp, which ensures that long cDNAs are not lost during the PCR steps, a problem previously associated with subtractive hybridisation techniques (Brady & Iscove, 1993; Hillarby *et al.,* 1996). Subtractive hybridisation does not rely on prior sequencing of the genes isolated and so is able to identify novel genes (Carulli *et al.,* 1998a). It has been used successfully to identify genes upregulated during osteoblastic differentiation, including novel genes (Brown, 1999; Candeliere *et al.,* 1999).

The method is illustrated in figure 3.4 and relies on hybridisation with each other of cDNA sequences present in both of the two samples compared, due to complimentarity of their base-pair sequences. Denatured double stranded (ds) cDNA from the two samples to be compared are hybridised so that sequences common to the samples are 'subtracted' from the solution, leaving a population of cDNA species enriched for sequences preferentially expressed in one or other of the samples. Briefly, cDNA from one sample (driver) is hybridised with cDNA from another (tracer) and an avidin biotin method used to remove driver/tracer hybrids. The driver cDNA is photobiotinylated by irradiation using a sunlamp, and TE-saturated 2-butanol and chloroform used to extract biotinylated product. Biotinylation of the driver promotes strand breakage during the hybridisation step, the resultant smaller driver fragments hybridising faster than the large ones. Following hybridisation the biotinylated driver cDNA species, both hybridised and unhybridised, are removed by streptavidin and phenol and the subtracted product used in between 2 to 3 further rounds of subtraction. Alternative methods for removal of common sequences include use of use of a biotinylated primer in the PCR amplification of driver cDNA, the use of streptavidin coated magnetic beads to remove biotinylated driver cDNA, and the ligation of a specific primer to the tracer cDNA, such that only fragments derived from the tracer are amplified and cloned. The first round of subtraction removes the rapidly hybidising repetitive classes of DNA (approximately 40% of the genome), whilst the subsequent second and third rounds remove more slowly hybridising sequences, leaving only genes unique or massively upregulated in the tracer. If the technique has worked well less than 5% of the tracer cDNA sequences will be left.

The difficulty of subtractive hybridisation and related methods derives from the nature of subtraction kinetics. Abundant genes hybridise faster and to a greater level of completion than low abundance genes, making them more amenable to subtraction, but, unfortunately, many interesting regulatory genes are of low abundance. Furthermore, exhaustive

hybridisation is generally necessary in order to avoid retention of sequences that are not differentially expressed. Notwithstanding these strictures however, subtractive hybridisation yields fewer false positives than differential display (Carulli *et al.,* 1998a).

3.1.3.5.1Combined subtractive and display techniques

Differential display and subtractive hybridisation have been used in combination in differential subtractive display, and in subtracted differential display (reviewed in Pardinas *et al.,* 1998). In the former, the amplicons (tags) are electrophoresed as in normal differential display, followed by excision of differentially expressed bands. These are amplified by PCR, the 'driver' sample biotinylated, and used in a single round of subtractive hybridisation. After streptavidin extraction of driver species, the subtraction product is amplified by PCR prior to further analysis, as in normal subtractive hybridisation. In subtracted differential display, two populations of cDNA are first compared by subtractive hybridisation, and the enriched cDNAs subjected to differential display. Both techniques give a similar enhancement of reproducibility, increase in sensitivity, and reduction in false positives compared with differential display.

3.1.4 Aims and objectives of the investigation

There is a relative lack of molecular markers for cells early in the osteoblastic lineage. Consequently study of the normal process of osteoblastic differentiation, and its perturbation in disease, particularly in osteoporosis, has been hampered. Since a proportion of cases of osteoporosis involve a failure of recruitment of osteoblasts from the precursor pool it is important to understand the normal process if one is to unravel its alteration in osteoporosis. In order to facilitate study of these processes it is necessary to identify stage specific markers of early osteoblastic differentiation. In particular, genotypic markers will not only enable identification of early cells, but are also likely to prove more useful for understanding the processes controlling early osteoblastic differentiation than phenotypic markers, such as STRO-1 and HOP-26, since, with the exception of SB-10, such markers cannot be directly related to molecular events, whilst genotypic markers by definition can.

The overall aim of the investigation was therefore to identify genes involved in early osteoblastic differentiation of bone marrow stromal cells, in order to provide genotypic markers that could be used to identify both the cells and the processes controlling their differentiation. This was achieved using subtractive hybridisation to isolate genes differentially expressed during early osteoblastic differentiation of primitive bone marrow

stromal cells. Clonal culture of primary human cells was used to eliminate the possibility that the genetic changes identified were due to immortalisation, to intrinsic genetic heterogeneity in the cell populations used, or applicable only to an animal species. Since such cultures produced very low numbers of cells poly (A) RT-PCR was used to amplify the mRNA species present. Additionally, initial cell sorting for the STRO-1 epitope was used to enrich the cultures for cells early in the osteoblastic lineage.

Specifically the objectives of the investigation were:

1) To generate primary, clonal cultures of STRO-1 positive human bone marrow stromal cells,
2) To stimulate osteoblastic differentiation of these clones and extract mRNA from them at a variety of time points during the process,
3) To isolate, using subtractive hybridisation, genes whose expression was upregulated during differentiation,
4) To identify the nature of these genes, and to verify their importance for osteoblastic differentiation by analysis of their temporal and spatial expression.

3.2 Methods

Materials were obtained from the following companies, unless otherwise stated: culture media, Gibco (Paisley, U.K.); plasticware, Costar (Cambridge, U.S.A.) and Nunc (Illinois, U.S.A.); micropipettes, Gelman (Ann Arbour, U.S.A.); reagents and immunochemicals , Sigma (Poole, U.S.A.). Fluorescence activated cell analysis was carried out using a Beckton Dickinson FAC Scan, and magnetic activated cell sorting using a Miltenyi Biotech Minimacs kit. Supernatant from the STRO-1 hybridoma was obtained from the Developmental Studies Hybridoma Bank, maintained by the Department of Pharmacology and Molecular Sciences, John Hopkins University School of Medicine, Baltimore, and the Department of Biological Sciences, University of Iowa.

3.2.1 Cell culture

3.2.1.1 Primary human bone marrow stromal cell culture

Human bone marrow (0.5 to 1ml) was harvested from the sterna of consenting normal adult volunteers at the time of median sternotomy during cardiac surgery and density gradient centrifugation over a histopaque-1077 layer used to isolate bone marrow stromal cells (Cheng *et al.*, 1994). The marrow was placed directly into 10ml of alpha modified minimal essential medium (αMEM), without addition of supplements, at the time of

harvest and immediately transported to the laboratory. All manipulations of the tissue were carried out in a class II biological cabinet. The marrow was removed from the transport medium using aseptic technique, placed in a small sterile petri dish and finely diced using a scalpel. The marrow was then resuspended in the transport medium and centrifuged at 500xg for 10 minutes at room temperature. The supernatant, including fat present at the top, was removed and the pellet resuspended in 5ml of αMEM in a 15ml centrifuge tube. Five millilitres of histopaque-1077 was added to the tube, beneath the medium, and the tube centrifuged at 500xg for 30 minutes. Following this a thin grey layer of cells, containing the mononuclear cells was present at the interface of the medium and the histopaque-1077, whilst fragments of bone and erythroid cells, including erythroid precursors, were present in the pellet at the base of the tube which was discarded. The mononuclear layer was removed by gentle aspiration, seeded in a $25cm^2$ culture flask, and 5ml of alpha modified minimal essential medium (αMEM), supplemented with 10%(v/v) heat inactivated fetal bovine serum, 1%(v/v) glutamine, 1%(v/v) ascorbate, 1%(v/v) penicillin, streptomycin and amphotericin and 0.1%(v/v) gentamicin (hereon designated "standard medium") added. The cells were incubated at $37^{O}C$ in 5% carbon dioxide and were left to settle for 1 week after which the medium was removed. The cells were washed briefly with phosphate buffered saline (PBS(A)) to remove non-adherent cells and fresh medium added. The medium was changed every 2-3 days thereafter. Adherent cells were left to proliferate for 2 weeks, after which time they were nearly confluent. They were then removed by cell scraping and their capacity for osteoblastic differentiation tested both histochemically and biochemically.

3.2.1.2 Culture of control cells

In addition, the following cells were cultured for use as controls. HCC1, an immortalised early human bone marrow stromal cell line, which has capacity for osteoblastic and adipocytic differentiation was obtained from Dr B Ashton, Oswestry and cultured in standard medium at $37^{O}C$ (Brown, 1999). Medium was changed every 2-3 days and the cells were subpassaged weekly (1:3 split). Osteoblasts were grown from samples of trabecular bone, harvested during hip and knee joint revision surgery. Bone samples were cut into 1 to 3mm large chips, washed in serum free alpha-MEM to remove marrow, and incubated for 30 minutes at $37^{O}C$ in serum free medium with crude collagenase (2mg/ml). The medium was discarded and the chips incubated in new crude collagenase at the same concentration for 2 hours at $37^{O}C$. Collagenase-released cells were removed from the supernatant and resuspended in αMEM. The chips were rinsed three times with αMEM, and each rinse added to the collagenase released cells. The collagenase-released cells/rinse

suspension was centrifuged at 150xg for 12 minutes and the pellet resuspended in alpha-MEM, followed by centrifugation at 150xg for 12 minutes. The pellet was resuspended in standard medium, the cells seeded and left to adhere for 1 week, after which the medium was changed every 2-3 days. A human fetal fibroblast cell line was obtained from Mr D Coupes, Manchester Royal infirmary, and cultured in standard medium, with medium changes every 2-3 days. An immortalised osteosarcoma cell line, SaOS-2 was obtained from ATCC (Maryland, U.S.A.) and cultured in McCoy's 5A medium.

3.2.1.3 Histochemical measurement of alkaline phosphatase expression

Adherent cells were released with trypsin/ethylenediaminetetraacetate (EDTA), reseeded in 4 well chamber slides and stimulated for 2 weeks with standard medium or with standard media supplemented with either dexamethasone (10^{-7}M), β-glycerophosphate (10mM), or both. The morphology of the cells in each well was noted and the slides stained cytochemically for alkaline phosphatase using an azo-dye method. Briefly the medium was drawn off and the cells fixed in acetone for 10 minutes at room temperature. The substrate, napthol AS-BI phosphate, was dissolved in 0.05M Tris buffer (pH 9.0) to give a final concentration of 0.1mg/ml, and the diazonium salt, fast blue RR salt, added at 1mg/ml, to give the assay mixture. The fixed cells were incubated with the assay mixture at 37^{O}C for 10 minutes, alkaline phosphatase giving a blue reaction product. The amount of mineralised tissue formed in each culture was estimated by staining fixed cells with the calcium stain Alizarin Red S at pH 4.2 for 5 minutes.

3.2.1.4 Biochemical measurement of alkaline phosphatase expression

After two weeks in culture adherent cells were split in two and cultured in 25cm^2 flasks for 2 weeks with standard medium or with standard media supplemented with dexamethasone (10^{-7}M). For each flask the amount of alkaline phosphatase was measured, the number of cells in each flask counted and the level per 1000 cells calculated. Alkaline phosphatase was measured in both by the hydrolysis of p-nitrophenyl phosphate. The cells were fixed with ethanol, incubated for 10 minutes at 37^{O}C with p-nitrophenyl phosphate and the amount of p-nitrophenol in the supernatant measured spectrophotometrically at 410 nm.

3.2.1.5 Measurement of STRO-1 expression

STRO-1 expression in the HCC1 cell line and the adherent stromal cells was assessed by fluorescence activated cell analysis (FACS), after 1, 2 and 6 weeks in primary culture. The cells released using trypsin / EDTA, washed in phosphate buffered saline (PBS) and resuspended in 0.5ml of STRO-1 supernatant, containing from 50 to 75μg/ml antibody, in which they were incubated for 1 hour at 37^{O}C. The cells were washed in PBS and resuspended in 0.5ml secondary antibody (rabbit IgG anti mouse IgM, FITC-isomer 1 conjugated, dilution 1:256) and incubated for 1 hour at 37^{O}C. The cells were then washed and fixed in 0.6ml of 2%(v/v) paraformaldehyde. Controls, omitting either both of the antibodies or the STRO-1 antibody, were used. The cell suspensions were analysed using a fluorescence activated cell analyser.

3.2.2 Production of STRO-1 positive clones

Magnetic activated cell sorting (MACS) was used to produce populations enriched in STRO-1 positive cells. Once nearly confluent (10 days post primary harvest), adherent cells were released with trypsin / EDTA and reacted with STRO-1 supernatant, followed by incubation with isomer 1 FITC labelled rabbit anti-mouse IgM(1:256), as described above. They were washed in PBS, resuspended in 100μl MACS buffer (PBS pH7.2, supplemented with 0.5%(v/v) bovine serum albumin and 2mM EDTA) and incubated with 0.5μl MACS anti-FITC microbeads at 4^{O}C for 15 minutes. The cells were then washed in PBS, resuspended in 500μl of MACS buffer, and passed through a separation column held in a MACS magnet. Negative cells were washed through with MACS buffer, after which the column was removed from the magnet and positive cells eluted with 1ml MACS buffer. The positive fraction was resuspended in standard medium and reseeded in a 6 well plate at low cell density (10 cells/cm^{2}) to produce clones.

In order to verify the nature of the enrichment produced by the MACS, an initial sort was carried out using the immortalised human bone marrow stromal cell line, HCC1, which is known to express STRO-1 (Brown, 1999). HCC1 cells were reacted with STRO-1 and subjected to MACS as detailed above. The positive and negative elutants from the MACS were collected and subjected to FACS, as described for the stromal cells.

3.2.2.1 Stimulation of osteoblastic differentiation

STRO-1 positive clones were identified 5 days after seeding at low density (10cells/cm^2). They were composed of between 50 to 100 cells and were widely separated from each other. They were isolated using glass cloning rings and released with trypsin/EDTA. Each clone was resuspended in standard medium and subpassaged into 6 wells of a 24 well plate. The cells were left for 24 hours to adhere, after which time the cells were removed from one well (T0), and the remainder stimulated by addition of dexamathasone (10^{-7}M). Cells were removed from the remaining wells after 1(T1), 2(T2), 3(T3), 7(T7), and either 10(T10) or 14(T14) days of stimulation with dexamethasone (10^{-7}M).

3.2.3 Poly (A) RT-PCR and verification of osteoblastic differentiation

Due to the strictures of subcloning primary cultures and the short time course of the protocol, it was only possible to remove very few cells (1-4) at each time point. In order to overcome this, mRNA was amplified from each time point using poly (A) RT-PCR, which is capable of amplifying mRNA from as little as one cell (Brady & Iscove, 1993) (figure 3.5), whilst preserving the relative abundance of each mRNA species amplified.

3.2.3.1 Poly (A) reverse-transcription polymerase chain reaction

Poly (A) RT-PCR was performed as previously described (Brady & Iscove, 1993). Briefly, cells (1-10 / time point) were released at each time point with trypsin/EDTA, resuspended in 50µl standard medium, centrifuged for 5 minutes at 1,000xg and resuspended in 4µl of pre-chilled first strand buffer (50nM Tris-HCl pH 8.3, 75mM KCl, 3mM $MgCl_2$, 2mM of each dNTP[A,T,G,C], 100ng/ml Inhibit Ace, 200units/ml RNAguard, 0.5%(v/v) NP-40). This was left on ice for 10 minutes, after which it was heated to 65^{O}C for 1 minute, allowed to cool to room temperature for 3 minutes and then returned to ice. Ten units of Reverse Transcriptase (Boehringer Mannheim) were added to each sample. The samples were incubated at 37^{O}C for 15 minutes and the reaction stopped by heat inactivation at 65^{O}C for 10 minutes, after which the samples were returned to ice. This step was time limited to 15 minutes to restrict the length of the cDNA to about 300-700bp. In the second step, a 3` oligo(dA) tail was added to the cDNA using terminal transferase. An equal volume of 2X Tailing buffer (200mM potassium cacodylate pH 7.2, 4mM $CoCl_2$, 0.4mM DTT, 200mM dATP) and 1 unit of Terminal Transferase (Boehringer Mannheim) were added to the cDNA and incubated for 30 minutes at 37^{O}C, followed by heat inactivation at 65^{O}C for 10 minutes. The DNA is now defined at both ends and amenable to amplification

1 - cDNA preparation

---------- AAAAAAA mRNA
←——— /TT TTTT T cDNA-1
primer

2 - Poly (A) tailing

---------- AAAAAAA mRNA
AAAAAAA----------TTTTTTTTT cDNA-1

3 - PCR amplification

1st cycle

PCR Primer
X-TTTTTTT----------AA cDNA-2
AAAAAAA----------TTTTTTT cDNA-1

2nd cycle

X-TTTTTTT----------AAAAAA cDNA-2
AAA----------T TTTTT-X cDNA-3
PCR Primer

Figure 3.5 Summary and outline of poly (A) amplification of mRNA (Adapted from Brady & Iscove 1993). X-TTT = poly (T) primer, with defined oligonucleotide tail (X) containing restriction / cloning site. 1-First strand cDNA is synthesised from mRNA by reverse transcription using a poly (T) primer hybridised to the poly (A) at the 3'end of each mRNA species, 2-First strand cDNA is poly adenylated at its 3'end, rendering it suitable for further amplification (3) by PCR using a poly (T) primer.

by PCR using a single oligo(dT) containing primer. Polymerase chain reaction (PCR) was carried out using the following mix: 10µl Taq Polymerase buffer (100mM Tris-HCl pH 8.3, 500mM KCl, 25mM $MgCl_2$, 1mg/ml bovine serum albumin), added to a final concentration of 1 OD_{260}/ml of NOT 1 d(T) oligo (GCG GGC CGC $(T)_{24}$), (Oswel DNA Synthesis), 25mM dNTP's (Boehringer Mannheim), and sterile distilled water to 49µl, with Taq polymerase (Boehringer Mannheim) to 5 units/50µl. Half the volume of the product of the terminal tailing reaction i.e. 5-6µl, was used at template in the PCR. Samples were initially denatured for 30 seconds at 94°C, and then amplified for 35 cycles of 15 seconds at 94°C, 20 seconds at 42°C and 30 seconds at 72°C in an Idaho Technology rapid thermal cycler. The size range of the poly (A) PCR products (300 – 700 bp) was confirmed by electrophoresis in a 1.5%(w/v) agarose gel. Poly (A) cDNA products were generated from the osteoblast-like, fibroblast and osteosarcoma cultures using the same protocol as above.

3.2.3.1.1 Verification of efficiency of poly (A) PCR

In order to establish the efficiency of poly (A) PCR for amplification of mRNA from as little as a single cell, which contains approximately 25pg of RNA, known amounts of RNA, extracted from cultures of SaOS-2 cells, were serially diluted to 25pg/µl, amplified by poly (A) PCR and hybridised with a 3'cDNA probe to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

3.2.3.1.2 Extraction of mRNA from SaOS-2 cells

Confluent cultures in T75 (surface area 75cm^2) flasks were generated, as detailed in 3.2.1.2. The cells were washed three times with diethyl pyrocarbonate (DEPC) treated PBS(A) and harvested by scraping into 1.5ml RNAzol (Biogenesis), in which they were stored at minus 80°C until use. Following gently thawing on ice a tenth volume i.e. 150µl of cold chloroform was added to the cells which were then vortexed for 15 s and placed at –20°C for 15 min. They were then centrifuged at 4°C at 15,000xg for 15 min and the clear upper phase (approximately 800µl) transferred to a new 1.5ml eppendorf tube and an equal volume of cold isopropanol added. The mixture was then vortexed for 15 s and placed at –20°C for 15 min. It was then centrifuged again at 4°C at 15,000xg for 15 min, after which a glassy pellet was present at the bottom of the tube. This was washed twice with 1ml of 75%(v/v) cold ethanol, at 8,000xg at 4°C for 15 min each. The pellet was air dried for 10 min and resuspended in 20µl of DEPC treated distilled H_2O.

3.2.3.1.3 Quantitation of mRNA

The amount of RNA present in the sample was measured spectrophotometrically using an Hitachi spectrophotometer, according to the manufacturer's instructions. Briefly, 1µl of the sample was resuspended in 1ml of DEPC treated distilled H_2O, mixed and, after the spectrophotometer had been zeroed against DEPC treated distilled H_2O, the absorbance measured at 260nm and 280nm. The ratio of the absorbance at the two wavelengths indicated the purity of the RNA present, whilst that at 260nm was multiplied by 40 to obtain the amount, in µg, present in the 1µl sample used.

Following quantitation, the purity of the RNA present in the sample was assessed by subjecting 10µg, approximately 2µl, to electrophoresis on a 1.5%(w/v) agarose gel, prepared using DEPC treated solutions and run in DEPC treated buffer. The sample was loaded on the gel with 2µl of RNA loading buffer (40%(v/v)glycerol, 0.2M EDTA, 2mg/ml bromophenol blue) and, after electrophoresis, visualised by trans-illumination with ultra-violet light.

3.2.3.1.4 Poly (A) reverse-transcription polymerase chain reaction

The RNA in the sample was serially diluted, with DEPC treated distilled H_2O, to provide aliquots at concentrations of 1µg/µl, 500ng/µl, 1ng/µl, 500pg/µl, 100pg/µl, 50pg/µl and 25pg/µl. From each of these 1µl was used as template in a poly (A) reverse-transcription polymerase chain reaction, as detailed in 3.2.3.1, by addition to 4µl of pre-chilled first strand buffer; the remainder of the protocol used was the same as described in section 3.2.3.1.

3.2.3.1.5 Southern blotting of cDNA products

The cDNA products generated by poly (A) reverse-transcription polymerase chain reaction of each aliquot were each suspended in loading buffer and loaded onto a 0.8%(w/v) agarose gel. They were electrophoresed briefly, in order to minimise differential migration of the different band-widths, and the quantity of cDNA present in each lane compared visually by trans-illumination with ultra-violet light. The quantities of cDNA in each lane were adjusted, and further gels loaded with the cDNA pools until each contained similar amounts. Once this was achieved, the gel was used in Southern blotting, using a vacuum blotter. Briefly, a piece of nitrocellulose nucleic acid transfer membrane (Hybond N) was pre-soaked in 10x standard saline citrate (SSC) [1.5M NaCl, 0.15M trisodium citrate] and then placed on the bed of the vacuum blotter, taking care not to introduce any air bubbles between the membrane and the bed. A plastic sheet, with an aperture cut in it to allow

passage of the cDNA from the gel to the membrane, was placed over the bed, with the aperture over the membrane. The edges of the sheet extended to the sides of the vacuum aparatus, and the aperture within it was slightly smaller than the gel, so as to form an air-tight seal. The gel was then placed over the aperture in the plastic sheet, such that the lanes containing the cDNA lay over, and were in direct contact with, the nitrocellulose membrane, taking care not to introduce any air bubbles between the membrane and the gel. The gel was then covered with denaturing solution (1.5M NaCl, 0.5M NaOH) and the vacuum pump turned on. The denaturing solution was drawn through the gel by the resultant vacuum in approximately 20 minutes, after which any excess solution was removed with a pipette and the gel covered with neutralising solution (1.5M NaCl, 0.5M Tris-HCl pH 7.0). This was left to be drawn through the gel for 20 minutes after which the gel was the covered with 4x SSC, which was also exposed to vacuum for 20 minutes. Following this the nitrocellulose membrane was removed, air-dried for 20 minutes and the cDNA upon it crosslinked by exposure to ultraviolet (UV) light. Efficiency of removal of the cDNA from the gel was assessed by trans-illumination of the gel with UV light.

3.2.3.1.6 Prehybridisation of cDNA Southern blots

Prehybridisation mixture was freshly prepared [50% deionised formamide(v/v), 4x SSC(v/v) , 0.1% SDS(v/v), 1x Denhardts(Sigma)(v/v), and 1%(w/v) herring sperm DNA (Sigma)] and denatured by boiling in a Falcon tube. Twenty millilitres of prehybridisation mixture was used. Following boiling the mixture was cooled rapidly on ice prior to addition to the cDNA Southern blot, which had been pre-soaked in 10xSSC for 10 minutes. The blot was incubated in the prehybridisation mixture, in a Techne tube with continuous agitation, overnight at $42^{O}C$.

3.2.3.1.7 Isotopic labelling of cDNA GAPDH probe

Purified cDNA GAPDH probe (prepared as detailed below in section 3.2.3.2) was thawed and 100ng (1 to 2µl) removed for labelling, added to 10µl of primer, boiled at $100^{O}C$ for 5 minutes in a water bath and then returned to room temperature. Eight microlitres each of dATP, dGTP and dTTP were added, followed by addition of 10µl of P^{32} labelled dCTP and 4µl of Klenow fragment. The reaction mixture was incubated at $37^{O}C$ for 1 hour and the reaction terminated by addition of 5µl of 0.2M EDTA. Unincorporated nucleotides were removed by passage through push columns and purified probe eluted with STE (sucrose/Triton/EDTA) buffer and stored at $-20^{O}C$ prior to use.

3.2.3.1.8 Hybridisation of cDNA Southern blots

The labelled cDNA GAPDH probe was thawed and added to 20ml of fresh prehybridisation mixture in a Falcon tube. The prehybridisation/probe mix was denatured by boiling for 15 minutes, cooled rapidly on ice prior to addition, in a Techne tube, to the prehybridised cDNA Southern blots, and hybridisation carried out overnight at 42^{O}C with continuous agitation.

3.2.3.1.9 Post Hybridisation washes and visualisation of hybridisation signal

After hybridisation the dot-blot filters were washed at increasing stringency in 2xSSC / 0.1% sodium dodecyl-sulphate (SDS) for 30 minutes at 37^{O}C, followed by 0.5xSSC / 0.1%SDS for 30 minutes at 55^{O}C, 60^{O}C, 65^{O}C and 70^{O}C. The radioactivity of the filters was monitored and hybridisation signal viualised using a phospho-imager plate (read on a Basix bioimager) between washes. Following final stringency washing the filters were placed against autoradiographic film in radiographic cassettes (fitted with intensifier screens) and stored at –80^{O}C for between 2 to 7 days. The autoradiographs were developed in developer for 5 minutes, rinsed in cold tap water for 5 minutes and fixed in fixer for 5 minutes. They were then rinsed once more in cold tap water and air-dried.

3.2.3.2 Assessment of osteoblastic gene expression of STRO-1 positive clones

In order to verify the osteoblastic nature of the clone the amplified mRNA products from each time point were hybridised with isotopically labelled 3` end cDNA probes to collagen type I, osteocalcin and osteopontin. All cDNA probes were designed to the 3′ untranslated region and verified by both restriction analysis and sequencing (Brown, 1999); GAPDH was used as a housekeeping gene.

3.2.3.2.1 Generation of cDNA probes to collagen type I, osteocalcin, osteopontin and GAPDH

Probes were prepared by cloning PCR products into a plasmid vector. Positive recombinations were identified, picked with an inoculation loop and incubated overnight at 37^{O}C, with agitation, in 10 ml of L-broth (10g/l bacto-tryptone, 5g/l bacto-yeast extract, 10g/l NaCl in 1l distilled H_2O) supplemented with freshly thawed stock ampicillin to a final concentration of 12 μg/ml. After incubation 2ml of the culture was taken for glycerol stock and the plasmid extracted from the remainder by a mini-prep protocol.

3.2.3.2.1.1 Preparation of glycerol stocks

The culture was centrifuged at 10,000xg for 5 minutes and the supernatant removed. The pellet was resuspended in 1ml of fresh L-broth, with addition of 250µl of 10x Hogness buffer, mixed by gentle inversion and stored at –80^{O}C.

3.2.3.2.1.2 Mini-prep extraction of plasmid

Plasmid was isolated using a Qiagen plasmid mini-prep kit. The culture was centrifuged at 8,000xg for 5 minutes, the supernatant removed and the pellet resuspended, in a 2ml eppendorf tube, in 500µl of P1(resuspension) buffer (50mM Tris-HCl, pH 8.0; 10mM EDTA; 100µ g/ml RNase A)(Qiagen), followed by addition of 500µl of P2 (lysis) buffer (200mM NaOH, 1% SDS), which was mixed by gentle inversion. This was incubated for 5 minutes at room temperature and 500µl of P3 (neutralisation) buffer (3.0m potassium acetate, pH 5.5) added, followed by incubation on ice for 5 minutes. The sample was then centrifuged at 18,000xg for 10 minutes and, taking care not to dislodge the pellet, the supernatant removed and re-centrifuged at 18,000xg for 10 minutes. The supernatant was then run through a freshly equilibriated anion-exchange resin column (Qiagen-tip 20), which was then washed with 4x 1ml washes with QC buffer (850mM NaCl, 50mM Mops, pH 7.0); columns were equilibrated by flushing with 1ml of QBT buffer (400mM NaCl, 40mM Mops, pH 7.0). The plasmid was then eluted from the column with 800µl of QF buffer (1.2M NaCl, 50mM Mops, 1.5% (v/v) ethanol, pH 8.0) and precipitated by addition of a 0.1 x volume of 3M NaAc (80µl) and a 0.8 x volume (640µl) of isopropanol, followed by storage on ice for 20 minutes and centrifugation at 4^{O}C for 15 minutes. The pellet was washed with 70%(v/v) ethanol, followed by re-centrifugation to facilitate removal of the ethanol and air-drying. The pellet was air-dried for 5 minutes, resuspended in 12µl of distilled H_2O and the quantity of plasmid present assessed by electrophoresis of 2µl (suspended in 2µl of loading buffer) on a 1.5% (w/v) agarose gel containing 5% (v/v) ethidium bromide. Purified plasmid was stored at minus 20^{O}C.

3.2.3.2.1.3 Restriction digestion of purified plasmid

Restriction digestion was used to excise the cDNA probe from the plasmid. Restriction digests were set up using 1µl of plasmid as template together with 2.5µl each of the appropriate restriction enzyme and buffer, and made up to a final reaction volume of 25µl with distilled H_2O. The reaction mixture was incubated at 37^{O}C for 2 hours and the reaction terminated by addition of 5µl of loading buffer, followed by electrophoresis on a 0.8% (w/v) agarose gel. Visualisation under UV light was used to confirm restriction

digestion and the lower band, representing the excised cDNA probe, cut from the gel using a new scalpel blade. The cDNA was extracted from the excised portion of gel by centrifugation through glass wool at 18,000xg for 10 minutes, followed by addition of a $1/10^{th}$ volume of 3M NaAc and a 2.5x volume of 100% ethanol and overnight precipitation at $-20^{O}C$. The DNA was then pelleted by centrifugation at 18,000xg for 15 minutes, the pellet washed with 70%(v/v) ethanol, air-dried for 5 minutes and resuspended in 12µl of distilled H_2O. The quantity of cDNA present was assessed by electrophoresis of 2µl (suspended in 2µl of loading buffer) on a 1.5% (w/v) agarose gel containing 5% (v/v) ethidium bromide. Purified cDNA was stored at $-20^{O}C$.

3.2.3.2.2 Isotopic labelling of cDNA probes

Purified cDNA was thawed and 100ng (1 to 2µl) removed for labelling, added to 10µl of primer, boiled at $100^{O}C$ for 5 minutes in a water bath and then returned to room temperature. Eight microlitres each of dATP, dGTP and dTTP were added, followed by addition of 10µl of P^{32} labelled dCTP and 4µl of Klenow fragment. The reaction mixture was incubated at $37^{O}C$ for 1 hour and the reaction terminated by addition of 5µl of 0.2M EDTA. Unincorporated nucleotides were removed by passage through push columns and purified probe eluted with STE buffer and stored at $-20^{O}C$ prior to use.

3.2.3.2.3 Preparation of cDNA dot-blots

Two microlitres of each of the cDNA samples from the time points, i.e. T0 to T10 or T14, for each clone were heat denatured at $100^{O}C$ for 5 minutes, cooled on ice and dot blotted onto hybond N filters. The filters were then air-dried for 20 minutes, crosslinked with ultra-violet (UV) light and stored prior to use. Additionally, probe cDNA, cDNA from fibroblasts, trabecular bone and osteosarcoma cells, and poly (A) PCR control cDNA were treated in the same manner and dot-blotted onto each filter as positive and negative controls respectively. Each filter was then probed with the same probe as the cDNA probe control that it bore, separate filters having been made for each probe.

3.2.3.2.4 Prehybridisation of cDNA dot-blots

Prehybridisation mixture was freshly prepared [50% deionised formamide(v/v), 4x SSC(v/v), 0.1% SDS(v/v), 1x Denhardts(Sigma)(v/v), and 1%(w/v) herring sperm DNA (Sigma)] and denatured by boiling in a Falcon tube. Twenty millilitres of prehybridisation mixture was used for each dot-blot. Following boiling the mixture was cooled rapidly on ice prior to addition to the cDNA dot-blots, which had been pre-soaked in 10xSSC for 10

minutes. The dot-blots were incubated in the prehybridisation mixture, in Techne tubes with continuous agitation, overnight at 42^{O}C.

3.2.3.2.5 Hybridisation of cDNA dot-blots with cDNA probes

Labelled cDNA probes were thawed and added to 20ml of fresh prehybridisation mixture each in Falcon tubes. The prehybridisation/probe mix was denatured by boiling for 15 minutes, cooled rapidly on ice prior to addition, in Techne tubes, to the prehybridised cDNA dot-blots, and hybridisation carried out overnight at 42^{O}C with continuous agitation.

3.2.3.2.6 Post Hybridisation washes and visualisation of hybridisation signal

After hybridisation the dot-blot filters were washed at increasing stringency in 2xSSC / 0.1%SDS for 30 minutes at 37^{O}C, followed by 0.5xSSC / 0.1%SDS for 30 minutes at 55^{O}C, 60^{O}C, 65^{O}C and 70^{O}C. The radioactivity of the filters was monitored and hybridisation signal visualised using a phospho-imager plate (read on a Basix bioimager) between washes. Following final stringency washing the filters were placed against autoradiographic film in radiographic cassettes (fitted with intensifier screens) and stored at –80^{O}C for between 2 to 7 days. The autoradiographs were developed in developer for 5 minutes, rinsed in cold tap water for 5 minutes and fixed in fixer for 5 minutes. They were then rinsed once more in cold tap water and air-dried.

3.2.3.2.7 Polymerase chain reaction for detection of collagen type I

In addition to hybridisation of the cDNA from each time point for each clone, polymerase chain reaction amplification of cDNA from each time point was performed using primers for the 3' region of collagen type I (Forward: 5'-TCCTTGTTTTGTTTTGTTCAT-3', Reverse:5-TGCTTTATTTCATTTTTTTTTTCA-3'). For each time point 1µl of test cDNA was added to a 50µl reaction with a final concentration of 0.5x Taq buffer (2mM $MgCl_2$), 10mM dNTPs, 1.5µl (100pM) each of forward and reverse primers, and 1U of Taq polymerase. Samples were initially denatured by 30s at 94^{O}C and then amplified by 40 cycles of 15s at 94^{O}C, 20s at 42^{O}C, and 30s at 72^{O}C in an Idaho Technology rapid thermal cycler. The products were visualised on a 1.5%(w/v) agarose gel.

3.2.4 Subtractive hybridisation to isolate genes upregulated during early osteoblastic differentiation

Analysis of the above hybridisation studies demonstrated upregulation of osteocalcin at day 4 in clone 1 (see section 3.3.3.2 for details). Subtractive hybridiation was therefore

used to isolate a pool of poly (A) cDNAs encoding mRNA differentially expressed specifically by cells at 4 days (T4), compared to 0 days (T0).

3.2.4.1 Amplification of driver (T0) and tracer (T4) cDNA pools

Several rounds of poly (A) PCR, using 1µl of T0 or T4 cDNA as template, as described in section 3.2.3.1, were used to generate a 20 fold excess of T0 cDNA (driver) to T4 cDNA (tracer). Specifically, 20 x 50µl reactions were used to amplify tracer cDNA and 80 x 50µl reactions to amplify driver cDNA. The quantity present after amplification was assessed crudely, prior to more precise quantitation after purification, by electrophoresis on a 1.5%(w/v) gel.

3.2.4.2 Purification of driver and tracer cDNA pools

The same protocol was used for purification of both driver and tracer cDNA pools. Amplified driver poly (A) cDNA was pooled. The 80 x 50µl reactions were pooled in glass hydridisation bottles, using 10 reactions / bottle. The final volume in each bottle was made up to 0.8ml by addition of 300µl of distilled H_2O and 2ml of QBT buffer added to each bottle. The contents of each bottle was then loaded onto a Qiagen anion-exchange resin column (freshly flushed with 4ml of QBT buffer) and let to run through. Each column was then flushed with 6 x 1ml of QC buffer, following which the cDNA was eluted from the column into a 2ml eppendorf tube by addition of 1.6ml of QF buffer to the column. The elutant was split into 2 equal fractions (0.8ml) and the cDNA precipitated by addition of a 0.1 x volume of 3M Na Ac and 0.8 x volume of isopropanol, followed by storage on ice for 20 minutes and centrifugation at $4^{O}C$ for 15 minutes. The resultant pellet was washed with 70% ethanol, air-dried and resuspended. The driver cDNA was resuspended in 30µl of HE (10mM HEPES (*N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid]), 1mM EDTA) buffer (pH 8.0) and the tracer cDNA in 30µl of distilled H_2O.

3.2.4.3 Quantitation of purified driver and tracer cDNA

Each of the purified fractions of driver and tracer cDNA was quantitated both by visualisation after gel electrophoresis and spectrophotometrically. A 1.5%(w/v) agarose gel was used. For each fraction, 2µl of cDNA was suspended in 2µl of loading buffer; DNA ladder was used to verify cDNA size. Spectrophometric quantitation was carried out on a Hitachi spectrophotometer, using UV solutions analysis software. For each fraction 1µl of cDNA was resuspended in 1ml of distilled H_2O and the absorbance at 260nm and 280nm measured and multiplied by 50 to give the quantity of cDNA present in the sample in µg/ml.

3.3.4.4 Photobiotinylation of purified driver cDNA

Photobiotin (Sigma) was resuspended in distilled H_2O at 1mg/ml, aliquoted (40µl) in opaque eppendorf tubes and stored at $-20^{O}C$ prior to use. Each fraction of purified driver cDNA (approximately 25µl) was transferred to a 1.5ml eppendorf tube, boiled for 2 minutes and then placed on ice. To each fraction 40µl of photobiotin (1mg/ml) was added and mixed by gently flicking the side of the tube. The tube was then placed open on ice, in a vertical position, 10 cm directly beneath a GE sunlamp (250W) for 5 minutes, after which the reaction mixture was again gently mixed before returning the tube beneath the lamp for another 5 minutes. Forty microlitres of fresh photobiotin was added to the reaction mixture, followed by a final 5 minutes irradiation beneath the sunlamp. The reaction was then stopped by addition of an equal volume (105µl) of 200mM Tris-HCl (pH 9.0), followed by addition of 105µl of 7.5M Ammonium Acetate and 780µl of 100% ethanol. The fractions were then placed on ice for 15 minutes and centrifuged at 18,000xg for 15 minutes. The resultant pellet was washed with 80% ethanol, air-dried and resuspended in 20µl of HE buffer. Each fraction was then re-quantitated as detailed in section 3.2.4.3.

3.3.4.5 Hybridisation of driver and tracer cDNA

Subtractive hybridisation was carried out using a 20x fold excess of driver cDNA over tracer cDNA in four rounds of subtraction. For the first round 40 µl of driver cDNA (2ug), 2 µl of tracer cDNA (100ng), and 1 µl (2.5µg) of transfer RNA (tRNA) were mixed and the reaction mixture made up to a total volume of 80 µl with HE buffer. The reaction mixture was incubated at $100^{O}C$ for 2 min, followed by ethanol precipitation, by addition of 0.1 x volume of 3M NaAc (pH 5.2) and 2.5 x volume 100% ethanol, on ice. This was then centrifuged at $4^{O}C$ at 18,000xg for 15 minutes and the resultant pellet washed in 70% ethanol. This was resuspended in hybridisation mixture [3 µl of 3x Hyb buffer (50mM EPPS pH 8.5, 10mM EDTA pH 8.0, 0.3%(v/v) SDS), 3 µl 5M Na Cl, 3 µl 40%(v/v) PEG 8000], covered with mineral oil, and incubated in a hot block for 5min at $98^{O}C$, 5 min at $80^{O}C$, and 1hour at $60^{O}C$. Following this 90µl of freshly thawed, pre-heated ($68^{O}C$) extraction buffer (50mM EPPS pH 8.5, 0.5M NaCl, 5mM EDTA pH 8.0) was added to the reaction mixture, prior to removal from the hot block.

3.2.4.6 Phenol / chloroform extraction of cDNA

TE-saturated phenol/chloroform was prepared by the following method. Briefly, 700µl of phenol/chloroform was mixed with 700µl of TE buffer (pH 8.0), vortexed and centrifuged

for 1 minute at 10,000xg. The upper, aqueous, phase, was removed and a further 700µl of TE buffer mixed with the remaining phases. The mixture was again vortexed and centrifuged prior to removal of the aqueous phase, and the procedure repeated a further 3 times i.e. 4 times in total. Following the fourth removal of the aqueous phase the lower phase, which consists of TE-saturated phenol/chloroform, was retained for use, as detailed below.

The reaction mixture from section 3.2.4.5 was pulse centrifuged to combine all aqueous layers and the mineral oil removed from the top. The bottom aqueous layer, containing the reaction mixture and extraction buffer, was then removed, transferred to a new eppendorf tube, and a further 21µl of freshly thawed extraction buffer, and 4µl (4µg/µl) of streptavidin added. This was incubated at room temperature for 2 minutes, followed by addition of 100µl of TE-saturated phenol/chloroform, and then by centrifugation at 18,000xg at room temperature for 5 minutes. The top/aqueous phase was transferred to a new tube, taking care not to include any of the interphase layer, and 50µl of chloroform added, followed by further centrifugation with the same conditions. The top/aqueous phase was once again retained, and represented the first round of subtraction product (labelled S1). This fraction was split into two. Ten microlitres of it was stored at $-20^{O}C$, whilst the remainder was used in the second round of subtraction, in which it took the place of the tracer in the initial reaction mixture, which therefore contained approximately 60µl of S1 cDNA, 40µl of driver cDNA, and 1µl of tRNA; the reaction volume was approximately 100µl and no HE buffer was added. The procedure detailed above for the first round of subtraction was repeated for the second round, and the resultant subtraction product (labelled S2) again split into 2 fractions, one of which (10µl) was stored, whilst the other was used in the third round of subtraction, in the same way as detailed for the use of S1 in the second round. The process was repeated a fourth time and the subtraction product of the fourth round of subtraction (labelled S4) retained in its entirity.

3.2.4.7 Amplification of subtraction products

Each of the subtraction products (S1-S4) was amplified using poly (A) PCR (see section 3.2.3.1), using 1µl of each as template. The PCR products were visualised by gel electrophoresis as detailed in section 3.2.3.1.

3.2.4.8 Verification of subtractive hybridisation

In order to assess efficiency of the subtraction the subtraction products (S1-S4) were hybridised with isotopically labelled cDNA probes for the driver and S4 cDNA pools.

3.2.4.8.1 Southern blotting of cDNA pools

The driver, tracer and subtraction (S1-S4) cDNA pools were each suspended in loading buffer and loaded onto a 0.8%(w/v) agarose gel. They were electrophoresed briefly, in order to minimise differential migration of the different band-widths, and the quantity of cDNA present in each lane compared visually. The quanitities of cDNA in each lane were adjusted, and further gels loaded with the cDNA pools until each contained similar amounts. Once this was achieved, the gel was used in Southern blotting, using a vacuum blotter. Briefly, a piece of nitrocellulose nucleic acid transfer membrane (Hybond N) was pre-soaked in 10xSSC and then placed on the bed of the vacuum blotter, taking care not to introduce any air bubbles between the membrane and the bed. A plastic sheet, with an aperture cut in it to allow passage of the cDNA from the gel to the membrane, was placed over the bed, with the aperture over the membrane. The edges of the sheet extended to the sides of the vacuum aparatus, and the aperture within it was slightly smaller than the gel, so as to form an air-tight seal. The gel was then placed over the aperture in the plastic sheet, such that the lanes containing the cDNA lay over, and were in direct contact with, the nitrocellulose membrane, taking care not to introduce any air bubbles between the membrane and the gel. The gel was then covered with denaturing solution and the vacuum pump turned on. The denaturing solution was drawn through the gel by the resultant vacuum in approximately 20 minutes, after which any excess solution was removed with a pipette and the gel covered with neutralising solution. This was left to be drawn through the gel for 20 minutes after which the gel was then covered with 4xSSC, which was also exposed to vacuum for 20 minutes. Following this the nitrocellulose membrane was removed, air-dried for 20 minutes and the cDNA upon it crosslinked by exposure to UV light. Efficiency of removal of the cDNA from the gel was assessed by trans-illumination of the gel with UV light. Two Southern blots were prepared, one for hybridisation with driver cDNA and one for hybridisation with S4 cDNA.

3.2.4.8.2 Prehybridisation of cDNA Southern blots

Both of the Southern blots were prehybridised using the method detailed in section 3.2.3.2.4.

3.2.4.8.3 Isotopic labelling of cDNA probes

Isotopically labelled cDNA probes to driver and S4 cDNA pools were prepared using the method detailed in section 3.2.3.2.2, using 1μl of driver and S4 cDNA respectively as template.

3.2.4.8.4 Hybridisation of cDNA Southern blots

The Southern blots were hybridised, one with probe to driver and one with probe to S4, as detailed in section 3.2.3.2.5.

3.2.4.8.5 Post Hybridisation washes and visualisation of hybridisation signal

Post hybridisation washes were carried out at increasing stringency as detailed in section 3.2.3.2.6, and visualised by exposure to a phosphoimager plate.

3.2.5 Library Screening

A cDNA library was constructed from pooled mRNA extracted from HCC1 cells both before and during stimulation of both adipocytic and osteoblastic differentiation (as detailed in Brown, 1999)(Stratagene) and therefore contained cDNA representative of the total mRNA present in human marrow stromal cells undergoing osteoblastic differentiation. The library was prepared commercially using the lambda vector Zap Express II (Stratagene). The product of the fourth round of subtraction (S4) was labelled with P^{32} and used to screen approximately $50x10^3$ plaques of the library using standard plaque hybridisation techniques and duplicate filters exposed to auto-radiographic film. Clones which hybridised to the S4 product were isolated and purified by secondary screening.

3.2.5.1 Generation and packaging of library

The lambda Zap Express vector (Stratagene) has 12 cloning sites and will accommodate cDNA inserts up to 1.2 kb long. The HCC1 bone cDNA expression library had been prepared previously (Brown, 1999). It was packaged in this vector commercially and was constructed using RNA extracted, using standard techniques, from cultures of HCC1 cells prior to and during stimulation of adipocytic and osteoblastic differentiation.

3.2.5.2 Preparation of plating bacteria

A glycerol stock of MRF' (rec A^- E.coli host strain)(Stratagene) cells was thawed out on ice, streaked out onto LB tetracycline (12.5mg/ml) agar plates and incubated overnight at 37^oC. Single colonies were picked the following day and cultured overnight at 30^oC, with agitation, in 20ml of LB low salt medium (supplemented with maltose (0.2% w/v) and 10mM $MgSO_4$). The next day 500µl of the culture was removed, added to a cryogenic tube containing 500µl of glycerol and stored at -70°C, whilst the remainder was transferred to a fresh sterile 50ml falcon tube and centrifuged at 3,000xg for 10 minutes at room temperature. The cell pellet was gently resuspended in 10ml of 10mM $MgSO_4$, and the

cells diluted to OD_{600} = 0.5, with 10mM $MgSO_4$. The diluted cells were stored for up to 3 days at 4°C prior to use.

3.2.5.3 Titre of library

Agar plates (150mm diameter) were prepared (21g/l NYZ broth, 15g/l agar, 1l distilled H_2O) and left to set at room temperature for four days prior to use. Top agar was prepared (21g/l NYZ broth, 7g/l (0.7%w/v) agarose, 1l distilled H_2O), and autoclaved. The library was gently thawed on ice and serial dilutions of $1/10^1$, $1/10^2$, $1/10^3$, $1/10^4$, and $1/10^5$ prepared in SM (5.8g NaCl, 2.0g $MgSO_4.7H_2O$, 50ml 1M Tris-HCl pH 7.5, 5ml 2%(w/v) gelatin, all in 1l distilled H_2O) buffer. One microlitre of each of these was added a separate 200µl aliquot of MRF' cells (OD_{600}=0.5), mixed by gentle flicking and incubated at 37°C for 10 minutes. Following this each aliquot of infected MRF' cells was added to 7.5ml of pre-heated (55°C) top agar and rapidly poured and spread over the surface of an agar plate. The required number of agar plates were incubated at 37°C for 4 hours prior to use to prevent the top agar from solidifying before it had been evenly spread. Similarly the top agar was pre-heated to 55°C; higher temperatures will kill the MRF' cells. The plates were left for 10 minutes for the top agar to solidify and were then incubated overnight at 37°C. The following day the number of plaques on each plate were counted and the plaque-forming units per millilitre (pfu/ml) calculated:

$$\frac{\text{Number of plaques per plate x Dilution factor}}{\text{Volume of cells plated}} \times 1000$$

Where dilution factor = 10^1, 10^2, 10^3, 10^4, or 10^5
And volume of cells plated = 1 microlitre

At least 50,000 plaques are required to represent the entire library, therefore the number of 150mm plates needed to achieve this was calculated at each dilution.

3.2.5.4 Primary library screening

Once the optimum library titre, and number of plates, required to represent the library had been established the method detailed in section 3.2.5.3 was repeated at the chosen library dilution. The plates were incubated overnight as above and the following day the plates were sealed with Nesco film, inverted and stored at 4°C for 2 days in order to set prior to plaque lifting.

3.2.5.5 Plaque lifting

The plasmid DNA present in each plaque was transferred to circular (150mm diameter) nitrocellulose nucleic transfer membranes. For each plate of the primary screen two nitrocellulose membranes were used in order to produce duplicate filters. Each membrane was labelled and placed gently on top of the agar plate for 3 minutes and orientated with respect to the plate by puncture at a number of points with a sterile hypodermic needle, which was pushed into the agar beneath the puncture site. The position of the holes in the agar was then marked on the bottom of the plate with a permanent marker pen. The membrane was then transferred, DNA side up, onto a piece of 3mm Whatman filter paper soaked in 4xSSC. It was left on this for 3 minutes and then transferred, again for 3 minutes, onto filter paper soaked in denaturing solution, followed by transfer onto filter paper soaked in neutralising solution for a final 3 minutes, after which it was air-dried for 20 minutes prior to UV crosslinking; the membrane was kept DNA side up to this point. The procedure was repeated with a second nitrocellulose membrane for each plate, using the positions of the holes in the agar, marked on the bottom of the plate, to identify the location of orientation holes in the membrane, which were made in the membrane using a sterile hypodermic needle.

3.2.5.6 Prehybridisation of nitrocellulose filters

The nitrocellulose filters were placed in a plastic sandwich box and prehybridised with 250ml of fresh prehybridisation mixture. The prehybridisation mixture was prepared, boiled and ice-cooled prior to addition to the filters, which had been pre-soaked in 10xSSC for 10 minutes, as detailed in section 3.2.3.2.4.

3.2.5.7 Hybridisation of filters

Isotopically labelled probe to the S4 subtraction product was prepared as detailed in section 3.2.3.2.2, using 1µl of PCR amplified S4 product as template. The radiolabelled probe was added to fresh prehybridisation mixture and hybridised with the nitrocellulose filters as detailed in section 3.2.3.2.5.

3.2.5.8 Post Hybridisation stringency washes

Post-hybridisation washes were carried out at increasing stringency in 2xSSC / 0.1%SDS for 30 minutes at 37^{O}C, followed by 0.5xSSC / 0.1%SDS for 30 minutes at 55^{O}C, 60^{O}C, 65^{O}C and 70^{O}C, until the radioactive count for each filter had fallen to 100. The filters were then vacuum packed in plastic bags and exposed to autoradiographic film at –80^{O}C for 2 days.

3.2.5.9 Visualisation of hybridisation signal and identification of positive plaques

The autoradiographs were developed as detailed in section 3.2.3.2.6 and the autoradiographic images of each membrane orientated with respect to the puncture marks on the membranes by marking the position of the holes on the autoradiographs. This enabled orientation of the duplicate filters for each plate. Once this had been done a light box was used to trans-illuminate the autoradiographs, and hybridisation signals present in the same position on each of the duplicate images identified. These 'double positives' were marked on one of the autoradiographic images for each plate.

3.2.5.10 Isolation of positive plaques

The autoradiographic images were orientated with the plates, using the marks of the holes in the membranes marked on each of them, and, the plaques on the plates corresponding to the 'double positives' marked on the autoradiographs identified. These plaques were then picked from the plate using a pastuer pipette and transferred to SM buffer. Each plaque was transferred to a separate eppendorf tube containing 1ml SM buffer and 50µl chloroform, which was briefly vortexed to dislodge plasmid from the surface of the agar plug into the SM buffer, and stored at $4^{O}C$.

3.2.5.11 Titre of primary plaques

The primary plaques were titred as detailed in section 3.2.5.3, using dilutions of the SM buffer containing plasmid instead of the entire library. The number of plaques per plate were counted and the dilution producing 500 plaques/plate selected for use in secondary screening.

3.2.5.12 Secondary screening

Following selection of the optimum dilution of each of the primary plaques, the plasmid, in SM buffer, from each of the primary positive plaques was used to infect MRF' cells and plated onto agar plates as detailed for the primary screen. The plasmid from one plaque was plated onto one plate. The plates were incubated overnight at $37^{O}C$ as before, followed by transfer to $4^{O}C$ after sealing with Nesco film.

3.2.5.13 Plaque lifting

The DNA present on the plaques was transferred onto nitrocellulose membranes as detailed in section 3.2.5.5.

3.2.5.14 Prehybridisation of nitrocellulose filters

The nitrocellulose filters were prehybridised as detailed in section 3.2.5.6.

3.2.5.15 Hybridisation of filters

The nitrocellulose filters were hybridised with isotopically labelled probe to the S4 subtraction product as detailed in section 3.2.5.7.

3.2.5.16 Post Hybridisation stringency washes

The nitrocellulose filters were washed at increasing stringency as detailed in section 3.2.5.8.

3.2.5.17 Visualisation of hybridisation signal and identification of positive plaques

Hybridisation signal was visualised autoradiographically and 'double positives' identified as detailed in section 3.2.5.9.

3.2.5.18 Isolation of positive plaques

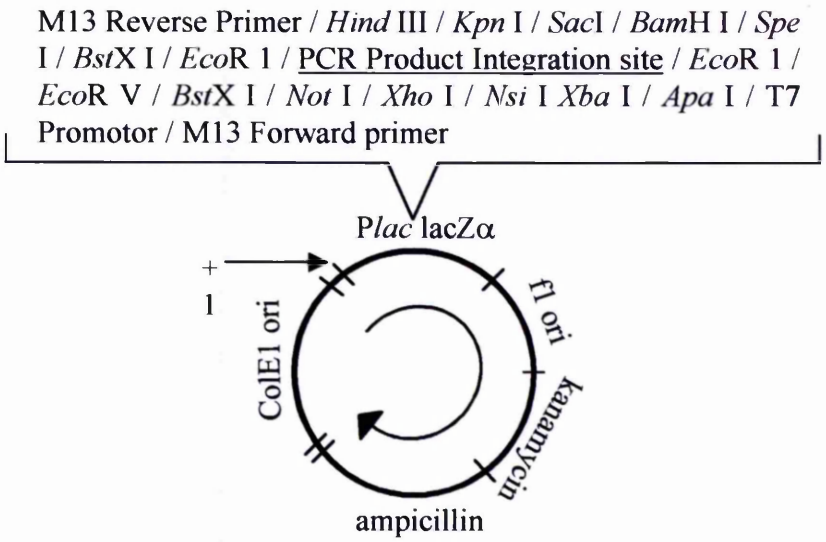
Plaques corresponding to double positives on the autoradiographic images were isolated and transferred to SM buffer and chloroform as detailed in section 3.2.5.10.

3.2.6 Gene sequencing

The plasmid insert present in each of the double positive plaques identified by secondary screening was amplifiied by PCR, cloned and sequenced.

3.2.6.1 Amplification of secondary clone inserts

The cDNA insert present in the plasmid in each positive plaque was amplified by PCR prior to cloning. Briefly, 1 µl of SM buffer, containing plaque plasmid, was used as template in a 25µl reaction mixture together with 1 µl of T3 primer (5'-AAT TAA CCC TCA CTA AAG GG-3'), 1 µl of T7 primer (5'-GTA ATA CGA CTC ACT ATA GGG-3') and 22 µl of PCR master mix (containing 0.63 units of Taq DNA polymerase, 0.2mM dNTP's, 75mM Tris-HCl (pH 8.8), 20mM $(NH_4)_2SO_4$, 1.5mM $MgCl_2$ and 0.01%(v/v) Tween 20). The reaction mixture was overlain with 50µl of mineral oil and PCR carried out with a thermal profile of 45 cycles of 94^{O}C for 45 sec, 54^{O}C for 45 sec and 72^{O}C for 1 min 20 sec, followed by a final extension at 72^{O}C for 7 min. Positive and negative controls, of 1 µl of a known plasmid (containing T3 and T7 primer sites) and 1 µl of distilled H_2O respectively, were used. Five microlitres of each of the PCR products were mixed with 2 µl of loading buffer, subjected to agarose gel electrophoresis, using a 1.5%(w/v) gel, and the size of the insert measured in each case.



pCR 2.1-TOPO

3908 nucleotides

LacZα fragment: bases 1-571 M13

reverse priming site: bases 205-221

Multiple cloning site: bases 234-357

T7 promotor/priming site: bases 364-383

M13 Forward priming site: bases 391-406 & 411-426

f1 origin: bases 548-962

kanamycin resistance: bases 1296-2090

ampicillin resistance: bases 2108-2968

ColE1 origin: bases 3113-3786

Figure 3.6

Map of pCR 2.1-TOPO cloning vector (Invitrogen, Carlsbad, CA, U.S.A.) showing sites of restriction enzyme cleavage, antibiotic resistance genes, primer sites and site of PCR product integration during cloning

3.2.6.2 Cloning of secondary clone inserts

Cloning was perfomed using TOPO-cloning (Invitrogen, California, U.S.A.) according to the manufacturers instructions and recombinants incubated overnight in L broth (supplemented with ampicillin) at 37^{O}C. The TOPO cloning vector pCR 2.1-TOPO, the details of which are given in figure 3.6, was used. A cloning reaction was set up containing 2μl of PCR product, from amplification of clones (see 3.2.6.1), 1μl of pCR-TOPO cloning vector, and distilled H_2O to a final volume of 5μl (3μl). The reaction volume was mixed gently and incubated for 5 minutes at room temperature, after which it was transferred to ice. Transformation of competent cells was carried out according to the manufacturer's instructions. Briefly, 2μl of 0.5M β- mercaptoethanol was added to each vial of competent cells (E.coli, with genotype: F' {*lac*I^{q} Tn10(Tet^{R})} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*K *rps*L (Str^{R}) *end*A1 *nup*G) and mixed by gentle pipetting. Two microlitres of the cloning reaction mixture was added, mixed and the cells incubated on ice for 30 minutes. Transformation was terminated by heat shock at 42^{O}C for 30 seconds and the cells then immediately incubated on ice for 2 minutes. SOC medium (20g tryptone, 5g yeast, 0.5g NaCl, and 10ml 250mM KCl in 1l distilled H_2O, adjusted to pH 7.5, autoclaved and then supplemented with 10ml 1M $MgCl_2$ and 7.2ml 50%(v/v) glucose) (250μl) was added at room temperature and the cells then incubated, with agitation, at 37^{O}C for 30 minutes, after which they were transferred to ice prior to plating. LB agar plates (1.0%(w/v) tryptone, 0.5%(w/v) yeast extract, 1.0%(w/v) NaCl, pH 7.0) were prepared prior to cloning and spread with 40 μl of X-Gal (40mg/ml) and with 40 μl of 100mM IPTG (isopropyl-thio-galactopyranoside) in turn immediately before plating. Between 50-100μl from each transformation was spread evenly on a prepared plate and the plate incubated overnight at 37^{O}C. Two plates were spread from each transformation .

3.2.6.3 Identification and analysis of recombinant clones

Following overnight incubation the plates were placed at 4^{O}C and then inspected. Positive recombinant colonies were white whilst non-recombinants were blue. This is due to disruption of the *lacZ'* gene, which codes for the first 146 amino acids of β-galactosidase, by insertion of the PCR product between the gene and its promotor, with consequent failure of conversion of X-Gal (colourless) to a blue product by the action of β-galactosidase. White colonies were picked and each one inoculated in 5ml of L broth. A PCR reaction was performed using 1μl of the inoculated broth in a 25μl reaction (detailed in section 3.2.6.1). The PCR products were analysed by gel and the product size compared

with that of the corresponding product generated in section 3.2.6.1. Those colonies for which the product size was the same for the two corresponding PCR reactions were adjudged to have undergone efficient recombination, and the respective inoculants incubated overnight at 37^{O}C with continuous agitation.

3.2.6.4 Minipreps of recombinant clones

For each of the cultures glycerol stocks were prepared and plasmid isolated using a miniprep, as detailed in sections 3.2.3.2.1.1 and 3.2.3.2.1.2. Isolated plasmid was resuspended in 12µl of distilled H_2O, and the quantity and size present verified by agarose gel electrophoresis on a 1.5%(w/v) gel.

3.2.6.5 Sequencing of isolated plasmid

Isolated plasmid contained cloned cDNA insert flanked by M13 forward and reverse primer sites and was sequenced by PCR using Big Dye Terminators (ABI Prism) and M13 forward (5'-TGT AAA ACG ACG GCC AGT-3') and reverse (5'-AAC AGC TAT GAC CAT G-3') primers. Each sequencing reaction mix contained plasmid DNA template, primer (M13 forward or reverse) to a final concentration of 0.3pmol/µl, 4µl of Big Dye Terminator Ready Reaction Mix (ABI Prism,), 1 µl of dimethysulphoxide (DMSO) and distilled H_2O, to a total volume of 10 µl. The amount of template added was dependent upon the total size of the plasmid used in each case, using 100ng of plasmid for each 1kb of plasmid length. The reaction mixture was overlain with 100µl of mineral oil and PCR carried out using a thermal profile with a hot start of 96^{O}C for 5 minutes, followed by 30 cycles of 98^{O}C for 30 seconds, 50^{O}C for 15 seconds and 60^{O}C for 4 minutes. The PCR products from the sequencing reaction were removed, diluted with 90µl of distilled H_2O (final volume 100µl) and ethanol precipitated with 10µl of 3M NaAc (pH 4.5), 250µl of 95% ethanol and 0.5µl of glycogen (20mg/ml), at –20^{O}C, followed by centrifugation at 18,000xg for 15 minutes at room temperature. The pellet was washed with 200µl of cold 75% ethanol, centrifuged at 18,000xg and air-dried. Each pellet was resuspended in loading buffer and subjected to gel electrophoresis on an ABI Prism 377 DNA Sequencer, according to the manufacturer's instructions.

3.2.6.6 Analysis of sequence data and identification of gene homology

The base sequence was derived semi-automatically from the fluorescence pattern of each sample. For each sample the EcoR1 site was identified in the sequence obtained and the sequence beyond it compared with known sequences held in the public domain by submission

to BLAST inquiry at the National Institute of Health, Bethesda, Maryland, U.S.A. (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST). For each positive clone sequenced sequence data generated by PCR with both forward and reverse M13 primers was submitted in this manner.

3.2.7 Expression of isolated genes

For each of the genes identified 3' end cDNA probes were generated from plasmid and their temporal and spatial patterns of expression established. This was achieved by hybridisation with cDNA from each of the time points for each clone, and by *in-situ* hybridisation with tissue sections of normal and diseased human bone. In addition, for one of the genes immunohistochemistry was performed on cytospins of human bone marrow stromal cells both prior to and following 4 days of dexamethasone stimulation, whilst for another of the genes the expression pattern in a wide range of human tissues was assessed by hybridisation with a commercially obtained human mRNA master blot.

3.2.7.1 Hybridisation with time point cDNA

In order to verify upregulation of gene expression following stimulation of the STRO-1 clones with dexamethasone, probes to each of the genes isolated were hybridised with dot-blots of the cDNA from each of the time points from clone 1. Following post-hybridisation washes and visualisation of hybridisation signal, as detailed below, the dot-blot used for each gene was stripped, by boiling in 0.1xSSC / 0.1% SDS for 10 minutes, and re-probed with a 3'end GAPDH cDNA probe, to allow comparison of signal intensity for each of the genes with that for GAPDH.

3.2.7.1.1Generation and isotopic labelling of 3'end cDNA probes

For each of the genes identified by sequence homology search the plasmid used as template in the sequencing reaction was used to produce a 3'end cDNA probe. For each gene the corresponding glycerol stock was defrosted, inoculated in 10ml of L broth (supplemented with ampicillin) and incubated overnight at 37^{O}C. Plasmid was isolated from the culture using a mini-prep (detailed in section 3.2.3.2.1.2) and a fresh glycerol stock taken (detailed in section 3.2.3.2.1.1). The cloned cDNA insert was excised from the plasmid by restriction enzyme digestion (detailed in section 3.2.3.2.1.3), visualised by agarose gel electrophoresis and used as template in a labelling reaction (detailed in section 3.2.3.2.2). The probe to GAPDH was prepared in the same manner.

3.2.7.1.2 Production of cDNA dot-blots

Dot-blots of cDNA from each of the time-points for each of the clones were prepared as detailed in section 3.2.3.2.3, with inclusion of PCR control and excised insert as negative and positive controls respectively.

3.2.7.1.3 Prehybridisation and hybridisation of cDNA dot-blots

Prehybridisation and hybridisation were carried out as detailed in sections 3.2.3.2.4 and 3.2.3.2.5 respectively.

3.2.7.1.4 Post Hybridisation washes and visualisation of hybridisation signal

The dot-blots were washed at increasing stringency and hybridisation signal visualised as detailed in section 3.2.3.2.6. In addition the intensity of hybridisation signal for each probe (isolated gene and GAPDH) was measured using a densitometer, and the values obtained for each time point normalised to those for GAPDH.

3.2.7.2 Immunohistochemistry with cytospin preparations of stimulated marrow stromal cells

In order to eliminate the possibility that the modulation of gene expression determined above in section 3.2.7.1 was due to a PCR artefact, a polyclonal antibody to one of the genes, 14-3-3 zeta, was used on cytospin preparations of primary cultures of human bone marrow stromal cells.

3.2.7.2.3 Primary culture of human bone marrow stromal cells

Primary culture of human bone marrow stromal cells was established as detailed in section 3.2.1.1. After 2 weeks of culture the cells were split and reseeded in two T25 ($25cm^2$) tissue culture flasks, using a cell scrapper, and left to adhere. One was maintained with standard medium (alpha-modified minimal esssential medium, supplemented with 10%(v/v) heat inactivated fetal calf serum, 1%(v/v) glutamate, 1%(v/v) ascorbic acid, 1%(v/v) penicillin, 1%(v/v) streptomysin, 1%(v/v) amphotericin) and the other with standard medium supplemeted with dexamethasone (10^{-7} M).

3.2.7.2.2 Cytospin preparations of primary stromal cells

The cells were maintained in standard or osteogenic media for 4 days, as detailed above, after which the medium was removed from both flasks and the cells in each flask washed 3 times in PBS. The cells in each flask were then scrapped off into 10ml of PBS and pelleted by centrifugation at 1,500xg for 5 minutes. The pellets were resuspended in 1200µl of PBS, 100µl aliquots of which were loaded into each well of a cytospin centrifuge. The

cells were then cytospun onto glass slides by centifugation at 1,000xg for 30 mins. The cytospin preparations were fixed in cold 100% methanol for 10 minutes, air-dried and stored in foil at $4^{O}C$ prior to use.

3.2.7.2.3 Immunohistochemistry for 14-3-3 zeta protein

The expression of one of the isolated genes, 14-3-3 zeta, was assessed at the protein level by immunohistochemistry. A rabbit polyclonal antibody specific for the zeta isoform of 14-3-3 was generously provided by Dr Alistair Aitken, Univerity of Edinburgh, and used at a 1/100 dilution in 1%(v/v) bovine serum albumin (BSA).

Both stimulated and unstimulated cytospin sections were then transferred to Tris buffered saline (TBS) (pH 7.6), and blocked with 1%(v/v) H_2O_2 in TBS (291ml TBS, 9ml 30%(v/v) H_2O_2, and 5 drops concentrated HCl) for 30 minutes. All slides were washed in distilled H_2O, transferred to TBS, marked with a DAKO pen to outline the area bearing the cells on each, and then blocked with 100µl 20%(v/v) goat serum in TBS at room temperature for 30 minutes. This and all subsequent incubations were carried out in a wet-box. Following this the primary antibody (50µl/slide) was added (diluted to the appropriate concentration in 1%(v/v) BSA and incubated at room temperature for 1 hour; 1%(v/v) BSA alone was used as negative control on both trephine sections and cytospins. The primary antibody was removed by two 5 minute washes in TBS, the secondary antibody (100µl/slide), a goat anti-rabbit (Dako) diluted 1/200 in 1%(v/v) BSA, applied, and incubated at room temperature for 30 minutes. It was then removed with two 5 minute TBS washes, and the slides transferred to a solution of freshly prepared and filtered DAB (20ml DAB, 280ml TBS, 5 drops 30%(v/v) H_2O_2) for 10 minutes. The slides were then washed in distilled H_2O for 5 minutes, stained in filtered Mayer's haematoxylin for 30 seconds and blued in TBS and tap H_2O. All slides were dehydrated in industrial methylated spirit (IMS) and mounted in xylene.

3.2.7.3 In-situ hybridisation with human bone

In-situ hybridisation was performed to establish the tissue distribution of the genes identified. cDNA probes were radiolabeled with ^{35}S by random prime labelling. Briefly, the probes were prepared using excised insert from purified plasmid (detailed in sections 3.2.3.2.1.1 and 3.2.3.2.1.2) as template in a random prime labelling reaction. A labelling reaction containing 50ng of cDNA probe (1-2µl), 10µl of primer and distilled H_2O to final volume of 52µl was boiled for 5 minutes, followed by addition, at room temperature, of 10µl reaction buffer, 8µl of each of dATP, dGTP and dTTP, 10µl of ^{35}S-dCTP and 4µl of

Klenow fragment. The reaction mixture was incubated at 37°C for 1 hour and the labelling reaction terminated by addition of 5µl of 0.2M EDTA (pH 8.0). Labelled probe was purified by push columns and purified probe stored at –20°C prior to use.

3.2.7.3.1 Case selection and tissue processing

Tissue expression of mRNA was determined in sections cut from archived paraffin blocks of normal adult bone, post-menopausal bone, both pre- and post-hormone replacement therapy, osteophytic bone, hyperparathyroid bone, Pagetic bone, healing fracture callus, non-union fracture and normal and abnormal fetal bone. The case details are given in table 3.2a. In each case tissue was fixed at the time of receipt in 10%(v/v) buffered formalin for 24 hours, followed by decalcifiction in 20%(v/v) EDTA (pH 7.4), until calcification was absent on radiographic analysis, and processed to paraffin, using standard protocols. For one of the genes isolated samples from a wider range of normal fetal long bones, each including the growth plate, and from cases with intra-uterine growth retardation (IUGR) and from a variety of skeletal dysplasias, detailed in table 3.2b, were also studied. Sections were cut at 7µm and floated out on DEPC treated H_2O and mounted on silanated slides, followed by baking at 60°C. Slides were stored at room temperature in foil prior to use.

3.2.7.3.2 Prehybridisation and hybridisation

Sections were dewaxed in xylene for 3 x 5 minutes, rehydrated in IMS for 4 x 2 minutes and washed in DEPC treated H_2O for 10 minutes, followed by treatment with 0.2M HCl for 20 minutes, 2xSSC for 2 x 3 minutes and 0.05M Tris HCl (pH 7.4) for 3 minutes. The sections were outlined with a DAKO pen and proteinase K (10µg/ml in 0.05M Tris HCl, pH 7.4) applied to the sections, followed by incubation at 37°C for 1 hour. The sections were then rinsed in DEPC H_2O for 2 x 3 minutes, dehydrated in IMS for 3 minutes and RNase (0.1mg/ml in RNase buffer), DNase (1 unit/ml in DNase buffer), and RNase (0.mg/ml in RNase buffer) / DNase (1 unit/ml in DNase buffer) pre-treatments applied to control sections, followed by incubation at 37°C for 1 hour. Control and test slides were rinsed with PBS for 3 minutes, post-fixed with cold 0.4%(v/v) paraformaldehyde/PBS for 20 minutes, rinsed with DEPC H_2O for 5 minutes and incubated in 0.1M triethanolamine (pH 8.0) for 5 minutes, followed by treatment with a freshly prepared solution of acetic anhydride/triethanolamine for 20 minutes. The sections were rinsed with DEPC H_2O for 5 minutes and each prehybridised with 50µl of freshly prepared prehybridisation mixture (50%(v/v) deionised formamide, 0.6M NaCl, 10mM Tris-HCl pH 7.4, 0.5mM EDTA, 10% dextran sulphate, 0.2mg/ml salmon sperm DNA, and 10mM DTT(dithiothreitol)) at 37°C for 2 hours. Sections were hybridised with 50ng of heat denatured probe in 50%(v/v)

Biopsy number	Diagnosis	Age / Sex
B95-0655	Normal bone	35yr / female
B96-1048	Healing fracture	32yr / male
B95-0892	Osteoporosis – pre HRT	37yr / female
B96-1171	Osteoporosis – post HRT	49yr / female
B97-0719	Paget's disease of bone	73yr / male
B95-0635	Hyperparathyroid bone disease	27yr / female

Table 3.2a Case details of adult specimens used for *in-situ* studies of expression of each of the genes isolated.

Biopsy number	Diagnosis	Gestation / Sex
A12 / 98 +2	Normal bone	15wks / male
F296 / 97 +3	Normal bone	21wks / female
A11 / 98 +3	Normal bone	24wks / female
A10 / 98 +4	Normal bone	28wks / male
A188 / 97 +2	Normal bone	36wks / female
A57 / 97 +1	Intra-uterine growth retardation	26wks / male
A160 / 96 +2	Intra-uterine growth retardation	34wks / female
F79 / 96 +3	Osteogenesis imperfecta	20wks / female
A291 / 98 +3	Chondrodysplasia calcificans congenita	23wks / female
F55 / 96 +4	Short rib polydactyly syndrome	17wks / male
F95 / 124 +2	Diastrophic dysplasia	19wks / female
F120 / 96 +2	Camptomelic dysplasia	20wks / female

Table 3.2b Case details of fetal specimens used for *in-situ* studies of expression of 14-3-3 zeta.

deionised formamide, 0.6M NaCl, 10mM Tris-HCl pH 7.4, 0.5mM EDTA, 10%(w/v) dextran sulphate, 0.2mg/ml salmon sperm DNA, 10mM DTT. Sections were covered with plastic coverslips and hybridised overnight at 37^{O}C in a wet-box.

3.2.7.3.3 Post-hybridisation washes

A series of increasingly stringent washes were carried out. All washes were carried out on an orbital shaker. Coverslips were removed by floating off in 4xSSC and the sections washed in 0.5xSSC, 1mM EDTA, 10mM DTT twice for 5 minutes, rinsed in 0.5xSSC, 1mM EDTA twice for 5 minutes and then in 50%(v/v) formamide / 50%(v/v) 0.15M NaCl, 5mM Tris HCl (pH 7.4), 0.5mM EDTA (pH 8) for 10 minutes in a fume cupboard. The sections were then rinsed in 0.5xSSC at 55^{O}C four times for 5 minutes, in 0.5xSSC at room temperature for 5 minutes. They were dehydrated in IMS for 3 minutes, air-dried, coated with freshly prepared photographic emulsion (Ilford K5 emulsion melted at 40^{O}C and diluted 1:1 with warm distilled H_2O) in a darkroom, and exposed at 4^{O}C for approximately 14 days in the darkroom.

3.2.7.3.4 Development

Slides were developed after between 10 and 14 days. They were developed in D-19 developer (Kodak) for 5 minutes, rinsed in cold H_2O, and fixed in AM Fix fixer (Kodak) for 5 minutes. The slides were counterstained with haematoxylin and eosin and mounted in XAM.

3.2.7.3.5 Analysis of hybridisation signal

The slides were examined using a Leica RMDB research microscope and for each section pattern and intensity of hybridisation signal, identified by the presence of black silver grains, was assessed independently by two observers (Drs Judith Hoyland & Richard Byers). Images were captured on P600 image analysis system via a Sony DXC 930P 3 chip colour video camera and saved as TIFF files on compact disc.

3.2.7.4 Hybridisation with human RNA master blot

For the genes isolated for which a tissue distribution was not documented in the literature, cDNA probes, prepared and isotopically labelled as detailed in section 3.2.3.2.2 were used to probe a commercially obtained human RNA master blot (Clontech, California, U.S.A.). The master blot was prepared by dot-blotting poly (A) RNA samples from a variety of human tissues, shown in figure 3.7. The type and position of the poly (A) RNAs and the

	1	2	3	4	5	6	7	8
A	Whole brain	Amygdala	Caudate nucleus	Cerebellum	Cerebral cortex	Frontal lobe	Hippo-campus	Medulla oblongata
B	Occipital lobe	Putamen	Substantia nigra	Temporal lobe	Thalamus	Acumens	Spinal cord	
C	Heart	Aorta	Skeletal muscle	Colon	Bladder	uterus	Prostate	Stomach
D	testis	Ovary	Pancreas	Pituitary gland	Adrenal gland	Thyroid gland	Salivary gland	Mammary Gland
E	Kidney	Liver	Small intestine	Spleen	Thymus	Peripheral leucocyte	Lymph node	Bone marrow
F	Appendix	Lung	Trachea	Placenta				
G	Fetal brain	Fetal heart	Fetal kidney	Fetal liver	Fetal spleen	Fetal thymus	Fetal lung	
H	Yeast total RNA	Yeast tRNA	E.coli rRNA	E.coli DNA	Poly r(A)	Human Cot1 DNA	Human DNA	Human DNA

Figure 3.7 Positional plan of tissue RNA samples on RNA master blot

controls dotted on the membrane are shown. For each dot the amount of poly (A) RNA present ranged from 100-500ng.

3.2.7.4.1 Prehybridisation of RNA master blot

The master blot was defrosted slowly, taking care not to let it dry, and placed in a hybridisation tube at 42°C, to which 10ml of pre-prepared ExpressHyb Hybridisation Solution (Clontech, California, U.S.A.) at 60°C was added. Briefly, 15mg of salmon sperm DNA was added to 1ml of distilled water and denatured by boiling at 100°C for 5 minutes, followed by rapid cooling on ice. It was then added immediately to 15ml of ExpressHyb at 60°C, and 10ml of the resultant solution used for prehybridisation, as noted above, whilst the remaining 5ml was retained for addition of the cDNA probe. The master blot was prehybridised at 65°C for 3 hours prior to addition of the cDNA probe.

3.2.7.4.2 Hybridisation of RNA blot with cDNA probe

The labelled cDNA probe used was boiled at 100°C in a capped 1.5ml eppendorf tube for 5 minutes, rapidly cooled on ice and then added to the retained 5ml of pre-prepared ExpressHyb solution. The prehybridisation solution was discarded and the new solution added to the master blot. Hybridisation was carried out at 42°C overnight with continuous agitation in a rotating hybridisation tube.

3.2.7.4.3 Post Hybridisation washes and visualisation of hybridisation signal

Post hybridisation stringency washes were carried out according to the manufacturer's instructions, namely, five times with 2xSSC/1%SDS for 5 minutes at room temperature, followed by one wash with 2xSSC/1%SDS at 42°C for 1hour and 20 minutes, and finally by increasingly high temperature washes in 0.1xSSC/1%SDS to a final stringency of 70°C for 15 minutes, in order to remove all non-specific hybridisation to the poly (A) dot-blot (location H5, figure 3.7). Between each step the pattern of hybridisation signal present was visualised by exposure to a phosphoimager plate for 1hour, and, after the final wash hybridisation signal was characterised by exposure to autoradiographic film, as detailed in section 3.2.3.2.6.

3.3 Results

3.3.1 Primary human bone marrow stromal cell culture

3.3.1.1 Induction of osteoblastic differentiation

Triplicate cultures all yielded the same results. Cells in standard medium formed sheets of spindle shaped fibroblast cells (figure 3.8a), whilst cells stimulated with dexamethasone (Dex) were less slender and demonstrated a slightly nodular growth pattern (figure 3.8b). Cells stimulated with beta-glycerophosphate showed similar changes, whilst the cells stimulated with both were plump and demonstrated a marked nodular growth pattern (figure 3.8c). Expression of alkaline phosphatase staining was increased by stimulation with dexamethasone. Positive histochemical staining was present in less than 10% (range 5 – 9%) of the cells grown in standard medium (figure 3.8d) but was present in over 50% (range 55-70%) of those stimulated with dexamethasone (figure 3.8e). An increase in the histochemical staining for alkaline phosphatase was also apparent macroscopically (figure 3.8f). Increased alkaline phosphatase expression following dexamethasone stimulation was also demonstrated biochemically (16.4 nmol p-nitrophenol liberated/min per 1000 cells (range 11.1 - 17.3) in Dex vs. 5.6 nmol (range 3.4 – 6.5) in control). Alizarin red staining for mineralised calcium was increased by stimulation with beta-glycerophosphate. It was absent in the cells grown in standard medium (figure 3.8g), and was maximal in those supplemented with both dexamethasone and beta-glycerophosphate (figure 3.8h): these increases were also apparent macroscopically (figure 3.8i).

3.3.1.2 STRO-1 expression

Adherent cells showed variable STRO-1 ligand expression, dependent on length of time in culture. The level in unstimulated adherent cells increased from 28% (n=3, SE 5%) at 1 week to 40% at 2 weeks (n=4, SE 7%) (figure 3.9) and then fell to 8% at 6 weeks (n=2, 6-9%) (figure 3.9). Controls omitting either primary antibody alone or both primary and secondary antibody were negative in each case (mean positivity 0.5%, range 0 – 1.7).

3.3.2 Production of STRO-1 positive clones and stimulation of osteoblastic differentiation

MACS of HCC1 cells for STRO-1 produced a twenty-nine fold enrichment (n=1) (figure 3.10), confirming the utility of the system for generation of STRO-1 enriched cultures: the positive fraction showed 11.7 per cent positivity versus 0.4 per cent for the negative fraction. MACS sorting of human marrow stromal cells from primary culture gave a low yield of cells, which produced 2 clones after low density seeding. Both survived

Figure 3.8a

Figure 3.8b

Figure 3.8c

Figure 3.8d

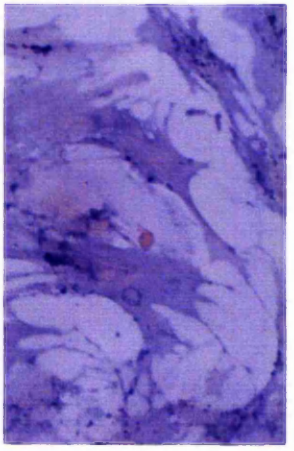


Figure 3.8e

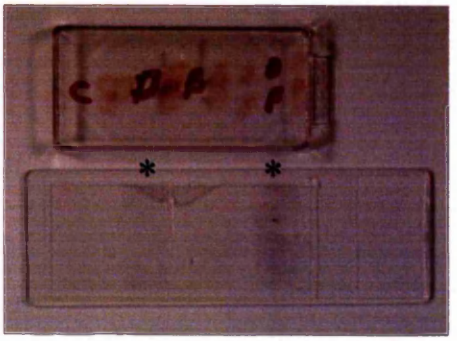


Figure 3.8f

Figure 3.8g

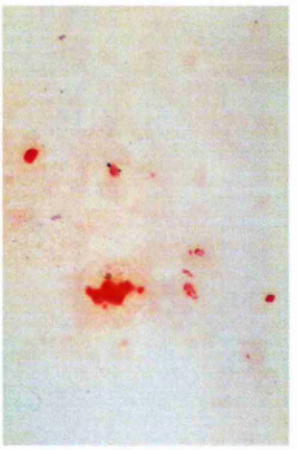


Figure 3.8h

C D β Dβ

Figure 3.8i

Figure 3.8

Phase contrast photomicrographs of cultured bone marrow stromal cells grown in standard medium (a) without supplementation, (b) supplemented with dexamethasone(Dex), or (c) supplemented with dexamethasone & beta-glycerophosphate(β-GP). Unstimulated cells exhibited fibroblast-like morphology and grew in sheets, whilst stimulation with Dex resulted in plumper cells, and with β-GP in a nodular growth pattern. Alkaline phosphatase background staining of stromal cells demonstrated low level of staining in cells cultured in control medium (d), compared with frequent strongly positive cells when stimulated with Dex (e). The same upregulation of alkaline phosphatase expression was apparent macroscopically (f), in those cells (*) stimulated with Dex (D), either alone or together with β-GP(β), compared to control medium (C). Alizarin red staining for the presence of mineralised calcium in stromal cells demonstrated lack of staining in cells cultured in control medium (g), and frequent positive cells in medium supplemented with Dex & β-GP (h). Similarly, macroscopically (i) mineralised calcium was maximal in those cells (**) cultured in both Dex & β-GP (D/β). Magnification x100 for all photomicrographs.

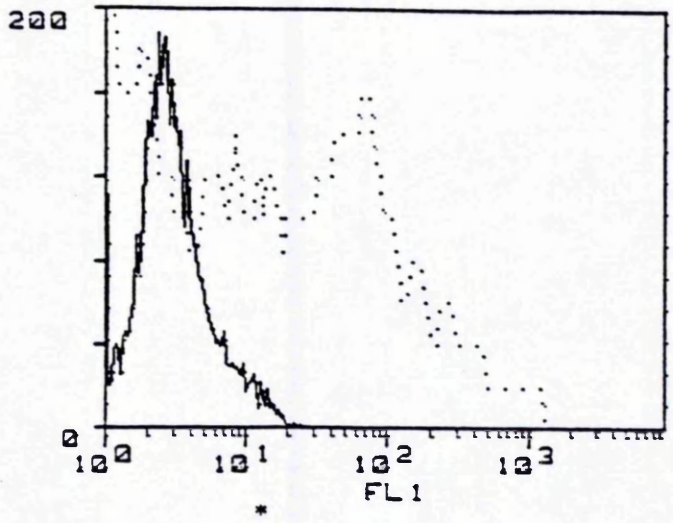


Figure 3.9a

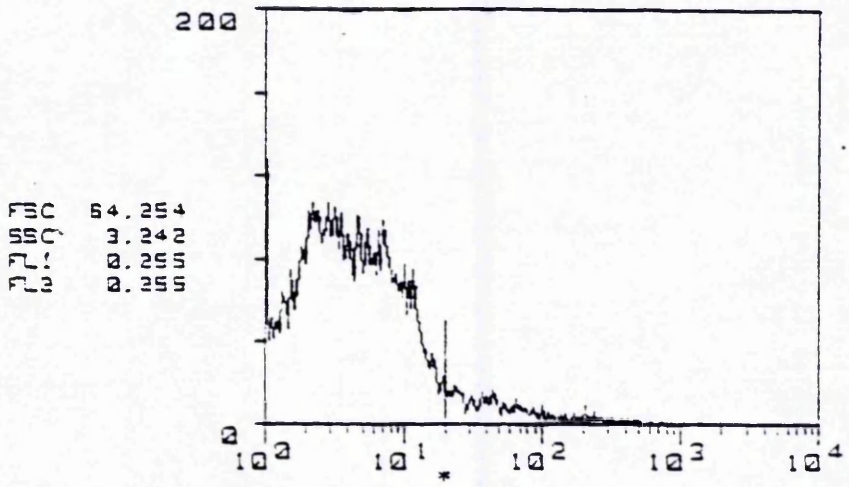


Figure 3.9b

Figure 3.9

Fluorescence activated cell analysis for STRO-1 positivity of adherent bone marrow stromal cells (a) 2weeks (dotted line) and, (b) 6 weeks (solid line) after harvest, demonstrating positivity in 40% and 8% of the cells respectively. In each case the fluorescence intensity is given along the x-axis and the number of cells of each intensity on the y-axis. In each case the number of positive cells was counted to the right of the negative cut-off threshold (*), set at the extreme right of the tail for the control sample (omitting secondary antibody), as shown by the solid line in figure 3.9a.

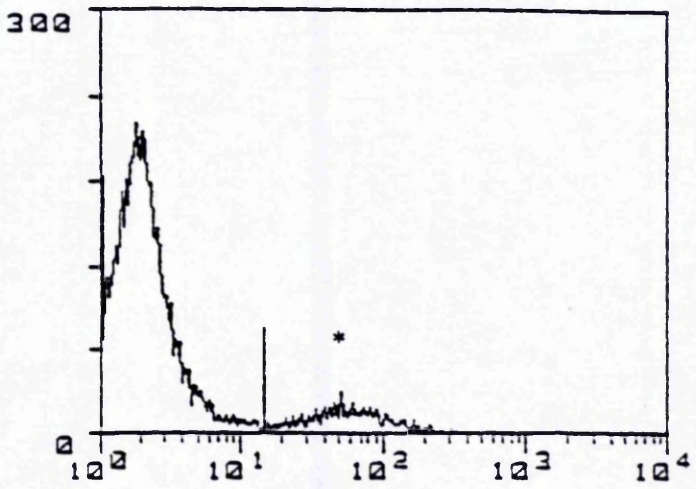


Figure 3.10a

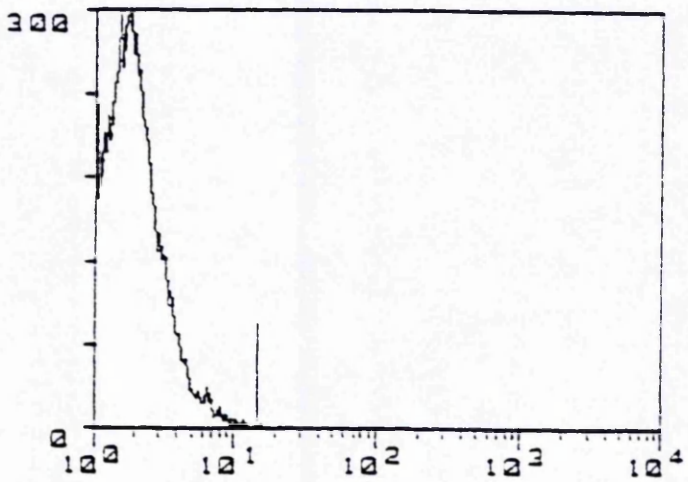


Figure 3.10b

Figure 3.10

Fluorescence activated cell analysis, for STRO-1 positivity, of (a) postive and, (b) negative fractions of HCC1 cells after magnetic activated cell sorting for STRO-1, demonstrating positive cells (*) in the positive fraction only. In each case the fluorescence intensity ii given along the x-axis and the number of cells of each intensity on the y-axis. In each case the number of positive cells was counted to the right of the negative cut-off threshold (vertical line), which was set at the extreme right of the tail for the control sample (omitting secondary antibody).

subpassage using cloning rings and demonstrated similar morphological changes as those described above when stimulated with dexamethasone.

3.3.3 Poly (A) RT-PCR and verification of osteoblastic differentiation

3.3.3.1 Verification of efficiency of poly (A) PCR

3.3.3.1.1 Extraction of mRNA from SaOS-2 cells

Efficient RNA extraction from the SaOS-2 cells was demonstrated by visualisation of the resuspended extraction product on a 1.5% (w/v) agarose gel (figure 3.11).

3.3.3.1.2 Quantitation of mRNA

The absorbance of the resuspended extraction product at 260nm was 0.145, whilst that at 280nm was 0.089, yielding a 260:280 ratio of 1.64 (>1.60 indicates adequate purity). From the absorbance at 260nm the quantity of RNA present was calculated to be 5.8 μg/μl.

3.3.3.1.3 Poly (A) reverse-transcription polymerase chain reaction

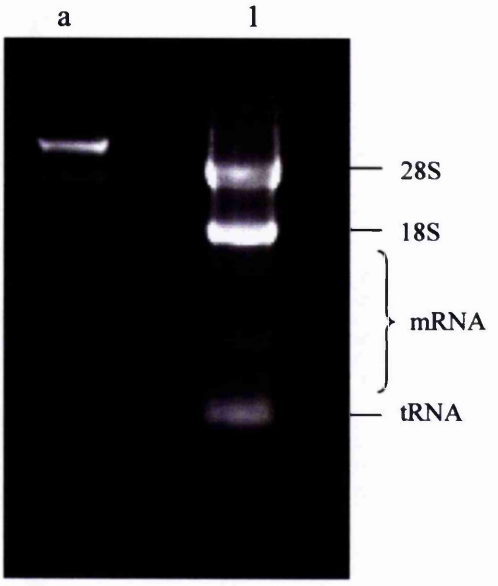
Serial dilutions of the extracted RNA were used in poly (A) reverse-transcription polymerase chain reaction, as detailed in section 3.2.3.1.4. For each of the dilutions PCR products between 100 and 700 bp long were generated (figure 3.12). The negative RNA control also generated a smear on gel electophoresis, but neither the open or closed PCR control tubes contained PCR product (figure 3.12).

3.3.3.1.4 Hybridisation of cDNA Southern blots with GAPDH probe

GADPH hybridised with the PCR products from the higher concentrations of RNA template, but not with those generated from the lower concentrations (figure 3.13). In addition, the smear present for the negative RNA control failed to hybridise with GAPDH, indicating that it was artefactual (figure 3.13).

3.3.3.2 Assessment of osteoblastic gene expression of STRO-1 positive clones

Poly (A) cDNA samples were generated from each time coursed mRNA extraction in each clone (figure 3.14). In each case the PCR products generated were between 100 and 500 bp in size and both open and closed control PCR tubes contained no PCR product. These PCR products hybridised with probes for osteocalcin (figure 3.15a) and osteopontin (figure 3.15b) confirming the osteoblastic nature of the clones; though the results are not quantitive. Collagen type I expression however was detectable by PCR, in clone 1 at day 14 and in clone 2 at day 7 (figure 3.16). GAPDH was present in each time point (figure 3.15c). PCR controls were negative and probe controls positive. Osteocalcin and



Lane a: 1Kb Lambda size marker

Lane 1: RNA extraction product from SaOS-2 cells

Figure 3.11
Ethidium bromide stained agarose gel electrophoresis fractions of extracted RNA from SaOS-2 cells, indicating presence of S28 and S18 ribosomal bands, and size of mRNA (messenger RNA) and tRNA (transfer RNA) fractions, and thereby demonstrating integrity of the RNA present.

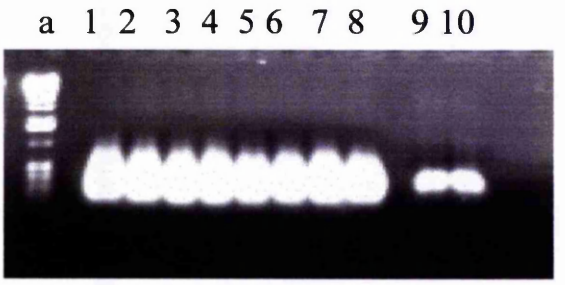


Figure 3.12
Ethidium bromide stained agarose gel electrophoresis fractions of poly (A) reverse-transcription polymerase chain reaction (RT-PCR) products from serial dilutions of extracted RNA from SaOS-2 cells. PCR products were between 100 and 800 base pairs long for each of the reactions. Serial dilutions of RNA used/μl : lane 1=1μg, lane2=500ng, lane 3=1ng, lane 4=500pg, lane 5=100pg, lane 6=50pg, lane 7=25pg. Lane a: 1Kb Lambda size marker, Lanes 1 to 7: Products from poly (A) RT-PCR of serial dilutions of RNA from SaOS-2 cells, Lane 8: Product from poly (A) RT-PCR negative control, Lanes 9 & 10: Products from open and closed poly (A) negative controls respectively.

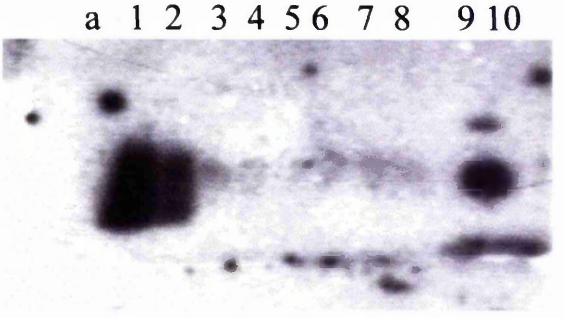
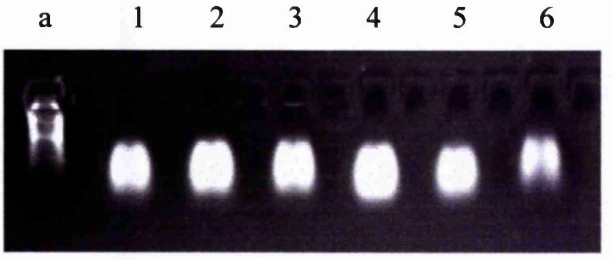
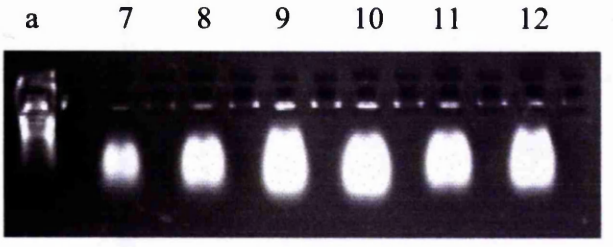


Figure 3.13
Auto-radiograph of hybridisation signal of ^{32}P labelled GAPDH probe with poly (A) RT-PCR products from serial dilutions of extracted RNA from SaOS-2 cells, demonstrating hybridisation with products from dilutions of RNA at 1μg/μl (lane1), and 500ng/μg (lane2). Lane 8 (poly (A) RT-PCR negative control) contained no hybridisation signal indicating that the smear present in this location on gel electrophoresis (figure 3.12) was artefactual .



3.14a Clone 1



3.14b Clone 2

Lane a: 1Kb Lambda size marker

Lanes 1 to 12: clones 1(figure 3.14a)[lanes 1 to 6] and 2 (figure 3.14b)[lanes 7 to 12], prior to and following stimulation with dexamethasone. Pre-stimulation (lanes 1 & 7) and post-stimulation after 1(lanes 2 & 8), 2(lanes 3 & 9), 4(lanes 4 & 10), 7(lanes 5 & 11) 10(lane 6), and 14(12) days.

Figure 3.14
Ethidium bromide stained agarose gel electrophoresis fractions of poly (A) reverse-transcription polymerase chain reaction (RT-PCR) products from clones 1(figure 3.14a)[lanes 1 to 6] and 2 (figure 3.14b)[lanes 7 to 12], prior to and following stimulation with dexamethasone. Pre-stimulation (lanes 1 & 7) and post-stimulation after 1(lanes 2 & 8), 2(lanes 3 & 9), 4(lanes 4 & 10), 7(lanes 5 & 11) 10(lane 6), and 14(12) days.

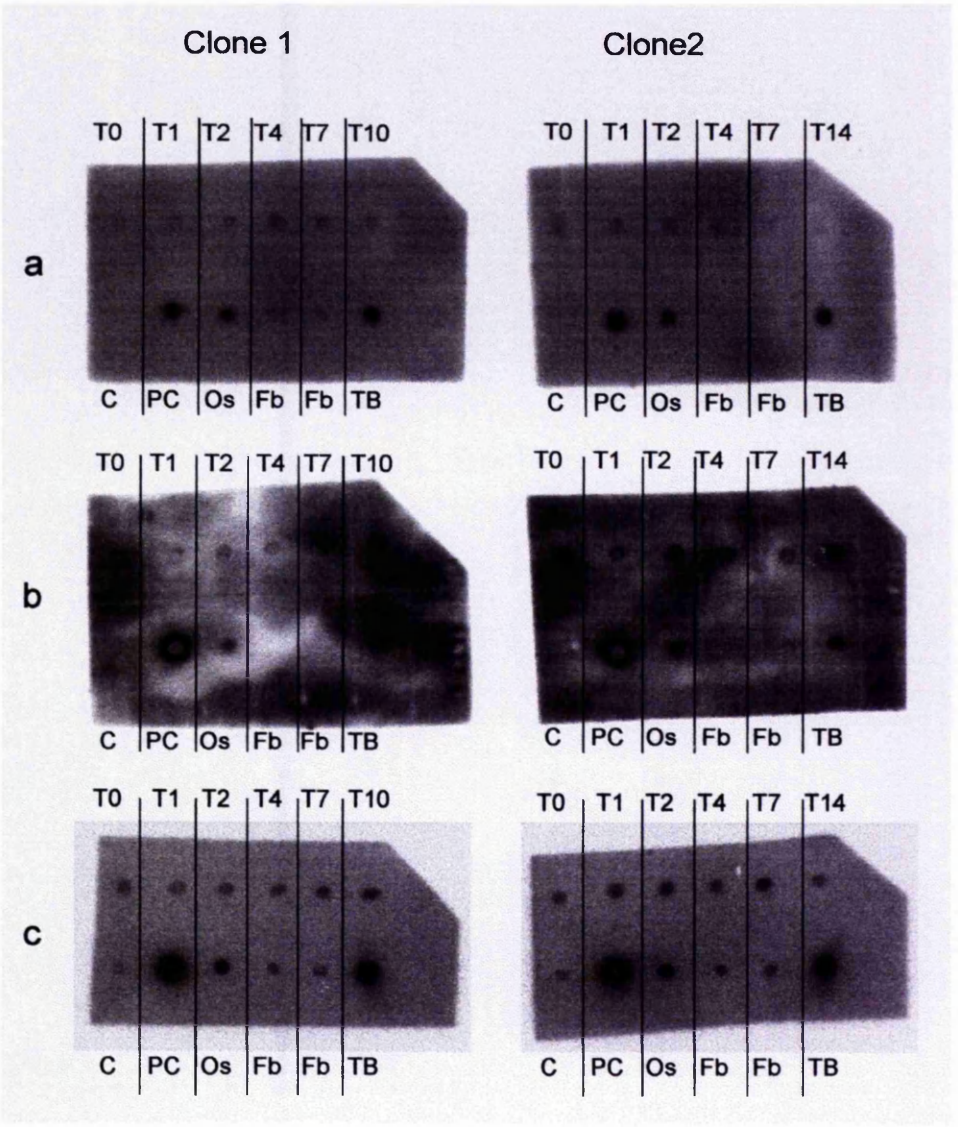


Figure 3.15 Hybridisation of poly (A) cDNA products from both clones with probes for (a) osteocalcin(OCN), (b) osteopontin(OPN), and (c) GAPDH. Pre-stimulation and post-stimulation after 1(T1), 2(T2), 4(T4), 7(T7) 10(T10), and 14(T14) days of stimulation with dexamethasone. C=PCR control; PC=probe control; Os=osteosarcoma; Fb=fibroblast; TB=trabecular bone. Osteocalcin is upregulated markedly at T4 in clone 1, whilst osteopontin is upregulated at T7 in the same clone.

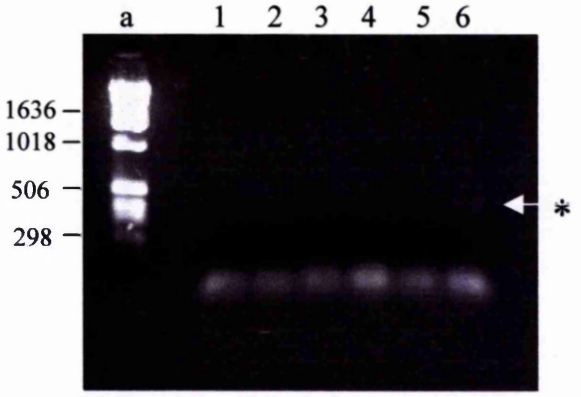


Figure 3.16a Clone 1

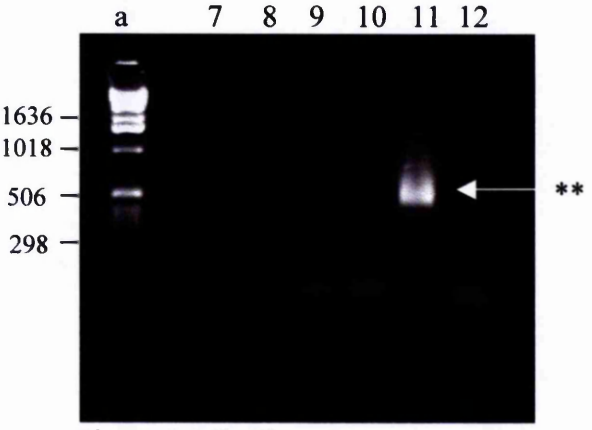


Figure 3.16b Clone 2

Lane a: 1Kb Lambda size marker Lanes 1 to 12: clones 1(figure 3.14a)[lanes 1 to 6] and 2 (figure 3.14b)[lanes 7 to 12], prior to and following stimulation with dexamethasone. Pre-stimulation (lanes 1 & 7) and post-stimulation after 1(lanes 2 & 8), 2(lanes 3 & 9), 4(lanes 4 & 10), 7(lanes 5 & 11) 10(lane 6), and 14(12) days.

Figure 3.16

Ethidium bromide stained agarose gel electrophoresis PCR products for collagen type I from clones 1(figure 3.16a)[lanes 1 to 6] and 2 (figure 3.16b)[lanes 7 to 12], prior to and following stimulation with dexamethasone, showing weak positive band(*) after 10 days (clone 1) and strong positive band(**) after 7 days (clone 2) of dexamethasone stimualtion. Pre-stimulation (lanes 1 & 7) and post-stimulation after 1(lanes 2 & 8), 2(lanes 3 & 9), 4(lanes 4 & 10), 7(lanes 5 & 11) 10(lane 6), and 14(12) days.

osteopontin hybridised with mRNA products from osteoblast and osteosarcoma cultures, but not that from the fibroblasts, consistent with the reported phenotype of the control cultures (figure 3.15). Precise quantitation of mRNA levels was not carried out for the bone specific proteins.

3.3.4 Subtractive hybridisation to isolate genes upregulated during early osteoblastic differentiation

3.3.4.1 Generation of subtraction products

Four subtraction products were generated by the four rounds of subtractive hybridisation undertaken, and designated S1, S2, S3 and S4. Each yielded a PCR product upon amplification by poly (A) PCR (figure 3.17): the PCR products were between 100 to 500 bp long and both the open and closed PCR negative control tubes contained no PCR product.

3.3.4.2 Verification of efficiency of subtraction

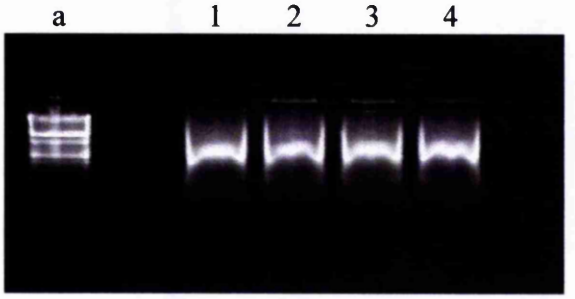
Driver cDNA did not hybridise to S4 cDNA, verifying that the S4 subtraction product contained only those genes upregulated during osteoblastic differentiation, confirming efficient subtraction (figure 3.18a): only very weak driver binding was noted (figure 3.18a). However, since some binding of S4 to driver was present, the S4 product was not composed entirely of genes unique to T4 (day 4 time-point) (figure 3.18b).

3.3.5 Library Screening

Primary library screening yielded 157 positive clones, stocks from each of which have been prepared and stored at $4^{O}C$ as detailed in section 3.2.5. Four of these successfully underwent secondary screening and sequencing. Representative pairs of duplicate plaque lift filters from the primary and secondary screens are shown in figures 3.19a & 3.19b.

3.3.6 DNA sequencing

The genes identified by BLAST homology search, together with the base pair length in which homology was present, and the percentage homology present, are shown in table 3.3. The sequence alignments for each gene are detailed in appendix 2.



Lane a: 1Kb Lambda size marker

Lanes 1 to 4: Products from poly (A) PCR of subtraction products S1 to S4 respectively (1 to 4)

Figure 3.17
Ethidium bromide stained agarose gel electrophoresis fractions of poly (A) PCR products from subtraction products S1 to S4.

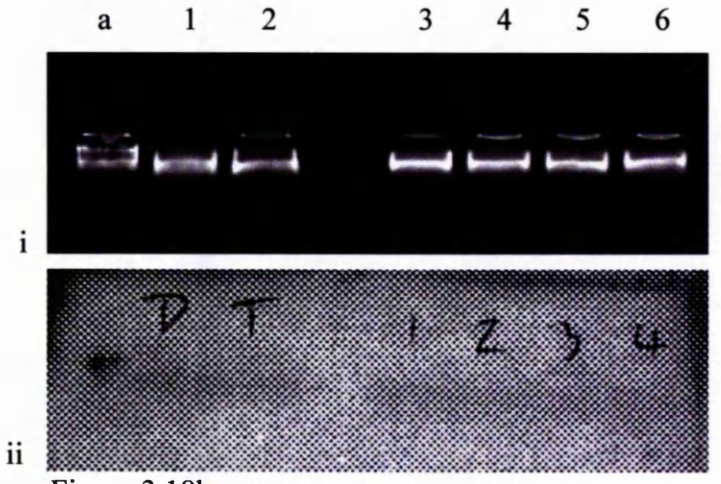


Figure 3.18b

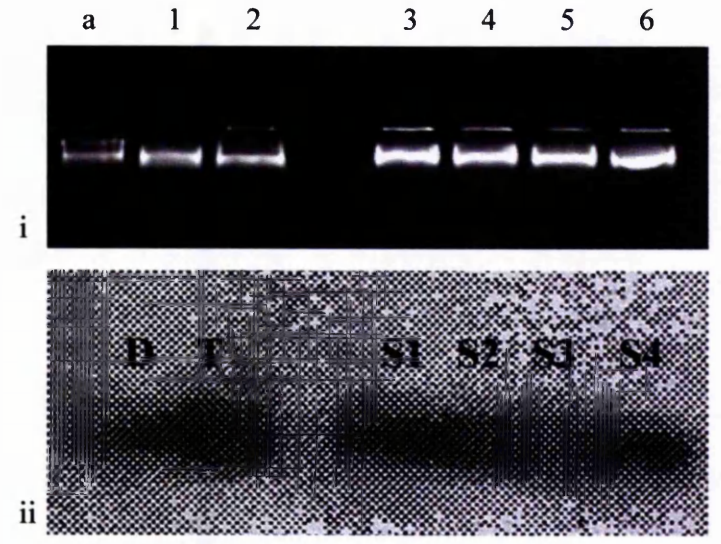


Figure 3.18b

Lane a: 1Kb Lambda size marker
Lane 1: Poly (A) PCR product from driver cDNA (T0)
Lane 2: Poly (A) PCR product from tracer cDNA (T4)
Lanes 3 to 6: Products from poly (A) PCR of subtraction products S1 to S4 respectively

Figure 3.18
Ethidium bromide stained agarose gel electrophoresis fractions (i) of poly (A) polymerase chain reaction products from driver (T0), tracer (T4) and subtraction products S1 to 4 and corresponding autoradiographs (ii) of Southern blots prepared from them after hybridisation with ^{32}P labelled probes to (a) driver cDNA, and (b) S4 cDNA.

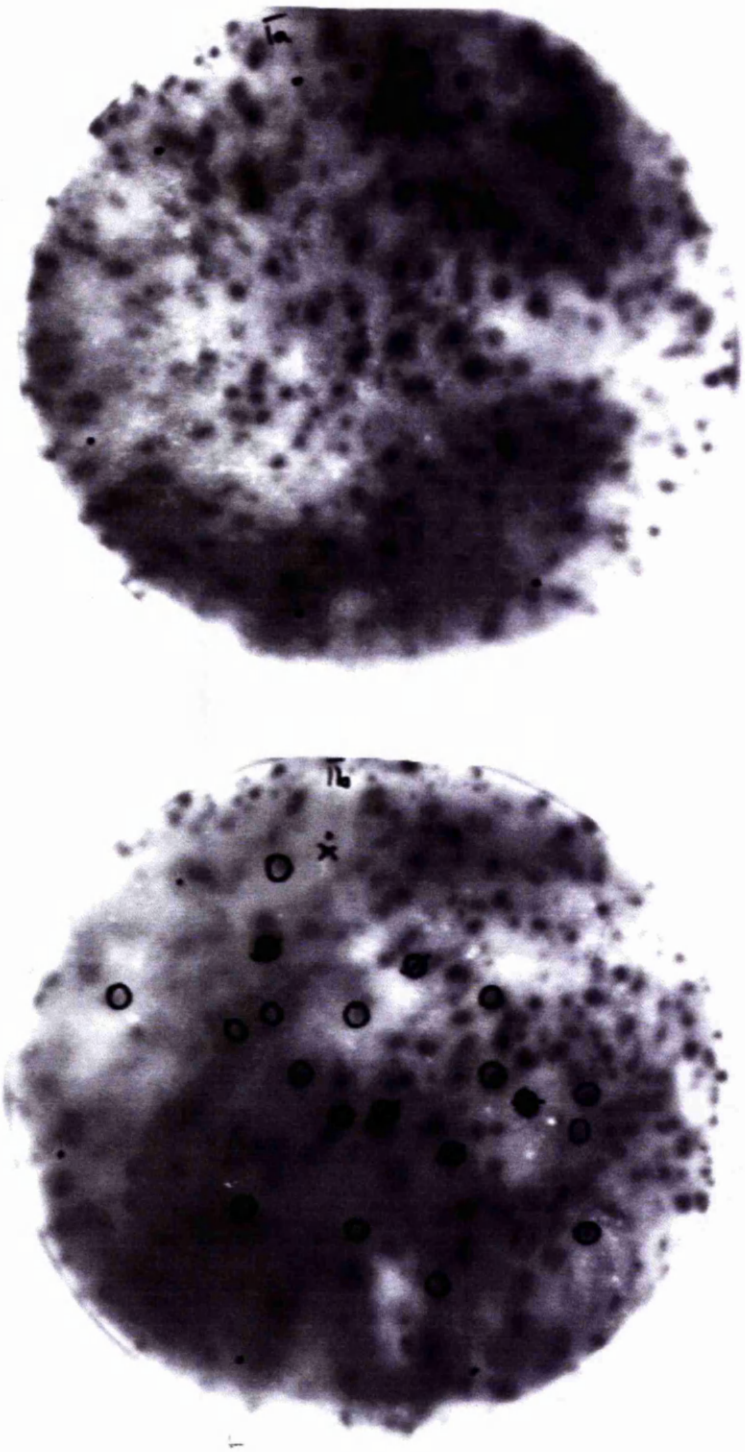


Figure 3.19a
Representative auto-radiographs of duplicate plaque lifts from primary library screen, hybridised with ^{32}P labelled probe S4 subtraction product. The location of the double positive plaques picked are circled on one of the autoradiographs.

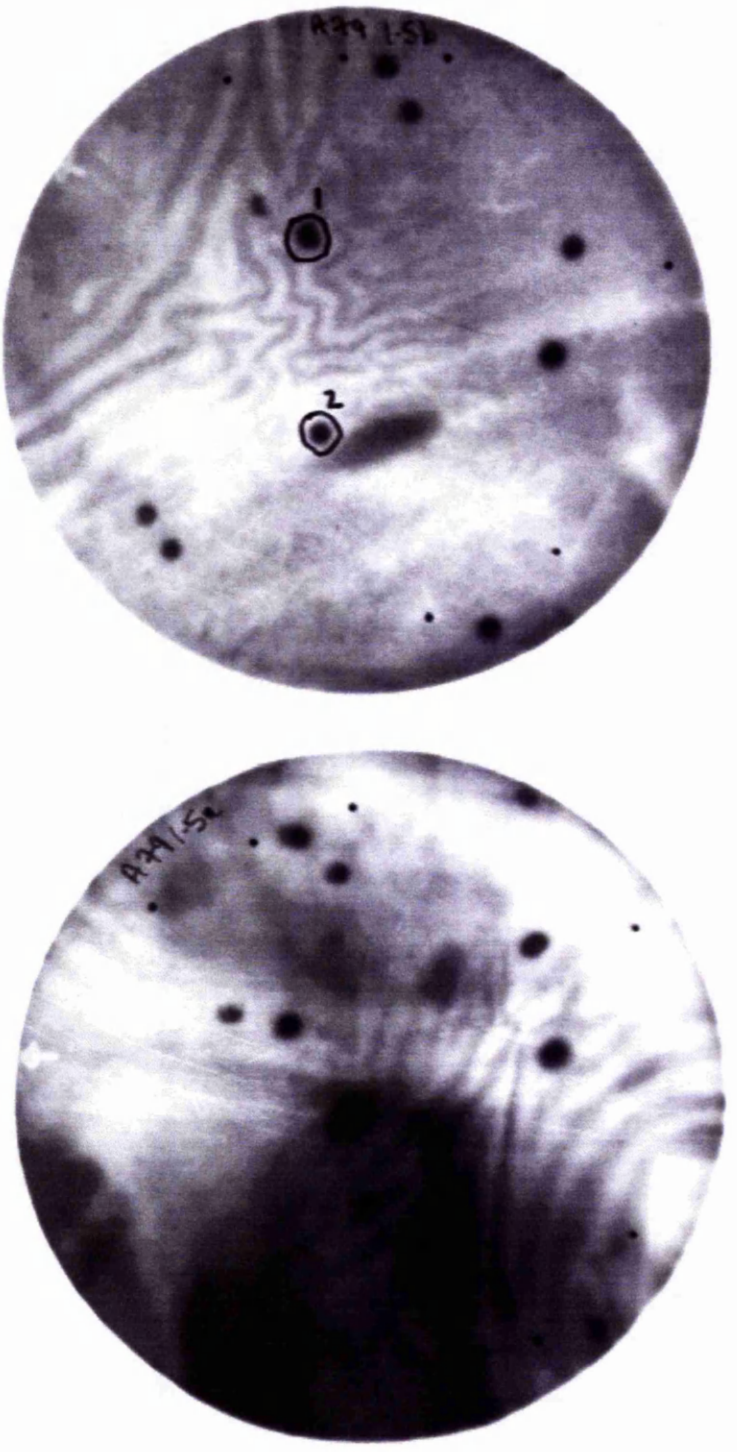


Figure 3.19b
Representative auto-radiographs of duplicate plaque lifts from secondary library screen, hybridised with ^{32}P labelled probe S4 subtraction product. The location of the double positive plaques picked are circled on one of the autoradiographs.

Clone	Gene homology	% identity
1.3	14-3-3 zeta	F – 97% over 537bp R - 96% over 516bp
1.6	Alpha enolase	F – 81% over 131bp R – 100% over 516bp
1.7	Non-skeletal tropomyosin	R – 99% over 548bp
1.9	KIAA0081	R – 99% over 601bp

Table 3.3 Genes identified by BLAST homology searches

For each gene identified, the secondary screen clone from which it was sequenced (local notation), its homology in the BLAST database, and both the % and base pair (bp) length over which the homology was present, for both forward (F) and reverse (R) are given; for further details see appendix 2.

3.3.7 Expression of isolated genes

3.3.7.1 Hybridisation with time point cDNA

For each of the four genes identified hybridisation with cDNA from the time points confirmed upregulation during stimulation with dexamethasone, as indicated in figure 3.20(a to d). For each of the genes the degree of hybridisation with the time-point cDNA was greater for the time-points following stimulation with dexamethasone (T1-T10), compared with that prior to stimulation (T0) (figure 3.20 a to d). The extent of upregulation was most marked for 14-3-3 zeta (figure 3.20a). The dot-blots are shown in figure 3.20, and the results of densitometric analysis, normalised to GAPDH expression, in table 3.4 confirming upregulation of 14-3-3 zeta, non-skeletal tropomyosin and KIAA0081 following stimulation with dexamethasone. Densitometry failed to confirm upregulation of alpha enolase expression (table 3.4).

3.3.7.2 Hybridisation with cytospin preparations of stimulated marrow stromal cells

For one of the genes, 14-3-3 zeta, immunohistochemistry was carried out on cytospins of cells prior to and following 4 days of stimulation with dexamethasone. Protein expression was increased in the latter cells (figure 3.21), confirming upregulation of the gene during early osteoblastic differentiation: negative controls showed no reactivity.

3.3.7.3 In-situ hybridisation with human bone

The results of *in-situ* hybridisation are given below for each of the genes studied, and the expression pattern of each summarised in tables 3.5 & 3.6. RNase controls were negative. For each case hybridisation was assessed over the entirety of three test sections.

3.3.7.3.1 14-3-3 zeta

14-3-3 ζ (zeta) demonstrated a basal level of expression in the haematopoietic stroma in normal bone, together with a low level of expression over osteoblasts (figure 3.22). Osteoclasts were negative in all sections examined (figure 3.23). The expression was decreased in osteoporotic bone, both pre- and post-HRT, in the osteoblasts but not over the haematopoietic stroma (figure 3.24). Hyperparathyroid bone demonstrated increased expression over the haematopoietic stroma, whilst osteoblasts and osteoclasts were negative (figure 3.25). Expression was increased compared to that in normal bone in both fetal bone and healing fracture callus. In fetal bone from a range of gestations intense signal was present over the osteoblasts, particularly those lining the primary and secondary

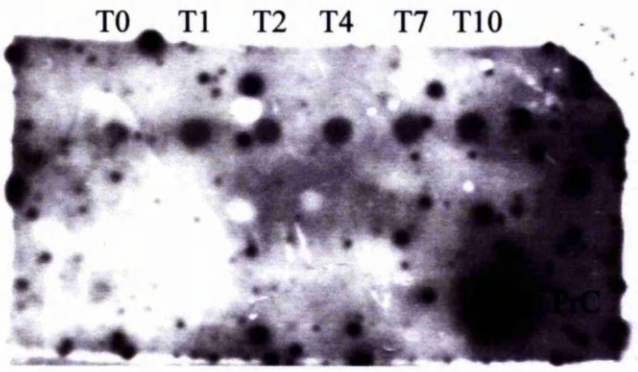


Figure 3.20a (i)

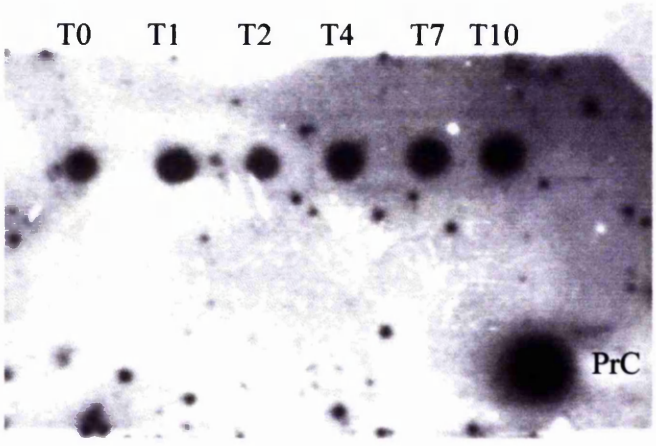


Figure 3.20a (ii)

Figure 3.20a
Auto-radiographs of hybridisation signal of ^{32}P labelled probes to (i) 14-3-3 zeta, and (ii) GAPDH probe with poly (A) RT-PCR products from clone 1 prior to (T0) and following 1(T1), 2(T2), 4(T4), 7(T7) and 10(T10) days of dexamethasone stimulation. Upregulation of expression of 14-3-3 zeta at T4, compared to T0, is demonstrated. By comparison GAPDH hybridisation is equal over each of the time-points.

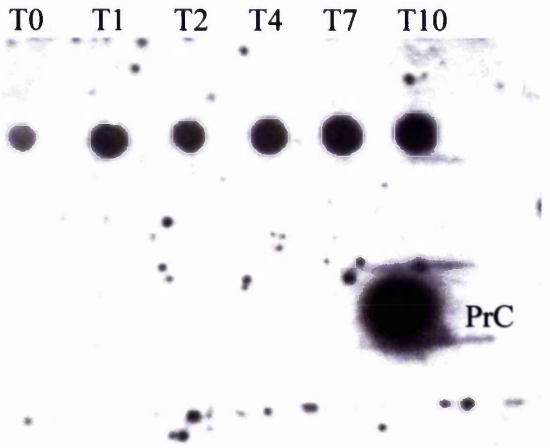


Figure 3.20b (i)

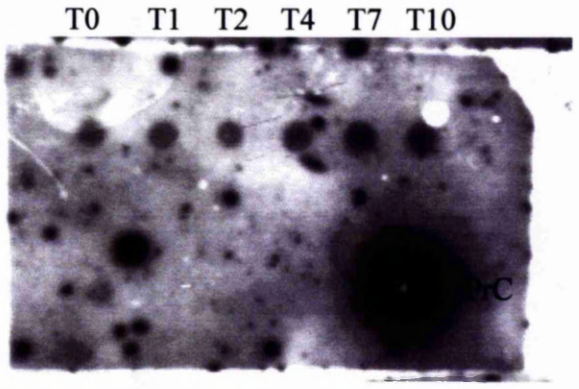


Figure 3.20b (ii)

Figure 3.20b
Auto-radiographs of hybridisation signal of ^{32}P labelled probes to (i) alpha enolase, and (ii) GAPDH probe with poly (A) RT-PCR products from clone 1 prior to (T0) and following 1(T1), 2(T2), 4(T4), 7(T7) and 10(T10) days of dexamethasone stimulation. Upregulation of expression of alpha enolase at T4, compared to T0, is demonstrated. By comparison GAPDH hybridisation is equal over each of the time-points. PrC = probe control

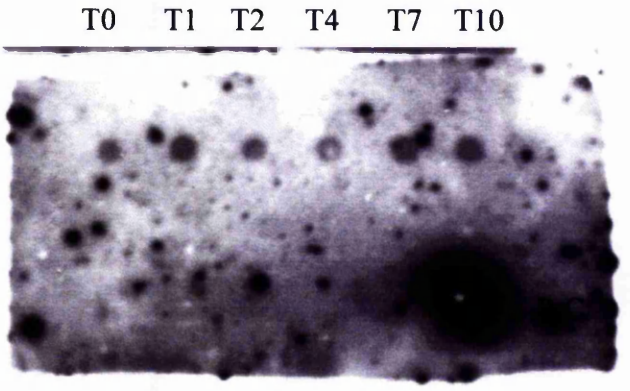


Figure 3.20c (i)

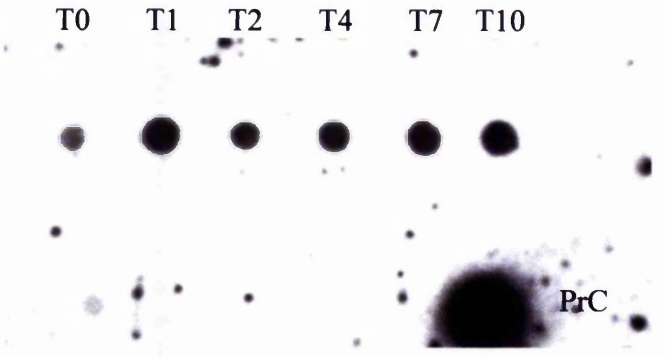


Figure 3.20c (ii)

Figure 3.20c
Auto-radiographs of hybridisation signal of ^{32}P labelled probes to (i) non-skeletal tropomyosin, and (ii) GAPDH probe with poly (A) RT-PCR products from clone 1 prior to (T0) and following 1(T1), 2(T2), 4(T4), 7(T7) and 10(T10) days of dexamethasone stimulation. Upregulation of expression of non-skeletal tropomyosin at T1 and T7, compared to T0, is demonstrated, though the level at T4 is not . By comparison GAPDH hybridisation is equal over the time-points T1 to T10. PrC = probe control

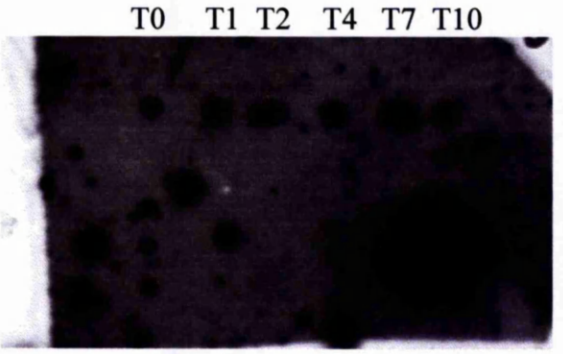


Figure 3.20d (i)

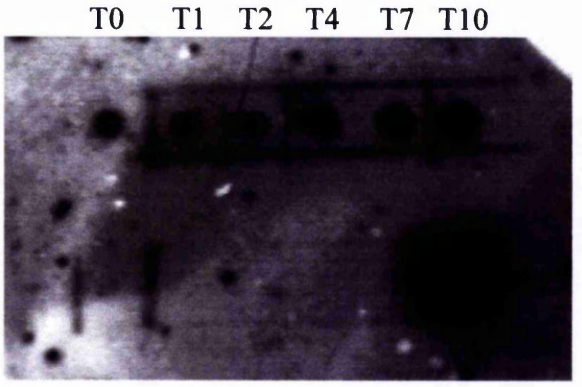


Figure 3.20d (ii)

Figure 3.20d
Auto-radiographs of hybridisation signal of ^{32}P labelled probes to (i) KIAA0081, and (ii) GAPDH probe with poly (A) RT-PCR products from from clones 1 prior to (T0) and following 1(T1), 2(T2), 4(T4), 7(T7) and 10(T10) days of deaxmethasone stimulation. Upregulation of expression of non-skeletal tropomyosin at T1 and T7, compared to T0, is demonstrated, as is the level at T4. By comparison GAPDH hybridisation is equal over each of the time-points. PrC = probe control

Gene	T0	T1	T2	T4	T7	T10
14-3-3 zeta	100	110	127	120	121	136
Alpha enolase	100	76	73	84	94	87
Non-skeletal tropomyosin	100	118	102	119	100	108
KIAA0081	100	125	133	105	133	85

Table 3.4

Densitometric measurements of hybridisation intensity of P^{32} labelled probes for each of the genes isolated with dot-blotted cDNA from each of the time-points (T0 to T10) from clone 1. Each measurement was normalised to the hybridisation intensity of P^{32} labelled probe for GAPDH to the same sample of cDNA, and expressed as a percentage of the normalised intensity for the gene at T0. Upregulation of expression, normalised to expression of GAPDH, following stimulation with dexamethasone is confirmed for 14-3-3 zeta, non-skeletal tropomyosin and KIAA0081, though the expression of alpha enolase fell when compared to that for GAPDH.

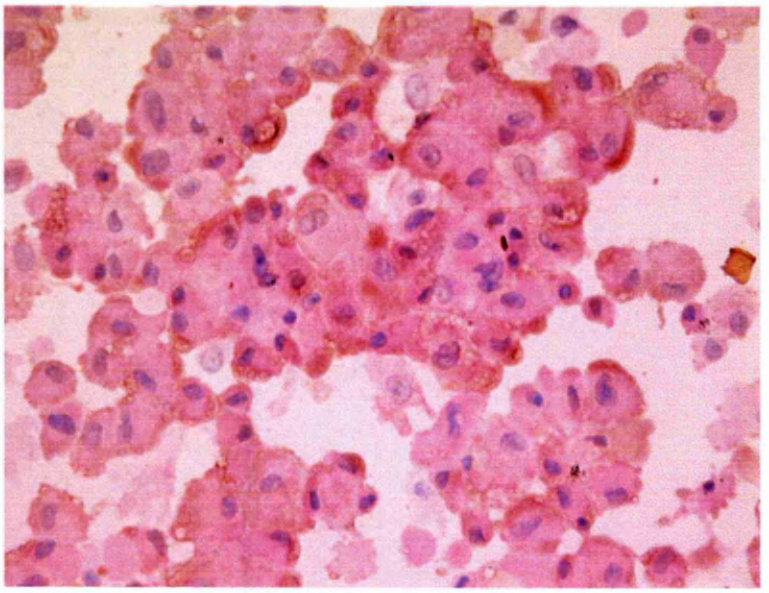


Figure 3.21a

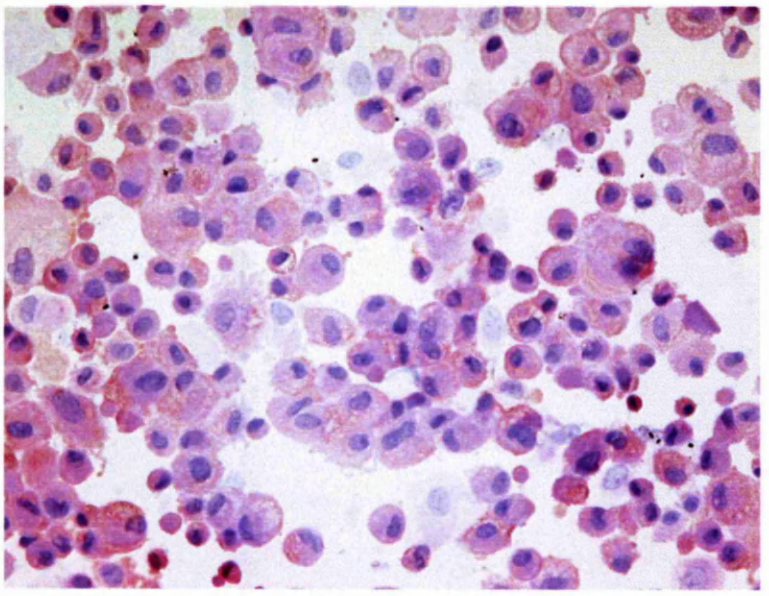


Figure 3.21b

Figure 3.21
Immunohistochemical detection of 14-3-3 zeta in cytospin preparations of bone marrow stromal cells, demonstrating (a) upregulation of protein expression (x400) in cells stimulated with dexamethasone, compared with (b) unstimulated cells (x400). H&E, protein expression disclosed with DAB.

Bone Status (adult bone)	Haematopoietic stroma	Osteoblasts	Osteoclasts	Condensing mesenchyme
Normal	++	+	neg	NA
Healing fracture	NA	+++	neg	+++
OP pre-HRT	+	neg	neg	NA
OP post-HRT	+	neg	neg	NA
Paget's disease	++	neg	neg	NA
HPT bone disease	+++	neg	neg	NA

Table 3.5a *In-situ* expression pattern for 14-3-3 zeta

Normal	++	+	neg	NA
Healing fracture	NA	+++	neg	+++
OP pre-HRT	+	neg	neg	NA
OP post-HRT	+	neg	neg	NA
Paget's disease	+	neg	+/++	NA
HPT bone disease	++	+	neg	NA

Table 3.5b *In-situ* expression pattern for KIAA0081

Normal	+	neg	neg	NA
Healing fracture	NA	neg	neg	neg
OP pre-HRT	+	neg	neg	NA
OP post-HRT	+	neg	neg	NA
Paget's disease	+	neg	neg	NA
HPT bone disease	+	neg	neg	NA

Table 3.5c *In-situ* expression pattern for alpha enolase

Normal	+	neg	neg	NA
Healing fracture	NA	+++	neg	+++
OP pre-HRT	+	neg	neg	NA
OP post-HRT	+	neg	neg	NA
Paget's disease	+	neg	++	
HPT bone disease	+++	+	neg	NA

Table 3.5d *In-situ* expression pattern for non-skeletal tropomyosin

For each gene expression assessed as low (+), moderate (++), high (+++) or absent (neg); HPT=hyperparathyroid, OP=osteoporotic, NA=not applicable

Bone status	Haematopoietic stroma	Osteoblasts	Osteoclast	Growth plate	Periosteal mesenchyme	Cartilage
Normal	+ / ++	+++	neg	++ / +++	++	neg
IUGR	+ / ++	+	neg	+	+	neg
OI	+ / ++	+++	neg	++ / +++	++	neg
CCC	+ / ++	neg	neg	neg	+	neg
DD	+ / ++	+	neg	+	neg	neg
CD	+ / ++	+	neg	+	+	neg
SRPS	+ / ++	++++	neg	++++	++	neg

Table 3.6 *In-situ* expression pattern for 14-3-3 zeta in fetal bone, expressed as low (+), moderate (++), high (+++), maximal (++++) or absent (neg). Expression was assessed in a range of normal fetal bones (n=5), and bones from cases of intra-uterine growth retardation (IUGR), and a variety of skeletal dysplasias, namely

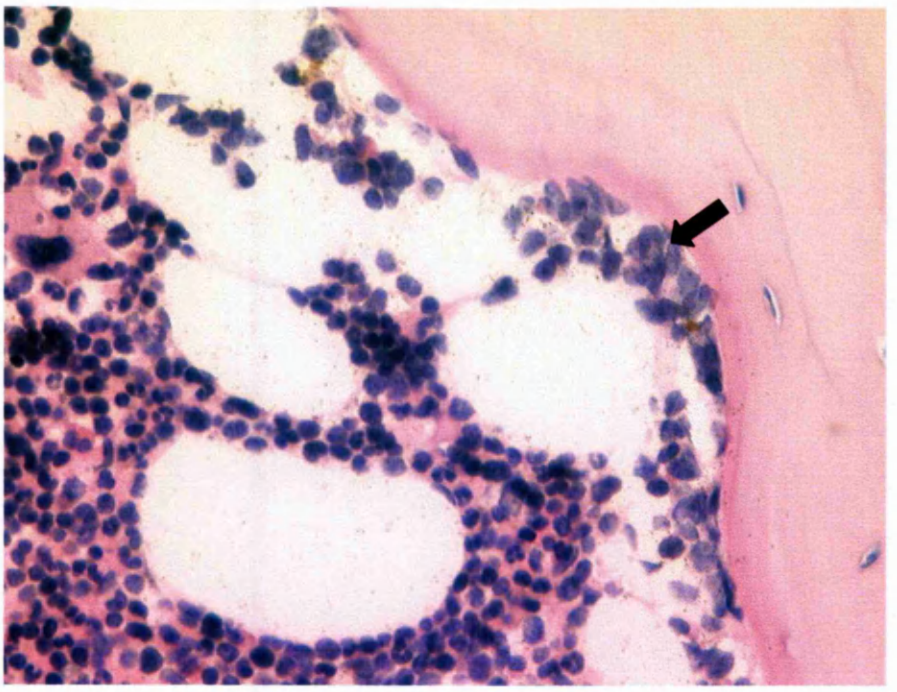


Figure 3.22a

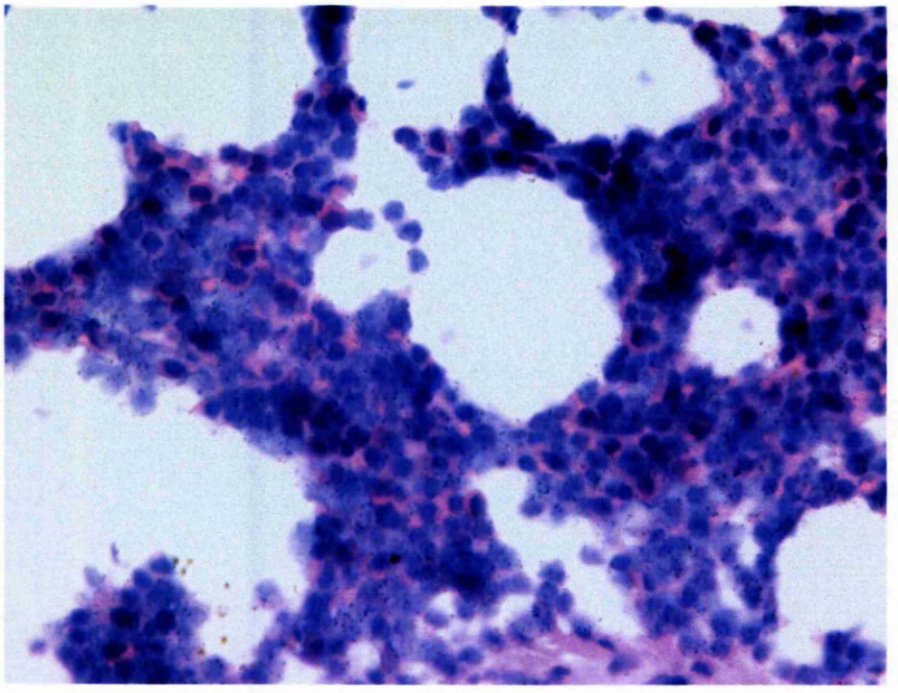


Figure 3.22b

Figure 3.22
Photomicrographs showing low basal level of expression of 14-3-3 zeta mRNA in (a) osteoblasts (arrow), and (b) haematopoietic stroma, in normal bone. H&E, magnification x400.

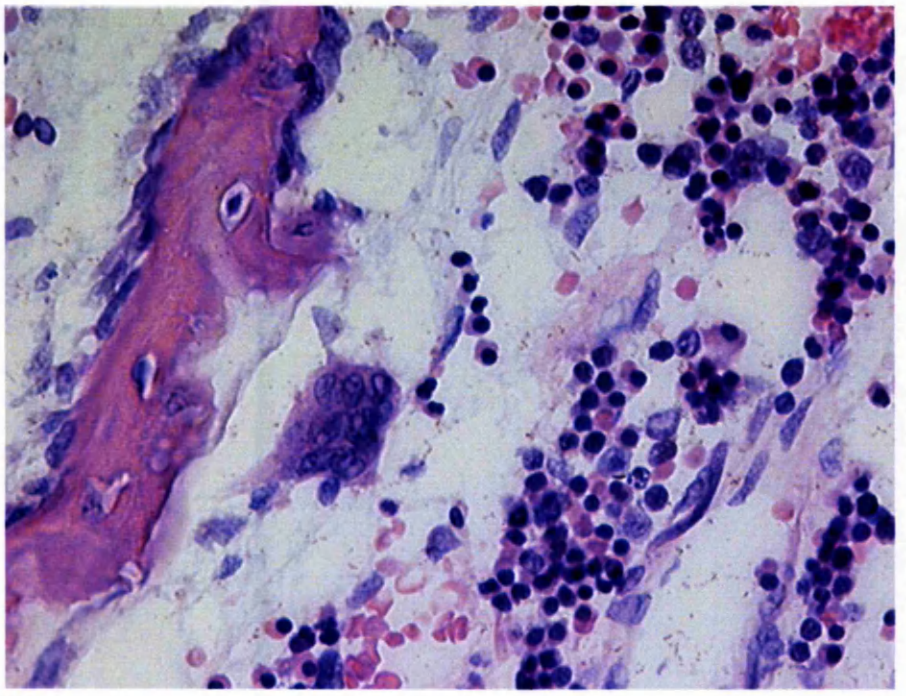


Figure 3.23
Photomicrograph showing absence of expression of 14-3-3 zeta mRNA in osteoclast in Pagetic bone. H&E, magnification x400.

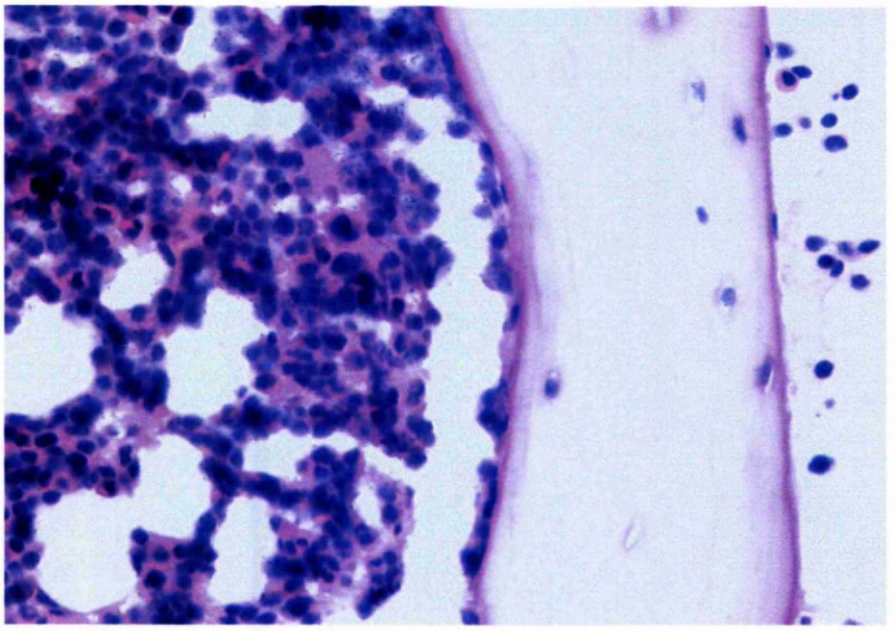


Figure 3.24
Photomicrograph showing14-3-3 zeta mRNA expression in osteoporosis, showing absence over osteoblasts (arrow), and persistence over the haematopoietic stroma (*). H&E, magnification x400.

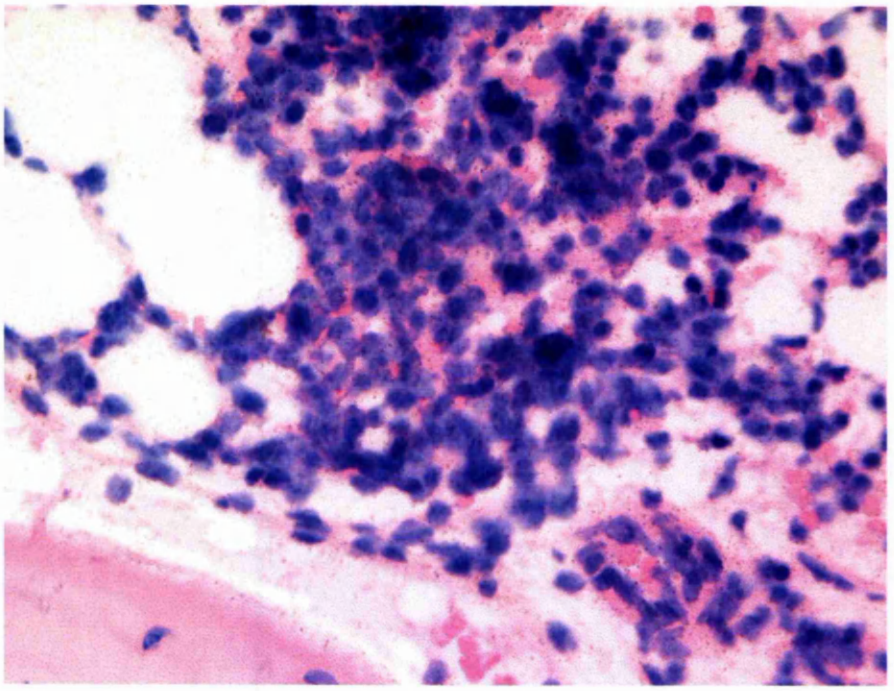


Figure 3.25

Figure 3.25
Photomicrograph showing expression of 14-3-3 zeta mRNA over haematopoietic stroma, but absence of expression over bone surfaces, in hyperparathyroid bone. H&E, magnification x400.

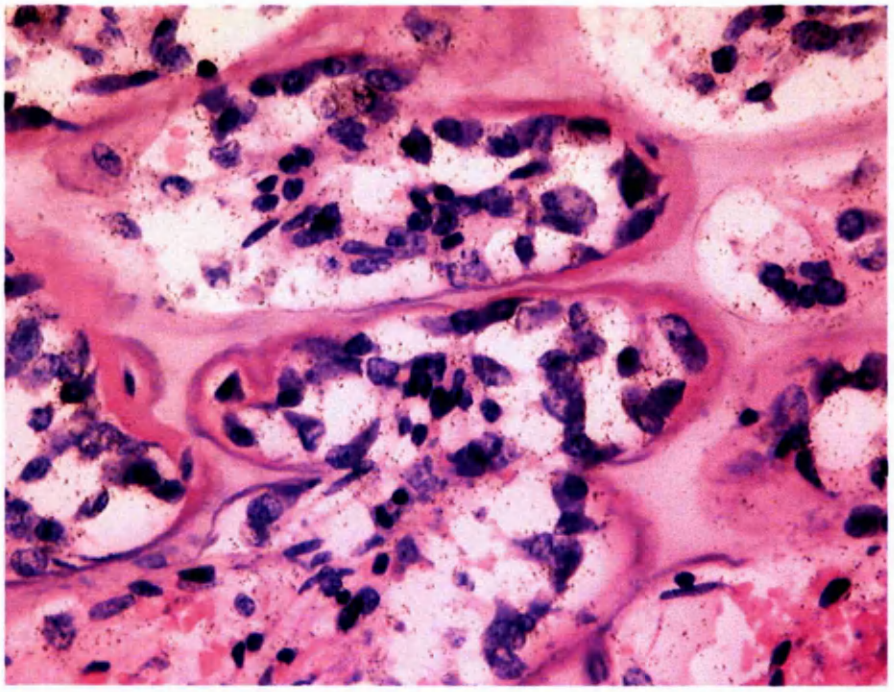


Figure 3.26a

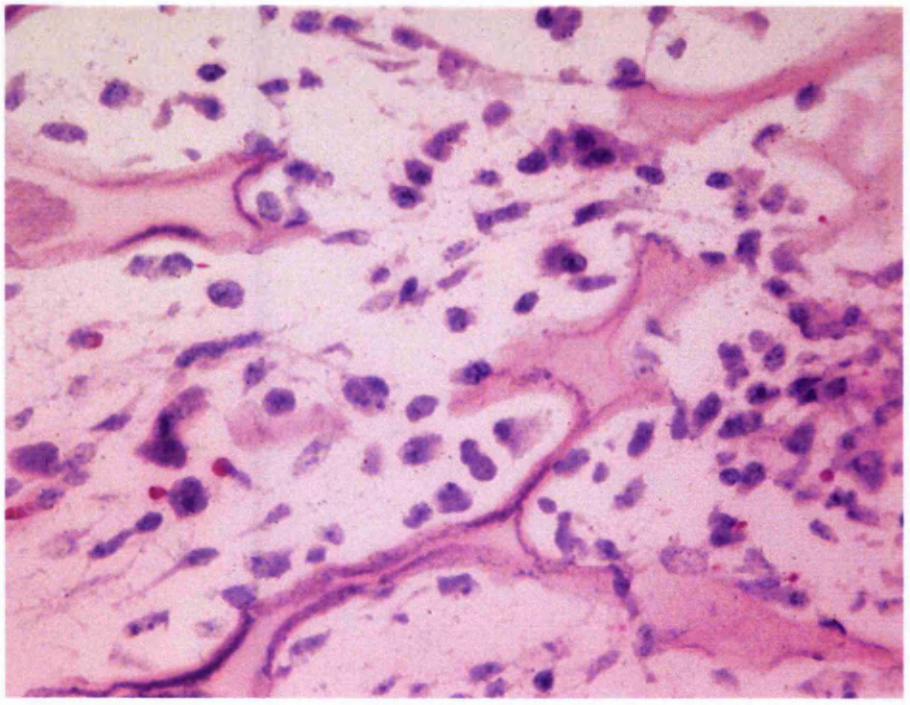


Figure 3.26b

Figure 3.26
Photomicrographs showing (a) high expression of 14-3-3 zeta mRNA in osteoblasts lining primary spongiosa in fetal bone, and (b) lack of hybridisation signal in RNase control section. H&E, magnification x400

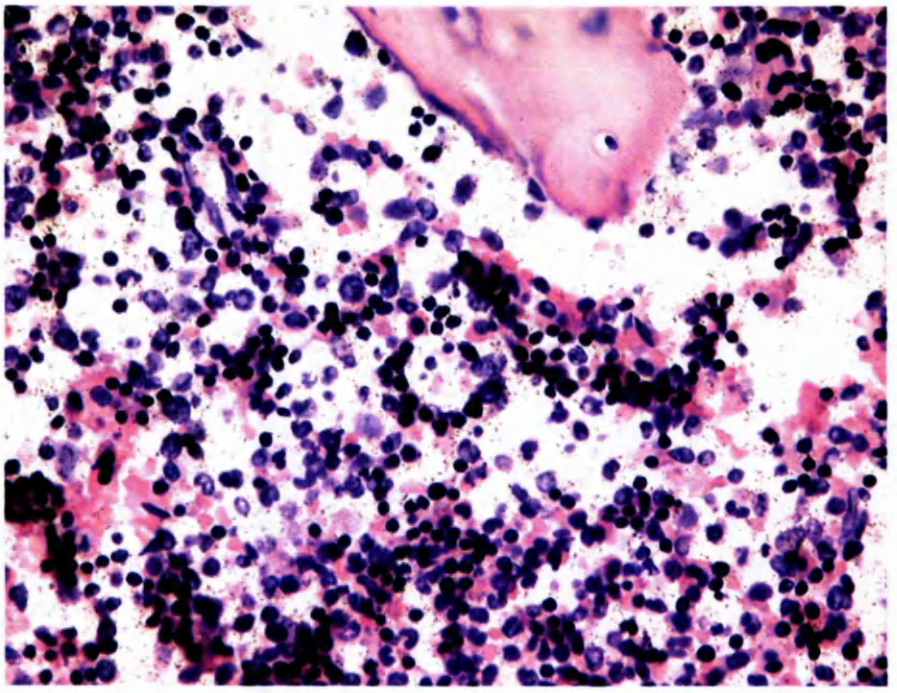


Figure 3.27
Photomicrograph showing low basal expression of 14-3-3 zeta mRNA in haematopoietic stroma in fetal bone. H&E, magnification x200.

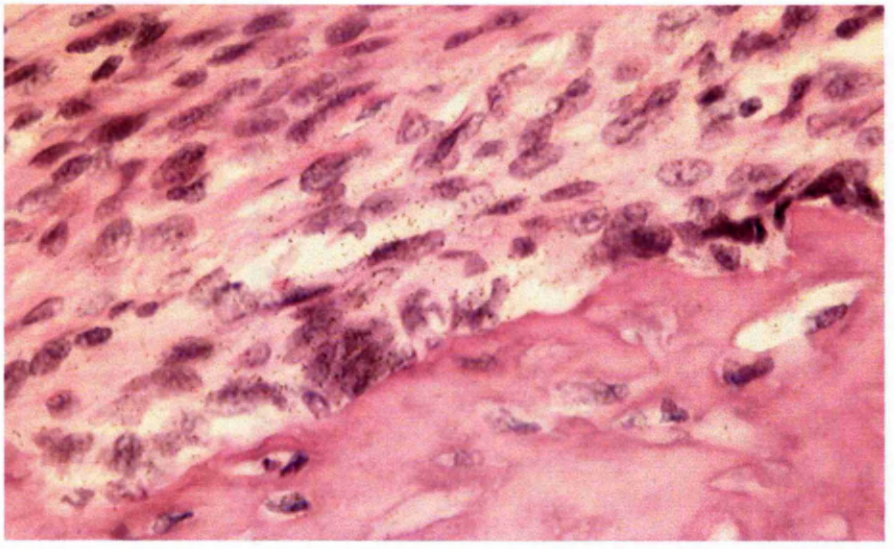


Figure 3.28
Photomicrograph showing high expression of 14-3-3 zeta mRNA in periosteal mesenchyme adjacent to endosteal surface of cortical bone in fetal long bone. H&E, magnification x400

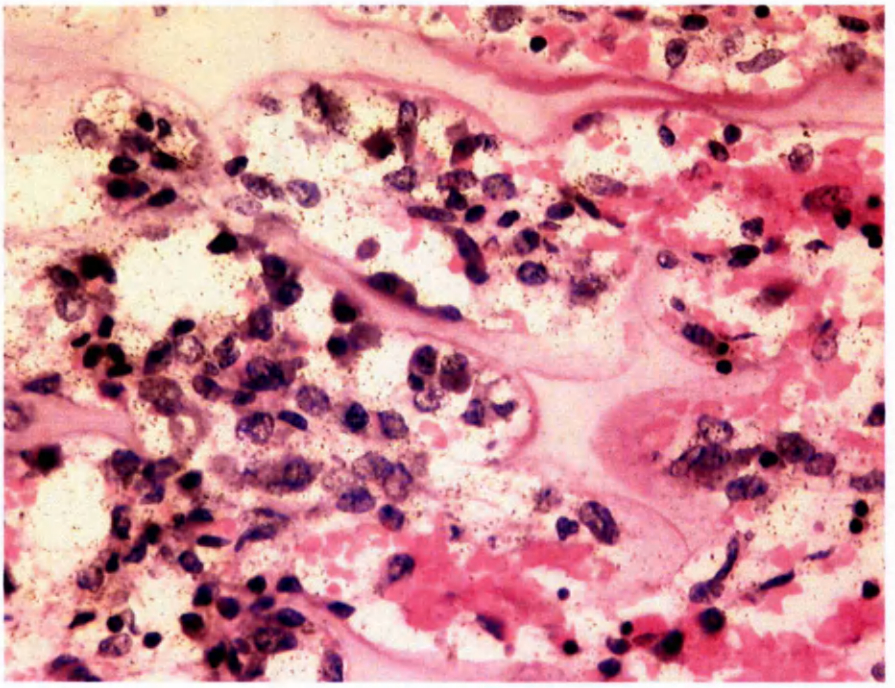


Figure 3.29
Photomicrograph showing strong expression of 14-3-3 zeta mRNA in growth plate of fetal long bone. H&E, magnification x400

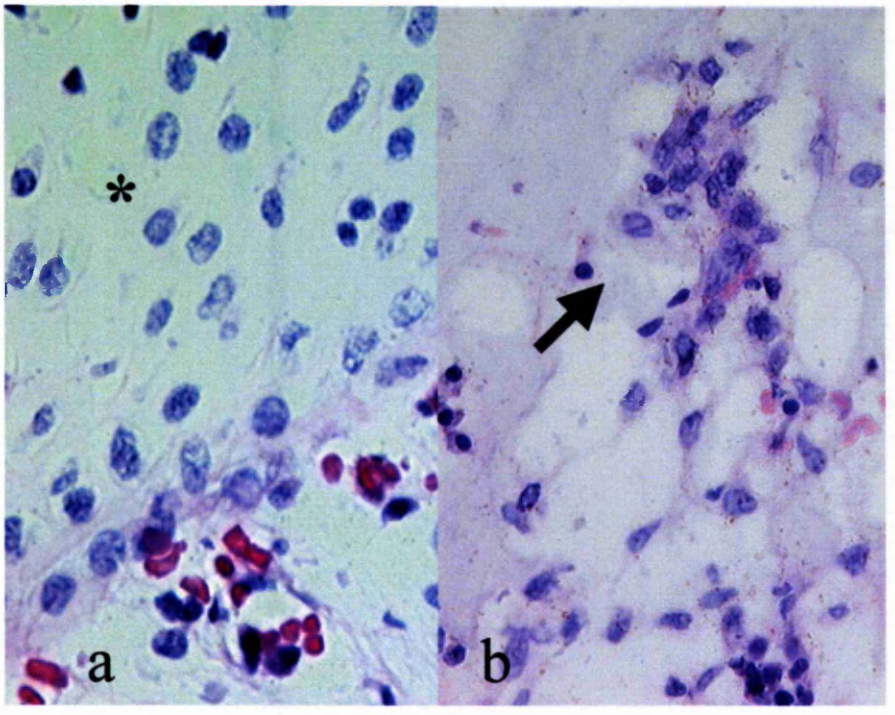


Figure 3.30
Absence of hybridisation signal for 14-3-3 zeta mRNA in (a) cartilage(*) at edge of growth plate, compared with expression in (b) adjacent osteoblastic (arrow) cells in fetal long bone. H&E, x400.

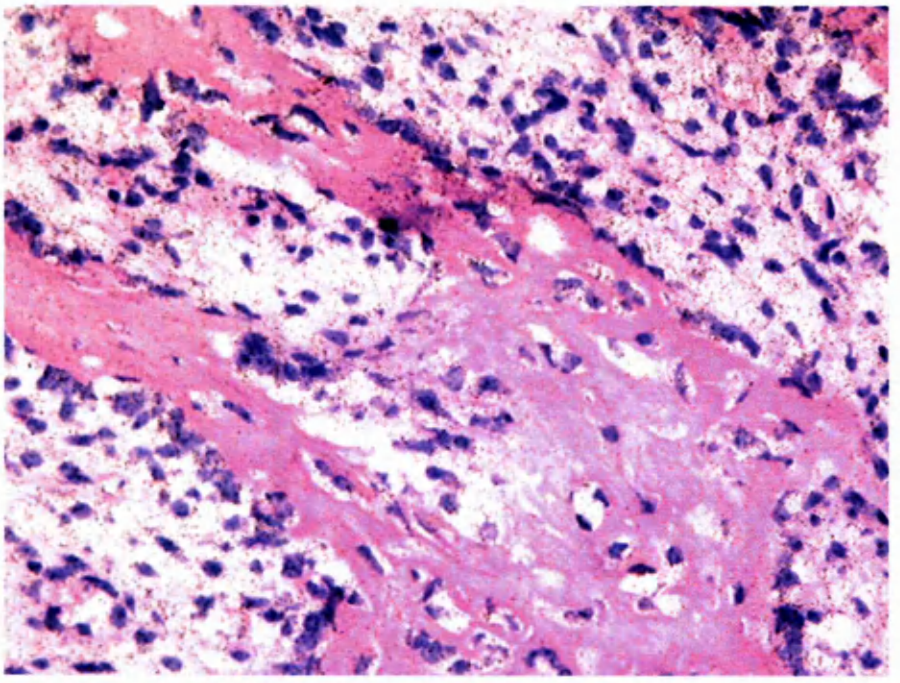


Figure 3.31a

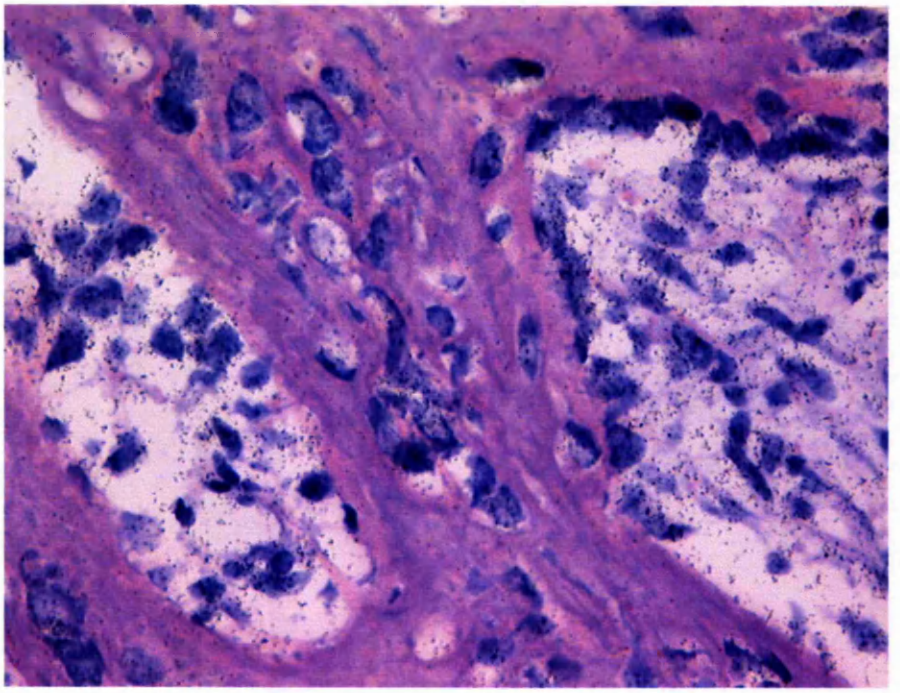


Figure 3.31b

Figure 3.31
Photomicrographs (a&b) showing strong expression of 14-3-3 zeta mRNA in woven bone, both in adjacent mesenchyme and in osteoblasts, in healing fracture. H&E, magnification (a) x200,(b) x400

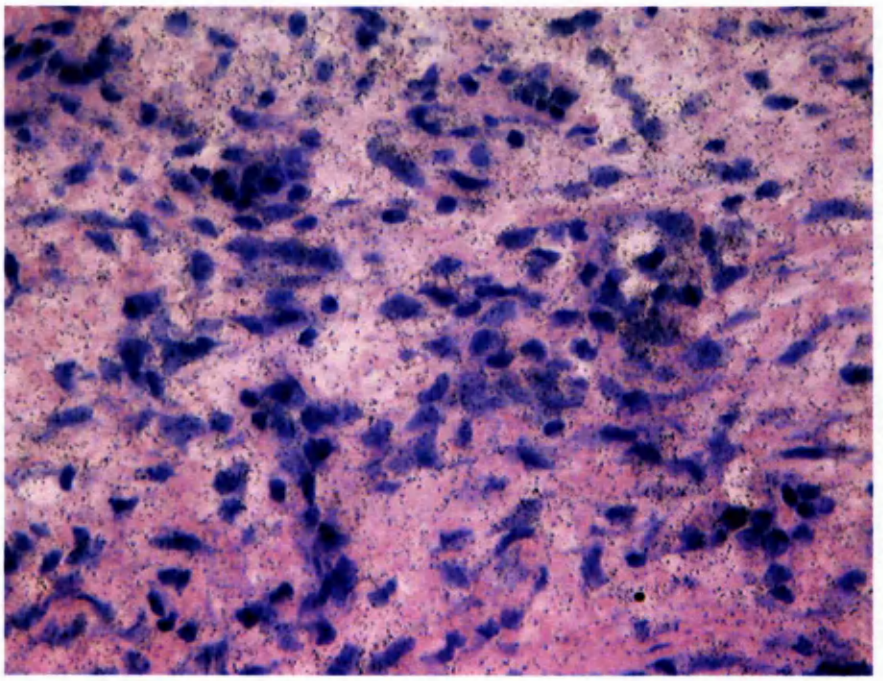


Figure 3.32a

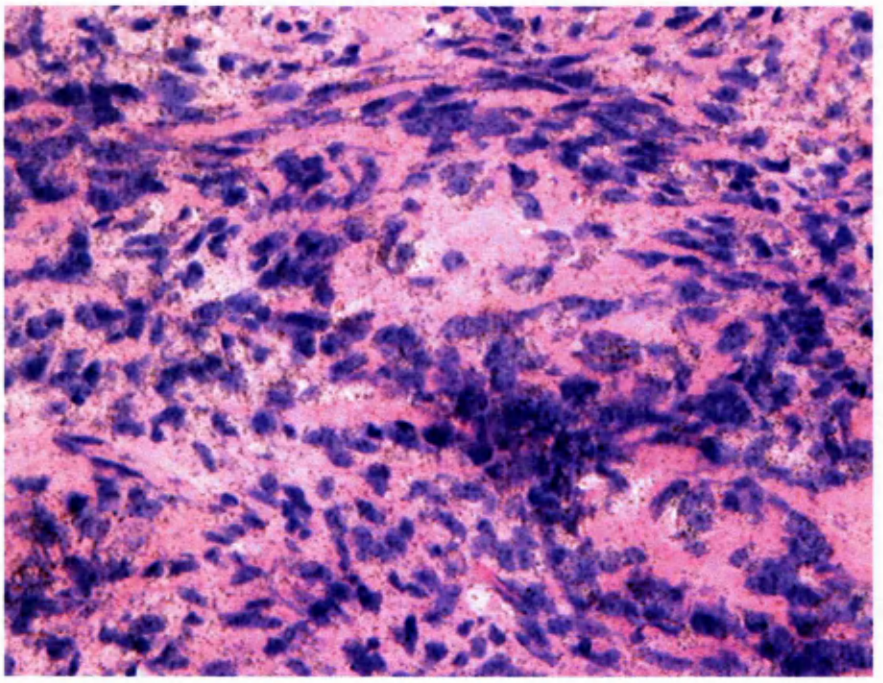


Figure 3.32b

Figure 3.32
Photomicrographs showing strong expression of 14-3-3 zeta mRNA in condensing mesenchyme in healing fracture. H&E, magnification x400.

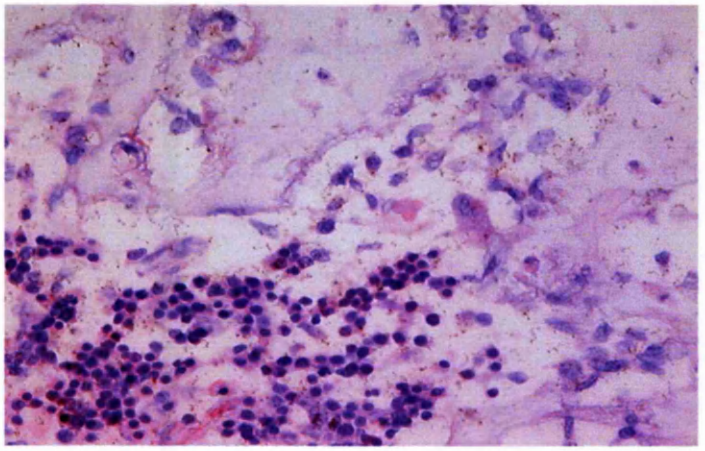


Figure 3.33a

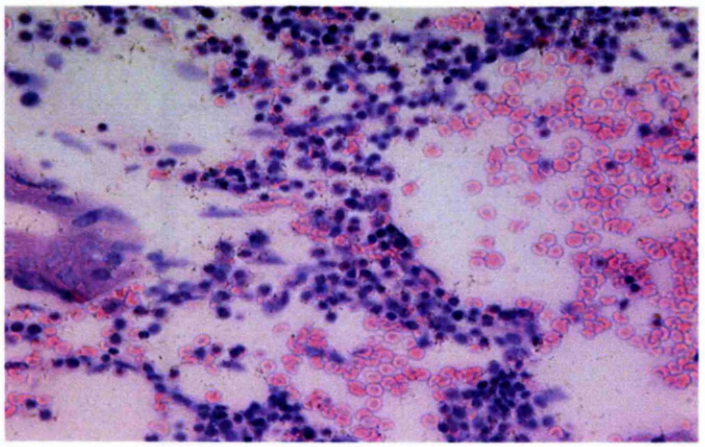


Figure 3.33b

Figure 3.33
Photomicrographs showing reduced expression, compared to normal fetal bone, of 14-3-3 zeta mRNA in (a) growth plate; expression over haematopoietic stroma was normal (b). H&E, magnification x200.

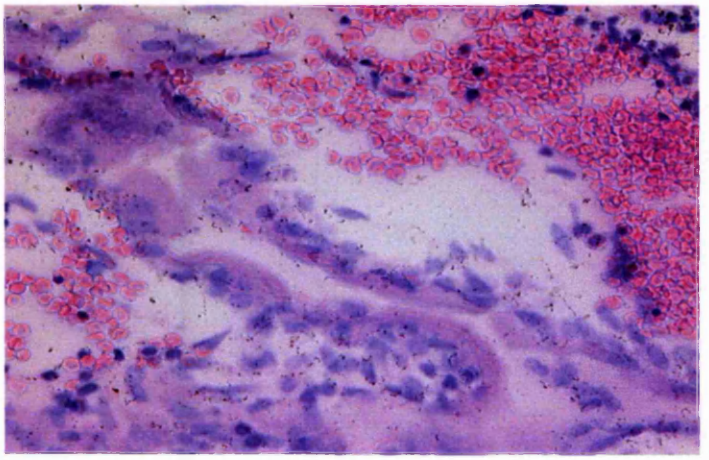


Figure 3.34a

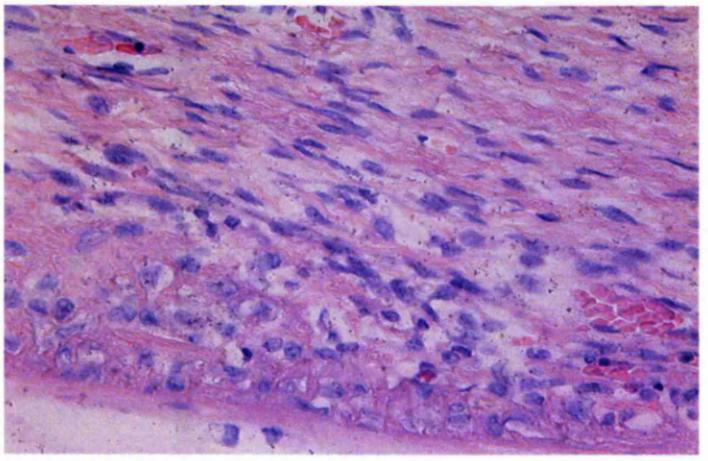


Figure 3.34b

Figure 3.34
Photomicrographs showing a normal level, compared to normal fetal bone, of expression of 14-3-3 zeta mRNA in (a) osteoblasts lining primary spongiosa, and (b) periosteal mesenchyme in osteogenesis imperfecta. H&E, magnification x200

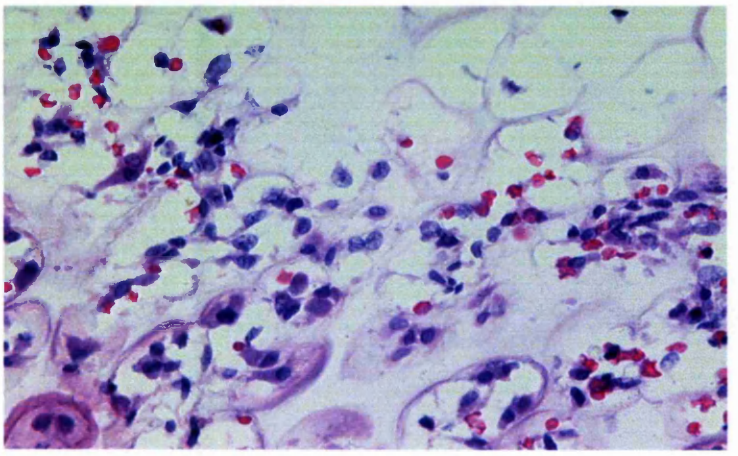


Figure 3.35a

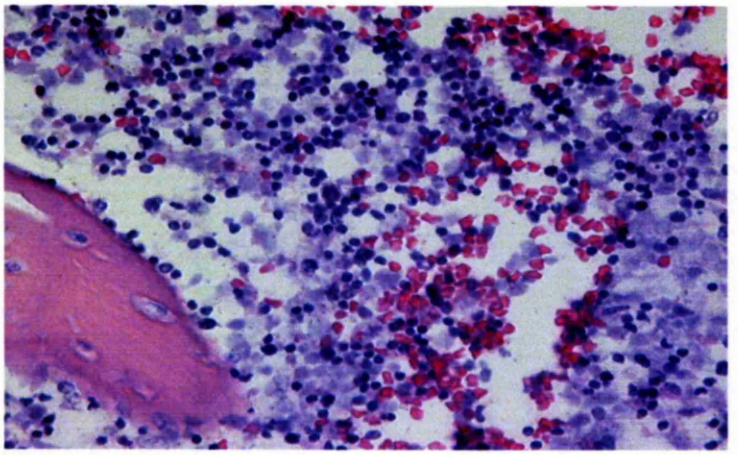


Figure 3.35b

Figure 3.35
Photomicrographs showing no detectable expression of 14-3-3 zeta mRNA in fetal bones in chondrodysplasia calcificans congenita; (a) growth plate (x400), and (b) osteoblasts lining primary spongiosa (x200). H&E.

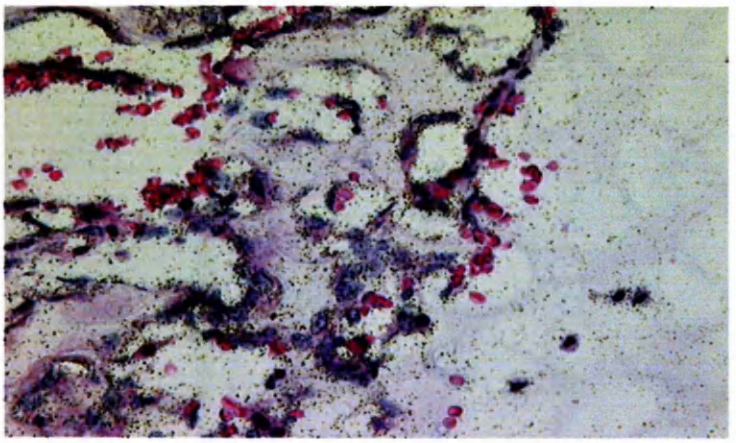


Figure 3. 36a

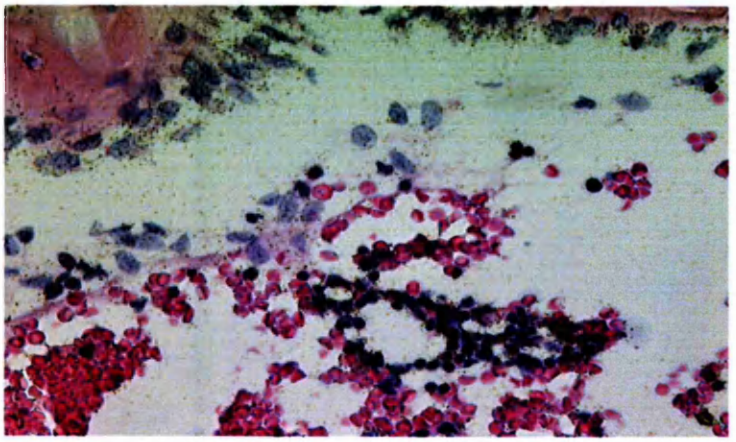


Figure 3.36b

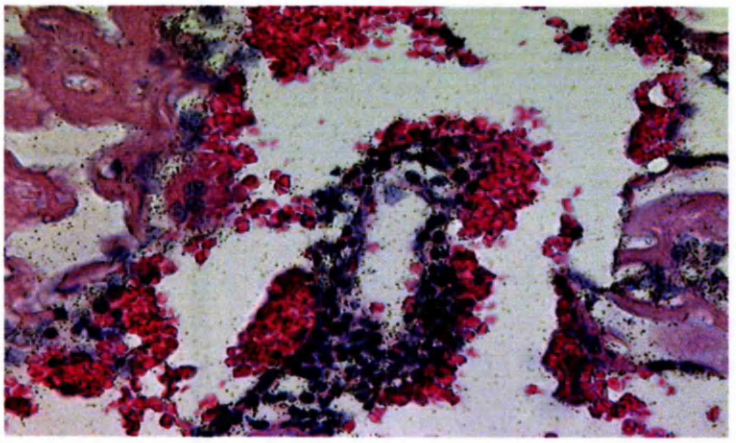


Figure 3.36c

Figure 3.36
Photomicrographs showing high expression of 14-3-3 zeta mRNA in fetal bone in short rib polydactyly syndrome; (a) osteoblasts lining primary spongiosa (x200), (b) periosteal mesenchyme (x400), and (c) growth plate (x400). H&E.

spongiosa (figure 3.26). It was present to a lesser degree over the haematopoietic stroma (figure 3.27), but was increased over the periosteal mesenchyme (figure 3.28). Hybridisation signal was present in large amounts over the osteoblasts in the growth plate (figure 3.29), but the epiphyseal cartilage was negative (figure 3.30). The same pattern of expression was present in fetal long bones from a wide variety of gestations (15-36 weeks) and the osteoclasts were again negative. In healing fracture callus expression was markedly increased over osteoblasts lining new, woven bone (figure 3.31), and over areas of condensing mesenchyme (figure 3.32). Signal was present, though not at high levels, over areas of mature fibrous tissue, whilst attached skeletal muscle was negative.

The expression of 14-3-3 ζ was also determined in cases of intra-uterine growth retardation (IUGR) and in a variety of skeletal dysplasias. The results are best considered in two parts, those for (IUGR) and those for the skeletal dysplasias. Compared to normal fetal bone, in the cases with IUGR expression of 14-3-3 ζ was reduced to low levels in both the growth plate and the osteoblasts lining the primary spongiosa, and reduced, though not to the same extent in the periosteal mesenchyme (figure 3.33). The level of expression over the haematopoietic stroma was normal, indicating that the levels in the other tissues had not been altered by autolysis (figure 3.33). The expression profile was different in each of the skeletal dysplasias. In the case of osteogenesis imperfecta expression was at the same level as in normal fetal bone in the growth plate, the osteoblasts lining the primary spongiosa, and in the periosteal mesenchyme (figure 3.34). As in the normal bone, the epiphyseal cartilage was negative, whilst a low basal level of expression was present over the haematopoietic stroma. In the cases of chondrodysplasia calcificans congenita, diastrophic dysplasia and camptomelic dysplasia (table 3.6 & figure 3.35) expression was reduced in the growth plate and in the osteoblasts lining the primary spongiosa; the latter were negative in the case of chondrodysplasia calcificans congenita. In each of these the expression was also reduced in the periosteal mesenchyme; in the case of diatrophic dysplasia it was absent. In all three cases expression was present over the haematopoietic stroma. In contrast, in the case of short rib polydactyly syndrome expression was increased in both the growth plate and in the osteoblasts compared to normal (figure 3.36), whilst expression in the other tissues, i.e. the haematopoietic stroma, the periosteal mesenchyme, and the cartilage and osteoclasts, both of which were negative, was normal.

3.3.7.3.2 KIAA0081

A similar expression pattern to that observed for 14-3-3 zeta was seen for KIAA0081. In particular, expression was present at a low basal level over haematopoietic tissue in normal bone, and low over bone surfaces (figure 3.37). It was absent in osteoporotic bone, both pre- and post- HRT, and increased in healing fracture, both in osteoblasts adjacent to new pieces of woven bone and over areas of condensing mesenchyme. It was also expressed in osteoclasts in Pagetic bone.

3.3.7.3.3 Alpha enolase

Alpha enolase expression was low over all cell types in each of the samples studied (table 3.5c).

3.3.7.3.4 Non-skeletal tropomyosin

Non-skeletal tropomyosin was expressed at a low basal level over the haematopoietic stroma in normal adult bone, in which it was absent over the osteoblasts and osteoclasts (figure 3.38). The same expression profile was observed in osteoporotic bone, both pre- and post-HRT, whilst in Pagetic bone it was expressed in osteoclasts and over the haematopoietic stroma at low levels. It was upregulated over the stroma in hyperparathyroid bone and over mesenchyme in healing fracture (figure 3.38), in a similar pattern to that observed for 14-3-3 zeta.

3.3.7.4 Hybridisation with human RNA master blot

Of the four genes isolated KIAA0081 was the only one for which no previous comprehensive tissue distribution was present in the literature, and for which, in particular, its presence in bone or bone marrow had not previously been reported. The pattern of hybridisation is shown in figure 3.39. Expression was highest in kidney and placenta, high in heart, brain, liver, lung, pancreas, small intestine and pituitary gland, and less strong in aorta, skeletal muscle, colon, bladder, uterus, prostate, stomach and the adrenal, thyroid and salivary glands. It was present at low levels in the mammary gland, and in the lymph node and bone marrow, and virtually absent in peripheral leucocytes and the trachea. The expression pattern in the fetal tissues recapitulated that of the adult tissues. Positive control human DNA was positive, whilst the negative control yeast total RNA, E.coli rRNA, Poly r(A) and human cot 1DNA samples showed no hybridisation. The E.coli DNA was positive; this is a recognised artefact of the system (Brown, 1999).

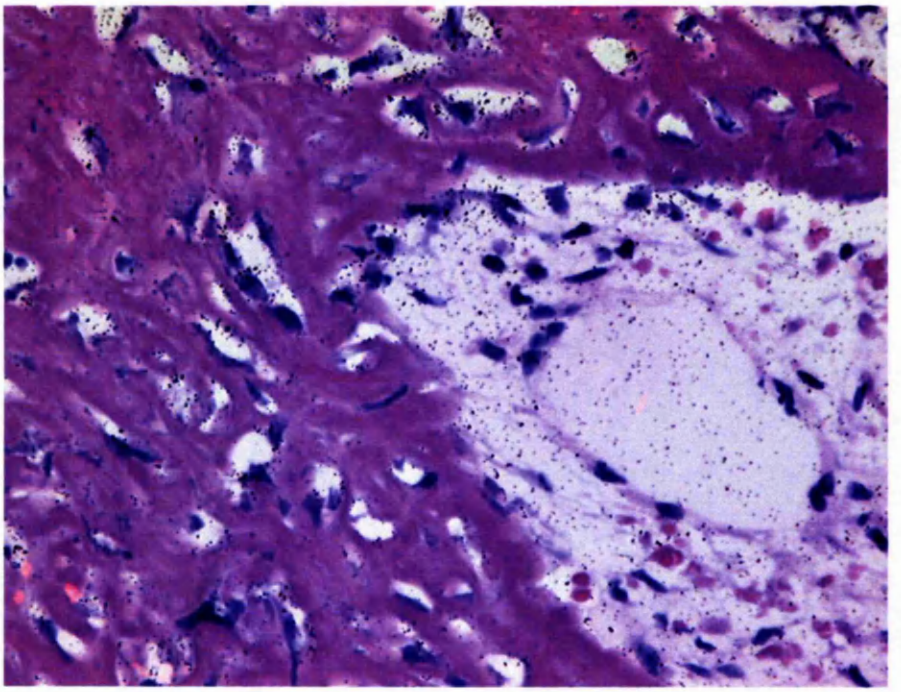


Figure 3.37a

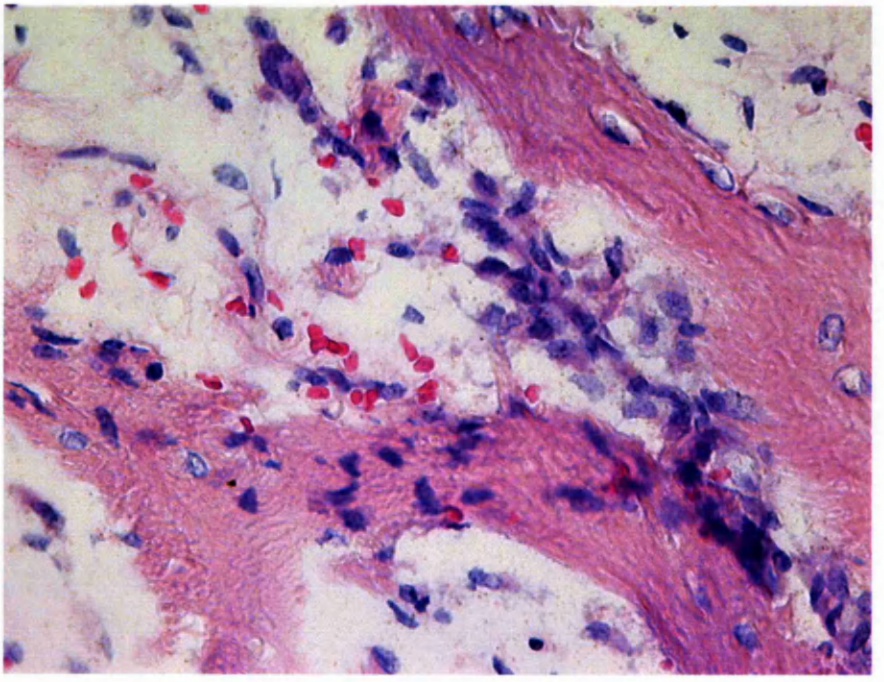


Figure 3.37b

Figure 3.37
Photomicrographs showing (a) high level of expression of KIAA0081 mRNA over osteoblasts in and adjacent to woven bone in healing fracture, and (b) absence of hybridisation signal in RNase control. H&E, magnification x400.

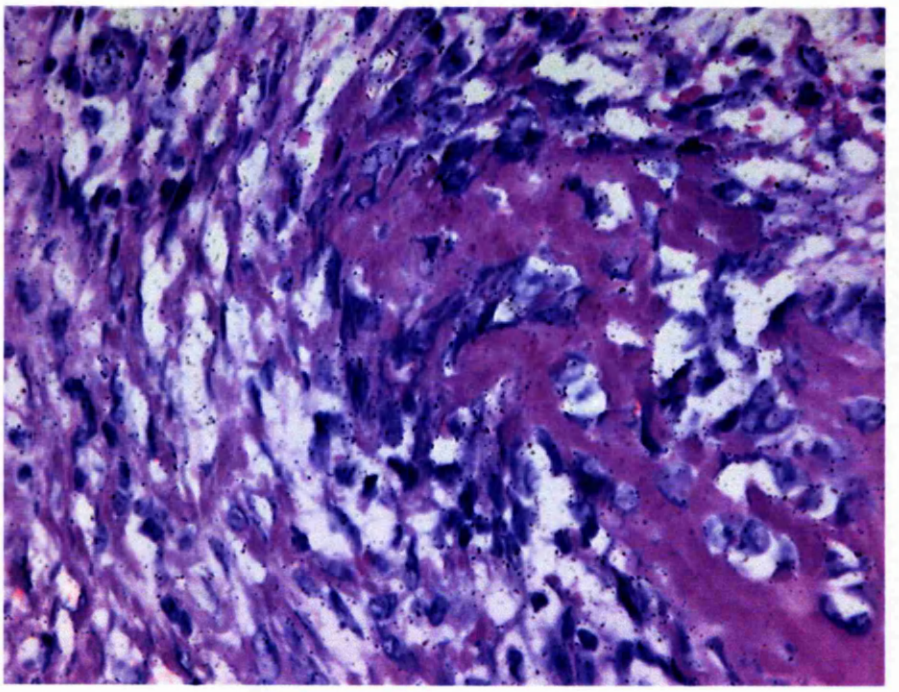


Figure 3.37c

Figure 3.37c
Photomicrograph showing increased level of expression of KIAA008 mRNA over condensing mesenchyme adjacent to woven bone in healing fracture. H&E, magnification x400.

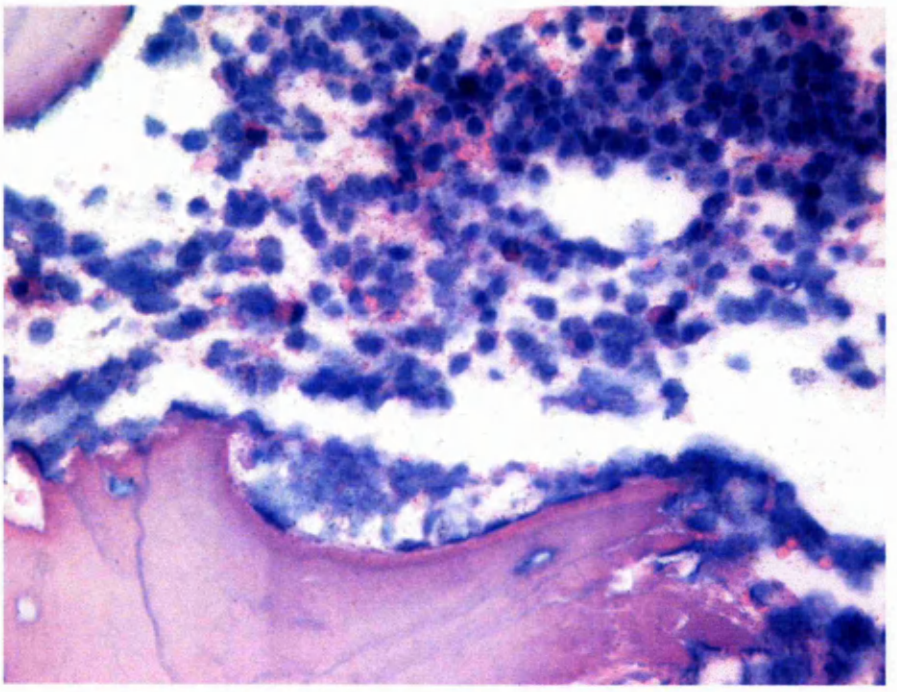


Figure 3.38a

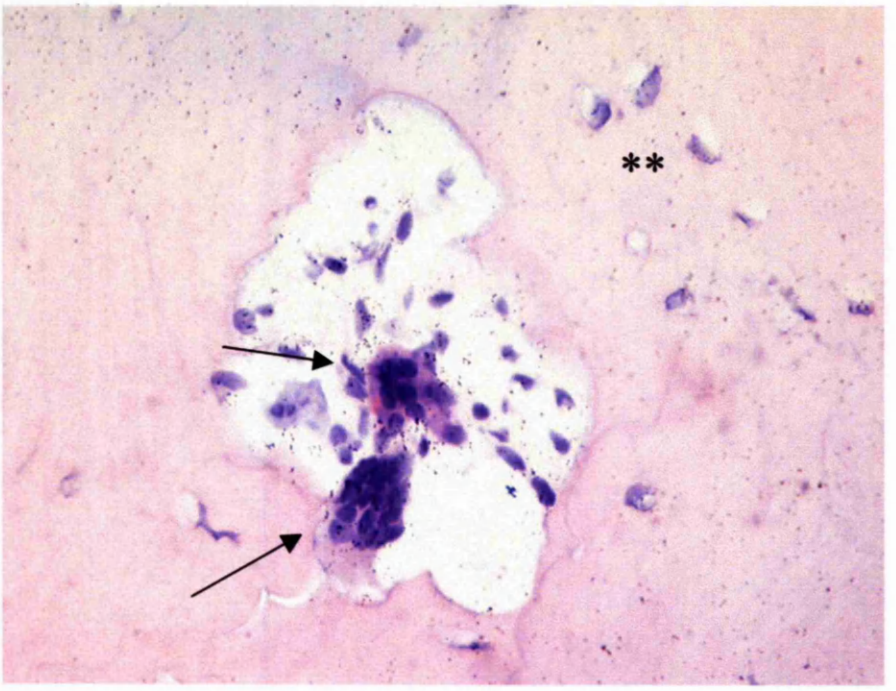


Figure 3.38b

Figure 3.38a & b

Photomicrographs showing high expression of non-skeletal tropomyosin mRNA over (a) haematopoietic stroma in hyperparathyroid bone (x400), and (b) over osteoclasts in Pagetic bone (arrow), whilst osteocytes (**) were negative (x400). H&E.

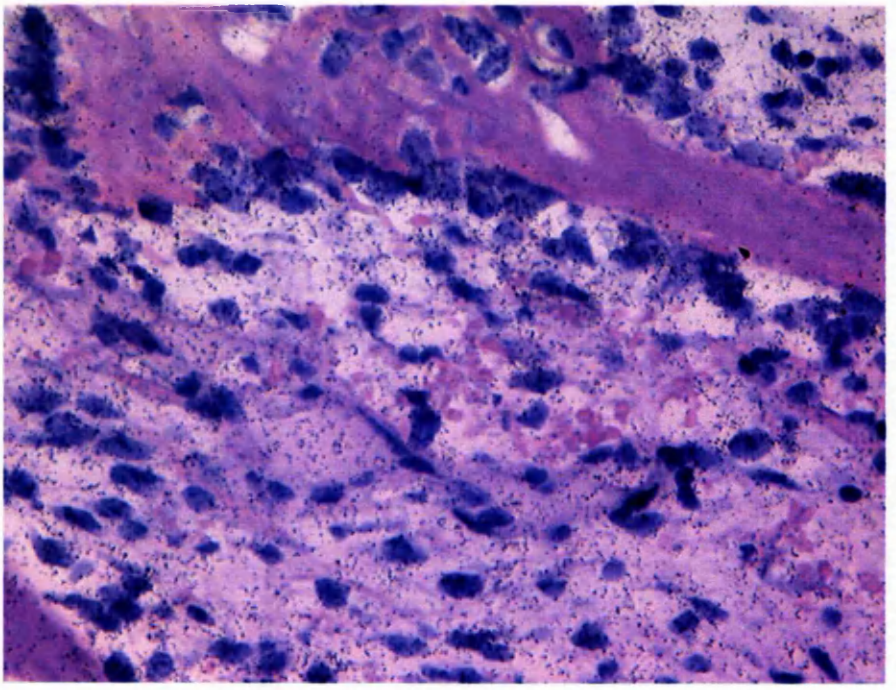


Figure 3.38c

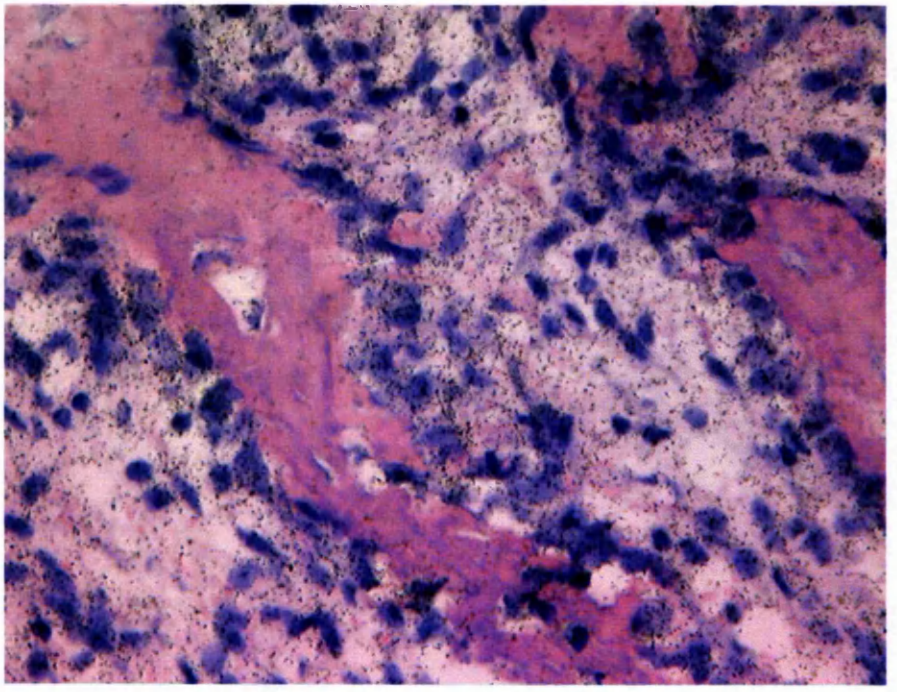


Figure 3.38d

Figure 3.38c & d
Photomicrographs showing high expression of non-skeletal tropomyosin mRNA (c) over condensing stroma adjacent to (x400), and (d) over osteoblasts in, woven bone in healing fracture (x400). H&E.

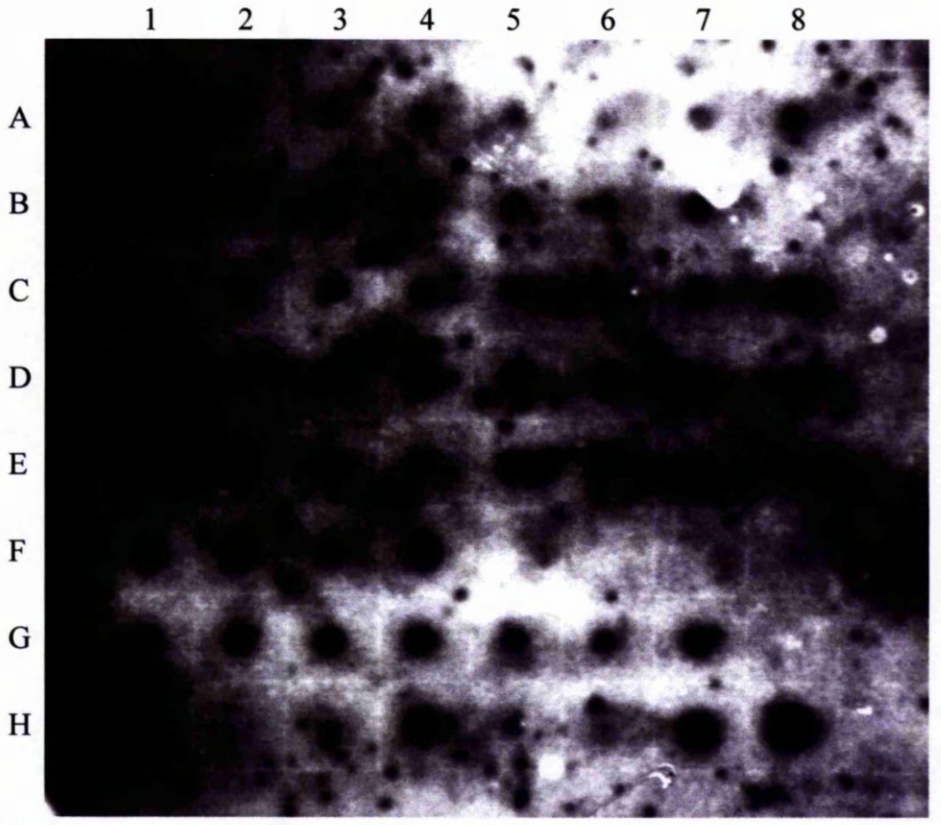


Figure 3.39
Auto-radiograph of hybridisation signal of ^{32}P labelled probe to KIAA0081 with human RNA master blot, demonstrating variable expression levels in different tissues (see figure 3.7 for positional map). Expression was highest in kidney and placenta, high in heart, brain, liver, lung, pancreas, small intestine and pituitary gland, and less strong in aorta, skeletal muscle, colon, bladder, uterus, prostate, stomach and the adrenal, thyroid and salivary glands. It was present at low levels in the mammary gland, and in the lymph node and bone marrow, and virtually absent in peripheral leucocytes and the trachea. The expression pattern in the fetal tissues recapitulated that of the adult tissues. Positive control human DNA was positive, whilst the negative control yeast total RNA, E.coli rRNA, Poly r(A) and human cot 1DNA samples showed no hybridisation. The E.coli DNA was positive; this is a recognised artefact of the system.

3.4 Discussion

The discussion will be presented in three parts. Firstly, the utility and significance of the method used to generate differentiation stage-specific cDNA gene pools from clonal populations of STRO-1 positive human bone marrow stromal cells will be addressed. Secondly, the efficiency of the procedure used to isolate those genes upregulated during early osteoblastic differentiation will be discussed, and finally the genes isolated, together with their expression pattern will be discussed.

3.4.1 Osteoblastic differentiation and mRNA analysis of STRO-1 positive human bone marrow stromal cells

Osteoblasts are thought to differentiate from stem cells of the CFU-F, but though the latter stages in the lineage are well characterised, there is a relative lack of markers, and, in particular of molecular markers, for cells early in the lineage. Until such markers are identified it will be impossible to locate early osteoblastic cells *in vivo*, hampering understanding of both basic bone biology and its alteration in disease. Previous attempts to identify such genes have relied on animal, immortalised, or heterogeneous human cultures, each of uncertain relevance to normal human bone development. In order to overcome these limitations poly (A) cDNA was generated from clonal populations of primary human marrow stromal cells, for a range of time points immediately prior to and following stimulation of osteoblastic differentiation. These cDNA products were used in subtractive hybridisation to isolate genes upregulated during early osteoblastic differentiation.

The primary culture system used was adapted from that described by Cheng *at al* (1994), and, in common with many the methods used by many other groups, used the glucocorticoid dexamethasone, at 10^{-7}M, in the presence of 10% (v/v) fetal calf serum and 1% (v/v) ascorbate, to induce osteoblastic differentiation in bone marrow stromal cells. Human cultures were used throughout to strengthen the relevance of any findings to normal human physiology. Standard assays of osteoblastic differentiation, namely histochemical and biochemical evidence of upregulation of alkaline phosphatase, together with the ability of the cells to produce calcified matrix when additionally stimulated with beta-glycerophosphate, confirmed the osteoblastic capacity of the cells used. The ability to form bone can only be reliably assessed *in vivo*, either by using diffusion chambers (Ashton *et al.*, 1980), or, less easily, by labelling and tracking of cells injected into either the circulation (Pereira *et al.*, 1998) or the marrow space (Onyia *et al.*, 1998). However, the ability of the cells to form calcified matrix, as in this study, is usually taken as evidence of differentiation along the osteoblastic lineage *in vitro* (Beresford *et al.*, 1993). The results were similar to those

previously reported by other groups (Cheng *et al.*, 1994; Beresford *et al.*, 1993; Liu *et al.*, 1994), confirming the utility used for investigation of early osteoblastic differentiation. Whilst alkaline phosphatase is a reliable marker of the osteoblastic phenotype it is less specific and discriminatory of stage of differentiation than other markers of osteoblastic differentiation, such as osteopontin, osteocalcin and type I collagen expression. However, it was adequate at this step in the study since it was first necessary only to establish the capacity of the system for osteoblastic differentiation overall, whilst the other markers were used later to establish the stage of differentiation of the cells more precisely prior to choice of cell populations for use in subtractive hybridisation. In addition to these markers of osteoblastic differentiation, the level of STRO-1 positivity of the cells was investigated, showing a basal level of 28% one week after seeding, rising to 40% at two weeks and then declining to under 10% after six weeks of culture. These levels are similar to those initially reported by Torok-Storb & Simmons (1991), who concluded that the alteration in the level of expression was due to differentiation of the cells in culture rather than an artefact of the culture system. The level of STRO-1 found confirmed the usefulness of the cultures generated for study of the CFU-F.

The use of clonal populations of cells allows the assignment of any genetic differences identified between two populations of cells to be attributed to the stimuli, and the resultant processes induced, in this case osteoblastic differentiation following stimulation with dexamethasone. Consequently the cDNA pools generated represent differences in gene expression expressly due to this process rather than to genetic differences inherent in the cells at the different time-points, which could be the case if heterogeneous, non-clonal populations of cells had been used instead. However, having established this it must also be acknowledged that the bone marrow stromal cell cultures initially generated were heterogeneous, a point that has been demonstrated by Liu *et al* (1994), and others (Owen *et al*). As a result, before these cells could be used to for study of the early stages of osteoblastic differentiation it was necessary firstly to enrich for the cells early in the lineage, in order to ensure that the clones studied represented the progeny of the CFU-F. In this case this was achieved by magnetic activated cell sorting for the only monoclonal antibody reported to be present on the earliest cells at the time the work was done, namely STRO-1. Subsequently two further antibodies have been developed against cells early in the osteoblastic lineage, HOP 26 and SB10, and further studies using these instead would be interesting.

The possibility that the clonal populations of STRO-1 positive cells produced, and which were used to generate the cDNA used in the subtractive hybridisation, were still in the proliferative phase, as a consequence of non-confluence, and therefore not differentiated

despite the addition of the osteoinductive agent dexamethasone (Cheng *et al.*, 1994; Beresford *et al.*, 1993), must be considered. Other groups have studied temporal patterns of gene expression during early osteoblastic differentiation of clonal, separated, and, therefore, non-confluent cultures of marrow stromal cells (Liu *et al.*, 1994; Candeliere *et al.*, 1999), using rat fetal calvarial cell cultures. Consequently, whilst most groups have previously used confluent cultures for the study of the processes controlling proliferation and differentiation of osteoblastic precursors (Stein & Lian, 1993), a strategy in part dictated by the need for large quantities of cellular material for mRNA and protein expression studies, the possibility and validity of the culture method used in the present study has prior reference in the literature (Liu *et al.*, 1994; Aubin *et al.*, 1995). Cultures of marrow stromal cells undergo limited phenotypic change at confluence (Cheng *et al.*, 1994), but this effect is dramatically increased by addition of dexamethasone, in the presence of appropriate serum supplementation. Conversely non-confluent groups of cells usually exhibit marked proliferative capacity, with little differentiation. However, as Liu *et al* (1994) and Candeliere *et al* (1999) have demonstrated, this does not preclude the possibility of induction of differentiation in such culture systems, and the use of such systems for study of genotypic changes during differentiation. On the basis of this evidence the system chosen was appropriate.

Having chosen such a system however, the capacity of the system for differentiaion along the osteoblastic lineage, when appropriately stimulated (Cheng *et al.*, 1994), must be established. Use of larger cultures allows this to be carried out at the level of mRNA expression, using Northern analysis following extraction of total RNA. The use of clonal populations of primary cells, for the reasons already given above, precludes this strategy and forces reliance on hybridisation of probes to cDNA (Liu *et al.*, 1994), with correlation of the results to more comprehensive studies on larger scale cultures generated from the same starting material. This was achieved in the present study by hybridisation of probes for osteocalcin, osteopontin to, and polymerase chain reaction for type I collagen in, the cDNA samples from each time-point. This demonstrated modest upregulation for osteocalcin at day 4 in clone 1, compared to day 0, together with acquisition of collagen type I gene expression, at day 10 in clone 1. Though the degree of upregulation of osteocalcin was not high, its presence, together with latter expression of collagen type I after a longer duration of stimulation, indicated that the clone had undergone early osteoblastic differentiation by day 4: marked upregulation of osteocalcin at day 4, such as would be seen after differentiation to a mature osteoblastic phenotype, was not necessary given the intention to study early osteoblastic differentiation. It would however, be interesting to study the mRNA levels of bone specific proteins in

confluent cultures of the same primary human marrow stromal cells, and would provide a further level of verification of the validity of the technique used.

Magnetic activated cells sorting was used in preference to fluorescence activated cell sorting principally on ground of cost and ease of use, though is recommended for enrichment of small numbers of cells. Since the yield of cells was low the efficiency of the method was tested using the immortalised cell line HCC1, which shows a low level of STRO-1 positivity (Brown, 1999). Fluorescence activated cell analysis of positive and negative fractions of cells following magnetic activated cell sorting for STRO-1 demonstrated a nearly 30-fold enrichment, confirming the utility of the system for enrichment of cultures in cells of the CFU-F. Since the primary cells used in enrichment were 10-days post-harvest, at which time STRO-1 positivity is approximately 40%, both in the cells studied and in other reports, such enrichment would produce an almost pure population of STRO-1 positive cells.

The use of primary culture followed by magnetic activated cell sorting for STRO-1 positivity yielded very few positive cells. This reflects cell loss during the multiple wash steps present in the protocol. However, since only very small numbers of cells were required for both production of clones, and for analysis of gene expression by poly (A) RT-PCR, this was not problematic. These were cultured at clonal densities much lower than those previously reported, i.e. 10 cells/cm^2 compared to between 100 to 1000 cells/cm^2 in others studies (Liu *et al.*, 1994; Cheng *et al.*, 1994). Culture at low densities was necessary to produce clones. This has been produced in other studies by limiting dilution, and by seeding single cells in each well of a 96-well plate, but in these studies supplementation of the media with growth factors was needed. The use of such supplements was not admissable in the present study since control of differentiation was required and may have been altered by the presence of additional growth factors. The clones produced were therefore composed of very few cells (between 50 and 100), rendering them inadequate in quantity for conventional genotypic analysis. As a result poly (A) PCR was used to generate cDNA pools representative of all the mRNA expressed in each of the time-points. This method is extremely sensitive and is capable of generating cDNA from as little as one cell, whilst in this case between only 4 to 10 cells were removed from each time-point for generation of cDNA. The efficiency of the system was verified in an initial study using known dilutions of RNA extracted from SaOS-2 cells. This demonstrated the generation of cDNA from 500 ng of RNA. The cDNA generated hybridised with GAPDH, eliminating the possibility that it was artefactual. Conversely however, the smear present for the RNA negative control, which by trans-illumination with ultraviolet light in an agarose gel was identical to the smears for each of the dilutions, did not

hybridise with GAPDH, indicating that it was an artefact of the system, and demonstrating the importance of hybridisation of any cDNA products generated by poly (A) PCR with GAPDH prior to further studies. A similar finding has been reported by Brady & Iscove (1993) and whilst the precise mechanism responsible for the artefact is not known it may be due to production of concatamers formed from the primer used in the reaction. The poly (A) PCR products generated from each of the time-points each hybridised with GAPDH, confirming that they represented real cDNA products from the cells used as template. Since poly (A) PCR is an extremely sensitive technique the possibility of contamination was eliminated at each round of PCR by inclusion of negative controls. Poly (A) PCR is capable of generating cDNA representative of all the mRNA species in as little as one cell. Furthermore, truncation of the reverse transcription step after generation of first strand cDNA of between 300 and 700 bp ensures that subsequent amplification of the cDNA pool is equally efficient for all the mRNA cDNA constructs produced. Consequently mRNAs of different lengths in the starting sample remain equally represented in the final amplified cDNA pool (Brady & Iscove, 1993). In addition, the cDNAs produced by the method are representative of the relative abundance of each mRNA species present in the starting sample, and mRNA species accounting for as little as 0.025% of the total mRNA in the starting sample are represented in the cDNA produced. Since all the cDNAs produced are amplified equally, and can be so indefinitely, the relative abundance of each mRNA, and the presence of rare mRNA species, are preserved in the cDNA produced. As such the method is ideally suited to the analysis of the numbers of cells cultured in the present study, and in particular, to the identification of genes present at low abundance.

Osteoblastic differentiation during the course of dexamethasone stimulation was demonstrated by increased expression of osteocalcin and osteopontin, especially in clone one, which showed a very low level of hybridisation with osteocalcin at T0 compared with higher hybridisation after 4 days: by comparison each time-point showed equal hybridisation with GAPDH. Collagen type I expression was not demonstrable by hybridisation but was shown by PCR using specific primers for the 3'end. It was present at low levels at day 10 in clone one , and at day 7 in clone two. Whilst some maintain that the presence of 1,25 vitamin D3 is necessary for induction of both osteocalcin and type I collagen expression, Liu *et al* (1994), using a similarly sensitive poly (A) PCR method to that in the present study, also found both using the same culture conditions, in their case in a mouse model, as used in this investigation.

3.4.2 Subtractive hybridisation

Hybridisation of poly (A) PCR amplified subtraction cDNA products with driver (T0) and S4 (subtraction product from fourth round of subtraction) respectively confirmed efficiency of subtraction. Driver cDNA did not hybridise to S4 cDNA, verifying that the S4 subtraction product contained those genes upregulated during osteoblastic differentiation, though the fact that in the same blot the driver probe only bound weakly to the driver cDNA is unusual. The blot had been washed at very high stringency, which partly explains this. In addition, since some hybridisation of S4 to driver was present, the S4 product was not composed entirely of genes unique to the T4 time-point, compared with the unstimulated (T0) cDNA pool. Differences in hybridisation of the two probes to driver and subtracted cDNA samples are common in other reports of subtractive hybridisation (personal communication, Dr Brady, University of Manchester), appropriate hybridisation on one of the blots being sufficient to justify isolation of the genes therein. It must be stressed however that this then necessitates further verification of the upregulation of the genes isolated, by *in vitro* and *in vivo* gene expression studies, which ideally should be supplemented by protein expression studies. This strategy was used in the present study to further verify, in the face of incomplete Southern blot hybridisation evidence, the upregulation of the genes isolated. The results of these expression studies are discussed in detail below.

The subtractive hybridisation procedure is a kinetic reaction and consequently will isolate both upregulated and unique genes in one population compared to another. The isolation of the latter subgroup is easily apparent, since the absence of a unique gene in one pool of cDNA will rapidly lead, by kinetics, to its enrichment in the subtraction product. Conversely, genes more abundant in the tracer pool, but still present in the driver cDNA pool, albeit to a much lesser extent, will also be preferentially represented in the subtraction product. This will occur when the relative abundance of the upregulated gene in the tracer cDNA pool compared to the driver cDNA exceeds the capacity of the 20 fold excess of driver cDNA to quench its persistence after subtraction. Since four rounds of subtraction were carried out the relative abundance of the gene in the tracer compared to the driver cDNA pool must be greatly increased for it to be represented in the subtraction product. Consequently, such genes, whilst still expressed to a small extent in the driver cDNA, are still likely to be physiologically relevant to the process of early osteoblastic differentiation.

The difference in the pattern of hybridisation noted above between driver against S4, compared to S4 against driver, suggests that the S4 product does contain genes both upregulated and unique to human bone marrow stromal cells consequent upon early osteoblastic differentiation, both of which are useful candidate groups for the understanding of both normal and abnormal osteogenesis. Since the upregulated genes in the S4 fraction will be represented to a minor degree, due to the kinetics of subtractive hybridisation noted above, in the driver cDNA, then S4 will hybridise weakly to driver. Conversely, since very few driver species will be present in the S4 fraction, very little hybridisation of driver will occur to S4. Indeed, since the subtractive hybridisation process is a kinetic one, it is impossible to eliminate all genes common to the two cDNA pools without also removing all other cDNAs from the subtraction product.

On the basis of the above considerations it was decided to identify the genes present in the S4 product. This was achieved by library screening and semi-automated gene sequencing, using standard protocols, as detailed above. The utility of the genes isolated as candidates for the understanding of normal and pathological osteogenesis was further investigated by analysis of their expression pattern, as discussed below.

3.4.3 Genes upregulated during early osteoblastic differentiation of human bone marrow stromal cells

Following primary screening, which yielded 157 positive clones, secondary screening and gene sequencing isolated four genes upregulated during early osteoblastic differentiation, with sequence homology to 14-3-3 zeta, alpha enolase, non-skeletal tropomyosin and KIAA0081. Each of these will be discussed in turn. For each of these densitometric analysis of upregulation indicated only modest increases (up to 30%), which is surprising given that the genes were isolated by subtractive hybridisation. This may be a chance result given that only 4 genes, from the 157 primary clones identified at primary screening, were sequenced, and the others may therefore include genes more markedly upregulated. Alternatively it could be an artefact of the hybridisation to poly (A) generated cDNA. The former possibility would easily be addressed in future studies by isolation and characterisation of a wider range of genes, whilst the second could be investigated by comparison with Northern analysis of RNA extracted from larger scale cultures. It may, however, be the case that despite only modest upregulation the genes nevertheless are of functional importance, an assertion that is testable with a combination of *in vivo* expression and functional studies, as in chapter five.

3.4.3.1 14-3-3 zeta

In-situ hybridisation confirmed the localisation of 14-3-3 zeta to areas of increased bone formation, especially in fetal bone and healing fracture callus. In both of these its expression was particularly strong in osteoblasts, though it was also increased over periosteal mesenchyme in fetal bone and condensing mesenchyme in healing fracture. It was present over haematopoietic stroma in all of the sections tested, though at a lower level. Expression was reduced in osteoporotic bone. A similar pattern of expression was observed for KIAA0081, for which a more comprehensive tissue expression pattern than that previously reported was also obtained (see 3.4.3.4).

The 14-3-3 proteins are a highly conserved, widely distributed eukaryotic protein family. Initially isolated from mammalian brain tissue and called '14-3-3' because of their migration pattern in two-dimensional cellulose chromatography and starch gel electrophoresis, several isoforms exist. There are seven major mammalian brain forms, designated $\alpha - \eta$; the α and δ isoforms are phophorylated forms of the β and ζ (zeta) isoforms respectively. A wide range of cellular processes are thought to be controlled by them, including catecholamine biosynthesis, exocytosis and secretion, ADP ribosylation, and control of the cell cycle. Recently, interest has focussed on their interaction with the protein kinases PKC, cRaf, cBcr, and Bcr-Abl and their role in signal transduction.

The 14-3-3 proteins interact with the protein kinase Raf, which is activated by an increase in the level of GTP-bound Ras, downsteam of binding of receptor tryosine kinases to their ligands (figure 3.40). Ras-GTP then interacts with Raf, causing translocation from the cytosol to the plasma membrane. The β and ζ isoforms of 14-3-3 interact with Raf and, since Raf plays a critical role in signal transduction downstream of receptor tyrosine kinases, the relationship between the Raf – 14-3-3 interaction and Raf kinase activity is clearly important. 14-3-3 is not thought to directly stimulate Raf activity but to stabilise the conformation of the active kinase, and to protect it from inactivation by phosphatases, by rendering specific phosphate groups on Raf inaccessible. Consequently, overexpression of 14-3-3 has the net effect of upregulating Raf activity.

Raf occupies a critical point in the mitogen-activated protein kinase cascade (MAPK), convergent for different signals regulating growth factor and hormone effects of differentiation and proliferation. Consequently, formation of a stabilised complex of 14-3-3, Raf and Ras-GTP due to stimulation by growth factors or hormones induces downstream

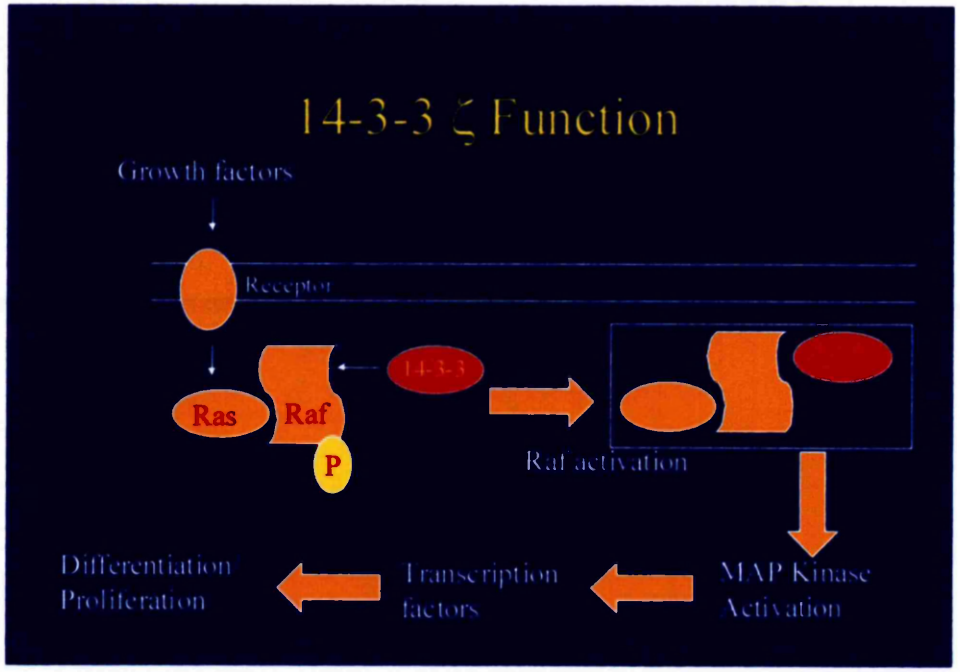


Figure 3.40 Raf plays a key role in the mitogen activated protein kinase (MAPK) cascade and is at a pivotal point for different signals that regulate growth factor and hormone effects on differentiation and proliferation. Raf forms a multiprotein complex, which can be translocated to the plasma membrane in the presence of Ras-GTP. 14-3-3 β- and ζ- isoforms are part of this complex and have been shown to activate Raf. The association of 14-3-3 with the complex of Ras-phosphorylated Raf results in stabilisation of the complex, which in turn initiates downstream events in the MAPK cascade, with resultant modualtion of gene expression and alteration of differentiation and / or proliferation. (Adapted from Aitken *et al.*, 1995)

activation of the MAPK cascade, resulting in an increase in transcription factors and finally differentiation or proliferation. 14-3-3 proteins are also known to modulate protein kinase C activity, either up or down depending on the study. Since Raf is also activated by protein kinase C, this may provide a further physiological point for indirect control of Raf activity by 14-3-3.

Increased expression of 14-3-3 zeta was observed in osteoblasts in situations of increased bone formation, verifying the efficacy of the subtractive hybridisation, but also demonstrating the lineage specificity of the result. In both the fetal bone and healing fracture callus other tissues undergoing rapid growth adjacent to the bone, notably the cartilage in the fetal bone and fibrous tissue in the healing fracture, did not show upregulation, indeed signal was absent in the cartilage. Furthermore, consistent with the isolation of the gene from primitive stromal cells, the areas of condensing mesenchyme, which constitute the starting point for new bone formation, in the centre of the healing fracture and subjacent to the periosteum in the fetal bone, demonstrated increased signal. Other reports of upregulation of 14-3-3 isoforms during fetal development, or in response to injury, also found differential tissue expression. In a balloon angioplasty model of vascular smooth muscle injury Autieri *et al* (1995 & 1996) found increased expression of 14-3-3 gamma, which was localised to the smooth muscle cells. Stimulation of human vascular smooth muscle cells with inflammatory cytokines *in vitro* also increased their expression of 14-3-3, but stimulation of other cell types with the same cytokines failed to upregulate it. Similarly, using differential display to identify genes upregulated during murine nephrogenesis, McConnell *et al* (1995) demonstrated upregulation of the epsilon isoform. *In-situ* hybridisation to localise it in whole mount murine fetuses demonstrated highest expression in the mesenchyme, particularly that which went on to form bone and cartilage. The role of the 14-3-3 proteins in stabilising activated proteins, and Raf in particular, with consequent induction of the MAPK cascade, was implicated as the mechanism responsible for their upregulation in both systems, and the same probably holds for the system described in the present study. However, this is the first direct report of upregulation of 14-3-3, and of the zeta isoform in particular, in osteoblast differentiation. The *in vitro* system used to isolate the gene was designed to approximate as closely as possible to the normal human situation, but the possibility of culture artefact remains present in all such studies. However, confirmation of a role *in vivo* is provided by the upregulation of 14-3-3 zeta *in-situ* in situations of increased bone formation, and osteoblast recruitment, further supporting the utility of the system used to study human disease. The lineage specificity of the pattern of upregulation, and the absence of signal in osteoporotic

bone, suggests that the gene is an important transducer of differentiation *in vivo*, and that it acts specifically to modulate the effects of bone acting growth factors *in vivo*. A similar effect was described by Autieri *et al* (1996) when PDGF and IL-1β were used *in vitro* to induce upregulation of 14-3-3 gamma in vascular smooth muscle cells, and mouse T lymphocytes. The former were responsive, whilst the latter were not. The therapeutic possibility therefore exists of modulating osteoblast differentiation via upregulation of 14-3-3 zeta though a more thorough tissue expression profile would clearly be needed before such a possibility could be entertained. Furthermore, the differential expression of 14-3-3 zeta, and its relation to level of bone formation, could reflect either abnormal stimulation by growth factors, or abnormal response to them. Elucidation of the correct cause is problematical due to the multiple interactions of 14-3-3 at the start of the MAPK cascade and functional studies, including both sense and anti-sense transfections, to upregulate and downregulate its expression respectively, are needed to address this issue. 14-3-3 zeta, is markedly upregulated *in vivo* in situations of increased bone formation and osteoblast recruitment, but downregulated in osteoporosis. Its marked upregulation in the osteoblastic lineage and position at a pivotal point in the control of the mitogen activated protein kinase cascade renders it a candidate for development of more targeted and effective therapies for osteoporosis.

Intriguingly however, most studies investigating the effect of glucocorticoids on the mitogen activated protein kinase system report an inhibition of the system by dexamethasone (Hulley *et al.,* 1998; Rider *et al.,* 1996; Hansson *et al.,* 1996). Hulley *et al* (1998) used a mouse pre-osteoblastic cell line MBA 15.4, in which dexamethasone induced differentiation at the expense of proliferation. Since this cell line was derived from mouse bone marrow stromal cells it would appear to be a suitable model for the process investigated in the present study, though species differences have been shown in the response of the mitogen activated protein kinase system to a variety of stimuli, including bFGF, IGF-I and PDGF (Chaudhary & Avioli, 1998). Hulley *et al* (1998) demonstrated that the effect of dexamethasone was mediated by up-regulation of tyrosine phosphatases, which inactivate MAPK by dephosphorylation of tyrosine and threonine residues. The precise action of dexamethasone on the mitogen activated kinase system therefore needs to be determined by functional studies in primary cultures of human bone marrow stromal cells. In support of the findings in the present investigation Wakui *et al* (1997) reported interaction of the glucocorticoid receptor with 14-3-3 eta, which was also induced by glucocorticoid agonists, including dexamethasone.

3.4.3.2 Alpha enolase

Though initial isolation suggested that alpha enolase was upregulated during early osteoblastic differentiation of marrow stromal cells, *in-situ* hybridisation demonstrated it at only low levels; interestingly it was also the only one of the four genes identified for which upregulation was not confirmed by densitometric analysis. This suggests that it was a false positive result, underlining the importance of verification of the expression of genes isolated by subtractive hybridisation, using appropriate expression systems.

Enolase is a glycolytic enzyme, which exists in a number of isoforms (Tanaka *et al.,* 1995a). Each isomer is a dimer composed of α, β, or γ subunits. In chicken the alpha, but not the beta, isoform contains a Src-dependent tyrosine–phophorylation site, and is preferentially expressed compared to the beta isoform in embryonic chick muscle, with a switch to the beta isoform occurring just after hatching (Tanaka *et al.,* 1995a). Keller *et al.,* (1995) also found an increase in the ratio of the alpha to the beta isoform in rat fetal or hypertrophic heart, and suggested that this was linked to beneficial energetic changes in contractile properties occurring during cardiac hypertrophy. Wallace and colleagues (unpublished communication) have demonstrated, using subtractive hybridisation, up-regulation of alpha enolase during chondrocytic differentiation, but also noted its expression in osteoblasts adjacent to the growth plate by *in-situ* hybridisation, but to date it has not been reported in connection with osteogenesis. Interestingly, in a meeting abstract, Chaudhary *et al* (1996) reported inverse expression of alpha enolase and c-myc during differentiation of HL-60 cells. The c-myc promoter binding protein (MBP-1) binds to c-myc and has a 1.4kb long cDNA that is identical to the 1.8kb cDNA of human alpha enolase in its 1.4kb 3' region, suggesting that alpha enolase has c-myc promoter binding activity, and that alpha enolase / MBP-1 represents an important intersection of the growth control and energy utilisation pathways (Chaudhary *et al.,* 1996). Since c-myc is involved in osteogenesis (Onyia *et al.,* 1999; Sakano *et al.,* 1997) reduction in its level due to binding by alpha enolase could result in alteration of differentiation. Further studies, to establish whether the 1.4kb or the 1.8kb transcript of alpha enolase / MBP-1 is upregulated, are needed in order to resolve whether the up-regulation of alpha enolase found is involved in modulation of the glycolytic pathway or of nuclear transcription, via c-myc. Since the poly (A) PCR generates cDNA from the 3' end it is not possible to distinguish between the two on the basis of the gene sequence isolated.

3.4.3.3 Non-skeletal tropomyosin

Non-skeletal tropomyosin was upregulated in healing fracture callus, in a similar pattern to that for 14-3-3 zeta. It is a cytoskeletal protein of 30kD encoded by a 2.5kb mRNA, alternate splicing of which results in a number of different muscle and non-muscle tropomyosin isoforms, including a 1.3kb mRNA that encodes a 285 amino acid alpha-tropomyosin found in skeletal muscle (MacLeod *et al.,* 1986). The cytoskeleton is now known to play a role in differentiation, via interaction of adhesion molecules and their ligands, though the induction of the protein is more likely to reflect phenotypic reponse to a stimulus rather than be reponsible by itself for differentiation. Shibanuma *et al* (1993) reported up-regulation of tropomyosin in a mouse osteoblastic cell line, MC3T3-E1, by transforming growth factor beta, whilst Sternberg *et al* (1996) demonstrated a decrease in its protein expression in marrow stromal cells upon stimulation with bFGF. In this study stromal cell lines used to support haematopoiesis, namely 14F1.1 endothelial adipocytes, the MBA-15 osteogenic cell line and the MBA-13 fibroendothelial cell line (Sternberg *et al.,* 1996) were used. Whilst bFGF induced a more spindle shaped morphology in each of the cell lines the effect on osteoblastic markers was not assessed (Sternberg *et al.,* 1996). The role of tropomyosin in osteogenesis is therefore unclear.

3.4.3.3 KIAA0081

KIAA0081 was upregulated in healing fracture, in a similar pattern to 14-3-3 zeta, but not to the same extent. Of the four genes isolated least is known about it, and only one report of it exists in the literature. It was identified as part of a large project to accumulate information on the structure of human genes that analysed cDNA clones from the human myeloid cell line KG-1. Part of the results of the project was published in a series of three papers, each of which detailed 40 of the first 120 genes identified by the project (Nagase *et al.,* 1995, Nomura *et al.,* 1994a, Nomura *et al.,* 1994b). Each gene was numbered with the prefix KIAA, KIAA0081 being reported in the third paper in the series (Nagase *et al.,* 1995). KIAA0081 is encoded by a 4,169 bp long cDNA and comprises 233 amino acid residues. Within the amino acid structure no known functional sites are present and consequently its function is unknown. Its tissue distribution was assessed by Nagase *et al.,* (1995), who found it in brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, and colon, in all of which it was present at a low level. The only tissue in which it was not detected by Nagase *et al.,* (1995) was peripheral blood, whilst bone and bone marrow were not analysed. The tissue distribution found by hybridisation with the mRNA master blot in the present study accords with that of Nagase *et al.,* (1995), and expands upon it by providing a greater

degree of stratification of level of expression, more specific localisation for some of the nervous tissues and a wider determination of expression in other tissues, most notably bone marrow. In particular, the highest level of expression on the master blot was mainly in those tissues reported positive by Nagase *et al* (1995), whilst that reported as negative, peripheral blood, were also negative in the present study, i.e. peripheral leucocytes. The level of expression was low in bone marrow. This is consistent with the findings of the present study, in which it was present in low amounts in unstimulated, physiologically normal marrow, but increased in stimulated, differentiating marrow, an increase reflected by its isolation by subtractive hybridisation and by its increased expression in healing fracture callus.

3.4.4 Conclusions

Poly (A) based subtractive hybridisation was used to identify genotypic differentiation stage specific markers of early osteoblastic differentiation. Primary human bone marrow stromal cell culture was used, with the generation of clonal populations of STRO-1 cells. Isolation of genes for these cells will enable identification of cells early in the osteoblastic lineage, i.e. cells of the CFU-F, enabling their role in both normal and abnormal osteogenesis, specifically osteoporosis, to be determined. This provides for the first time the opportunity to study the causes behind the failure of recruitment of osteoblasts from the precursor pool in osteoporosis. However, possibly of greater importance, the genes identified not only serve as phenotypic markers, albeit not tissue or osteoblast specific, but also point to functional processes occurring during early osteogenesis. Consequently, the fact that the most striking results were obtained in healing fracture and fetal bone, rather than in osteoporosis, is not obviated by the fact that these were not the situations it was set out to study, for notwithstanding this fact they are examplars of osteogenesis. Interestingly, for one of the genes in particular, 14-3-3 zeta, expression was increased markedly in both, indicating recapitulation of some of the processes responsible for fetal osteogenesis in repair of adult bone. In order to establish the importance of 14-3-3 zeta in osteogenesis anti-sense transfections were carried out, as detailed in chapter 5, whilst an attempt to identify genotypic markers for cells even earlier in the osteoblastic lineage than those studied above was also made, using a similar procedure, as detailed in chapter 4.

Chapter 4

Identification of genes differentially expressed during adhesion of non-adherent human bone marrow stromal cells

4.1 Introduction

As detailed in chapter 3, until very recently, the only reliable marker of the earliest cells in the lineage was the monoclonal antibody STRO-1 (Simmons & Torok-Storb, 1991). Osteoblast precursors are thought to be present exclusively in the STRO-1 positive fraction (Simmons & Torok-Storb, 1991), and the STRO-1 positive fraction of adult human bone marrow has been shown to contain osteogenic precursors (Gronthos *et al.,* 1994). STRO-1 recognises a cell surface trypsin resistant ligand, the precise nature and function of which is presently unknown. Initially the STRO-1 ligand was thought to be present on the earliest cell in the osteoblast lineage (Simmons & Torok-Storb, 1991), but recent work suggests that the STRO-1 positive fraction of marrow stromal cells is recruited from a STRO-1 negative fraction (Stewart *et al.,* 1996). In addition there is recent *in vitro* evidence that stimulation of non-adherent bone marrow stromal cells results in recruitment of adherent cells early in the osteoblast lineage (Scutt & Bertram, 1995). Further evidence has shown that the presence of non-adherent cells augments osteoblast differentiation of rat marrow stromal cells (Rickard *et al.,* 1995), possibly via the secretion of an unidentified growth factor, whilst others have also identified the presence of cells capable of subsequent adherence and osteoblastic differentiation in the non-adherent fraction of marrow stromal cells (Long *et al.,* 1995). Parenthetically, condensation of stromal cells, which is dependent on normal adhesion molecule expression, is the first step in normal embryological bone formation (Dunlop & Hall, 1995). Adhesion molecules, are expressed at an early stage of osteoblast differentiation (Hughes *et al.,* 1993; Okazaki *et al.,* 1994; Cheng *et al.,* 1998), and expression of tenascin is an early event in bone formation (Vakeva *et al.,* 1990). This suggests that adhesion molecules, as in other systems, are important in the early stages of osteoblast differentiation (Gumbiner, 1996).

4.1.1 Non-adherent bone marrow stromal cells

Whilst the studies quoted above in chapters one and three demonstrate that osteoblasts arise from stromal cells, which by convention are recognisable *in vitro* as slender adherent fibroblast-like cells (Hughes & Aubin, 1997), recent lines of evidence have implicated non-adherent stromal cells in early osteogenesis. The evidence for both the hypothesis that non-adherent cells are osteogenic, along with that for the antithesis that the adherent cells alone give rise to cells of the osteoblastic lineage is noted below, in order to present a balanced view.

Friedenstein (1976) used culture of marrow mononuclear cells in the initial investigations that led to the development of the concept of the existence of the CFU-F, originally termed a fibroblast colony-forming cell (FCFC) (Friedenstein, 1976). Since these initial studies the concept of the CFU-F has gained wide acceptance (reviewed in chapters one, two and three) (Beresford, 1989). The cultures used by Friedenstein were adherent and generated after only two hours of adherence, after which the non-adherent cells were removed. The osteogenic cells were contained entirely within the adherent fraction, whilst the non-adherent pool showed no evidence of osteogenic capacity, cogently arguing against the possibility that non-adherent marrow stromal cells may be osteogenic. This finding has been confirmed in other studies (reviewed in Beresford, 1989) and shown to apply equally to *in vitro* and *in vivo* culture (Friedenstein *et al.*, 1982). Furthermore, the suggestion by Pereira *et al* (1998) that systemically infused transplanted marrow cells can give rise to osteogenic cells runs counter to the large body of work investigating osteoclastogenesis, in which chimeras were used to establish the origin of the osteoclast in the macrophage/monocyte lineage (reviewed in section 1.3.1.1; Kahn & Simmons, 1975). In chimeras and parabiotic animals in which transplanted marrow gave rise to osteoclasts no osteogenic potential of the donor marrow was detected. The assertion that non-adherent marrow cells may be osteogenic has therefore to be tested against this body of work and is therefore presently controversial.

Subsequent to the work of Friedenstein (1976) most workers have ignored the non-adherent fraction, but, amongst the small group of workers who have noted osteogenic capacity of these cells, such cells have been designated either "non-adherent cells" (Rickard *et al.,* 1995), "floaters" (Scutt & Bertram, 1995), or "non-adherent low-density cells" (NALD) (Long *et al.,* 1995). It is important to remember that it is the *in vitro* phenotype that is non-adherent, and that *in vivo* these cells need not be floating in the marrow spaces. However, it is certainly the case that they possess different adhesive properties to the more mature adherent cells typical of conventional osteoblast and stromal cell culture. Their existence and role in early osteogenesis is complementary rather than contradictory to the conventional model, and, specifically, one of the reports (Scutt & Bertram, 1995) suggests that they acquire adhesive characteristics and then progress along the usual path of osteoblastic differentiation. In this section their phenotypic characteristics and the evidence for their role in early osteogenesis will be reviewed, together with a brief discussion of the role of adhesion molecules in osteogenesis.

4.1.1.1 Phenotypic characteristics

Non-adherent cells have been investigated by Long *et al* (1990 & 1995), and Scutt & Bertram (1995). Long *et al* (1990) isolated a population of non-adherent low-density cells (NALD) from human bone marrow aspirates. Plastic adherence was used to remove adherent cells from the mononuclear layer after density gradient centrifugation, and the resultant non-adherent cells grown in serum-free medium (McCoy's 5A with 1% ITS-plus). These cells gave rise to an adherent population of cells, comprising 6-7% of the total, after 7 days, and whilst the latter were positive immunohistochemically for the bone-related proteins osteonectin and BGP, the non-adherent cells used to initiate the culture were negative. The expression of My-10 (which types to CD34) on the non-adherent cells was assayed by a complement mediated cytotoxic assay and by immuno-adherence; both indicated that the non-adherent cells were MY-10 negative. Whilst the non-adherent cells were negative for osteonectin and BGP, they showed weak expression of SaOS-2-P80, a membrane-specific antigen present on SaOS-2 cells. This increased upon adherence suggesting that the antigen may be important in osteogenesis (Long *et al.,* 1990).

Scutt & Bertram (1995) used a rat model, in which bone marrow cells were flushed from the femora of Wistar rats and seeded in medium (Dulbecco's MEM) for 24 hours, after which the non-adherent cells are transferred to a fresh petri dish (first "pour-offs"). This process can be repeated daily for up to 5 days, and at each stage adherent fibroblast-like cells precipitate from the non-adherent population of 'floaters'. The adherent cells generated at each stage were positive for bone-specific proteins whilst the floaters were negative. Recruitment of the adherent cells from the non-adherent population was increased dose-dependently by incubation with prostaglandin E2, with consequent increase in CFU-F and cAMP. Recent data from the group has shown that the floaters are negative for CD45, but express receptors for prostaglandin EP2 and EP4 (Still *et al.,* 1998). This suggests that, in contrast to adherent stromal cells, for which many studies have demonstrated heterogeneity in expression of bone-proteins, most notably alkaline phosphatase, the non-adherent cells may constitute a relatively homogeneous population. Long *et al* (1990) raised the same possibility, though they did not have direct evidence in its support.

4.1.1.2 Role of non-adherent cells in osteogenesis

Long *et al* (1995) reported the use of immuno-adherence to enrich non-adherent populations for cells positive for osteonectin and osteocalcin, despite an earlier report that

such cells were negative for these proteins (Long *et al.,* 1990). The immune-adherent cells were analysed by flow cytometry or cultured in serum-free media, with or without supplementation with growth factors. Flow cytometry identified two sub-populations within these cells, the first comprising 95% of the cells and consisting of small lymphocyte sized cells and the second only 4% of the cells and larger; both were osteocalcin and alkaline phosphatase positive. Stimulation with TGF-beta caused differentiation of the smaller cells into the larger, osteoblast-like cells. Colony formation studies in semi-solid media indicated that the cells formed two types of colonies, one consisting of small clusters of cells containing 20-50 Ocn positive cells and a second of several hundred Ocn positive cells, representing an osteoprogenitor cell with increased proliferative potential. Both colony types showed appropriate responses to TGF-beta, 1,25-vitamin D3 and BMP-2, confirming their role in osteogenesis. Long *et al* (1990) went on to suggest that osteoprogenitor cells are not, as usually thought, associated with the endosteal surface but exist in the marrow space, where they are capable of migration to the bone surface upon acquisition of certain properties, namely the expression of bone protein and matrix receptors. They postulated that these cells may also transmigrate to other sites in the body via the circulation, a process facilitated by their nonadhesive phenotype. Scutt & Bertram (1995) raised the same possibility and used it to explain the osteogenic response seen in marrow poor sites. The paucity with which precursor cells were found in cord blood need not be a problem given their large proliferative potential, and their rarity, combined with a round phenotype, could therefore explain the difficulty reported in visualising stromal cells *in vivo* (Simmons, 1996).

In support on the above hypothesis, Pereira *et al* (1998) have demonstrated the ability of murine marrow stromal cells systemically infused to transit the circulation and repopulate non-haematopoietic tissues, including bone, cartilage, lung, brain and skin. The donor origin of cells was demonstrated by PCR for the human mini-COL1A1 gene present in the donor, an inbred strain, and confirmed by FISH (fluoresence *in-situ* hybridisation) for the Y chromosome in primary cultures of lung, bone, bone marrow and skin, following infusion of male donor cells to female recipients. These results indicate that stromal cells are able to traverse the circulation and successfully give rise to cells at a number of sites, supporting the assertion that the earliest osteoprogenitor cell is non-adherent and non-fibroblast-like. Similarly, Onyia *et al* (1998) injected rat stromal cells transfected with retroviral genes for neomycin resistance and β-galactosidase into the surgically ablated marrow space of donor rats. The marker genes were later found in osteoblasts cultured from the adjacent bone, and *in vivo* in osteoblasts, osteocytes and chondrocytes.

Scutt & Bertram (1995) showed that glucocorticoids, and prostaglandin E2 (PGE2), can induce adhesion of non-adherent cells, and that this is associated with an increase in markers of osteoblastic phenotype. The effect was dose related and also occurred with calcitriol and PTH. Furthermore, when the adherent and non-adherent cells were cultured separately, PGE2 had no effect on the former, indicating that the anabolic effect of prostaglandin *in vivo* is via recruitment of 'adherent' fibroblast-like cells from a 'non-adherent' precursor pool. Other groups have hinted at the role of such cells in osteogenesis. Falla *et al* (1993) noted that when 5-flourouracil was used to enrich the stem cell population in mice nearly all the cells with osteogenic potential were in the non-adherent fraction. CFU-F have been reported in the supernatants of long term human bone marrow culture (Clarke & McCann 1991) and Rickard *et al.,* (1995) reported stimulation of both cell growth and alkaline phosphatase positivity of rat marrow stromal cells by conditioned media from cultures of non-adherent cells, suggesting that the non-adherent cells released a growth factor or factors.

Recently the hypothesis that osteoprogenitors can traverse the circulation and give rise to new bone has been tested clinically by transplantation of marrow-derived mesenchymal cells in children with osteogenesis imperfecta (Horwitz *et al.,* 1999). Unfractionated bone marrow from HLA-identical or single-antigen-mismatched siblings was infused after the recipients had received ablative conditioning therapy. Three months after engraftment only 1.5 to 2% of the osteoblasts collected from the recipients were of donor origin, but nevertheless, histological and clinical improvement was apparent, due to the ability of the small amount of normal collagen produced by these cells to offset the deleterious polymerisation of the mutant collagen (Horwitz *et al.,* 1999). Whilst no specific effort was made to split the cells infused into adherent and non-adherent fractions, the study indicated that osteoprogenitors were able to traverse the circulation, as suggested by Scutt & Bertram (1995) and demonstrated experimentally by Pereira *et al* (1998).

4.1.2 Adhesion molecules

The expression of adhesion molecules by osteoblasts has recently been studied extensively though, whilst the function of adhesion molecules in the control of osteoclastosis is now clearly understood the same is not true for osteoblast function (Horton, 1995). However, since cell adhesion, and in particular cell to cell and cell to extracellular matrix interactions, are now known be involved not only in providing structural integrity, but also in control of differentiation and morphogenesis (Gumbiner, 1996) it is clearly important to

have some understanding of the prinicipal adhesion molecules expressed by cells of the osteoblastic lineage.

In an investigation of embryonic avian osteogenesis, Dunlop & Hall (1995) found that mesenchymal condensation i.e. formation of the membranous skeleton, preceded osteoblast differentiation, and was in turn preceded by expression of tenascin in the mesoderm. In addition pre-osteoblasts appeared before condensation of the mesenchyme, but after the expression of tenascin, which was no longer present once condensation started. Furthermore, though the pre-osteoblasts formed in the mandible before the condensation of the membranous skeleton, they did so only in the presence of both mesoderm and epithelium, indicating that epithelial-mesodermal interactions are needed for initiation of osteogenesis (Dunlop & Hall, 1995). The same necessary interaction between the epithelium and the underlying tissue has been reported for tooth development (Ferguson *et al.,* 1998). In the model used by Dunlop & Hall (1995) tenascin was expressed at the earliest stage of the process, suggesting that it is involved in signal transduction from the epithelium to the mesoderm. In particular, the appearance of tenascin coincided with the onset of mesodermal proliferation. Others have demonstrated a central role for tenascin in regulating development of the central nervous system (reviewed in Crossin, 1996), whilst Vakeva *et al* (1990) also found tenascin expression prior to that of alkaline phosphatase.

The expression of integrins in human bone has been studied by Hughes *et al* (1993). All bone cells expressed β_1 and α_5 integrins, whilst α_4 and α_v were expressed only on osteoblasts. This points to the presence of VLA-4 ($\alpha_4\beta_1$) and VLA-5 ($\alpha_5\beta_1$) dimers in cells of the osteoblast lineage. VLA-5 binds to fibronectin and may be important for the osteoblast phenotype since inhibition of its function results in reduced expression of the osteoblastic phenotype in MG-63 osteosarcoma cells (Dedhar, 1989). VLA-4 also binds to fibronectin, but is also able to bind to VCAM-1 and ICAM-2, each of which have been implicated in control of osteoblast function via cytokine modulation (Tanaka *et al.,* 1995). Since osteoblasts synthesise fibronectin the expression of VLA-4 and VLA-5 provides the possibility of an autocrine effect (Hughes *et al.,* 1993). The role of integrin expression in control of osteoblastic differentiation was further investigated by Schneider *et al* (1999) who, in a fetal bovine mandible osteoblast culture model, reported β_3 integrin expression only in cells positive for bone sialoprotein, whilst β_1 integrin was expressed in a generalised pattern. The expression of β_3 integrin was restricted not only to those cells positive for bone sialoprotein, but was so only at the time of bone sialoprotein expression.

This restricted and coordinated expression of β_3 integrin and bone sialoprotein suggests that the adhesion molecule is involved in differentiation. A similar restriction of adhesion molecule expression to cells early in the osteoblastic lineage was reported for ALCAM / SB10 (Bruder *et al.*, 1997 & 1998).

Recently an osteoblast specific cadherin has been reported (Okazaki *et al.*, 1994), OB-cadherin or cadherin-11, which was the first of the cadherins to be found in bone. It is expressed in both pre-osteoblastic and osteoblastic cell lines, and in primary cultures of calvarial osteoblasts, and is expressed at the same time as alkaline phosphatase (Okazaki *et al.*, 1994). The cadherins are single-chain membrane glycoproteins that have recently been shown to act as morphogenetic regulators (Larue *et al.*, 1996), and specifically have been shown to control differentiation along epithelial and neuroepithelial lines. In order to study their role in osteogenesis Cheng *et al* (1998) investigated the expression of cadherin 11, cadherin 4 and N-cad in a variety of osteoblastic and pre-osteoblastic cells, and their modulation by rhBMP-2. Cadherin-11 and cadherin-4 were present in trabecular bone osteoblasts, bone marrow stromal cells and in the osteosarcoma cell lines SaOS-2 and MG-63, whilst cadherin-4 was present at low levels in the former two only. Cadherin-11 was the most abundant of the three, but neither its expression, nor that of N-cad, was altered by rhBMP-2, whilst cadherin-4 was down-regulated (Cheng *et al.*, 1998). Cadherin-11 may therefore be more important in regulating early cellular aggregation than response to later acting BMPs, such as BMP-2 which has more potent effects on mature osteoblasts than on stromal cells. Interestingly, Goomer *et al* (1998) found a marked reduction, of up to 90%, in the level of cadherin 11 in old compared with young rabbits. Since the results of Cheng *et al* (1998) indicated that cadherin-11 was probably more important at the earlier stages of osteoblastic differentiation, reduction in its expression with age may lead to failure of osteoblast recruitment from the precursor pool (Goomer *et al.*, 1998).

4.1.3 Aims and objectives

Adhesion plays an important role in osteogenesis and recent evidence, discussed above, has linked it to osteoblastic differentiation. However, little is known about the role it plays in early osteogenesis, and, in particular, the role of acquisition of an adhesive phenotype in the initiation of osteoblastic differentiation, if any. In order to investigate the hypothesis that the earliest cell in the osteoblastic lineage is non-adherent *in vitro*, and is STRO-1 negative, as suggested by the studies quoted above, primary bone marrow stromal cell culture, combined with poly (A) based subtractive hybridisation, was used to investigate the nature of the non-adherent fraction of cells at the phenotypic and genotypic level. The

isolation of genotypic markers for the putative non-adherent osteoprogenitors will enable both their identification *in vivo* and provide clues to the processes controlling their differentiation.

4.2 Methods

Materials were obtained from the following companies, unless otherwise stated: culture media, Gibco (Paisley, U.K.); plasticware, Costar (Cambridge, U.S.A.) and Nunc (Illinois, U.S.A.); micropipettes, Gelman (Ann Arbour, U.S.A.); reagents and immunochemicals, Sigma (Poole, U.S.A.). Fluorescence activated cell analysis was carried out using a Beckton Dickinson FAC Scan, and magnetic activated cell sorting using a Miltenyi Biotech Minimacs kit. Supernatant from the STRO-1 hybridoma was obtained from the Developmental Studies Hybridoma Bank, maintained by the Department of Pharmacology and Molecular Sciences, John Hopkins University School of Medicine, Baltimore, and the Department of Biological Sciences, University of Iowa.

4.2.1 Culture of non-adherent human bone marrow cells

A similar method to that detailed for the culture of adherent bone marrow stromal cells in section 3.2.1 was used. Human bone marrow was harvested from the sterna of consenting normal adult volunteers at the time of median sternotomy during cardiac surgery and density gradient centifugation over a histopaque-1077 layer used to isolate bone marrow stromal cells. These were seeded in a $25cm^2$ culture flask, 5ml of standard medium (detailed in section 3.2.1.1) and left to settle for 2 days. Following this the medium, which contained non-adherent cells (NACs) was removed, reseeded in a new $25cm^2$ flask, and fresh standard medium added to the adherent cells in the original flask. After reseeding the non-adherent cells, subsequently adherent cells precipitated out over the next week. The medium in the original flask was then changed every 2-3 days, and that in the second flask was left to settle for 1 week, after which it was also removed and changed every 2-3 days thereafter. The adherent cells in both flasks were left to proliferate for 2 weeks, after which time they were nearly confluent. They were then removed, and their capacity for osteoblastic differentiation tested both histochemically and biochemically.

4.2.1.1 Histochemical measurement of alkaline phosphatase expression

Both initially and subsequently adherent cells were released with trypsin/EDTA, reseeded in 4 well chamber slides and stimulated for 2 weeks with standard medium or with standard media supplemented with either dexamethasone ($10^{-7}M$), β-glycerophosphate

(10mM), or both. The morphology of the cells in each well was noted and the slides stained cytochemically for alkaline phosphatase using an azo-dye method (detailed in section 3.2.1.3). The amount of mineralised tissue formed in each culture was estimated by staining fixed cells with the calcium stain Alizarin Red S (detailed in section 3.2.1.3).

4.2.1.2 Biochemical measurement of alkaline phosphatase expression

Initially adherent cells were split in two, cultured for 2 weeks with standard medium or with standard media supplemented with dexamethasone (10^{-7}M) and the amount of alkaline phosphatase measured in the supernatant: the number of cells in each flask was counted and the level per 1000 cells calculated. Subsequently adherent cells were reseeded in a 96 well plate (1000 cells/well) and cultured for 2 weeks with standard medium or with standard media supplemented with dexamethasone (10^{-7}M). Alkaline phosphatase was measured in both by the hydrolysis of p-nitrophenyl phosphate. The cells were fixed with ethanol, incubated for 10 minutes at 37^{O}C with p-nitrophenyl phosphate and the amount of p-nitrophenol in the supernatant measured spectrophotometrically at 410 nm.

4.2.1.3 Measurement of colony-forming rate

The colony-forming rate of both the initially adherent and non-adherent cells was measured by seeding cells in 6 well plates at 1000 cells/well (well diameter $10cm^2$) and the effect of dexamethasone (10^{-7}M) tested. Cells were cultured for 1 week, colonies identified by haematoxylin staining and counted manually.

4.2.1.4 Measurement of STRO-1 expression

STRO-1 expression in both adherent and non-adherent cells was assessed by fluorescence activated cell analysis (FACS). Expression was measured in adherent cells after 1, 2 and 6 weeks in primary culture, and in non-adherent cells at the time of removal 1 week after initial seeding, and after reseeding and proliferation of subsequent adherent cells for 2 weeks. This was achieved by splitting the non-adherent fraction (on day 2 after initial seeding of the harvested stromal cells) and using half for measurement of STRO-1 expression, whilst the other half was reseeded as described in section 4.2.1, grown to confluence over two weeks, and then used for measurement of STRO-1 expression. The cells from each of these cultures were released using trypsin / EDTA, washed in phosphate buffered saline (PBS) and resuspended in 0.5ml of STRO-1 supernatant, containing from

50 to 75μg/ml antibody, in which they were incubated for 1 hour at 37°C. The cells were washed in PBS and resuspended in 0.5ml secondary antibody (rabbit IgG anti mouse IgM, FITC-isomer 1 conjugated, dilution 1:256) and incubated for 1 hour at 37°C. The cells were then washed and fixed in 0.6ml of 2% (v/v) paraformaldehyde. Controls omitting either both antibodies, or the STRO-1 antibody, were used. The cell suspensions were analysed using a fluorescence activated cell analyser (as detailed in section 3.2.1.5).

In a separate experiment the possibility that the non-adherent population may contain a small number of STRO-1 positive contaminant cells was excluded by complement mediated cell lysis. Non-adherent cells were removed and incubated in 0.5ml of neat (100%) STRO-1 supernatant on ice for 30 minutes. The cells were then washed in serum free alpha modified minimal essential medium and incubated at 37°C for 40 minutes in 1ml of serum free medium containing baby rabbit complement (1:20 dilution). The cells were then washed in PBS, reseeded in a 25cm^2 flask and left to adhere and grow to confluence (2 weeks) prior to FACS as described above.

4.2.1.5 Immunophenotype of non-adherent cells

The immunophenotype of the non-adherent cells was determined by immunohistochemical staining of cytospin preparations of non-adherent cells. Non-adherent cells were harvested 2 days after initial seeding of bone marrow stromal cells and pelleted by centrifugation at 1000xg for 5 minutes. The pellet was resuspended in 1.2 ml of PBS and cytopsin preparations made by addition on 100μl of suspended cells to each well of a cytospinner, followed by centrifugation at 1000xg for 30 minutes. The cytospins were fixed in cold methanol for 10 minutes, air-dried and stored at 4°C prior to use.

Immunohistochemistry was performed using antibodies against the following antigens, all obtained from DAKO (Ely, Cambridge, UK):

CD15	mouse monoclonal	IgM	1:40 dilution
CD34	mouse monoclonal	IgG	1:100 dilution
CD61	mouse monoclonal	IgG	1:40 dilution
CD45 (LCA)	rabbit polyclonal		1:100 dilution
Glycophorin A	mouse monoclonal	IgG	1:2000 dilution
Myeloperoxidase	mouse monoclonal	IgG	1:5000 dilution

Routinely processed, formalin fixed, EDTA decalcified, paraffin embedded bone marrow trephine sections from a case of myelodysplasia were used as positive controls. The following pre-treatments were carried out:

Trypsin	CD34, CD61 and myeloperoxidase
Microwave	CD15, CD45
None	Glycophorin A

Control (trephine) sections were dewaxed in xylene for 3 x 5 minutes and rehydrated through graded industrial methylated spirit (IMS) to H_2O. Control and test (cytospin) sections were then transferred to Tris buffered saline (TBS) (pH 7.6), and blocked with 1%(v/v) H_2O_2 in TBS for 30 minutes. All slides were washed in distilled H_2O and transferred to TBS. Control sections were then pretreated as appropriate with trypsin or by microwaving, as indicated above. Trypsin pretreatment was carried out in a 0.01%(v/v) solution of chymotrypsin (0.01%(v/v) chymotrypsin, 0.1% $CaCl_2$(v/v), in TBS) at 37^{O}C for 20 minutes. Microwave pretreatment was carried out by microwaving in 0.001M EDTA for 10 minutes at high power (pH 8.0), so that the solution boiled at the end of the period of microwaving. The sections were then left to stand for a further 10 minutes in the hot EDTA. The pretreated slides from both treatments were then washed in TBS. All slides were then marked with a DAKO pen, outlining the area bearing the cells on each, and blocked with 20%(v/v) goat serum at room temperature for 30 minutes. Following this the primary antibody was added (diluted to the appropriate concentration, as indicated above, in 1%(v/v) bovine serum albumin(BSA)) and incubated at room temperature for 1 hour; 1%(v/v) BSA alone was used as negative control on both trephine sections and cytospins. The primary antibody was removed by two five minute washes in TBS, the secondary antibody, biotinylated rabbit anti-mouse (1:200 in TBS) for all primary antibodies except CD45 or biotinylated swine anti-rabbit (1:300 in TBS) for CD45 (StreptABComplex/HRP kit, DAKO), applied, and incubated at room temperature for 30 minutes. It was then removed with two five minute TBS washes. Freshly prepared ABC complex (50µl of reagent A[streptavidin in 0.01M phosphate buffer, 0.15M NaCl, 15mM NaN_3 pH 7.2] and 50µl of reagent B[biotinylated horseradish peroxidase in 0.01M phosphate buffer, 0.15M NaCl, 15mM NaN_3 pH 7.2] in 5ml of 0.05M Tris/HCl, pH 7.6) (StreptABComplex/HRP kit, DAKO) was applied and incubated at room temperature for 30 minutes, followed by two further 5 minute washes in TBS. The slides were then transferred to a solution of freshly prepared and filtered DAB (20ml DAB, 280ml TBS, 5 drops 30%(v/v) H_2O_2) for 10 minutes, washed in distilled H_2O for 5 minutes, stained in filtered Mayer's

haematoxylin for 30 seconds and "blued" in TBS and tap H_2O. All slides were dehydrated in IMS and mounted in xylene.

4.2.2 Production of Glycophorin A negative clones

Non-adherent cells were harvested 2 days after initial seeding of marrow stromal cells and magnetic activated cell sorting used to remove glycophorin A positive cells. The glycophorin A negative fraction was then seeded at low density to generate clonal populations of glycophorin A negative cells. The method used is detailed below.

Non-adherent cells were removed, pelleted by centrifugation at 1000xg for 5 minutes, resuspended in PBS and repelleted. The pellet was then resuspended in 80µl of MACS buffer (PBS pH 7.2, supplemented with 0.5%(v/v) BSA and 2mM EDTA) and 20µl of anti-glycophorin A microbeads (MACS colloidal super-paramagnetic Microbeads conjugated to monoclonal mouse anti-human glycophorin A antibodies, isotype: mouse IgG1, clone: AC107.3) added, followed by incubation at $4^{O}C$ for 15 minutes. A further 1ml of MACS buffer was added, followed by centrifugation at 300xg for 10 minutes. The pellet was resuspended in 500µl of MACS buffer and run through a magnetised, pre-washed separation column (washed with 500µl of MACS buffer immediately prior to use). The negative cells were collected and the column washed twice with 500µl of MACS buffer. The effluent was collected and added to the negative cells. The column was then removed from the magnet, the positive cells eluted with 1ml of MACS buffer and the positive cells collected.

The positive and negative fractions were split as follows. From the negative fraction 400µl was removed, centrifuged at 300xg for 10 minutes and resuspended in 500µl of DEPC treated PBS, followed by centrifugation at 300 x g for 10 minutes. The pellet was resuspended in 0.5ml RNAzol and stored at $-80^{O}C$ prior to mRNA extraction. The number of cells in the remainder of the negative fraction was counted using a Coulter Counter, resuspended in standard medium, seeded at 1000cells/cm^2 in 5 x 6 well plates and left for 4 days to adhere. Cells remaining after this were cytospun as detailed previously (section 4.2.1.5). The positive fraction was cytospun and both the positive and negative cytospin preparations fixed in cold methanol for 10 minutes, air-dried and stored at $4^{O}C$ prior to use.

4.2.2.1 Verification of depletion of glycophorin A positive cells

In order to verify removal of glycophorin A positive cells by MACS, immunohistochemistry for glycophorin A was performed on the cytospin preparations of the positive and negative fractions of cells as detailed in 4.2.1.5.

4.2.3 Stimulation of osteoblastic differentiation

Clones were identified 4 days after seeding at low density (1000cells/cm2). Only one colony, of between 3 to 16 cells was present in each well. The colonies grew to between 7 to 50 cells each by 10 days post-seeding and at this stage each was released from the well with trypsin/EDTA, split, a fraction taken for mRNA extraction and amplification, and the remainder reseeded. Briefly, the medium was removed from the well, followed by three washes with PBS, and the cells incubated in 1ml of trypsin (10%v/v) at 37^{O}C for 5 minutes. Trypsinisation was terminated by the addition of 0.5ml of standard medium and the medium, containing the cells removed and centrifuged at 1000xg for 5 minutes. The supernatant was removed carefully and the cells resuspended in 100µl of DEPC treated PBS, 80µl of which was placed carefully, as a single drop, in the centre of a well of a new 6 well plate, whilst the remaining 20µl was used for mRNA extraction and amplification, by poly (A) RT-PCR (as detailed in section 4.2.6).

The reseeded cells were left to adhere for 3 hours, after which 0.5ml of standard medium was added gently. The following day the cells were inspected to verify adherence, and osteoblastic differentiation stimulated by addition of dexamethasone to the medium (10^{-7}M). The medium was changed every 2 to 3 days and the cells harvested, for extraction and amplification of mRNA (as detailed in section 4.2.6), after 10 days of dexamethasone stimulation.

4.2.4 Poly (A) RT-PCR of cells from adherent clones

The mRNA from the unstimulated and stimulated cell fractions of each clone was extracted and amplified by poly (A) RT-PCR, as previously described (section 3.2.3). The reagents and conditions used were the same as those detailed in section 3.2.3. Briefly, cells (1-10 / time point) were released at each time point with trypsin/EDTA, resuspended in 50µl standard medium, centrifuged for 5 minutes at 1000xg and resuspended in 15µl of pre-chilled first strand buffer (50nM Tris-HCl pH 8.3, 75mM KCl, 3mM $MgCl_2$, 2mM of each dNTP[A,T,G,C], 100ng/ml Inhibit Ace, 200u/ml RNAguard, 0.5%(v/v) NP-40). This was left on ice for 10 minutes, after which it was heated to 65^{O}C for 1 minute, allowed to cool to room

temperature for 3 minutes and then returned to ice. Ten units of Reverse Transcriptase (AMV) (Boehringer Mannheim) were added to each sample. The samples were incubated at 37^{O}C for 15 minutes and the reaction stopped by heat inactivation at 65^{O}C for 10 minutes, after which the samples were returned to ice. This step was time limited to 15 minutes to restrict the length of the cDNA to about 300-700bp. In the second step, a 3` oligo(dA) tail was added to the cDNA using terminal transferase. An equal volume of 2X Tailing buffer (200mM potassium cacodylate pH 7.2, 4mM $CoCl_2$, 0.4mM DTT, 200μM dATP) and 1 unit of Terminal Transferase (Boehringer Mannheim) were added to the cDNA and incubated for 30 minutes at 37^{O}C, followed by heat inactivation at 65^{O}C for 10 minutes. The DNA is now defined at both ends and amenable to amplification by PCR using a single oligo(dT) containing primer. Polymerase chain reaction was carried out using the following mix: 10μl Taq Polymerase buffer in 2.5mg/ml Bovine Serum Albumin, added to a final concentration of 1OD/ml of NOT 1dT oligo (GCG GGC CGC $(T)_{24}$), (Oswel DNA Synthesis, Southampton, UK), 25mM dNTP's (Boehringer Mannheim), and sterile distilled water to 49μl, with Taq polymerase (Boehringer Mannheim) to 5 units/50μl. The entire reaction volume from the synthesis of the first strand cDNA was used as template. Samples were initially denatured for 30 seconds at 94°C, and then amplified for 35 cycles of 15 seconds at 94^{O}C, 20 seconds at 42^{O}C and 30 seconds at 72^{O}C in an Idaho Technology rapid thermal cycler. The size range of the poly (A) PCR products (300 – 700 bp) was confirmed by electrophoresis on a 1.5%(v/v) agarose gel.

4.2.5 Verification of osteoblastic differentiation

In order to verify the osteoblastic nature of the clone the amplified cDNA products from each adherent clone, before and after stimulation with dexamethasone, were hybridised with isotopically labelled 3` end cDNA probes to alkaline phosphatase and GAPDH. Probe labelling, preparation, prehybridisation, hybridisation, post-hybridisation washes and visualisation of hybridisation signal was carried out as detailed in section 3.2.3.2. The poly (A) cDNA product from osteoblast culture was used as positive control, and a negative PCR control included for each probe.

4.2.6 Extraction and amplication of mRNA from non-adherent cells

The mRNA was extracted from the cells stored in RNAzol during generation of glycophorin A negative clones. The RNAzol was thawed slowly on ice, 1/10th volume

(150µl) of cold chloroform added, the mixture vortexed and placed at –20^{O}C for 15 minutes, followed by centrifugation at 13000xg for 15 minutes at 4^{O}C. The clear upper phase was removed and an equal volume of cold isopropanol added. The mixture was vortexed, placed at –20^{O}C for 15 minutes and centrifuged at 13000xg for 15 minutes at 4^{O}C. The resultant pellet was washed with 1 ml cold 75% ethanol, centrifuged at 6500xg for 15 minutes at 4^{O}C, air-dried and resuspended in 20µl of distilled H_2O. The amount of mRNA present was determined spectrophotometrically at 260nm and the purity of the mRNA assessed by visualisastion on an agarose gel prepared using DEPC treated TBE and using DEPC treated electrophoretic equipment (as detailed in section 3.2.3.1.3).

4.2.6.1 Poly (A) RT-PCR of non-adherent cells

The mRNA extracted from the non-adherent cells was used in poly (A) RT-PCR, as described in section 4.2.4. Between 50 to 100ng of mRNA (1-2µl) was used as template, with addition of 4µl of first strand lysis buffer as detailed in section 4.2.4, after which the same method as described in section 4.2.4 was used. The poly (A) products were visualised by agarose gel electrophoresis as detailed in section 3.2.3.1.

4.2.7 Subtractive Hybridisation

Subtractive hybridiation was used to isolate a pool of poly (A) cDNAs encoding mRNA differentially expressed specifically upon adherence of non-adherent cells.

4.2.7.1 Amplification of driver (non-adherent) and tracer (adherent) cDNA pools

Several rounds of poly (A) PCR, using 1µl of non-adherent or adherent cDNA as template, as described in section 3.2.4.1, were used to generate a 20 fold excess of non-adherent cDNA (driver) to adherent cDNA (tracer). Specifically, 20 x 50µl reactions were used to amplify tracer cDNA and 80 x 50µl reactions to amplify driver cDNA, as detailed in 3.2.4.1 with the exception that dUTP was substituted for dTTP in the dNTP mix for amplification of the driver (non-adherent) cDNA. The quantity present after amplification was assessed crudely, prior to more precise quantitation after purification, by electrophoresis on a 1.5%(v/v) agarose gel.

4.2.7.2 Purification of driver and tracer cDNA pools

The same protocol as detailed in section 3.2.4.2 was used for purification of both driver and tracer cDNA pools.

4.2.7.3 Quantitation of purified driver and tracer cDNA

Each of the purified fractions of driver and tracer cDNA was quantitated both by visualisation after gel electrophoresis and spectrophotometrically, as detailed in section 3.2.4.3.

4.2.7.4 Photobiotinylation of purified driver cDNA

Photobiotinylation of purified driver cDNA was carried out as detailed in section 3.2.4.4. Each fraction was then requantitated as detailed in section 3.2.4.3.

4.2.7.5 Hybridisation of driver and tracer cDNA

Subtractive hybridisation was carried out using a 20 fold excess of driver cDNA over tracer cDNA in three rounds of subtraction. The first round was performed as detailed in section 3.2.4.5.

4.2.7.6 Phenol / chloroform extraction of cDNA

Phenol / choroform extraction was carried out as detailed in section 3.2.4.6. The procedure detailed above for the first round of subtraction was repeated for the second round, and the resultant subtraction product (labelled S2) again split into 2 fractions, one of which (10μl) was stored, whilst the other was used in the third round of subtraction, in the same way as detailed for the use of S1 in the second round and the subtraction product of the third round of subtraction (labelled S3) retained in its entirety.

4.2.7.7 Amplification of subtraction products

Each of the subtraction products (S1-S3) was amplified using poly (A) PCR (see section 3.2.3.1), using 1μl of each as template, with the addition of uracil-DNA digestion using uracil-DNA glycosylate. For each of the subtraction products 1μl of uracil-DNA glycosylate (1U/μl) was added to the PCR reaction mixture just prior to PCR thermal cycling. It was incubated at room temperature for 10 minutes to allow uracil-DNA digestion, ensuring elimination of any residual driver cDNA species, following which the uracil-DNA glycosylate was inactivated by incubation at $95^{O}C$ for 2 minutes, immediately following which the previously detailed PCR thermal cycle (see section 3.2.3.1) was applied. The PCR products were visualised by gel electrophoresis as detailed in section 3.2.3.1.

4.2.7.8 Verification of subtractive hybridisation

In order to assess efficiency of the subtraction the subtraction products (S1-S3) were hybridised with isotopically labelled cDNA probes for the driver and S3 cDNA pools.

4.2.7.8.1 Southern blotting of cDNA pools

The driver, tracer and subtraction (S1-S3) cDNA pools were Southern blotted as detailed in section 3.2.4.8. Two Southern blots were prepared, one for hybridisation with driver cDNA and one for hybridisation with S3 cDNA.

4.2.7.8.2 Prehybridisation of cDNA Southern blots

Both of the Southern blots were prehybridised using the method detailed in section 3.2.4.8.2.

4.2.7.8.3 Isotopic labelling of cDNA probes

Isotopically labelled cDNA probes to driver and S3 cDNA pools were prepared using the method detailed in section 3.2.4.8.3, using 1µl of driver and S3 cDNA respectively as template.

4.2.7.8.4 Hybridisation of cDNA Southern blots

The Southern blots were hybridised, one with probe to driver and one with probe to S3, as detailed in section 3.2.4.8.4.

4.2.7.8.5 Post Hybridisation washes and visualisation of hybridisation signal

Post hybridisation washes were carried out at increasing stringency as detailed in section 3.2.4.8.5, and visualised by exposure to a phosphoimager plate.

4.2.8 Library Screening

The cDNA library detailed in section 3.2.5 was used. Clones which hybridised to the S3 product were isolated and purified by secondary screening.

4.2.8.1 Generation and packaging of library

The library was generated and packaged as detailed in section 3.2.5.1

4.2.8.2 Preparation of plating bacteria

Plating bacteria were plated as detailed in section 3.2.5.2.

4.2.8.3 Titre of library

The library was titred as detailed in section 3.2.5.3.

4.2.8.4 Primary library screening

Once the optimum library titre, and number of plates, required to represent the library had been established the method detailed in section 3.2.5.3 was repeated at the chosen library dilution, as detailed in section 3.2.5.4.

4.2.8.5 Plaque lifting

The plasmid DNA present in each plaque was transferred to circular (150mm diameter) nitrocellulose nucleic transfer membranes as detailed in section 3.2.5.5.

4.2.8.6 Prehybridisation of nitrocellulose filters

The nitrocellulose filters were prehybridised as detailed in section 3.2.5.6.

4.2.8.7 Hybridisation of filters

Isotopically labelled probe to the S4 subtraction product was prepared as detailed in section 3.2.3.2.2, and hybridised with the nitrocellulose filters as detailed in section 3.2.3.2.5.

4.2.8.8 Post Hybridisation stringency washes

Post-hybridisation washes were carried out as detailed in section 3.2.5.8.

4.2.8.9 Visualisation of hybridisation signal and identification of positive plaques

The autoradiographs were developed as detailed in section 3.2.3.2.6 and 'double positives' identified and marked as detailed in section 3.2.5.9.

4.2.8.10 Isolation of positive plaques

Positive plaques were identified as detailed in section 3.2.5.10.

4.2.8.11 Titre of primary plaques

The primary plaques were titred as detailed in section 3.2.5.3, using dilutions of the SM buffer containing plasmid instead of the entire library. The number of plaques per plate were counted and the dilution producing 500 plaques/plate selected for use in secondary screening.

4.2.8.12 Secondary screening

Following selection of the optimum dilution of each of the primary plaques, the plasmid, in SM buffer, from each of the primary positive plaques was used to infect MRF' cells and plated onto agar plates as detailed for the primary screen in section 3.2.5.4. Secondary screening was performed as detailed in section 3.2.5.12.

4.2.8.13 Plaque lifting

The DNA present on the plaques was transferred onto nitrocellulose membranes as detailed in section 3.2.5.5.

4.2.8.14 Prehybridisation of nitrocellulose filters

The nitrocellulose filters were prehybridised as detailed in section 3.2.5.6.

4.2.8.15 Hybridisation of filters

The nitrocellulose filters were hybridised with isotopically labelled probe to the S4 subtraction product as detailed in section 3.2.5.7.

4.2.8.16 Post Hybridisation stringency washes

The nitrocellulose filters were washed at increasing stringency as detailed in section 3.2.5.8.

4.2.8.17 Visualisation of hybridisation signal and identification of positive plaques

Hybridisation signal was visualised autoradiographically and 'double positives' identified as detailed in section 3.2.5.9.

4.2.8.18 Isolation of positive plaques

Plaques corresponding to double positives on the autoradiographic images were isolated and transferred to SM buffer and chloroform as detailed in section 3.2.5.10.

4.2.9 Gene sequencing

The plasmid insert present in each of the double positive plaques identified by secondary screening was amplified by PCR, cloned and sequenced.

4.2.9.1 Amplification of secondary clone inserts

The cDNA insert present in the plasmid in each positive plaque was amplified by PCR prior to cloning as detailed in section 3.2.6.1.

4.2.9.2 Cloning of secondary clone inserts

Cloning was perfomed as detailed in section 3.2.6.2.

4.2.9.3 Identification and analysis of recombinant clones

Following overnight incubation the plates were placed at $4^{o}C$ and then inspected. Positive recombinant colonies were identified as detailed in section 3.2.6.3. Recombinant colonies

were picked and each one inoculated in 5ml of L broth. PCR was performed using 1µl of the inoculated broth in a 25µl reaction (detailed in section 3.2.5.1). The PCR products were analysed by gel electrophoresis and the product size compared with that of the corresponding product generated in section 4.2.9.1. Those colonies for which the product size was the same for the two corresponding PCR reactions were adjudged to have undergone efficient recombination, and the respective inoculants incubated overnight at 37^{O}C with continuous agitation.

4.2.9.4 Minipreps of recombinant clones

For each of the cultures glycerol stocks were prepared and plasmid isolated using a miniprep procedure, as detailed in sections 3.2.3.2.1.1 and 3.2.3.2.1.2. Isolated plasmid was resuspended in 12µl of distilled H_2O and the quantity present and the size verified by electrophoresis on a 1.5%(v/v) agarose gel.

4.2.9.5 Sequencing of isolated plasmid

Isolated plasmid was sequenced by PCR using Big Dye Termintors and M13 forward and reverse primers as detailed in section 3.2.6.5.

4.2.9.6 Analysis of sequence data and identification of gene homology

The base sequence was derived semi-automatically from the fluorescence pattern of each sample and was analysed as detailed in section 3.2.6.6. For each sample the EcoR1 site was identified in the sequence obtained and the sequence beyond it compared with known sequences held in the public domain by submission to BLAST inquiry at NIH (www.ncbi.nlm.nih.gov/cgi-bin/BLAST). For each positive clone sequenced sequence data generated by PCR with both forward and reverse M13 primers was submitted in this way.

4.2.10 Expression of isolated genes

For each of the genes identified 3' end cDNA probes were generated from plasmid and their temporal and spatial patterns of expression established. This was achieved by hybridisation with cDNA from each of the time points for each clone, and by *in-situ* hybridisation with tissue sections of normal and diseased human bone.

4.2.10.1Hybridisation with time point cDNA

In order to verify upregulation of gene expression upon adhesion of the non-adherent cells, probes to each of the genes isolated were hybridised with dot-blots of the cDNA from the

non-adherent cells, and the subsequent adherent clones, both prior to and following stimulation of the latter with dexamethasone (10^{-7}M).

4.2.10.1.1 Generation and isotopic labelling of 3'end cDNA probes

For each of the genes identified by sequence homology search the plasmid used as template in the sequencing reaction was used to produce a 3'end cDNA probe. For each gene the corresponding glycerol stock was defrosted, inoculated in 10ml of L broth (supplemented with ampicillin) and incubated overnight at 37^{O}C. Plasmid was isolated from the culture using a mini-prep procedure (detailed in section 3.2.3.2.1.2) and a fresh glycerol stock taken (detailed in section 3.2.3.2.1.1). The cloned cDNA insert was excised from the plasmid by restriction enzyme digestion (detailed in section 3.2.3.2.1.3), visualised by agarose gel electrophoresis and used as template in a labelling reaction (detailed in section 3.2.3.2.2).

4.2.10.1.2 Production of cDNA dot-blots

Dot-blots of cDNA from each of the time-points for each of the clones were prepared as detailed in section 3.2.3.2.3, with inclusion of PCR control and excised insert as negative and positive controls respectively.

4.2.10.1.3 Prehybridisation and hybridisation of cDNA dot-blots

Prehybridisation and hybridisation were carried out as detailed in sections 3.2.3.2.4 and 3.2.3.2.5 respectively.

4.2.10.1.4 Post Hybridisation washes and visualisation of hybridisation signal

The dot-blots were washed at increasing stringency and hybridisation signal visualised as detailed in section 3.2.3.2.6.

4.2.10.2 In-situ hybridisation with human bone

In-situ hybridisation was performed to establish the tissue distribution of the genes identified. cDNA probes were radiolabeled with ^{35}S by random prime labelling. Briefly, the probes were prepared using excised insert from purified plasmid (detailed in sections 3.2.3.2.1.1 and 3.2.3.2.1.2) as template in a random prime labelling reaction. A labelling reaction containing 50ng of cDNA pobe (1-2µl), 10µl of primer and distilled H_2O to final volume of 52µl was boiled for 5 minutes, followed by addition, at room temperature, of 10µl reaction buffer, 8µl of each of dATP, dGTP and dTTP, 10µl of ^{35}S-dCTP and 4µl of Klenow fragment. The reaction mixture was incubated at 37^{O}C for 1 hour and the labelling

reaction terminated by addition of 5µl of 0.2M EDTA (pH 8.0). Labelled probe was purified by push columns and purified probe stored at –20^{O}C prior to use.

4.2.10.2.1 Case selection and tissue processing

Tissue expression of mRNA was determined in sections cut from archived paraffin blocks of healing fracture callus, and normal fetal bone. The case details are the same as those given in tables 3.2a & 3.2b. In each case tissue was fixed at the time of receipt in 10%(v/v) buffered formalin for 24 hours, followed by decalcifiction in 20%(v/v) EDTA (pH 7.4), until calcification was absent on radiographic analysis, and processed to paraffin, using standard protocols. Sections were cut at 7µm and floated out on DEPC treated H_2O and mounted on silanated slides, followed by baking at 60^{O}C. Slides were stored at room temperature in foil prior to use.

4.2.10.2.2 Prehybridisation and hybridisation

Sections were dewaxed in xylene for 3 x 5 minutes, rehydrated in IMS for 4 x 2 minutes and washed in DEPC treated H_2O for 10 minutes, followed by treatment with 0.2M HCl for 20 minutes, 2xSSC for 2 x 3 minutes and 0.05M Tris HCl (pH 7.4) for 3 minutes. The sections were outlined with a DAKO pen and proteinase K (10µg/ml in 0.05M Tris HCl, pH 7.4) applied to the sections, followed by incubation at 37^{O}C for 1 hour. The sections were then rinsed in DEPC H_2O for 2 x 3 minutes, dehydrated in IMS for 3 minutes and RNase (0.1mg/ml in RNase buffer), DNase (1 unit/ml in DNase buffer), and RNase (0.1mg/ml in RNase buffer) / DNase (1 unit/ml in DNase buffer) pre-treatments applied to control sections, followed by incubation at 37^{O}C for 1 hour. Control and test slides were rinsed with PBS for 3 minutes, post-fixed with cold 0.4%(v/v) paraformaldehyde/PBS for 20 minutes, rinsed with DEPC H_2O for 5 minutes and incubated in 0.1M triethanolamine (pH 8.0) for 5 minutes, followed by treatment with a freshly prepared solution of acetic anhydride/triethanolamine for 20 minutes. The sections were rinsed with DEPC H_2O for 5 minutes and each prehybridised with 50µl of freshly prepared prehybridisation mixture (50%(v/v) deionised formamide, 0.6M NaCl, 10mM Tris-HCl pH 7.4, 0.5mM EDTA, 10% dextran sulphate, 0.2mg/ml salmon sperm DNA, and 10mM DTT(dithiothreitol)) at 37^{O}C for 2 hours. Sections were hybridised with 50ng of heat denatured probe in 50%(v/v) deionised formamide, 0.6M NaCl, 10mM Tris-HCl pH 7.4, 0.5mM EDTA, 10%(w/v) dextran sulphate, 0.2mg/ml salmon sperm DNA, 10mM DTT. Sections were covered with plastic coverslips and hybridised overnight at 37^{O}C in a wet-box.

4.2.10.2.3 Post-hybridisation washes

A series of increasingly stringent washes were carried out. All washes were carried out on an orbital shaker. Coverslips were removed by floating off in 4xSSC and the sections washed in 0.5xSSC, 1mM EDTA, 10mM DTT twice for 5 minutes, rinsed in 0.5xSSC, 1mM EDTA twice for 5 minutes and then in 50%(v/v) formamide / 50%(v/v) 0.15M NaCl, 5mM Tris HCl (pH 7.4), 0.5mM EDTA (pH 8) for 10 minutes in a fume cupboard. The sections were then rinsed in 0.5xSSC at 55^{O}C four times for 5 minutes, in 0.5xSSC at room temperature for 5 minutes. They were dehydrated in IMS for 3 minutes, air-dried, coated with freshly prepared photographic emulsion (Ilford K5 emulsion melted at 40^{O}C and diluted 1:1 with warm distilled H_2O) in a darkroom, and exposed at 4^{O}C for approximately 14 days in the darkroom.

4.2.10.2.4 Development

Slides were developed after between 10 and 14 days. They were developed in D-19 developer (Kodak) for 5 minutes, rinsed in cold H_2O, and fixed in AM Fix fixer (Kodak) for 5 minutes. The slides were counterstained with haematoxylin and eosin and mounted in XAM.

4.2.10.2.5 Analysis of hybridisation signal

The slides were examined using a Leica RMDB research microscope and for each section pattern and intensity of hybridisation signal was assessed independently by two observers (Drs Judith Hoyland & Richard Byers). Images were captured on P600 image analysis system via a Sony DXC 930P 3 chip colour video camera and saved as TIFF files on compact disc.

4.3 Results

4.3.1 Stimulation of osteoblastic differentiation

Triplicate cultures exhibited the same results morphologically. After reseeding the subsequently adherent cells displayed a similar phenotype to that of the initially adherent cells (detailed in section 3.7.1), demonstrating capacity for osteoblastic differentiation when stimulated with dexamethasone. Cells grown in standard medium formed sheets of spindle shaped fibroblast cells (figure 4.1a), whilst the cells stimulated with dexamethasone were less slender and demonstrated a slightly nodular growth pattern (figure 4.1b). Cells stimulated with beta-glycerophosphate showed similar changes, whilst the cells stimulated with both were plump and demonstrated a marked nodular growth

Figure 4.1a

Figure 4.1b

Figure 4.1c

Figure 4.1d

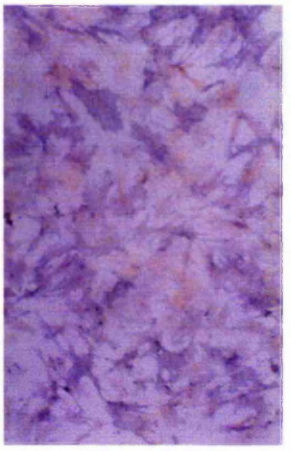


Figure 4.1e

Figure 4.1

Adherent cells resulting from reseeding of non-adherent marrow stromal cells displayed the same phenotype, indicating a similar capacity for osteoblastic differentiation, as the initially adherent stromal cells (see figure 3.8). Phase contrast photomicrographs of cultured bone marrow stromal cells grown in standard medium (a) without supplementation, (b) supplemented with dexamethasone(Dex), or (c) supplemented with dexamethasone & beta-glycerophosphate(β-GP). Unstimulated cells exhibited fibroblast-like morphology and grew in sheets, whilst stimulation with Dex resulted in plumper cells, and with β-GP in a nodular growth pattern. Alkaline phosphatase background staining of stromal cells demonstrated low level of staining in cells cultured in control medium (d), compared with frequent strongly positive cells when stimulated with Dex (e). Magnification x100.

Table 4.1 Mean alkaline phosphatase expression in adherent and non-adherent cells measured as nmol p-nitrophenol liberated/min/1000cells. Values given as mean, and (range): n=6.

	Control Medium	Dexamethasone (10^{-7}M)
Adherent	5.6 (3.4 – 6.5)	16.4 (11.1 – 17.3)
Non-adherent	5.6 (2.5 – 7.3)	15.5 (13.4 – 18.2)

Table 4.2 Colony-forming rate for adherent and non-adherent cells, per 1000 cells. Values given as mean, and (range): n=6.

	Control Medium	Dexamethasone (10^{-7}M)
Adherent	11.26 (1 – 19.25)	9.11(2 – 13.7)
Non-adherent	1.16 (0 – 2.72)	2.12 (0.1 – 4.25)

pattern (figure 4.1c). Expression of alkaline phosphatase staining was increased by stimulation with dexamethasone. Positive histochemical staining was present in less than 10% of the cells grown in standard medium (figure 4.1d) but was present in over 50% of those supplemented with dexamethasone (fig 4.1e) in both the initially adherent and non-adherent cells. Biochemical measurement also demonstrated increased alkaline phosphatase expression following dexamethasone stimulation (table 4.1), in both populations of cells.

The colony-forming rate was significantly higher for the adherent than for the non-adherent cells, ($p<0.1$), but was unchanged by stimulation with dexamethasone in either population of cells (table 4.2).

4.3.2 STRO-1 expression

The initially adherent cells showed a variable level of STRO-1 ligand expression, dependent on the length of time in culture, as detailed in section 3.7.1.2. The non-adherent cells were negative for the STRO-1 ligand with a mean positivity of 0.9%, range (0.3 – 1.8%), which was not significantly different from controls ($p>0.5$), but after reseeding a mean of approximately 19% of the subsequently adherent cells were positive (n=3, range 17.1 – 22.5%) (figure 4.2a). The non-adherent cells subjected to complement mediated cell lysis showed 22% STRO-1 ligand expression after reseeding and adherence (n=1) (figure 4.2b). Controls omitting either primary antibody alone or both primary and secondary antibody were negative in each case (mean positivity 0.5%, range 0 – 1.7).

4.3.3 Immunophenotype of non-adherent cells

The non-adherent cells were positive for glycophorin A (figure 4.3), but failed to react with each of the other antibodies applied (CD15, CD34, CD61, CD45 or myeloperoxidase). Each of the antibodies used reacted appropriately with cells in the control trephine sections, whilst the negative controls showed weak background reactivity only. For each antibody used 3 cytospin preparations were examined.

4.3.4 Production of glycophorin A negative clones

Immunohistochemistry for glycophorin A confirmed the elimination of reactive cells from the negative fraction after magnetic activated cell sorting, whilst the positive fraction was positive (figure 4.4): for each fraction 6 cytospin preparations were examined. Very few adherent clones (approximately 1 per 1000 cells reseeded) arose from the negative fraction.

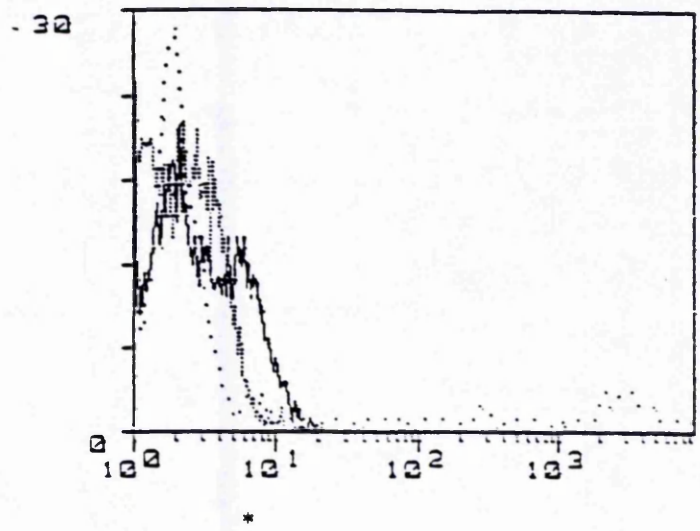


Figure 4.2a

Fluorescence activated cell analysis, for STRO-1 positivity, of non-adherent cells prior to (close dotted line,....), and following reseeding and subsequent adherence (wide dotted line,. . . .), demonstrating acquisition of STRO-1 positivity (control ommiting secondary antibody shown as solid line), to right of negative cut-off threshold (*). Fluorescence intensity given along x-axis and number of cells of each intensity on y-axis.

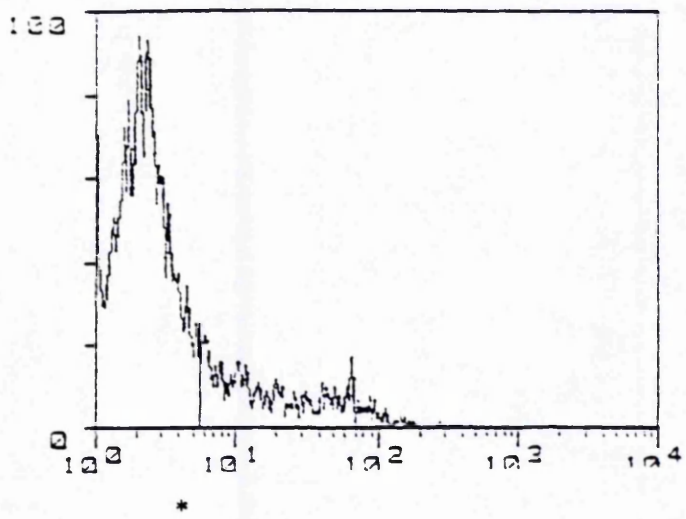


Figure 4.2b

Fluorescence activated cell analysis, for STRO-1 positivity, of adherent cells resulting from reseeding of non-adherent cells after complement activated cell lysis, to remove STRO-1 positive cells from the non-adherent population. Positive cells were counted to the right of the negative cut-off threshold (*), indicating positivity in 22% of the resultant adherent population. Fluorescence intensity given along x-axis and number of cells of each intensity on y-axis.

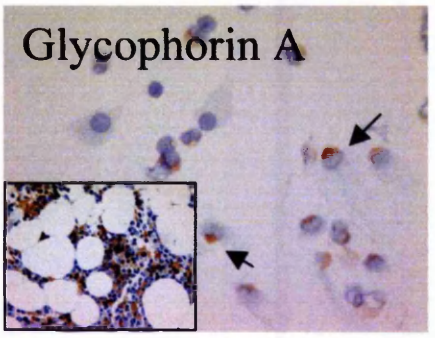


Figure 4.3a

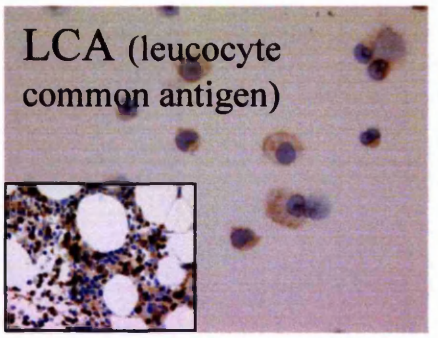


Figure 4.3b

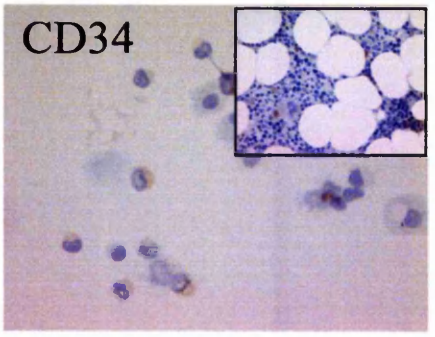


Figure 4.3c

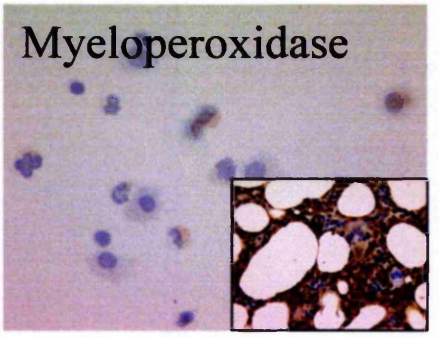


Figure 4.3d

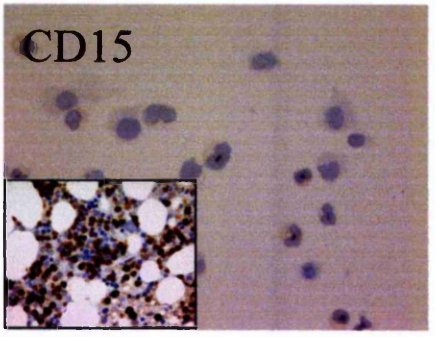


Figure 4.3e

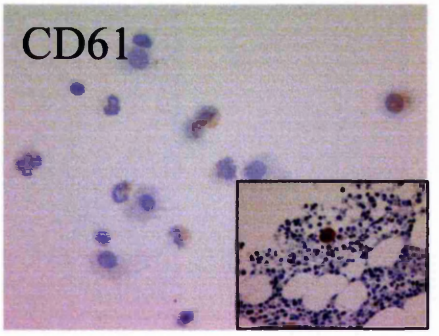


Figure 4.3f

Figure 4.3 Immunohistochemistry in non-adherent cells (x400) for a range of haematopoietic markers, showing positivity for glycophorin A (arrows, figure 4.3a) alone; positive controls inset. The non-adherent cells were strongly positive for glycophorin A, but negative for the other haematopoietic markers tested, including the lymphoid marker leucocyte common antigen, the myeloid markers CD15, CD34 and myeloperoxidase, and the megakaryocytic marker CD61; only weak background staining was present in the cells reacted with each of these antibodies

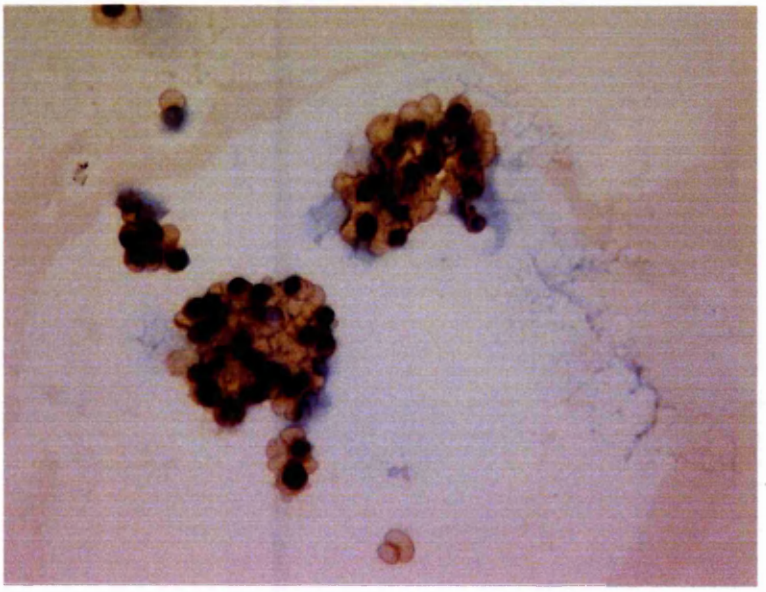


Figure 4.4a

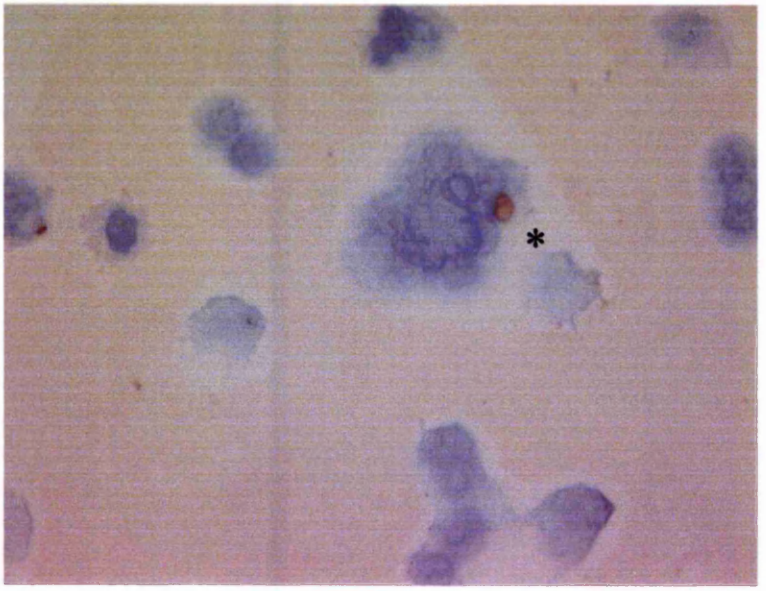


Figure 4.4b

Figure 4.4
Immunohistochemistry for glycophorin A after MACS sorting of non-adherent bone marrow stromal cells for glycophorin A, demonstrated restriction of cells expressing glycophorin A to the positive fraction (a), and their absence from the negative fraction (b), in which a contaminant red blood cell (*) is present as an internal control. Cytospin preparations,H&E, x400, protein expression disclosed with DAB.

Each of these grew slowly, and only one survived both subpassage and subsequent stimulation of osteoblastic differentiation.

4.3.5 Poly (A) RT-PCR and verification of osteoblastic differentiation

Poly (A) cDNA samples were generated from each of the time coursed mRNA extractions, namely from the non-adherent cells and from the subsequent adherent clones, both prior to and following stimulation of the latter with dexamethasone (figure 4.5). In each case the PCR products generated were between 100 and 500 bp in size and both open and closed control PCR tubes contained no PCR product. These PCR products hybridised with probes for GAPDH (figure 4.6a), and alkaline phosphatase (figure 4.6b), which was only positive in the cDNA from the adherent clones following dexamethasone stimulation, confirming the capacity of the clone for osteoblastic differentiation, and indicating the existence of genotypic differences between the non-adherent and adherent cells. GAPDH was present in each time point. PCR controls were negative and probe controls positive in each case.

4.3.6 Subtractive Hybridisation

4.3.6.1 Generation of subtraction products

Three subtraction products were generated by the three rounds of subtractive hybridisation undertaken, and designated S1, S2, and S3. Each yielded a PCR product upon amplification by poly (A) PCR (figure 4.7): the PCR products were between 100 to 500 bp long and both the open and closed PCR negative control tubes contained no PCR product.

4.3.6.2 Verification of efficiency of subtraction

Driver cDNA did not hybridise to S3 cDNA, verifying that the S3 subtraction product contained only those genes upregulated during osteoblastic differentiation, confirming efficient subtraction (figure 4.8). However, since some binding of S3 to driver was present, the S3 product was not composed entirely of genes unique to adherent cells.

4.3.7 Library Screening

Primary library screening yielded 83 positive clones, stocks from each of which have been prepared and stored at $4^{O}C$ as detailed in section 3.2.5. Two of these successfully underwent secondary screening and sequencing.

4.3.8 DNA sequencing

Two genes were identified by BLAST homology search, namely aldolase A and CGI-120 protein. For aldolase A 83% homology was present over a 149 bp long segment for the

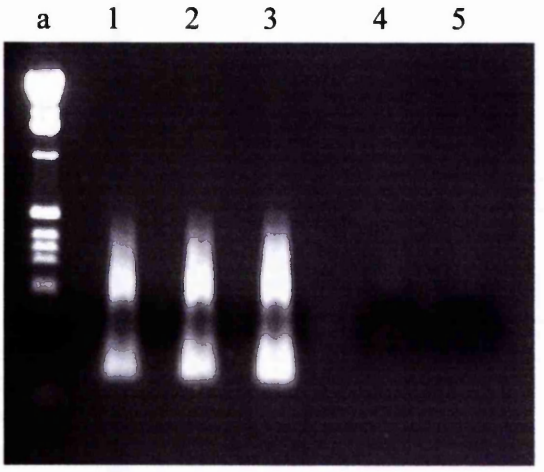


Figure 4.5
Ethidium bromide stained agarose gel electrophoresis fractions of poly (A) reverse-transcription polymerase chain reaction (RT-PCR) products from glycophorin A negative non-adherent cells and from resultant adherent clone, prior to and following dexamethasone stimulation. Lane a: 1Kb Lambda size marker, Lane 1: Products from poly (A) RT-PCR of glycophorin A negative non-adherent bone marrow stromal cells, Lane 2: Products from poly (A) RT-PCR of adherent clone prior to dexamethasone stimulation, Lane 3: Products from poly (A) RT-PCR of adherent clone following dexamethasone stimulation, Lanes 4 & 5: Open and closed poly (A) negative controls respectively.

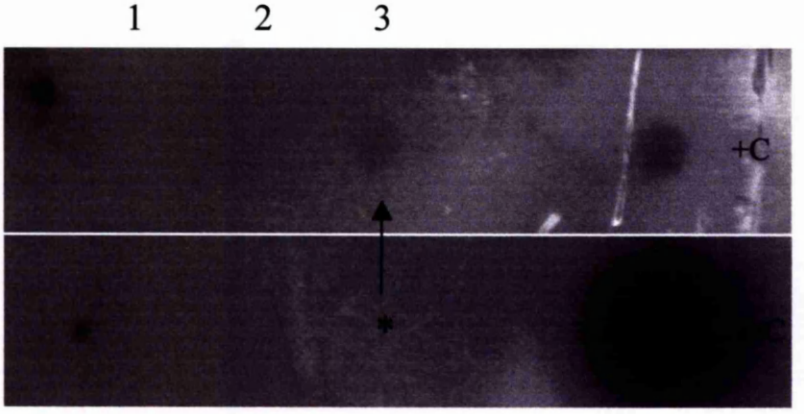


Figure 4.6 (i)

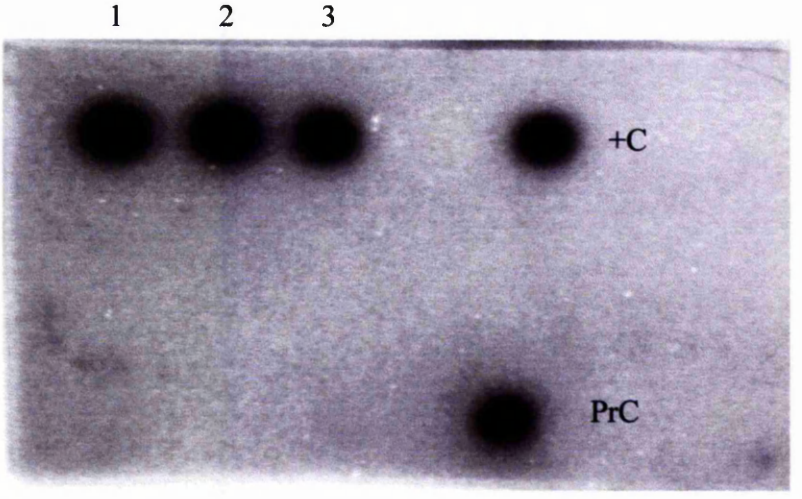
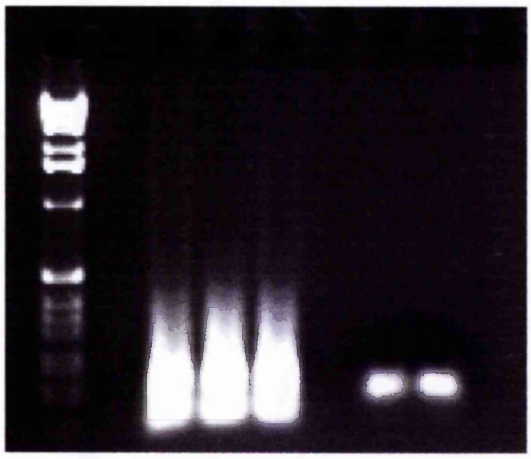


Figure 4.6 (ii)

Figure 4.6
Auto-radiographs of hybridisation signal of ^{32}P labelled probes to (i) alkaline phosphatase, and (ii) GAPDH probe with poly (A) RT-PCR products from glycophorin A negative non-adherent cells (1) and from resultant adherent clone, prior to (2) and following dexamethasone stimulation (3). GAPDH is expressed at the same level in each of the three cDNA pools, whilst alkaline phosphatase expression is absent from the non-adherent cells (1) and the adherent clone prior to dexamethasone stimulation (2), though present at a low level after stimulation (3 -*). PrC = probe control, +c = positive control



Lane a: 1Kb Lambda size marker

Lanes 1 to 3: Products from poly (A) PCR of subtraction products S1 to S3 respectively

Lanes 4 & 5: Open & closed PCR negative controls

Figure 4.7

Ethidium bromide stained agarose gel electrophoresis fractions of poly (A) PCR products from subtraction products S1 to S3.

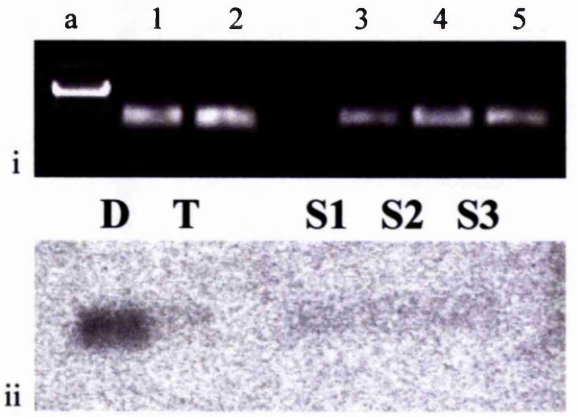


Figure 4.8a

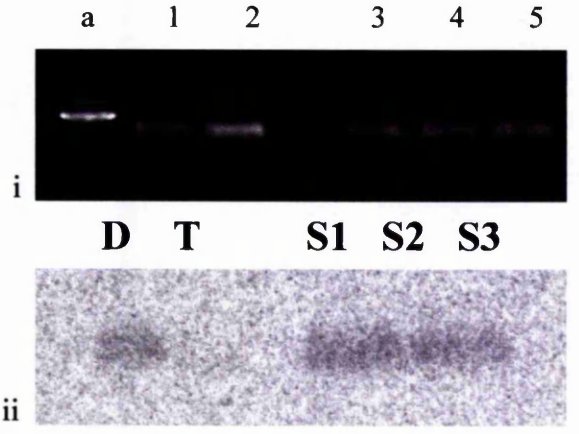


Figure 4.8b

Lane a: 1Kb Lambda size marker
Lane 1: Poly (A) PCR product from driver cDNA
Lane 2: Poly (A) PCR product from tracer cDNA
Lanes 3 to 5: Products from poly (A) PCR of subtraction products S1 to S3 respectively

Figure 4.8
Ethidium bromide stained agarose gel electrophoresis fractions (i) of poly (A) polymerase chain reaction products from driver (non-adherent cells), tracer (adherent cells) and subtraction products S1 to 3 and corresponding autoradiographs (ii) of Southern blots prepared from them after hybridisation with ^{32}P labelled probes to (a) driver cDNA, and (b) S3 cDNA.

forward sequence and 96% homology over a 656 bp segment for the reverse sequence. For CGI-120 protein 97% homology was present over a 666 bp segment. The sequence alignments for each gene are detailed in appendix 2.

4.3.9 Expression of isolated genes

4.3.9.1 Hybridisation with time point cDNA

For both of the genes identified hybridisation with cDNA from the time points confirmed upregulation during stimulation with dexamethasone, as indicated in figure 4.9. Both showed lack of hybridisation to the non-adherent cDNA (figure 4.9), indicating that both were unique to the adherent cells compared to the non-adherent cells.

4.3.9.2 In-situ hybridisation with human bone

The results of *in-situ* hybridisation are given below for the two genes studied; RNase controls were negative in all cases.

4.3.9.2.1 Aldolase A

Aldolase A was expressed at a low basal level over the haematopoietic stoma in normal fetal bone and was expressed at a moderately high level in osteoblasts lining the primary and secondary spongiosa, though osteoclasts were negative (figure 4.10). It was expressed in the periosteal mesenchyme and in the growth plate to a slightly lesser degree, whilst the cartilage was negative. It was upregulated in mesenchyme and in woven bone in healing fracture (figure 4.11), in a similar pattern to that observed for 14-3-3 zeta.

4.3.9.2.2 CG1-120 protein

CGI-120 protein mRNA was expressed at a moderate level in the periosteal mesenchyme adjacent to the outer surface of the bone in fetal long bone, and at a low level in osteoblasts, but was absent in the other cells in fetal bone (figure 4.12). It was not expressed at detectable levels in the sections of healing fracture callus studied.

4.4 Discussion

The discussion will be presented in two parts. Firstly the nature of the change from a non-adhesive to an adhesive phenotype, with acquisition of STRO-1 positivity, will be discussed, and secondly the nature of the genes upregulated during this process will be addressed.

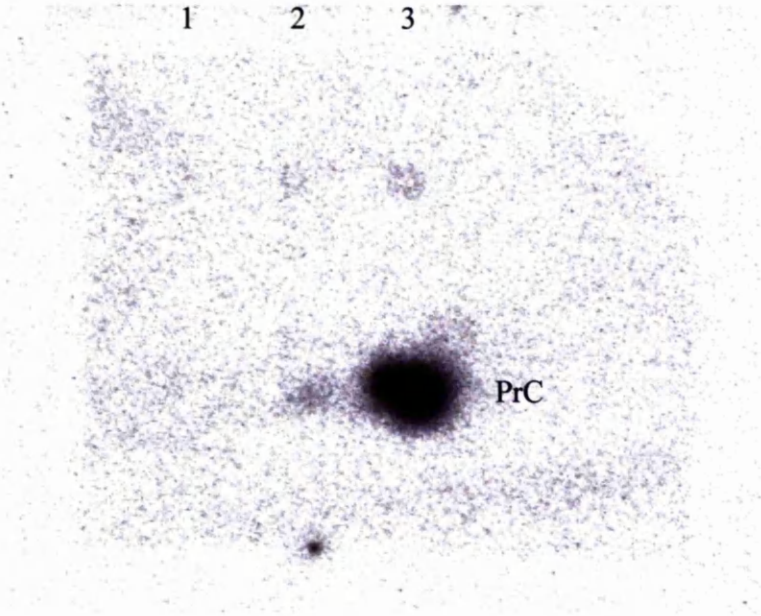


Figure 4.9a

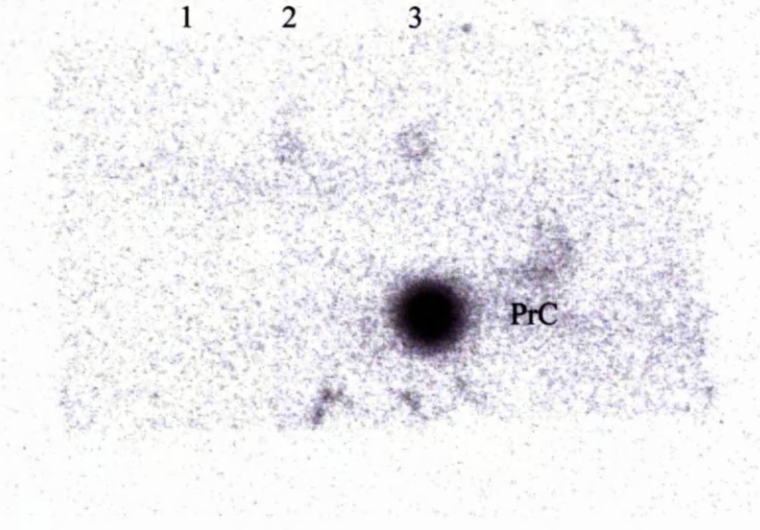


Figure 4.9b

Figure 4.9

Hybridisation of ^{32}P labelled probes to genes, (a) aldolase A, and (b) CGI-120 protein, isolated from subtraction between non-adherent cells and adherent cells, with poly (A) RT-PCR products from glycophorin A negative non-adherent cells (1) and from resultant adherent clone, prior to (2) and following dexamethasone stimulation (3). For both genes, hybridisation is present with cDNA products (2) & (3), but not with (1), indicating that both are uniquely present in the tracer (adherent cells) pool compared to the driver (non-adherent cells) one.

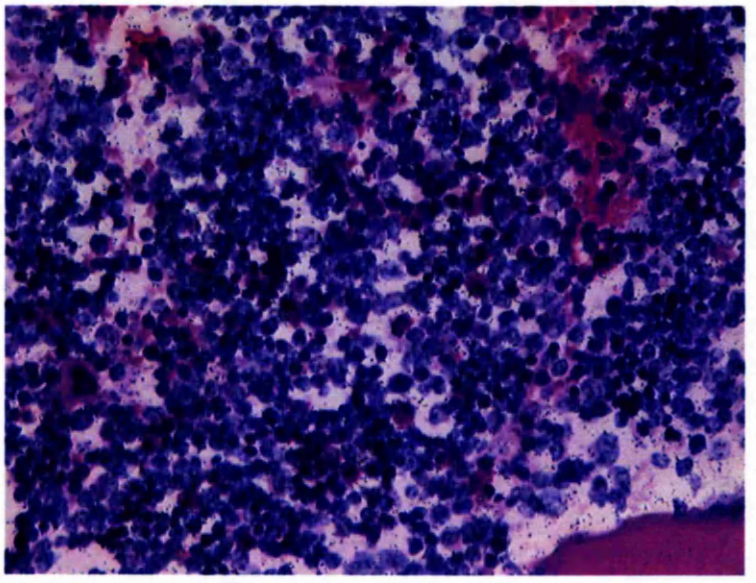


Figure 4.10a

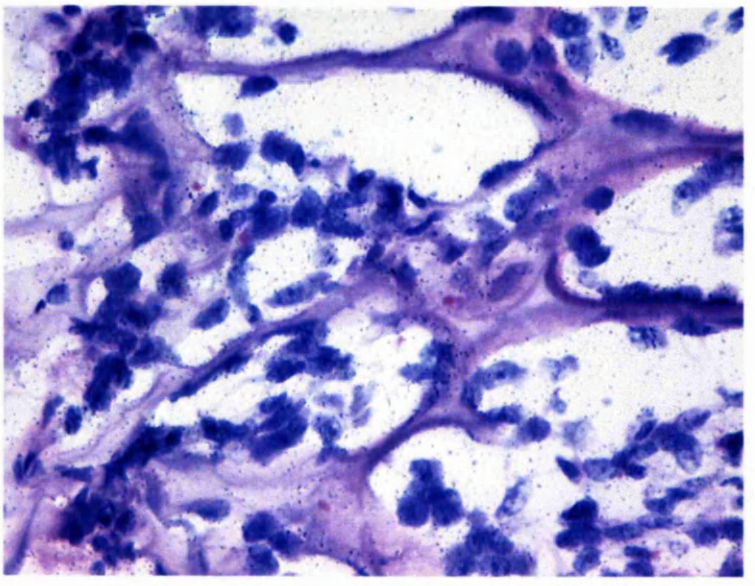


Figure 4.10b

Figure 4.10

Photomicrographs showing strong expression of aldolase A mRNA in (a) haematopoietic stroma (x400), and (b) over osteoblasts lining the primary spongiosa in fetal long bone. H&E.

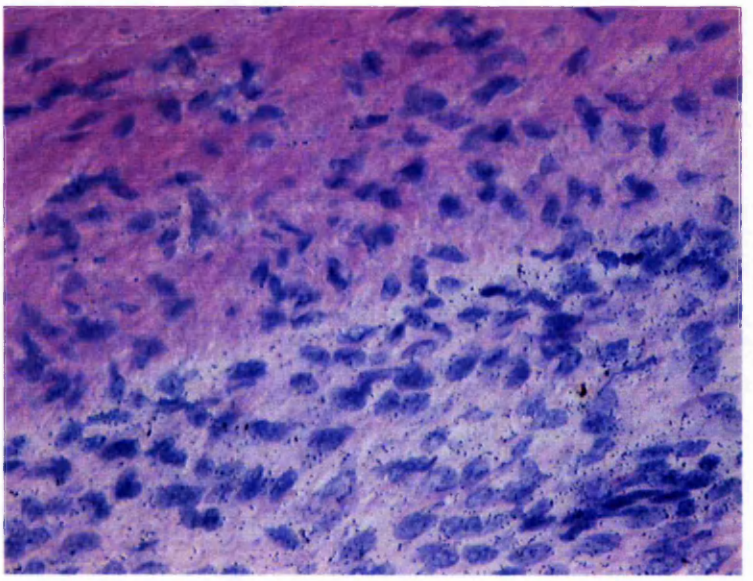


Figure 4.10c

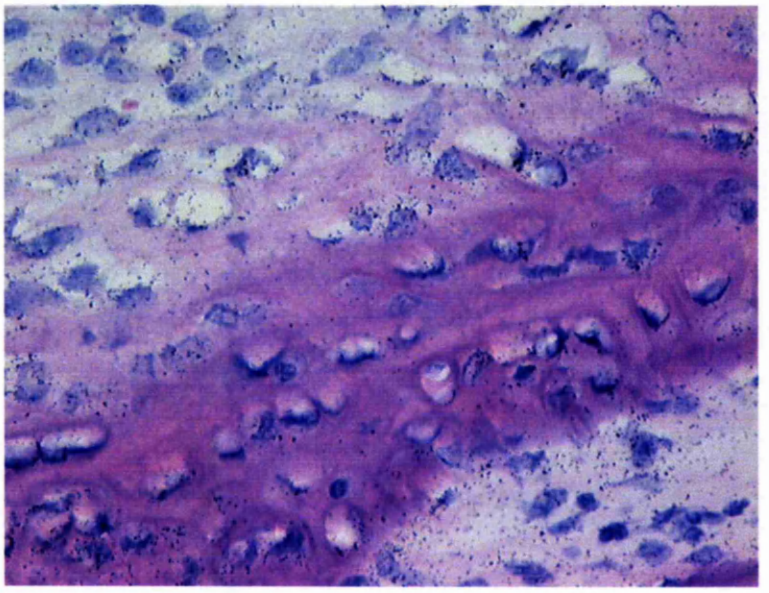


Figure 4.10d

Figure 4.10

Photomicrographs showing strong expression of aldolase A mRNA in (c) differentiating periosteal mesenchyme adjacent to the periosteal surface(x400), and (d) over osteoblasts at the periosteal surface in fetal long bone (x400). H&E.

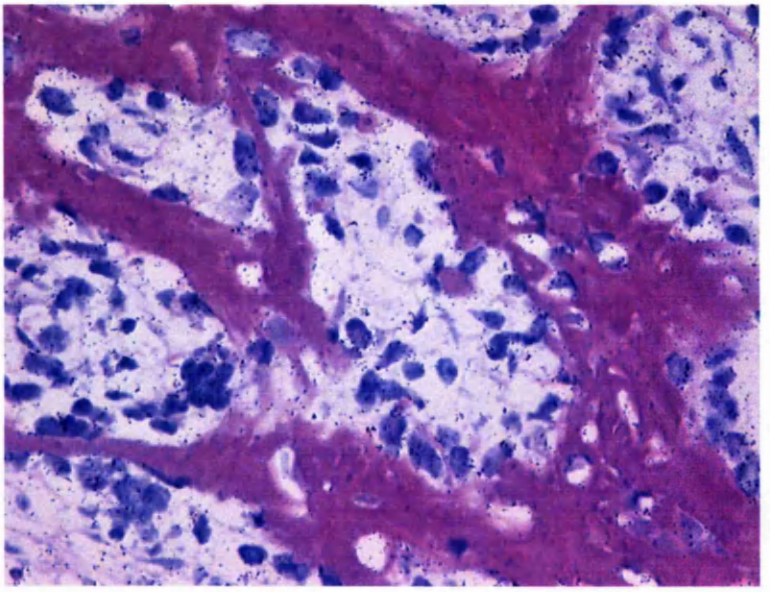


Figure 4.11a

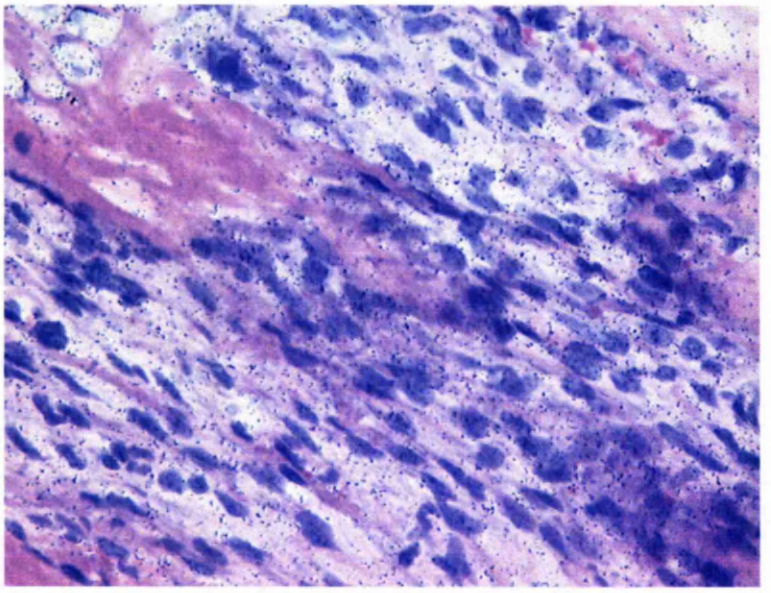


Figure 4.11b

Figure 4.11

Photomicrographs showing strong expression of aldolase A mRNA in (a) osteoblasts in woven bone(x400), and (b) in condensing mesenchyme adjacent to woven bone in healing fracture (x400). H&E.

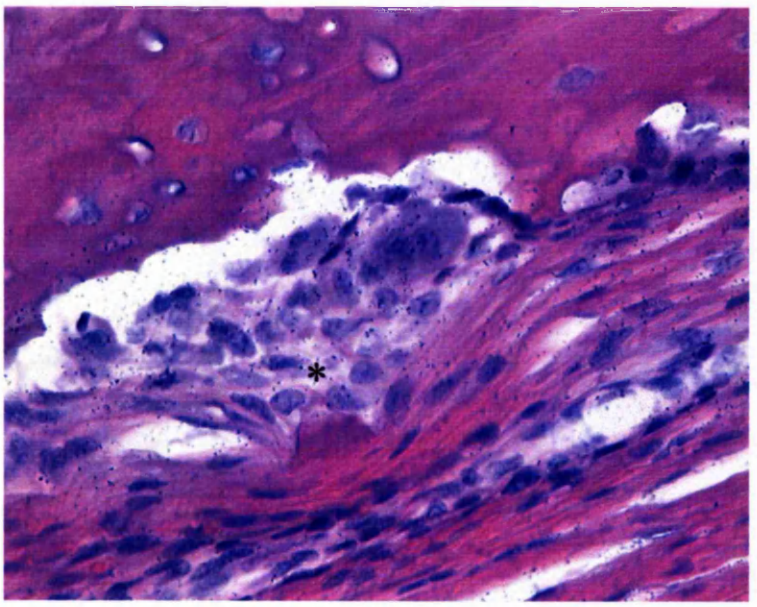


Figure 4.12
Photomicrograph showing strong expression of CGI-120 protein mRNA in periosteal mesenchyme in fetal bone (*). H&E, x400.

4.4.1 Acquisition of STRO-1 positivity upon adhesion of non-adherent marrow stromal cells

STRO-1 is a monoclonal mouse IgM anti-human antibody which recognises a trypsin resistant cell surface epitope present on all the assayable cells of the CFU-F (Simmons & Torok-Storb, 1991), including osteoprogenitors and, until the recent development of the antibodies HOP-26 (Joyner *et al.,* 1997) and SB-10 (Bruder *et al.,* 1997), was the only marker for the earliest cells of the CFU-F. The level of expression of STRO-1 in primary culture varies over time, from approximately 15% at 1 week to a maximum of nearly 60% at 2 weeks, followed by a fall to 30% at 3 weeks and 8% at 6 weeks (Simmons & Torok-Storb, 1991). Simmons and Torok-Storb (1991) were uncertain as to whether this variation was a culture epiphenomenon or due to loss of expression consequent upon differentiation of the cells, perhaps related to *in vitro* maturation of the stromal precursor cells into more differentiated cells. However, whilst they were unable to distinguish between these two possibilities, they noted that stromal cells expressing low or undetectable levels of STRO-1 demonstrated the highest levels of the fibroblast antigen TE7 or of smooth muscle actin, and by analogy with the reciprocal expression of the CD34 antigen and differentiation antigens in the haematopoetic system, suggested that this was in favour of STRO-1 loss occurring as a result of differentiation to a more mature phenotype. However, the nature of STRO-1 and whether it is present on the earliest stem cell or on a later cell, are uncertain.

Recent work has demonstrated alkaline phosphatase positive and negative subpopulations of STRO-1 positive cells within the CFU-F. Dexamathasone increased the size of the alkaline phosphatase positive subpopulation (Stewart *et al.,* 1996) indicating that STRO-1 reactivity spans a relatively wide span of differentiation, since alkaline phosphatase production is present from the late osteoprogenitor stage (Aubin *et al.,* 1995). The same study implicated that STRO-1 positive cells arise from a STRO-1 negative fraction. The data from the present study supports this, indicating that there is a population of STRO-1 negative stromal cells which subsequently become STRO-1 positive, and that the acquisition of STRO-1 positivity is associated with adherence. Simmons & Torok-Storb (1991) identified STRO-1 positivity on marrow stromal cells with osteogenic potential and, subsequently, Gronthos *et al* (1994) however found that CFU-F were restricted to the STRO-1 positive fraction of mononuclear cells, whilst STRO-1 negative cells contained no CFU-F. Similarly Gronthos *et al* (1999) assert that the STRO-1 positive / alkaline

phosphatase negative cell is the earliest in the osteoblast lineage (reviewed in detail below).

The system described demonstrates unequivocal capacity for osteoblastic differentiation (Cheng *et al.,* 1994), in both the initially adherent and non-adherent cells. As discussed in detail in chapter three a number of phenotypic and molecular markers, in this instance the expression of alkaline phosphatase after stimulation with dexamethasone, verified the utility of the system for investigation of early osteoblastic differentiation. The initial cultures exhibited a fibroblast-like morphology, grew in sheets, and expressed low levels of alkaline phosphatase. Stimulation with dexamethasone resulted in slighter plumper, more osteoblastic cells and increased expression of alkaline phosphatase (Cheng *et al.,* 1994), whilst stimulation with beta-glycerophosphate produced a nodular growth pattern similar to that seen in primary cultures of osteoblasts, together with formation of nodules of mineralised calcium (Beresford *et al.,* 1993). Similar results were observed for both the original and reseeded cultures, demonstrating equivalence of cell type within the two cultures indicating that cells of the CFU-F are present within the non-adherent population. The colony-forming rate was lower in the non-adherent, indicating higher adhesion and proliferation in the adherent cells. Most *in vitro* systems described use only the initially adherent cells and discard the non-adherent population, though Long *et al* (1995) demonstrated the presence of osteoprogenitor cells within a non-adherent population of bone marrow cells. However, in contrast to the present study, the non-adherent cells were isolated soon after initial seeding and then further immunoselected for expression of late osteoblastic markers, such as osteocalcin, osteonectin and alkaline phosphatase, calling into question the validity of the method for isolation of early osteoprogenitor cells in the study. Recently Scutt & Bertram (1995) observed change in stromal cells from a non-adherent to an adherent phenotype following stimulation with prostaglandin E2, and proposed a model of osteoblast differentiation in which active adherent osteoprogenitors were recruited from an inactive non-adherent pool (Scutt & Bertram, 1995), concordant with the findings in the present study. Non-adherent cells have also been shown to release a factor which stimulates differentiation of adherent stromal cells (Rickard *et al.,* 1995). Parenthetically, osteoid osteomas, which are composed of a central core of mesenchymal tissue and a dense peripheral zone of sclerotic bone secrete large amounts of prostaglandin (Wold *et al.,* 1988).

The level and variation over time of STRO-1 within the initially adherent cells was similar to those reported by others utilising the same methodology to prepare their primary stromal cell cultures (Simmons & Torok-Storb, 1991), demonstrating similarity of the system used to that of others. Simmons & Torok-Storb (1991) found that up to 95% of unfractionated marrow cells were of the erythroid lineage. However since erythroid precursors are largely excluded by the initial process of density gradient centrifugation and subsequently by adherence, double labelling of the cells against glycophorin A was not necessary in the initial part of the investigation. Interestingly, the non-adherent fraction still contained some cells of the erythroid lineage, as evidenced by glycophorin A positivity, indicating that adhesion does play a part in their removal in this system. The lack of STRO-1 reactivity in the non-adherent cells, and its subsequent expression in approximately 20% of the same cells following reseeding and consequent adherence, suggests that it is not present on the earliest cells of the CFU-F and that it is expressed following differentiation, probably to a more mature phenotype. The possibility that very low numbers of STRO-1 positive cells are present in the non-adherent population and that these precipitate and proliferate is unlikely given the low colony-forming rate of the cells (mean 1.25/1000cells), which was a log lower than that for the initially adherent cells. Stewart *et al* (1996) reported that the increase in the proportion of cells positive for alkaline phosphatase (either STRO-1 positive or negative) after stimulation with dexamethasone was preceded by an increase in the proportion of cells positive for the STRO-1 ligand, but negative for alkaline phosphatase. This implies recruitment of these cells from a pool of cells negative for both STRO-1 and alkaline phosphatase (Stewart *et al.,* 1996), though recently Gronthos *et al* (1999) have reported a comprehensive investigation into the differential expression of STRO-1 and alkaline phosphatase in which they assert that the STRO-1 positive / alkaline phosphatase negative cell is the earliest in the osteoblast lineage. According to this study this fraction was the only one capable of giving rise to all the other possible fractions, and did so in equal proportions to those isolated form uncultured marrow. However, despite this the physiological counterpart of the STRO-1 negative / alkaline phosphatase negative fraction remained undetermined, proposals including differentiated bone cells such as osteocytes and/or bone lining cells, though until the *in vivo* source of this fraction is identified the possibility that it presents an early stage of the osteoblastic lineage, as indicated in the present study, cannot be entirely ruled out. Furthermore, expression of the osteogenic transcription factor Cbfa1 was strongest in the STRO-1 negative / alkaline phosphatase negative fraction, and lowest in the STRO-1 positive / alkaline phosphatase negative fraction proposed as the earliest cell in the lineage (Gronthos *et al.,* 1999). Since Cbfa1 expression is necessary for commitment to the osteoblastic lineage (Ducy *et al.,*

1997), this would support the alternative that the STRO-1 negative / alkaline phosphatase negative cells are earlier in the lineage.

These results indicate that the STRO-1 ligand is upregulated during bone marrow stromal cell differentiation and adhesion, suggesting that it may be an adhesion molecule. Adhesion molecules are now known to be important in control of differentiation and morphogenesis (Gumbiner, 1996). Mesenchymal condensation is a part of normal embryogenesis, and recently initiation of osteogenesis at the pre-osteoblast stage has been shown to be controlled by the epithelial-mesenchymal interaction (Dunlop & Hall, 1995). Tenascin, an extracellular matrix glycoprotein (Crossin, 1996), is expressed before alkaline phosphatase in developing bone, which may reflect adhesion molecule interactions with the mesenchymal matrix (Dunlop & Hall, 1995; Vakeva *et al.,* 1990). The cadherins have been shown to be important in tissue formation (Larue *et al.,* 1996), though whilst integrin expression in osteoblasts has been described the role in differentiation is unclear (Hughes *et al.,* 1993). A recently cloned cadherin specific to osteoblasts, OB-cadherin, may prove to be involved in control of differentiation (Owazaki *et al.,* 1994).

It is intriguing as to why reseeding results in differentiation and precipitation of an additional pool of osteoprogenitor stromal stem cells. One possibility is that there exists within the bone marrow a large reservoir of osteogenic stromal stem cells, from which small numbers of activated cells, recognisable as cells of the CFU-F, arise (Simmons, 1996). This is consistent with recent evidence that osteogenicity is the property of perhaps as little as 2.5% of the fibroblastic stromal cells exhibiting clonal growth *in vitro* (Owen *et al.,* 1987) leaving a large amount of inactive cells. It is known that in situations of increased bone production, particularly during fracture repair, the number of active cells increases, and perhaps there is a requirement for fibroblast-like stromal cells to modulate to the osteoblast class only in the event of tissue damage and repair (Simmons, 1996). In the physiological steady state therefore the majority of fibroblast-like stromal cells would be inactive. Paracrine factors secreted by the small number of active cells could modulate further release of cells from the inactive reservoir.

These results demonstrate that non-adherent bone marrow stromal cells contain a STRO-1 negative population which when reseeded become STRO-1 positive and adherent (figure 4.13). Both the original and reseeded cultures are capable of osteoblastic differentiation.

This suggests that STRO-1, whilst being a differentiation antigen for early cells of the CFU-F, is not present on the earliest cell, and that it may act in cell adhesion. Furthermore, the existence of a non-adherent, STRO-1 negative, cell at an earlier stage in the osteoblastic lineage than the adherent, STRO-1 positive, cell presently thought to be the earliest osteoprogenitor cell, is suggested. An alternative explanation for the results obtained however exists, and is detailed in figure 4.14. This alternative arises from the qualification of the term 'non-adherent' which, in reference to the original work of Friedenstein (1976) is a cell not adherent two hours after initial seeding, whilst the studies of Simmons & colleagues noted above into the expression of STRO-1 on marrow stromal and mononuclear cells utilise freshly isolated marrow cells. Both of these systems therefore use much shorter periods of adherence selection than in either the present study, or in those of Scutt & Bertram (1995). It is possible therefore that, as indicated in figure 4.14, a STRO-1 positive marrow cell adheres rapidly and that subsequent non-adherence during cell proliferation and cycling is responsible for switching on and off of STRO-1 expression. This would be supported by the abstract of Stewart *et al* (1996) who demonstrated recruitment of a STRO-1 positive fraction from a STRO-1 negative fraction, whilst concording with the work of Gronthos *et al* (1999) who demonstrated that all other fractions could be generated only from the STRO-1 positive, alkaline phosphatase negative fraction. Such a hypothesis would also explain the upregulation in STRO-1 expression upon adherence of non-adherent cells seen in the present study.

Given the possibility of a STRO-1 negative precursor cell however it is necessary to establish whether or not such a cell exists *in vivo*, and to eliminate the possibility that it is not merely a culture artefact, it was necessary to identify markers for it. The identification of such markers may enable the above dichotomy to be investigated and tested *in vivo*, by gene expression studies in human bone. The strategy chosen, namely the use of poly (A) based subtractive hybridisation, identified genotypic markers which have the advantage of direct correlation with the molecular factors controlling the process. The details of this part of the investigation are discussed below.

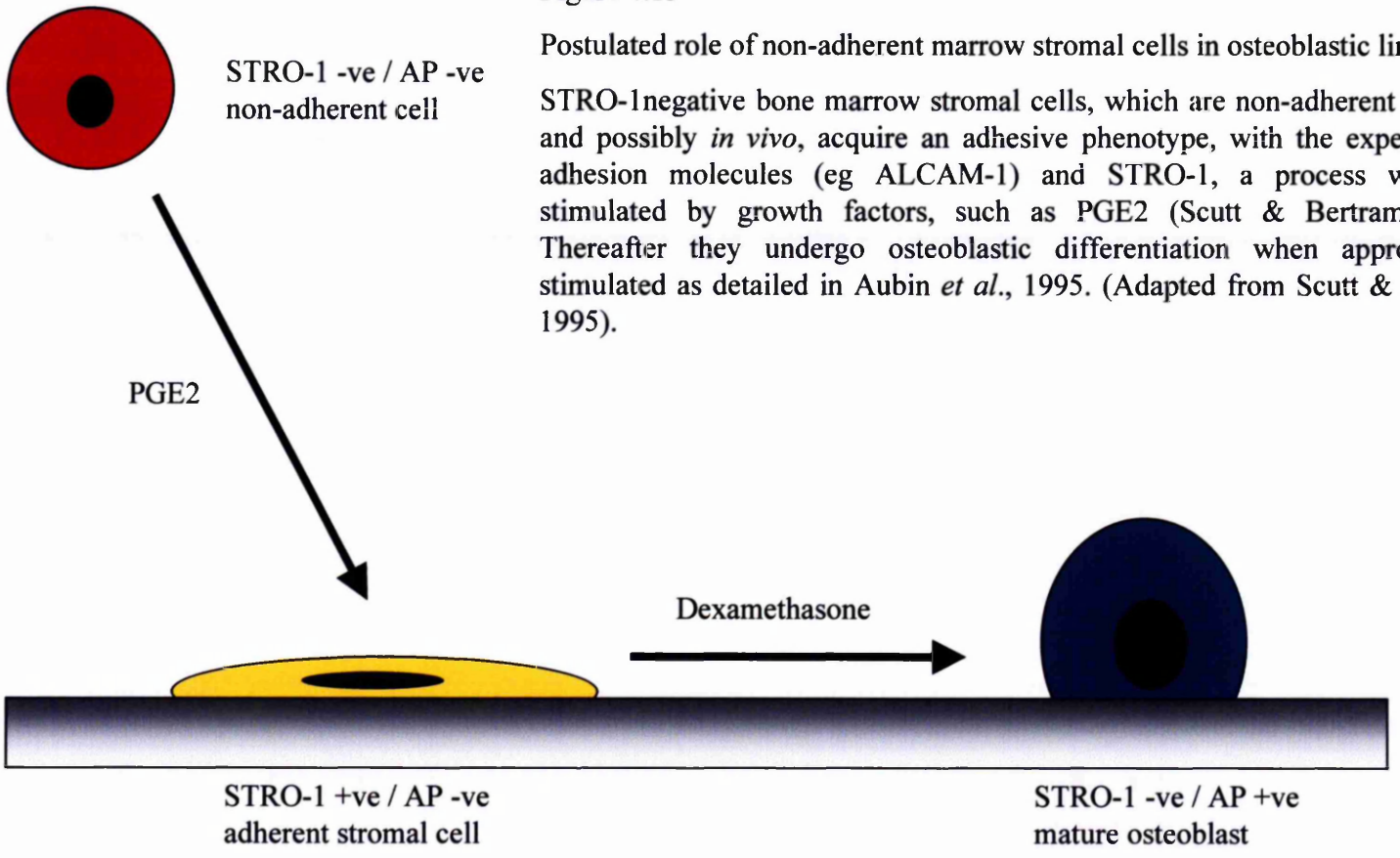


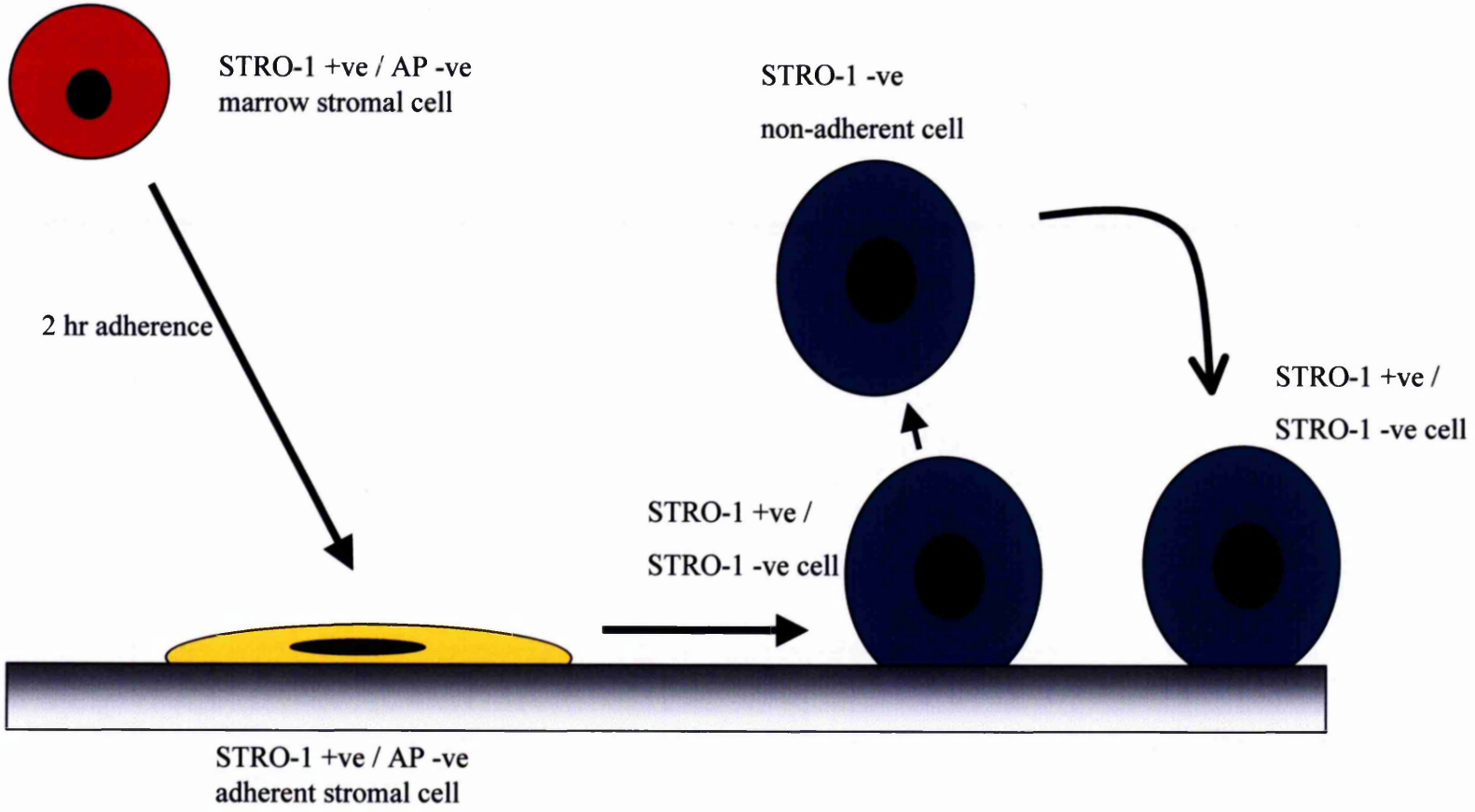
Figure 4.13

Postulated role of non-adherent marrow stromal cells in osteoblastic lineage

STRO-1negative bone marrow stromal cells, which are non-adherent *in vitro*, and possibly *in vivo*, acquire an adhesive phenotype, with the expession of adhesion molecules (eg ALCAM-1) and STRO-1, a process which is stimulated by growth factors, such as PGE2 (Scutt & Bertram 1995). Thereafter they undergo osteoblastic differentiation when appropriately stimulated as detailed in Aubin *et al.*, 1995. (Adapted from Scutt & Bertram 1995).

Figure 4.14

Alternative role of non-adherent marrow stromal cells in osteoblastic lineage



4.4.2 Genes upregulated during adhesion of non-adherent bone marrow stromal cells in early osteoblastic differentiation

As a prelude to the identification of genes upregulated during acquisition of an adhesive phenotype by marrow stromal cells, cell sorting and cloning was carried out, in a similar manner to that undertaken in the course of the work described in chapter three, in order to produce clonal populations of enriched cells. Since the non-adherent fraction may have possibly contained other elements of the bone marrow microenvironment, immunohistochemistry was used to establish which, if any, of these other elements were present in the non-adherent fraction, with an aim to their removal, by magnetic activated cell sorting, prior to the generation of clones. In contrast to the system described in chapter three, no markers have been reported for the putative non-adherent osteoprogenitor cell and consequently the most efficient strategy available was to deplete using known markers for all the other cell types known to reside in the marrow. These included the myeloid series, identified by myeloperoxidase, CD15 and CD34, the erythroid series, identified by glycophorin A, the lymphoid series, identified by CD45, and the megakaryocytic series, identified by CD61. Glycophorin A was the only one of the above antibodies that was positive with cells in the non-adherent fraction. Magnetic activated cell sorting was therefore used to remove these cells prior to generation of clones. Since the clone therefore arose from cells non-reactive against any of the markers for the other cells within the bone marrow it is reasonable to conclude that they arose from the stromal cell fraction, for which no markers presently exist. Other groups have attempted to immunophenotype the non-adherent cell. Long *et al* (1991) found that the non-adherent cells were negative for the My-10 antibody, which reacts against CD34 positive cells (Mercadal *et al.,* 1998), similarly to the result in the present study, whilst Still *et al* (1998) found that the non-adherent fraction was negative for CD45. Both these other reports concord with the, more extensive, immunophenotype established in the present study and support the strategy used for the generation of clonal populations of osteoprogenitor cells from the non-adherent fraction.

The poly (A) based subtractive hybridisation method used to isolate the genes upregulated, in this case during adhesion of the non-adherent cells, has been discussed in chapter three and need not be further considered at this point. A similar discrepancy in the hybridisation of probes for the S3 and driver cDNA to the composite Southern blot as noted, and discussed in detail, in chapter three was present, emphasising the need for verification of the upregulation of the genes isolated by the expression studies detailed below.

Two genes upregulated during adhesion of non-adherent osteoprogenitor cells were identified, namely aldolase A and CGI-120 protein. Aldolase A is a tetrameric glycolytic enzyme that catalyses a reversible aldose cleavage / condensation reaction in the glycolytic pathway, and which is found in high concentrations in skeletal muscle, heart muscle, cerebrum and lymphatic tissue (Okajima, 1990). The other two isoforms of the enzyme in mammalian tissue, the B-type and C-type enzymes, are found predominantly in liver and kidney, and in brain respectively. Recently however its expression has been linked to differentiation in a number of systems. Meighan-Mantha *et al* (1995) investigated the expression of aldolase A together with that of the other adult muscle fibre-specific genes, muscle creatine kinsase, fast skeletal troponin-T, and embryonic myosin heavy chain, in a model of chicken myoblast differentiation. Each of the genes showed a biphasic pattern of induction, with an initial rise in expression prior to the start of myoblast fusion, followed by a second rise following completion of myotuble fusion. Aldolase A also showed a biphasic pattern, but compared to the other genes its expression was delayed at both points. Reid & Masters (1985) reported preferential expression of the A-type isoform of aldolase, compared to the B- and C-types in development of mouse tissues from the embryo to adult, whilst Bernlohr *et al* (1985) found a moderate increase in the level of expression (between 2 to 4 fold) of fructose-1,6-bisphosphonate aldolase during differentiation of 3T3-L1 preadipocytes.

The differential expression of aldolase during differentiation may reflect differences in the glycolytic pathway consequent upon different energy requirements, in much the same way as was discussed for alpha enolase in chapter three. In support of this Gassman *et al* (1996) found increased expression of aldolase A in murine embryonic stem cells in the face of low oxygen tension, compared to normoxic controls. Interestingly the increased expression of aldolase A was accompanied by an increase in vascular endothelial growth factor, which is increased in a number of pathological situations, due to low oxygen. It was hypothesised that the low oxygen tension to which the embryo is exposed at the pre-implantation, until implantation and resultant vascularisation allow efficient oxygenation, results in differences in gene expression to accommodate the necessary changes in metabolism (Gassman *et al.,* 1996). Granstrom (1986) found a similar phenomenon in post implantation early rat embryonic facial development, in which anaerobic respiration predominates in the mandibular and maxillary processes. This stage was associated with predominant expression of the A-type isoform of aldolase (Granstrom, 1986).

The alternative hypothesis that the enzyme may act as a transcription factor has been reviewed by Ronai (1993), who described the localisation of four glycolytic enzymes, namely lactate dehydrogenase, phosphoglycerate kinase, aldolase and glyceraldehyde-3-phosphate dehydrogenase, to the nuclear compartment. The enzymes found in the nucleus were in a modified form from that found in the cytoplasmic compartment and all four were shown to bind to DNA (Ronai, 1993). Whilst no specific target DNA sequence was identifiable for any of the enzymes, their association with DNA may play a role in transcription and replication of DNA. In common with the effects of alpha enolase discussed in chapter three, aldolase may therefore act to modulate cell growth and differentiation via DNA-binding (Ronia, 1993).

Despite the wealth of literature on the role of aldolase in muscle however, little is known regarding its presence and function in bone. Popova (1983) reported its up-regulation in the bones of rats during spaceflight, and suggested that its role in glycogen splitting in the bone organic matrix was responsible for eliminating some of the adverse effects of weightlessness, though the mechanism by which this was stimulated was not stated. The only other report of a role for it in bone came from Ukaji *et al* (1999), who identified an autoantibody for the denatured form of aldolase A in the serum of patients with rheumatoid arthritis. The autoantibody was present in approximately 10% of such patients, but was not detected in the serum of either healthy controls, or patients with other arthropathies. Its presence in the serum of patients with rheumatoid arthritis was associated with the presence of severe bone erosion within the joints, linking it to bone destruction, though the mechanism whereby this may occur is not known (Ukaji *et al.*, 1999).

In the present study aldolase A was expressed in the adherent cells, but not in the non-adherent fraction, as indicated by its isolation by subtractive hybridisation between the two populations of cells and verified by hybridisation with cDNA from the two cell types. *In-situ* hybridisation demonstrated expression of its mRNA at a low basal level in the haematopoietic stroma in normal fetal bone and at a moderately high level in the osteoblasts lining the primary and secondary spongiosa. It was also expressed in the periosteal mesenchyme and in the growth plate to a slightly lesser degree, whilst the cartilage was negative. In addition it was upregulated in mesenchyme and woven bone in healing fracture callus, in a similar pattern to that observed for 14-3-3 zeta. This expression pattern confirms its involvement with osteogenesis, particularly in the condensation of mesenchyme occurring prior to bone formation and differentiation of osteoblasts in both fetal bone, at the periosteal surface, and in healing fracture callus. This is consistent with

the hypothesis that osteogenesis commences with mesenchymal condensation (Dunlop & Hall, 1995) of phenotypically fibroblastic stromal cells (Aubin *et al.,* 1995; Liu *et al* 1994), and supports the hypothesis that, concurrent with condensation, the stromal cells acquire an adhesive phenotype, as evidenced by upregulated expression of tenascin (Vakeva *et al.,* 1990) and OB-cadherin during early osteogenesis (Okazaki *et al.,* 1994). Whether the enzyme is involved only in modulation of energetic processes occurring during the differentiation of these cells, as in the case of rat fetal mandibular and maxillary processes, due to changes in oxygen tension (Granstrom, 1986; Gassman *et al.,* 1996), or whether it is also acting as a DNA-binding protein (Ronai 1993) is not possible to determine from the present study. None of the studies quoted above link it to acquisition of adhesion, but the fact that it has been linked to differentiation in other systems, and that its expression is localised to sites of mesenchymal condensation supports the hypothesis that acquisition of an adhesive phenotype is an early step in osteogenesis, and by extension therefore supports the hypothesis that the non-adherent cells represent a distinct population of cells *in vivo*.

In contrast to the extensive literature regarding aldolase A, apart from the registration of the encoding nucleotide sequence, localised to chromosome 12, with the GenBank Sequence Database at the National Institutes of Health, Bethesda, Maryland, USA, reference AF151878, no other references to CGI-120 protein are present in the literature. The mRNA detailed is 1900bp long and codes for a protein of 177 residues (AF151878.1), but further details of its structure, expression pattern or function are presently unknown. Its up-regulation was confirmed by hybridisation with cDNA from the adherent cells, but not with that from the non-adherent cells, whilst *in-situ* hybridisation demonstrated its expression at a moderate level in the periosteal mesenchyme in fetal long bone, in which it was also expressed at a low level in osteoblasts. In contrast to 14-3-3 zeta, and aldolase A it was not also expressed in sections of fracture callus, indicating heterogeneity of expression of the different genes isolated. Its spatial expression pattern is concordant with the remarks made above regarding the implications of the expression pattern of aldolase and, therefore, with the hypothesis that non-adherent cells are involved in the early stages of osteogenesis, but, in common with 14-3-3 zeta, alpha enolase and aldolase A, functional studies are needed before any conclusions as to its role can be drawn.

4.4.3 Conclusions

The hypothesis that the earliest cell in the osteoblastic lineage *in vivo* was tested by investigation of the phenotypic, namely STRO-1 expression, and genotypic characteristics of non-adherent marrow stromal cells *in vitro*, and the alterations occurring upon adhesion

of these cells. The capacity of the non-adherent cells to give rise to adherent cells of the osteoblastic lineage, and the acquisition of STRO-1 positivity consequent upon adhesion, was demonstrated, indicating either that the earliest cell in the osteoblastic lineage is non-adherent / STRO-1 negative, or that it is adherent / STRO-1 positive, with resultant modulation upon cell cycling. As a first step to resolving this dichotomy, and in order to establish genotypic markers for the identification of the non-adherent cells *in vivo,* poly (A) based subtractive hybridisation was used to isolate genes up-regulated during the adhesion of non-adherent cells. Two such genes were identified, namely aldolase A and CGI-120 protein. The former has been linked with differentiation in other systems, either via changes in the metabolism of cells during maturation, due to changes in oxygen tensions, or via DNA-binding, whilst the nature and role of CGI-120 protein is presently unknown. The spatial and temporal pattern of expression of the two genes support the hypothesis that the non-adherent cells observed *in vitro* represent a distinct population of osteoprogenitors *in vivo*, and that, as suggested by Scutt & Bertram (1995) and Long *et al* (1991 & 1995), the earliest cell in the osteoblast lineage is non-adherent *in vivo*.

Chapter 5

Investigation of the role of 14-3-3 zeta in early osteogenesis

5.1 Introduction

14-3-3 zeta was upregulated during early osteoblastic differentiation of human marrow stromal cells, and its expression profile *in-situ* suggests that it is important in the regulation of osteogenesis. In order to verify this hypothesis, and to exclude the possibility that it's upregulation is not due to association with another gene more pertinent to the process, it is necessary to demonstrate that it is both necessary and adequate for osteoblastic differentiation of marrow stromal cells. This can be achieved by transfection of sense or anti-sense sequences, to either upregulate or downregulate it's expression *in vitro*, a strategy that has been widely used for investigation of gene function (Inouye, 1988; Wagner, 1994). In deciding upon the protocol to be used the first step is to determine whether sense or anti-sense transfections are more likely to be informative. Preferably both should be performed, but in this case prior literature on the effect of over-expression of 14-3-3 *in vitro* (Reuther & Pendergast, 1996) indicates that anti-sense transfection would be more appropriate. If 14-3-3 is necessary for osteogenesis such transfections will abrogate osteoblastic diffferentiation.

5.1.1 The antisense concept

The antisense concept, in which mRNA translation is controlled by chemically synthesised complementary oligonucleotides (oligomers) was initially developed for study of gene function and is now attracting interest due to its potential for development of new medical therapies (Gibson, 1997), where key events in disease initiation or progression involve nucleic acid sequences. In essence the technique involves interruption of the normal sequence of transcription of mRNA from upregulated genes and translation of protein from the mRNA. This process is amenable to manipulation at several points. Consequently, since inception of the technique the range of methodologies employed has expanded rapidly, and presently encompasses the following strategies (Leonetti *et al.*, 1993):

1 – competition of transcription factors with double-stranded oligomers

2 – transcriptional blockade with triple-helix-forming oligomers

3 – inhibition of mRNA translation with antisense oligomers

4 – cleavage of mRNA by a ribozyme

5 – competition of RNA binding proteins with synthetic ribonucleotides

Of the above however, the most frequently used are inhibition of mRNA translation, using antisense oligomers, and cleavage of mRNA, using ribozymes.

5.1.2 Antisense technology

Most of the early studies were performed using oligodeoxyribonucleotides complementary to mRNAs (Zamecnik & Stephenson, 1978; Leonetti *et al.,* 1993); hence the antisense concept. Single stranded DNA oligomers are more frequently used now and bind to the complementary sequence on the mRNA, to up to single base fidelity (Wagner, 1994). The resultant DNA:RNA duplex prevents translation of the mRNA, either by physical restriction, or by activation of the RNase H enzyme, which acts to hydrolyse the RNA strand of the hybrid (Gibson, 1997). The antisense oligomer thereby acts as a powerful sequence-specific nuclease. The oligomer used is usually 16 bases long and designed to hybridise across the initiation codon of the mRNA (Giles *et al.,* 1995). It is therefore vital that the sequence chosen is not homologous with a segment of another gene. This is confirmed by database searches. Similarly, for each antisense oligomer used a control oligomer composed of the same proportions of each base, but in a random order should be used, and this random oligomer also checked against databases to ensure it will not hybridise to another gene (Gibson, 1997).

Apart from the design of the base pair sequence of the oligomer used, the stability of the oligomer *in vitro*, and assessment of its uptake into cells, must be considered. Antisense oligomers are susceptible to degradation by nucleases (Tidd, 1997). This is usually limited by phophorothioate modification, in which a non-bridging oxygen atom of the internucleoside linkage is replaced by sulphur (Tidd, 1997). According to some groups this is in itself helix destabilising, and consequently some recommend methylphosphonate-phosphodiester chimeras instead (Giles & Tidd, 1993). This involves addition of methylphosphonate groups to some of the linkage points in the oligomer backbone, and phophodiester groups to others. Entry of the oligomer into cells has been facilitated by a number of techniques, including microinjection, vector-mediated uptake, electroporation, streptolysin O permeabilisation, and calcium phosphate transfection, but increasingly cationic lipid transfection is being used in preference to these techniques (Twomey & Gibson, 1997). A number of cationic lipids are now available commercially, each of which is designed for delivery of certain DNA sequences, and into certain cell types (Malone, 1995; Malone *et al.,* 1989; Twomey & Gibson, 1997)). As a consequence for each cell type used the transfection technique used must be optimised.

Cationic lipids form small, unilamellar vesicles (average size 100 nm in diameter), the surface of which is positively charged (Malone *et al.,* 1989). As such, they interact spontaneously with both DNA and RNA. The nucleic acid is not encapsulated within the

liposome, as in conventional liposomes, but instead, 2-4 liposomes assemble with a 5kB plasmid, whilst several copies of smaller nucleic acids, i.e. oligomers, are associated with a single liposome (Malone *et al.*, 1989; Malone, 1995). The lipids are thought to neutralise the negative charge of the complexed nucleic acid, thereby facilitating fusion with the plasma membranes. Electron microscope studies of uptake of cationic lipids have shown attachment of the liposomes to the cell membrane, but the mechanism of subsequent uptake into the cell remains unresolved. It is thought to be mediated either by direct cytoplasmic delivery, or via an endocytic pathway.

Cationic lipids have several advantages over other sytems of transfection. They appear to be protective against nuclease degradation, both in cells and in serum, and the complexed nucleic acid is released slowly following uptake, such that the concentration of oligomer in the cell is sustained over a period of several days (Twomey & Gibson, 1997). Use of cationic lipids circumvents the problems associated with viral delivery systems. These include the possibility of recombination of the virus within the host genome and induction of an immune response against intrinsic viral antigens, thus making repeat administration less effective. The method is very versatile in terms of both the cell type and the nucleic acid to be transfected, and many cell types not previously transfectable by other methods have proved transfectable using cationic lipids (Malone, 1995). The method is simple, reliable and reproducible, but two major disadvantages must be overcome. Firstly, cationic lipids are cytotoxic, the degree of which varies with each cell type, so that toxicity profiles must be prepared for each cell type used. Secondly, initial incubation of cells with the lipid-nucleic acid complex must be carried out in a serum-free environment.

Uptake of oligomer into cells is usually assessed by use of a 5' fluoresceine (FITC) labelled oligomer (Twomey & Gibson, 1997). An FITC labelled oligomer is used in preliminary optimisation experiments and, since it is neither phosphorothioate or methylphosphonate modified, has no functional effect, but can be used to track uptake of the oligomer by the cells by visualisation with UV trans-illumination at a number of time-points. The efficiency of uptake is dependent upon the concentration of the oligomer and that of the cationic lipid used to effect it's introduction to the cell. However, since cationic lipids are also cytotoxic, the increased uptake of oligomer achieved with higher concentrations must be titrated against increasing loss of viable cells.

5.1.3 Ribozymes

Ribozymes are small RNA molecules that cleave other RNA molecules, and mRNA sequences in particular. Such catalytic RNA molecules are termed ribozymes and occur naturally. Three major classes exist (Sczakiel, 1997):

(i) Group I introns, which occur in the gene for the nuclear 26s rRNA in Tetrahymena thermophilia

(ii) Group II introns, which occur in organellar genes of lower eukaryotes and plants

(iii) Small plant pathogenic RNA molecules, occurring in satellite RNAs and virusoids, which play some role in viral replication.

This ability to cleave RNA has resulted in design of 'hammerhead' ribozymes, consisting of three base-paired stems with a consensus sequence, resembling a hammer (Sczakiel, 1997). Cleavage of the phosphodiester bond results in disruption of the mRNA. Design of the ribozyme must be done in consideration of the site of cleavage, which is restricted to key NUX sites in each sequence. Furthermore, the sequence either side of the cleavage region must be carefully chosen so as to prevent non-specific binding, but also to allow disassociation following cleavage, so that the ribozyme can cleave further copies of the mRNA species. The considerations have limited success of ribozyme use and antisense oligomers remain the simplest, must efficient system in terms of time (Sczakiel, 1997).

5.1.4 Aims and objectives of the investigation

In order to assess the functional importance of 14-3-3 zeta in osteoblastic differentiation of bone marrow stromal cells, anti-sense oligomers were used to block the expression of 14-3-3 zeta following stimulation with dexamethasone. In order to provide continuity and to strengthen the relevance of the findings to normal human osteogenesis, in recapitulation of the arguments for its use already forwarded in chapter three, the same primary human bone marrow stromal cell culture system as detailed in chapter three was used. The expression of 14-3-3 zeta in this system, at both the mRNA and protein level, both prior to and following dexamethasone stimulation has been detailed in chapter three; at both levels its expression is upregulated by stimulation with dexamethasone, in parallel with osteoblastic differentiation.

The overall aim of the investigation was to establish whether or not 14-3-3 zeta gene expression was necessary for osteoblastic differentiation of human bone marrow stromal cells. This was achieved by transient transfection of antisense oligomers into primary cultures of these cells, which in turn was accomplished via the following steps:

1- Design and modification of the antisense oligomers

2- Optimisation of cellular uptake of antisense oligomers

3- Assessment of modulation of osteoblastic differentiation by antisense oligomer

The protocols used for each of these steps are detailed below.

5.2 Methods

All reagents and laboratory glassware and plasticware were obtained from the following companies, unless otherwise stated: culture media, Gibco (Paisley, U.K.); plasticware, Costar (Cambridge, U.S.A.) and Nunc (Illinois, U.S.A.); micropipettes, Gelman (Ann Arbour, U.S.A.); reagents and immunochemicals, Sigma (Poole, U.S.A.). Anti-sense oligomers were obtained from MWG-Biotech AG (Milton Keynes, U. K.).

5.2.1 Design and modification of antisense oligomers

The antisense oligomer used was designed to hybridise to the initiation codon of 14-3-3 zeta, see figure 5.1. Two varients of 14-3-3 zeta, with alternate splicing of the 5'end, exist, but the two are identical downstream of a point proximal to the initiation codon, and therefore both yield the same protein product. Both varients are shown in figure 5.1, together with the site of the initiation codon. The antisense oligomer used is shown in figure 5.1, together with the corresponding random sequence. BLAST / Genbank database searches (at National Institutes of Health, Bethesda, Maryland, U.S.A) demonstrated lack of homology to other known human mRNA sequences. Both the antisense and random sequences were modified by phosphorothioate substitution, as detailed in section 5.1.2. In addition both of the sequences were also separately modified by addition of FITC at their 5' end. The FITC modified oligomers were used to assess uptake of the oligomer into the cells.

5.2.2 Optimisation of cellular uptake of antisense oligomers

A cationic lipid, Lipofectin (Gibco, Paisley, U. K.), was used to facilitate uptake of the oligomers into the cells. Lipofectin reagent is a 1:1 (w/w) liposome formulation of the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-n,n,n-triethylammonium chloride (DOTMA), and dioleoyl phosphotidylethanolamine (DOPE) in distilled water, and has been shown capable of transfection of DNA into a variety of cell types (Malone *et al.,* 1989). A range of concentrations of Lipofectin and FITC labelled oligomer were used to determine the optimum concentration of each for maximal uptake of oligomer into primary cultures of human bone marrow stromal cells, as detailed below.

1

gcctgtgagcagcgagatccagggacagagtctcagcctcgccgctgctgccgccgccgcccagagactgctgagcccgtccgtccgccg

ccaccacccactccggacacagg.acatc*cagtc*

a ◄——┘ *

127

*atggata*aaaatgagctggtgcagaaggccaagctggccgagcaggcagagcgatatgatgacatggcagcctgcatgaagtctgtcactgagc

M D K N E L V Q K A K L A E Q A E R Y D D M A A C M K S V T

agggagctgagctgtcgaatgaggagagaaaccttctctctgttgcttataaaaacgttgtaggagcccgtaggtcatcgtggagggtcgtctcaagt

E Q G A E L S N E E R N L L S F T V A Y K N V V G A R R S S W R

attgagcagaagacggaaggtgctgagaaaaagcagcagatggctcgagaatacagagagaagatcgagacggagctgcgtgacatctgcaac

V V S S I E Q K T E G A E K K Q Q M A R E Y R E K I E T E L R D I

gatgtactgtctcttttggaaaagttcttgatccccaatgcttcgcaaccagaaagcaaagtcttctatttgaaaatgaagggtgactactaccgttactg

C N D V L S L L E K F F T L I P N A S Q P E S K V F Y L K M K G D

gccgaggttgctgctggtgatgacaagaaaggaattgtggaccagtcacagcaagcataccaagaagcatttgaaatcagcaaaaaggagatgca

Y Y R Y L A E V A A G D D K K G I V D Q S Q Q A Y Q E A F E I S

gccgacacaccccatcagactgggtctggccctcaacttctctgtgttctattacgagatcctgaactccccagagaaagcctgctctcttgcaaaaa

K K E M Q P T F T H P I R L G L A L N F S V F Y Y E I L N S P E K A

cagctttcgatgaagccattgctgaacttgatacattaagtgaagagtcgtacaaagacagcacgctaataatcgagttactgagagacaacttaacat

C S L A K T A F D E A I A E L D T L S E E S Y K D S T L I I E L L R F

tgtggacatcggatacccaaggagatgaagcagaagcaggagaaggaggggaaaattaaccggccttccaacctttgtctgcctca

T D N L T L W T S D T Q G D E A E A G E G G E N

861

ttctaaaatttacacagtagaccatttgtcatccatgctgtcccacagatagttttttgtttacgatttatgacaggtttatgttacttctatttgaatttctatatttcccatg

tggtttttatgttttaatattaggg

b 1030

anti-sense sequence for initiation codon: 5' – tat cca tga ctg gat g – 3'
corresponding nonsense random sequence: 5' – tgt aca cga ctt gat g – 3'

Figure 5.1 Nucleotide sequence for human bone marrow 14-3-3 zeta (1030 base pairs), indicating segment in which two unique 5' untranslated regions (in placental and bone marrow cDNAs) reside, upstream of "." (*), together with the area flanking the initiation codon (*underlined in italics*). The 5' and 3' untranslated regions (a & b) and the corresponding amino acid sequence for the translated region, between 127 to 861 base pairs, (CAPITALS) are shown. The anti-sense oligomer and corresponding random sequence oligomer used are shown.

5.2.2.1 Primary human bone marrow stromal cell culture

As detailed in chapter three, human bone marrow (0.5 to 1ml) was harvested from the sterna of consenting normal adult volunteers at the time of median sternotomy during cardiac surgery and density gradient centrifugation over a histopaque-1077 layer used to isolate bone marrow stromal cells (Cheng *et al.*, 1994). The marrow was placed directly into 10ml of alpha modified minimal essential medium (αMEM), without addition of supplements, at the time of harvest and immediately transported to the laboratory. All manipulations of the tissue were carried out in a class II biological cabinet. The marrow was removed from the transport medium using aseptic technique, placed in a small sterile petri dish and finely diced using a scalpel. The marrow was then resuspended in the transport medium and centrifuged at 500xg for 10 minutes at room temperature. The supernatant, including fat present at the top, was removed and the pellet resuspended in 5ml of αMEM in a 15ml Falcon tube. Five millilitres of histopaque-1077 was added to the tube, beneath the medium, and the tube centrifuged at 500xg for 30 minutes. Following this a thin grey layer of cells, containing the mononuclear cells was present at the interface of the medium and the histopaque-1077, whilst fragments of bone and erythroid cells, including erythroid precursors, were present in the pellet which was discarded at the base of the tube. The mononuclear layer was removed by gentle aspiration, seeded in a $25cm^2$ culture flask, and 5ml of alpha modified minimal essential medium (αMEM), supplemented with 10%(v/v) heat inactivated fetal bovine serum, 1%(v/v) glutamine, 1%(v/v) ascorbate, 1%(v/v) penicillin, streptomycin and amphotericin and 0.1%(v/v) gentamicin (thereon designated "standard medium") added. The cells were incubated at $37^{O}C$ in 5% carbon dioxide and were left to settle for 1 week after which the medium was removed. The cells were washed briefly with PBS(A) to remove non-adherent cells and fresh medium added. The medium was changed every 2-3 days thereafter. Adherent cells were left to proliferate for 2 weeks, after which time they were nearly confluent. They were then removed by cell scraping and re-seeded on glass coverslips in wells of a 24 well plate, at 1000 cells/well, following which they were maintained in standard medium for up to three days prior to use. Care was taken to ensure that the cells were at approximately 40% to 60% confluency immediately prior to use in transfection experiments.

5.2.2.2 Transient transfection of bone marrow stromal cells

The cells seeded on glass coverslips were used to assess uptake of FTIC labelled oligomer, and optimum concentration of Lipofectin and oligomer. For each of the concentrations of

Lipofectin and oligomer used the protocol detailed below was used for induction of transient transfection.

1- Lipofectin (2, 4 or 5μl: stock concentration 1μg/μl) was added to serum free alpha modified minimal essential medium to a final volume of 50μl and left at room temperature for 30 minutes (Lf).
2- Between 5 and 10 μl of FITC labelled antisense oligomer (stock 100pmol/μl) was added to serum free alpha modified minimal essential medium to a final volume of 50μl at room temperature (Ol).
3- The Lf and Ol from steps 1 & 2 were added and incubated at room temperature for 15 minutes to allow complexes to form.
4- Whilst Lf and Ol were complexing, the medium in each of the wells to be used in the experiment was removed and the cells washed twice with serum free alpha modified minimal essential medium.
5- The Lf / Ol mixture was added to the wells and the cells incubated in it for 6 hours at $37^{O}C$, after which 100μl of osteogenic medium ("standard medium" supplemented with dexamethasone at $10^{-7}M$) was added to each well. Controls using addition of Lf or Ol alone, each made up to 100μl with 50μl of serum free alpha modified minimal essential medium, or 100μl of serum free alpha modified minimal essential medium alone, were used in each case.
6- The cells were incubated in the osteogenic medium at $37^{O}C$ a further 18 hours, and the uptake of FITC labelled oligomer assessed at 4 hours, 8 hours, and 24 hours following start of transfection, by performing each experiment in triplicate.

5.2.2.3 Assessment of uptake of FITC labelled oligomer

At each of the time points noted in step 6 above (5.2.2.2) the amount of FITC labelled oligomer transfected into the cells, and the percentage of cells transfected with it, was assessed by visualisation under ultra-violet light (494nm). The cells were washed three times in PBS, the coverslips removed from each well and mounted in a 2.5% (v/v) of DABCO (Sigma)[90% glycerol, 10% PBS, 2.5% DABCO (Sigma) pH 8.6] on glass microscope slides. Each coverslip was viewed under ultra-violet light (494nm), the amount of oligomer present assessed on a semi-quantitative basis, and the percentage of cells containing FITC label measured by counting the number of positive cells, together with the total number of cells, visualised with Nomarski phase. The degree of cytotoxicity of the Lipofectin was crudely assessed by a semi-quantitive assessment of the number of cells present. The same measurements were made at each time-point for the test cells and for

each of the controls. Once the optimal concentrations of Lipofectin and oligomer were established the effect of antisense 14-3-3 zeta oligomer on osteoblastic differentiation of the bone marrow stromal cells was measured.

5.2.3 Assessment of modulation of osteoblastic differentiation by antisense oligomer

5.2.3.1 Transfection with phosphothiorated 14-3-3 zeta antisense oligomer

Human bone marrow stromal cells were seeded at a density of 1000 cells per well into each well of a 24 well plate, maintained in standard medium at 37^{O}C, and left until 40-60% confluent (1-3 days). Transient transfection was then carried out, using phosphothiorated antisense 14-3-3 oligomer (5.2.1) using the protocol detailed in section 5.2.2.2, with substitution of the FITC labelled oligomer in step 2 with the phosphothiorated oligomer. For each well 1μg of oligomer was used. Since 5μg is equal to 1nmol, and the antisense oligomer was supplied at 0.1nmol/μl, 2μl of stock oligomer was used for each well (final concentration 4pmol/μl in 50μl of serum free medium). The optimal concentration of Lipofectin, as determined above, was used. The same controls as detailed in step 5 were used, and osteogenic medium was added at the same time as stated in step 5. The cells were maintained for 18 hours after addition of the osteogenic medium, at which time the transfection process was repeated, using the same protocol as on the first day. The process was repeated a third time 24 hours later and the degree of osteoblastic differentiation of the cells measured 24 hour later, i.e. following 72 hours of stimulation with osteogenic medium in the presence of 14-3-3 zeta antisense oligomer.

5.2.3.2 Measurement of osteoblastic differentiation

The degree of osteoblastic differentiation of the cells in each well was determined by biochemical measurement of alkaline phosphatase expression, as previously detailed in section 3.2.1.4. In detail, the cells were washed three times in PBS and 100μl of phosphatase substrate / alkaline buffer (prepared immediately prior to use by mixture of equal volumes of phosphatase substrate (p-nitrophenylphosphate at 4mg/ml) and alkaline buffer (1.5M 2-amino-2-methyl-1-propanol, pH 10.3)) added to each well, followed by incubation at 37^{O}C for 15 minutes. Following incubation 250μl of 0.05M NaOH was added to each well and the absorbancy of the reaction product in each well measured at 410nm using a colourimetric reader. Standard concentrations of p-nitrophenol were read alongside the test wells in order to allow calibration. The data was analysed using the Mann-Whitney test.

5.3 Results

5.3.1 Optimisation of cellular uptake of antisense oligomers

Cellular uptake of FITC labelled oligomer was clearly demonstrated (figure 5.2), and was maximal at a concentration of 10pmol/μl of oligomer. At concentrations in excess of 0.2μg/μl the cytotoxicity of Lipofectin led to death of more than 25% of the cells. The percentage of cells transfected was measured using a final concentration 0.1μg/μl of Lipofectin and a final concentration 10pmol/μl of FITC labelled oligomer. At 4 hours 18.6% of cells contained oligomer, whilst at 8 hours 70.8% of cells were positive; at 24 hours FITC signal was undetectable. The results indicated that, since approximately half the amount of FITC labelled oligomer is usually required in phosphothiorated form (Twomey & Gibson, 1997), the optimum concentrations for use in assessment of modulation of osteoblastic differentiation were 0.05μg/μl of Lipofectin and 4pmol/μl of phosphothiorated 14-3-3 antisense oligomer; the amount of Lipofectin was proportionately reduced aswell to reduce toxicity.

5.3.2 Assessment of modulation of osteoblastic differentiation by antisense oligomer

The results of biochemical assessment of the effect of 14-3-3 antisense oligomer, after three days of transient transfection, on osteoblastic differentiation, using alkaline phosphatase expression as a marker of osteoblastic differentiation, are detailed in table 5.1. The mean value for the activity of alkaline phosphatase was significantly reduced in those cells exposed to Lf / Ol (mean absorbance 0.180) compared to those exposed to Lf (mean absorbance 0.555) or Ol (mean absorbance 1.189) alone, or to those receiving neither Lf nor Ol (mean absorbance 0.974). This result was significant at the level of $p<0.001$, indicating that 14-3-3 antisense oligomer significantly abrogated osteoblastic differentiation of human bone marrow stromal cells. The level in those receiving Lf alone was less than in those receiving neither Lf or Ol, reflecting a cytotoxic effect of Lf. Notwithstanding this the reduction observed with Lf/Ol remained significantly reduced at the level of $p<0.001$ when compared with that of the cells receiving Lf alone, confirming the effect of the antisense oligomer. Furthermore, exposure to Ol alone had no significant effect on the level of alkaline phosphatase expression.

5.4 Discussion

The results indicate that 14-3-3 gene expression is necessary for induction of osteoblastic differentiation by dexamethasone in human bone marrow stromal cells, confirming the

Figure 5.2a

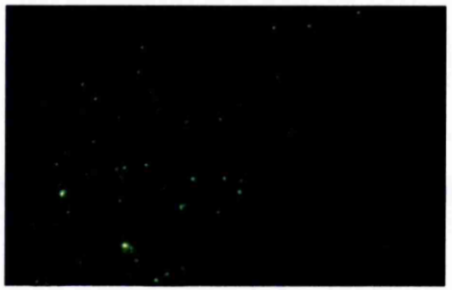


Figure 5.2b

Figure 5.2
Photomicrographs of adherent bone marrow stromal cells transiently transfected with FITC labelled 14-3-3 zeta antisense oligomer; (a) Nomarski phase microscopy demonstrating typical morphology and pattern of cells (x400, no counterstain), and (b) fluorescence microscopy demonstrating uptake of oligomer by the cells (x400, no counterstain).

Table 5.1 Alkaline phosphatase expression in bone marrow stromal cells, assessed biochemically, following transfection with 14-3-3 zeta antisense oligomer, with controls (Lipofectin, or oligomer alone, and neither Lipofectin or oligomer). Results given in arbitary absorbance units.

	Lipofectin only	Oligonucleotide only	Lipofectin with Oligonucleotide	Neither reagent (Control)
Absorbance	0.349	0.654	0.135	1.035
	0.164	1.074	0.143	1.229
	0.504	1.076	0.352	0.931
	0.846	1.082	0.196	1.220
	0.680	0.947	0.129	1.227
	0.344	0.968	0.154	1.199
	0.522	1.974	0.163	1.223
	0.679	1.277	0.148	1.168
	0.643	0.971	0.147	1.040
	0.266	1.040	0.140	1.296
	0.822	1.952	0.198	0.677
	0.910	1.075	0.191	0.628
	0.269	1.137	0.165	0.543
	0.293	1.130	0.151	0.830
	0.766	1.459	0.191	0.420
	0.824	1.212	0.281	0.919
Mean	0.555	1.189	0.180	0.974

involvement of the gene in the process of early osteogenesis implicated by the results detailed in chapter three. The capacity of the system used for osteoblastic differentiation was previously demonstrated in chapter three, and this process was significantly reduced by transfection with 14-3-3 zeta antisense oligomer. The optimisation studies carried out indicate that the level and extent of uptake of the oligomer by the cells was adequate, whilst the cytotoxic effect of Lipofectin was minimised, as evidenced by only a small reduction in the level of alkaline phosphatase activity in the presence of Lipofectin alone. Conversely, the reduction in activity observed in those cells exposed to both Lipofectin and oligomer remained significant when compared to that for those exposed to Lipofectin alone, indicating that the effect observed with both Lipofectin and oligomer was due to transfection rather than to cytotoxicity.

However, the results presented in this part of the study are only preliminary and further work is required to confirm their significance. In particular, the assessment of the effect of 14-3-3 zeta antisense oligomer needs to be repeated, and the results compared to those for the use of the corresponding random sequence oligomer, in order to eliminate the possibility that the effect is not merely a non-specific effect consequent upon the transfection of DNA *per se*. In addition, the effect of other antisense oligomers, to gene transcripts unrelated to either cell survival or osteoblastic differentiation, needs to be investigated to ensure that the effect is not due to a non-specific binding of the 14-3-3 antisense oligomer to other mRNAs. For the effect to be formally attributable to 14-3-3 a parallel reduction in 14-3-3 zeta protein expression should be demonstrated, in the absence of a similar effect for other proteins. Furthermore, the possibility that reduction in 14-3-3 zeta expression adversely effects cell survival, over and above its effects on osteoblastic differentiation, has not been ruled out. If the above critiques are all satisfactorily answered then the necessity of 14-3-3 zeta for osteoblastic differentiation will be confirmed, but sense transfections would then be needed to answer the parallel question as to whether or not it is sufficient for the process. The results presented above, in combination with the studies detailed in chapter three, indicate that such a series of experiments is justified.

Finally, the role of the mitogen activated protein kinase system in early osteogenesis requires further investigation, as indicated in the discussion at the end of chapter three. The identification of 14-3-3 zeta as a component of the process of early osteoblastic differentiation enables the direction of future investigation along this line, circumventing to a large degree the need for identification of associated factors, though, as indicated in chapter three, the contradictory results in the literature for the action of dexamethasone on

the mitogen activated kinase system, and its modulation during osteoblastic differentiation, still necessitates the use of transfection studies to investigate the effect of 14-3-3 zeta in the culture system used.

Chapter 6

General conclusions and future work

6.1 General conclusions

Osteoporosis, which is responsible for considerable morbidity and mortality, is characterised by a reduction in bone mass, due to a relative excess of bone resorption over formation. In the past attention has been focused on the increase in resorption, though in many cases there is also reduced formation. In order to investigate the nature of this reduction a histomorphometric analysis of osteoblastic activity, and of bone formation in the different bone compartments, in osteoporosis was carried out. In addition, the switch in differentiation of the cells of the CFU-F from the osteoblastic to the adipocytic lineage that has been hypothesised to occur in osteoporosis was examined by investigating the relationship between bone formation and the proportion of marrow adipose tissue in osteoporosis. The results of this initial analysis indicated that failure of osteoblast recruitment and maturation is present in nearly two thirds (62%) of cases of osteoporosis, and supported the hypothesis that there is a switch in the differentiation of the CFU-F from the osteoblastic to the adipocytic pathway. These results indicate that alteration of the normal pattern of osteoblast differentiation is an important factor in the pathogenesis of osteoporosis.

In order to investigate the mechanisms underlying these processes in osteoporosis it was first necessary to understand the normal process of osteogenesis. However, whilst the latter stages in the osteoblastic lineage are well characterised, the earlier stages in the lineage, which are the very stages involved in the processes noted above, remain an enigma, principally due to the relative lack of markers of cells early in the lineage. Previous attempts to identify such markers have used animal or immortalised culture systems, of uncertain relevance to normal human physiology, or heterogeneous cultures, the use of which precludes the assignment of changes identified to one population of cells or another.

In order to overcome these limitations, primary culture of clonal populations of STRO-1 (the only marker of cells early in the lineage at the time of the investigation) positive human bone marrow stromal cells was used to identify genes upregulated during the earliest stages of osteoblastic differentiation, in both adherent and non-adherent models of early osteogenesis. The methods used to produce clonal, and therefore non-heterogeneous, populations of primary human cells in both these models of osteogenesis resulted in very low cell numbers, the genotypic analysis of which necessitated the use of poly (A) reverse-transcription polymerase chain reaction. Indeed, the very low density at which the adherent

cells were grown in order to generate clonal populations has not previously been reported. Poly (A) RT-PCR based subtractive hybridisation isolated six genes, namely 14-3-3 zeta, alpha enolase, non-skeletal tropomyosin & KIAA0081 in the adherent model and aldolase A & CGI-120 protein in the non-adherent model.

The importance of these genes was investigated by hybridisation studies, both temporal to confirm upregulation and spatial to establish their expression *in vivo*, demonstrating upregulation of 14-3-3 zeta, and to lesser degree KIAA0081, non-skeletal tropomyosin and aldolase A in healing fracture and fetal bone, confirming their involvement in osteogenesis. In addition, each of these four genes showed increased expression in periosteal mesenchyme in fetal bone, and condensing mesenchyme in healing fracture callus, indicating activation at the early stages of osteoblastic differentiation, prior to differentiation of osteoblasts from both of these prior stromal condensations. As such, their expression, particularly that of aldolase A, in condensing stromal tissue, which has been linked to upregulation of adhesion molecules supports the hypothesis that acquisition of an adhesive phenotype is a first step in the process of osteoblastic differentiation, and that therefore the earliest cell in the lineage may be non-adherent.

The functional role of one of the genes, 14-3-3 zeta, was investigated by antisense transfection experiments which demonstrated abrogation of osteoblastic differentiation of primary human bone marrow stromal cells in the presence of antisense oligomer. This suggests that 14-3-3 zeta, which plays a pivotal role in stabilising Raf/Ras complexes at the start of the MAP kinase system, following activation of growth factor receptors by their ligands, is necessary for osteoblastic differentiation. Demonstration of its sufficiency for osteoblastic differentiation, however, requires the use of sense transfections, which were not carried out in this investigation. The role of the mitogen activated protein kinase system in early osteogenesis requires further investigation, particularly given the contradictory results in the literature for the action of dexamethasone on the mitogen activated kinase system, and its modulation during osteoblastic differentiation. Furthermore, since this pathway provides a direct link between binding of growth factors at the cell surface and subsequent events in the nucleus, its identification in the process of early osteogenesis is of particular relevance to the search for novel treatments of osteoporosis.

The initial histomorphometric analyses demonstrated reduction in osteoblast recruitment in osteoporosis, and suggested that there was a switch in differentiation of osteoblast

precursors from the osteoblastic to the adipocytic lineage. The results of the molecular and cellular biological investigations subsequently carried out to investigate the process of early osteoblastic differentiation, in order to establish a baseline for examination of its alteration in osteoporosis, identified a number of genes involved. So far however, the expression studies carried out, though demonstrating reduction in osteoporotic bone, have not explicitly indicated the nature of the defect in osteoblastic recruitment and differentiation present in osteoporosis. This is perhaps not surprising, since detection of upregulated expression, as demonstrated in the fetal bone and fracture callus, is more apparent than reduction. Notwithstanding this however, the identification of genes clearly involved, as discussed above, in early osteogenesis, provides candidates for more finely focused future investigation of the perturbation of early osteoblastic differentiation in osteoporosis. In particular, a wider range of expression studies will confirm the nature and extent of their reduction in the disease, whilst functional studies, such as have already proved promising for 14-3-3 zeta, will help elucidate the mechanisms involved. Furthermore, the role of the switch from the osteoblastic to the adipocytic lineage that is thought to occur in osteoporosis, could be usefully studied by investigating the expression of the genes identified during adipocytic differentiation. The process of adipocytic differentiation of bone marrow stromal cells fell outside the remit of the present investigation, but is clearly indicated in the future. Interestingly, Brown (1999) demonstrated equivalent expression during both osteoblastic and adipocytic differentiation of marrow stromal cells, for genes isolated in a model of osteoblastic differentiation. This finding calls into question the genotypic nature of the switch and underlines the importance of establishing the profile of the genes isolated in the two lineages.

Finally, the hypothesis that the earliest cell in the osteoblastic lineage is non-adherent *in vivo* was tested by investigation of the phenotypic, namely STRO-1 expression, and genotypic characteristics of non-adherent marrow stromal cells *in vitro*, and the alterations occurring upon adhesion of these cells. The capacity of the non-adherent cells to give rise to adherent cells of the osteoblastic lineage, and the acquistion of STRO-1 positivity consequent upon adhesion, was demonstrated. In order to establish genotypic markers for the identification of the non-adherent cells *in vivo* poly (A) based subtractive hybridisation was used to isolate genes upregulated during the adhesion of non-adherent cells. Two such genes were identified, namely aldolase A and CGI-120 protein. The former has been linked with differentiation in other systems, either via changes in the metabolism of cells during maturation, due to changes in oxygen tensions, or via DNA-binding, whilst the nature and role of CGI-120 protein is presently unknown. The spatial and temporal pattern of

expression of the two genes support the hypothesis that the non-adherent cells observed in vivo represent a distinct population of osteoprogenitors *in vivo*, and that, as suggested by Scutt & Bertram (1995) and Long *et al* (1991, 1995) the earliest cell in the osteoblast lineage is non-adherent *in vivo*.

Overall the investigations undertaken indicate that a reduction in osteoblast recruitment from the osteoblast precursor pool is present in many cases of osteoporosis. Genes upregulated during normal early osteoblastic differentiation, the involvement of which was confirmed by *in-situ* hybridisation studies, provide candidates for further study of the alteration of the process in osteoporosis. In particular, they provide markers for genotypic analysis of the hypothesised switch from osteoblastic to adipocytic differentiation in osteoporosis. Finally, the results of the non-adherent culture studies suggest that the earliest cell in the osteoblastic lineage, i.e. the osteoblast precursor pool, may be non-adherent *in vivo*, indicating that the process of early osteoblastic differentiation, both in normal and osteoporotic bone, may be different to that previously proposed.

6.2 Future work

The investigations undertaken indicate the following areas for future work:

1 For each of the two subtractions performed, i.e. between unstimulated / stimulated adherent bone marrow stromal cells and non-adherent and adherent bone marrow stromal cells, secondary screening and sequencing of the remainder of the clones isolated by primary screening in order to extend the range of genes involved in early osteogenesis. This will allow the identification of further candidate genes for investigation of the mechanisms underlying early osteogenesis, in both normal and osteoporotic bone, and would be expected to yield genes presently not known to be involved in the process, which have not been isolated to date.

2 Immunohistochemical determination of the expression pattern of each of the genes identified in normal and diseased adult and fetal bone, in order to establish whether or not the genes isolated are expressed at the protein, is a necessary step since alteration of function may be present at the post-translational stage. The possibility that the sequence identified as that of alpha enolase may represent either alpha enolase or c-myc binding protein particularly demonstrates the importance of this, and also raises the possible necessity of Northern hybridisation studies to establish transcript size, though such studies would require larger volume culture.

3 Confirmation of the necessity of 14-3-3 zeta expression for early osteoblastic differentiation, and assessment of its sufficiency, using sense transfections, is required. The reasons for this are detailed in the discussion in chapter 5 (see section 5.4) and are principally methodological. The investigations indicated include use of the random sequence oligomer, and of antisense oligomers to gene transcripts unrelated to either cell survival or osteoblastic differentiation, whilst for the effect to be formally attributable to 14-3-3 a parallel reduction in 14-3-3 zeta protein expression needs to be demonstrated, in the absence of a similar effect for other proteins. Furthermore, the possibility that reduction in 14-3-3 zeta expression adversely effects cell survival, over and above its effects on osteoblastic differentiation, needs to be excluded. Finally, sense transfections are needed to establish whether or not it is sufficient for the process.

4 Further investigation of the role of the mitogen activated protein kinase system in early osteogenesis, and subsequent identification of candidates for novel treatments of osteoporosis, is required, together with application of the same methods used to study the functional role of 14-3-3 zeta to the other genes isolated, in particular KIAA0081 and CGI-120 protein, neither of which have reported functions to date. Such studies will enable application of the results to investigation, and possibly treatment, of the alteration of early osteoblastic differentiation occurring in osteoporosis.

5 Investigation of the role of the postulated switch in differentiation of marrow stromal cells from the osteoblastic to adipocytic lineage in osteoporosis, using the genotypic markers isolated. This will establish the validity of the hypothesis, and indicate at which level it operates, together with the degree of plasticity present in the system. These studies will be important for understanding the nature of the mechanisms underlying reduced osteoblastic recruitment in osteoporosis, and need to be carried out in conjunction with a more extensive investigation of the expression of the genes isolated in cases of osteoporosis.

6 Finally, the nature of the precursor cell at the start of the osteoblastic lineage requires further investigation, and, in particular, the implication from chapter 4 that it is non-adherent *in vivo* needs to be tested. This will have widespread consequences for understanding of early osteogenesis, and its alteration in

osteoporosis, and if confirmed will explain the difficulty of identifying the osteoblastic precursor pool *in vivo* reported by others (Simmons, 1996).

The investigations listed above will support the conclusions derived above, and will extend the range of genes upregulated during early osteoblastic differentiation, thereby both allowing identification of early osteoprogenitor cells *in vivo*, and enabling further assessment of the role of non-adherent cells in the process. They will facilitate elucidation of the mechanisms controlling early osteoblastic differentiation, and their perturbation in disease, and may therefore lead to identification of novel treatments for osteoporosis.

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Appendix 1 Histomorphometric data

The histomorphometric data used in chapter 2 are detailed below. Specifically the data sets used for each component of the investigation are given:

Appendix 1a	Analysis of differential pattern of osteoblast dysfunction
Appendix 1b	Analysis of the differential pattern of altered bone formation in different bone compartments
Appendices 1c to 1i	Analysis of the alteration of the haematopoietic to adipocytic ratio

Appendix 1a Histomorphometric data - Parameters of osteoblastic function

		Osteoblastic number (ObS/BS %)				Osteoblastic activity (MS/BS[dLA+1/2sLS] %)				Osteoblastic efficiency (MS/OS[sLA+dLS] %)			
Case No	Biopsy No	Raw valu	Mean	2SD	Z score	Raw valu	Mean	2SD	Z score	Raw valu	Mean	2SD	Z score
1	94/1149	4.8		2	4.8	4.8	7.4	3.7	-1.405405405	66.1	79.9	14.5	-1.9034483
2	94/1412	0.3	4.6	1	-8.6	0	7.5	2.1	-7.142857143	0	79.4	11	-14.436364
3	94/2156	1	4.3	1.8	-3.66666667	2.8	7.2	4.7	-1.872340426	36.6	48.3	12.6	-1.8571429
4	94/2226	1.5	4.2	1	-5.4	1.2	6.8	4.1	-2.731707317	18.3	77.5	13.3	-8.9022556
5	94/2280	3.8	5.4	2	-1.6	5.1	7.4	3.7	-1.243243243	46.9	79.9	14.5	-4.5517241
6	94/1623	0	5.16	2.13	-4.84507042	0	7.43	3.75	-3.962666667	0	77.5	13.4	-11.567164
7	94/1620	1.2	6	11.1	-0.86486486	1.6	7.5	3.6	-3.277777778	63.2	78.4	12.9	-2.3565891
8	94/1516	1.1	5.16	2.13	-3.81220657	1.4	7.43	3.75	-3.216	51.8	77.5	13.4	-3.8358209
9	94/1655	4.58	6	1.8	-1.57777778	4.9	7.7	2.5	-2.24	70.8	80.1	15.1	-1.2317881
10	94/1687	5.8	4.3	1.8	1.66666667	0	7.2	4.7	-3.063829787	0	48.3	12.6	-7.6666667
11	94/1756	1.8	4.9	1.6	-3.875	3.7	7.4	2.1	-3.523809524	70.5	81	10.8	-1.9444444
12	94/1916	1.5	4.3	1.8	-3.11111111	0	7.2	4.7	-3.063829787	0	48.3	12.6	-7.6666667
13	94/1928	3.9	6	1.8	-2.33333333	4.1	7.7	2.5	-2.88	53.2	80.1	15.1	-3.5629139
14	94/1945	3.9	6	1.1	-3.81818182	4.9	7.5	3.6	-1.444444444	70.6	78.4	12.9	-1.2093023
15	94/2048	4.1	5.4	2	-1.3	4.4	7.4	3.7	-1.621621622	40.1	79.9	14.5	-5.4896552
16	94/2050	3.7	4.9	1.6	-1.5	7.6	7.4	2.1	0.19047619	83.2	81	10.8	0.40740741
17	94/2105	2.1	4.3	1.8	-2.44444444	6.8	7.2	4.7	-0.170212766	57.2	48.3	12.6	1.41269841
18	94/1255	2	5.16	2.13	-2.96713615	3.9	7.43	3.75	-1.882666667	45.7	77.5	13.4	-4.7462687
19	93/3112	0.2	4.6	1	-8.8	2.3	7.5	2.1	-4.952380952	68.1	79.4	11.1	-2.036036
20	94/0108	1.7	5.4	2	-3.7	0.9	7.4	3.7	-3.513513514	42.3	79.9	14.5	-5.1862069
21	95/1302	2.8	6	1.8	-3.55555556	0.9	7.7	2.5	-5.44	43.7	80.1	15.1	-4.8211921
22	94/0043	1.8	6	1.8	-4.66666667	4.3	7.7	2.5	-2.72	65.7	80.1	15.1	-1.9072848
23	94/0135	2.1	6	2.2	-3.54545455	2.8	7.4	2.2	-4.181818182	60.2	81	10.8	-3.8518519
24	94/0169	1	4.6	1	-7.2	2.7	7.5	2.1	-4.571428571	62	79.4	11.1	-3.1351351
25	94/0187	0	4.7	1.1	-8.54545455	0	7.6	3.7	-4.108108108	0	75.8	15.3	-9.9084967
26	94/0370	1.9	5.16	2.13	-3.06103286	3.3	7.43	3.75	-2.202666667	60.7	77.5	13.4	-2.5074627
27	94/0309	1.9	5.4	2	-3.5	6.8	7.4	3.7	-0.324324324	81.8	79.9	14.5	0.26206897
28	94/0441	0	4.3	1.8	-4.77777778	0	7.2	4.7	-3.063829787	0	48.3	12.6	-7.6666667
29	94/0461	0.4	4.6	1	-8.4	0	7.5	2.1	-7.142857143	0	79.4	11.1	-14.306306
30	94/0487	2.9	4.2	1	-2.6	3.8	6.8	4.1	-1.463414634	50.5	77.5	13.3	-4.0601504
31	94/0574	1	5.16	2.13	-3.90610329	0	7.43	3.75	-3.962666667	0	77.5	13.4	-11.567164

		Osteoblastic number (ObS/BS %)				Osteoblastic activity (MS/BS[dLA+1/2sLS] %)				Osteoblastic efficiency (MS/OS[sLA+dLS] %)			
Case No	Biopsy No	Raw valu	Mean	2SD	Z score	Raw valu	Mean	2SD	Z score	Raw valu	Mean	2SD	Z score
33	94/0591	2.1	5.4	2	-3.3	4.2	7.4	3.7	-1.72972973	46.2	79.9	14.5	-4.6482759
34	94/0602	3.7	6	1.8	-2.55555556	6	7.7	2.5	-1.36	69.3	80.1	15.1	-1.4304636
35	94/0664	0.7	4.9	1.6	-5.25	2.4	7.4	2.1	-4.761904762	2.2	81	10.8	-14.592593
36	94/0783	2.1	5.4	2	-3.3	3	7.4	3.7	-2.378378378	80.2	79.9	14.5	0.04137931
37	94/0840	0	4.9	1.6	-6.125	0	7.4	2.1	-7.047619048	0	81	10.8	-15
38	94/1044	14.9	4.6	1	20.6	10.2	7.5	2.1	2.571428571	64	79.4	11.1	-2.7747748
39	94/1063	5.8	56	1.8		4.5	7.7	2.5		51	80.1	15.1	
40	94/1096	2.9	4.6	1	-3.4	2.5	7.5	2.1	-4.761904762	46.1	79.4	11.1	-6
41	94/1342	3.4	5.4	2	-2	5.6	7.4	3.7	-0.972972973	51.8	79.9	14.5	-3.8758621
42	95/0017	2.1	5.16	2.13	-2.87323944	2	7.43	3.75	-2.896	41	77.5	13.4	-5.4477612
43	93/2999	2.7	5.16	2.13	-2.30985915	2.9	7.43	3.75	-2.416	12.6	77.5	13.4	-9.6865672
44	93/1431	3	4.3	1.8	-1.44444444	5.2	7.2	4.7	-0.85106383	61	48.3	12.6	2.01587302
45	93/1524	2.6	4.2	1	-3.2	4	6.8	4.1	-1.365853659	56.2	77.5	13.3	-3.2030075
46	93/1845	3	4.6	1	-3.2	6	7.5	2.1	-1.428571429	69.9	79.4	11.1	-1.7117117
47	93/1662	3.5	4.7	1.1	-2.18181818	19.5	7.6	3.7		96	75.8	15.3	
48	94/1067	2	4.7	1.1	-4.90909091	4.1	7.6	3.7	-1.891891892	47.1	75.8	15.3	-3.751634
49	93/1670	3.8	4.2	1	-0.8	5.3	6.8	4.1	-0.731707317	69.8	77.5	13.3	-1.1578947
50	93/1841	2	4.7	1.1	-4.90909091	4.7	7.6	3.7	-1.567567568	70	75.8	15.3	-0.7581699
51	93/1935	4.8	4.6	1	0.4	6	7.5	2.1	-1.428571429	67.7	79.4	11.1	-2.1081081
52	94/1983	6.1	5.4	2	0.7	5	7.4	3.7	-1.297297297	73.1	79.9	14.5	-0.937931
53	93/2193	2	5.4	2	-3.4	4.5	7.4	3.7	-1.567567568	71.5	79.9	14.5	-1.1586207
54	93/2204	3.1	6	1.8	-3.22222222	3.8	7.7	2.5	-3.12	67.1	80.1	15.1	-1.7218543
55	93/2217	7.1	6	1.8	1.22222222	6.8	7.7	2.5	-0.72	76.4	80.1	15.1	-0.4900662
56	95/0771	2.5	5.4	2	-2.9	0	7.4	3.7	-4	0	79.9	14.5	-11.02069
57	93/2436	15.8	6	1.1	17.8181818	11.3	7.5	3.6	2.111111111	79.4	78.4	12.9	0.15503876
58	93/2472	2.9	4.3	1.8	-1.55555556	2.6	7.2	4.7	-1.957446809	62.4	48.3	12.6	2.23809524
59	93/2483	0.9	6	1.1	-9.27272727	0	7.5	3.6	-4.166666667	0	78.4	12.9	-12.155039
60	93/2866	0	4.3	1.8	-4.77777778	0	7.2	4.7	-3.063829787	0	48.3	12.6	-7.6666667
61	93/2962	4.9	4.7	1.1	0.36363636	8.9	7.6	3.7	0.702702703	79.1	75.8	15.3	0.43137255
62	93/3032	7.9	4.6	1	6.6	8.2	7.5	2.1	0.666666667	86.1	79.4	11.1	1.20720721
63	93/2946	3.6	5.4	2	-1.8	4.6	7.4	3.7	-1.513513514	66.3	79.9	14.5	-1.8758621
64	95/0039	1.6	5.16	2.13	-3.342723	9.2	7.43	3.75	0.944	47	77.5	13.4	-4.5522388
65	95/0042	0	5.16	2.13	-4.84507042	0	7.43	3.75	-3.962666667	0	77.5	13.4	-11.567164
66	95/0261	0.1	4.2	1	-8.2	0	6.8	4.1	-3.317073171	0	77.5	13.3	-11.654135

		Osteoblastic number (ObS/BS %)				Osteoblastic activity (MS/BS[dLA+1/2sLS] %)				Osteoblastic efficiency (MS/OS[sLA+dLS] %)			
Case No	Biopsy No	Raw valu	Mean	2SD	Z score	Raw valu	Mean	2SD	Z score	Raw valu	Mean	2SD	Z score
68	95/0317	1.9	6	1.8	-4.55555556	1	7.7	2.5	-5.36	24.8	80.1	15.1	-7.3245033
69	95/0460	3.1	6	1.1	-5.27272727	4.2	7.5	3.6	-1.833333333	56.2	78.4	12.9	-3.4418605
70	95/0381	0	4.3	1.8	-4.77777778	0	7.2	4.7	-3.063829787	0	48.3	12.6	-7.6666667
71	95/0418	3.5	4.6	1	-2.2	4.1	7.5	2.1	-3.238095238	60.1	79.4	11.1	-3.4774775
72	95/0490	5.6	6	1.8	-0.44444444	8.2	7.7	2.5	0.4	93	80.1	15.1	1.70860927
73	95/0597	2	5.4	2	-3.4	3.1	7.4	3.7	-2.324324324	32.8	79.9	14.5	-6.4965517
74	95/0734	2.9	4.3	1.8	-1.55555556	4.6	7.2	4.7	-1.106382979	51.2	48.3	12.6	0.46031746
75	95/0784	3	5.4	2	-2.4	5.1	7.4	3.7	-1.243243243	66.1	79.9	14.5	-1.9034483
76	95/0856	2	4.7	1.1	-4.90909091	3.5	7.6	3.7	-2.216216216	50.2	75.8	15.3	-3.3464052
77	95/0825	2.6	4.6	1	-4	2.5	7.5	2.1	-4.761904762	89.9	79.4	11.1	1.89189189
78	95/1108	0.8	4.6	1	-7.6	1	7.5	2.1	-6.19047619	23.3	79.4	11.1	-10.108108
79	95/1175	0	4.3	1.8	-4.77777778	0	7.2	4.7	-3.063829787	0	48.3	12.6	-7.6666667
80	95/1221	0.9	6	1.8	-5.66666667	0.1	7.7	2.5	-6.08	20.5	80.1	15.1	-7.8940397
81	95/1409	0	4.3	1.8	-4.77777778	0	7.2	4.7	-3.063829787	0	48.3	12.6	-7.6666667
82	95/1539	3.8	6	1.8	-2.44444444	3.6	7.7	2.5	-3.28	66.6	80.1	15.1	-1.7880795
83	95/0017	2.1	5.16	2.13	-2.87323944	2	7.43	3.75	-2.896	41	77.5	13.4	-5.4477612
84	94/0308	3.1	5.16	2.13	-1.9342723	5.7	7.43	3.75	-0.922666667	70.3	77.5	13.4	-1.0746269
85	91/2179	2.43	4.98	1.56	-3.26923077	4.3	7.43	2.1	-2.980952381	63.8	81	10.8	-3.1851852
86	92/0028	4.7	4.3	1.8	0.44444444	5.2	7.2	4.7	-0.85106383	62.3	48.3	12.6	2.22222222
87	91/2267	1.7	4.3	1.8	-2.88888889	3.1	7.2	4.7	-1.744680851	61.1	48.3	12.6	2.03174603
88	94/0051	0.1	4.3	1.8	-4.66666667	0.5	7.2	4.7	-2.85106383	50	48.3	12.6	0.26984127
89	92/0125	0.4	4.3	1.8	-4.33333333	1.2	7.2	4.7	-2.553191489	38.1	48.3	12.6	-1.6190476
90	92/0403	4.1	6	1.8	-2.11111111	5.7	7.7	2.5	-1.6	68.1	80.1	15.1	-1.589404
91	92/0588	2.1	4.6	1	-5	3.2	7.5	2.1	-4.095238095	41.5	79.4	11.1	-6.8288288
92	92/0550	63.5	5.16	2.13	54.7793427	0	7.43	3.75		0	77.5	13.4	
93	92/0549	1.2	4.6	1	-6.8	4.9	7.5	2.1	-2.476190476	76.1	79.4	11.1	-0.5945946
94	92/0685	3.1	4.98	1.56	-2.41025641	6	7.43	2.1	-1.361904762	69.1	81	10.8	-2.2037037
95	92/0690	37.3	4.2	1	66.2	51.2	6.8	4.1		97.2	77.5	13.3	
96	92/0848	7.1	4.3	1.8	3.11111111	9.4	7.2	4.7	0.936170213	89.2	48.3	12.6	
97	95/0540	2.1	4.3	1.8	-2.44444444	3.2	7.2	4.7	-1.70212766	20.5	48.3	12.6	-4.4126984
98	92/1037	16.5	6	1.8	11.6666667	11.4	7.7	2.5	2.96	67.8	80.1	15.1	-1.6291391
99	92/1882	3.14	4.98	1.56	-2.35897436	5.78	7.43	2.1	-1.571428571	69.9	81	10.8	-2.0555556
100	92/1891	4.1	6	1.8	-2.11111111	6.1	7.7	2.5	-1.28	76.1	80.1	15.1	-0.5298013
101	92/1903	7.3	4.2	1	6.2	7.1	6.8	4.1	0.146341463	85.1	77.5	13.3	1.14285714

		Osteoblastic number (ObS/BS %)				Osteoblastic activity (MS/BS[dLA+1/2sLS] %)				Osteoblastic efficiency (MS/OS sLA+dLS] %)			
Case No	Biopsy No	Raw valu	Mean	2SD	Z score	Raw valu	Mean	2SD	Z score	Raw valu	Mean	2SD	Z score
103	92/2087	5.3	5.4	2	-0.1	7.1	7.4	3.7	-0.162162162	68.7	79.9	14.5	-1.5448276
104	92/2120	4.7	6	1.8	-1.44444444	5.1	7.7	2.5	-2.08	69	80.1	15.1	-1.4701987
105	92/2178	2.9	4.3	1.8	-1.55555556	2.9	7.2	4.7	-1.829787234	46.9	48.3	12.6	-0.2222222
106	92/2266	4.4	6	1.8	-1.77777778	4.1	7.7	2.5	-2.88	58.2	80.1	15.1	-2.9006623
107	92/2289	3	5.4	2	-2.4	5.1	7.4	3.7	-1.243243243	59.9	79.9	14.5	-2.7586207
108	93/0199	0.5	4.3	1.8	-4.22222222	0	7.2	4.7	-3.063829787	0	48.3	12.6	-7.6666667
109	93/0209	0.9	6	1.1	-9.27272727	4.9	7.5	3.6	-1.444444444	70.6	78.4	12.9	-1.2093023
110	93/0389	7.5	4.98	1.56	3.23076923	7.4	7.43	2.1	-0.028571429	70.8	81	10.8	-1.8888889
111	93/0528	1.1	4.6	1	-7	3.1	7.5	2.1	-4.19047619	71.2	79.4	11.1	-1.4774775
112	93/0602	2.3	6	1.8	-4.11111111	1.7	7.7	2.5	-4.8	73.1	80.1	15.1	-0.9271523
113	93/0686	1.83	5.16	2.13	-3.12676056	5	7.43	3.75	-1.296	63.8	77.5	13.4	-2.0447761
114	93/0848	2.83	5.16	2.13	-2.18779343	2.18	7.43	3.75	-2.8	81.9	77.5	13.4	0.65671642
115	93/0838	4.8	4.6	1	0.4	5.1	7.5	2.1	-2.285714286	64	79.4	11.1	-2.7747748
116	93/1136	3.7	6	1.1	-4.18181818	3.9	7.5	3.6	-2	65	78.4	12.9	-2.0775194
117	93/1150	3.3	5.4	2	-2.1	7.6	7.4	3.7	0.108108108	75.8	79.9	14.5	-0.5655172
118	93/1411	2.1	5.4	2	-3.3	1.8	7.4	3.7	-3.027027027	81.2	79.9	14.5	0.17931034
119	93/1788	4.1	4.3	1.8	-0.22222222	6.5	7.2	4.7	-0.29787234	41.4	48.3	12.6	-1.0952381
120	93/1568	2.3	4.3	1.8	-2.22222222	3.8	7.2	4.7	-1.446808511	42.6	48.3	12.6	-0.9047619
121	93/1447	1.2	4.3	1.8	-3.44444444	3.1	7.2	4.7	-1.744680851	39.5	48.3	12.6	-1.3968254
122	93/1279	2.4	6	1.8	-4	6.4	7.7	2.5	-1.04	46.7	80.1	15.1	-4.4238411
123	93/1232	0.3	4.3	1.8	-4.44444444	0.7	7.2	4.7	-2.765957447	40.2	48.3	12.6	-1.2857143
124	93/0659	5.2	6	1.8	-0.88888889	4.9	7.7	2.5	-2.24	60.1	80.1	15.1	-2.6490066
125	93/0450	2.7	5.4	2	-2.7	3.7	7.4	3.7	-2	60.2	79.9	14.5	-2.7172414
126	93/0183	3.8	4.2	1.2	-0.66666667	5.4	6.8	4.1	-0.682926829	65.8	77.5	13.3	-1.7593985
127	93/0049	2	4.3	1.8	-2.55555556	3.1	7.2	4.7	-1.744680851	36.5	48.3	12.6	-1.8730159
128	92/1170	3.9	6	1.8	-2.33333333	6.1	7.7	2.5	-1.28	73.5	80.1	15.1	-0.8741722
129	92/1169	2.6	4.3	1.8	-1.88888889	4	7.2	4.7	-1.361702128	33.3	48.3	12.6	-2.3809524
130	92/1101	2.1	4.3	1.8	-2.44444444	4.9	7.2	4.7	-0.978723404	39.9	48.3	12.6	-1.3333333
131	92/1039	1.2	6	1.8	-5.33333333	5.1	7.7	2.5	-2.08	63.2	80.1	15.1	-2.2384106
132	92/0982	4.3	6	1.8	-1.88888889	3.8	7.7	2.5	-3.12	47.9	80.1	15.1	-4.2649007
133	92/0905	4.7	6	1.8	-1.44444444	6	7.7	2.5	-1.36	73.2	80.1	15.1	-0.9139073
134	92/0903	4.7	6	1.8	-1.44444444	5.9	7.7	2.5	-1.44	64.3	80.1	15.1	-2.0927152
135	92/0791	4	4.2	1	-0.4	7.6	6.8	4.1	0.390243902	51.1	77.5	13.3	-3.9699248
136	92/0771	4.8	4.3	1.8	0.55555556	10.4	7.2	4.7	1.361702128	50.1	48.3	12.6	0.28571429

		Osteoblastic number (ObS/BS %)				Osteoblastic activity (MS/BS[dLA+1/2sLS] %)				Osteoblastic efficiency (MS/OS[sLA+dLS] %)			
Case No	Biopsy No	Raw valu	Mean	2SD	Z score	Raw valu	Mean	2SD	Z score	Raw valu	Mean	2SD	Z score
138	92/0715	1	4.3	1.8	-3.66666667	1.9	7.2	4.7	-2.255319149	17.2	48.3	12.6	-4.9365079
139	92/0504	2.5	4.3	1.8	-2	6.9	7.2	4.7	-0.127659574	54.7	48.3	12.6	1.01587302
140	92/0461	5.2	4.3	1.8	1	6.8	7.2	4.7	-0.170212766	52.7	48.3	12.6	0.6984127
141	92/0347	0.1	4.2	1	-8.2	0.1	6.8	4.1	-3.268292683	11.7	77.5	13.3	-9.8947368
142	92/0149	26.4	5.4	2		28.5	7.4	3.7		84.7	79.9	14.5	
143	92/0127	7	6	1.8	1.11111111	6.3	7.7	2.5	-1.12	61.1	80.1	15.1	-2.5165563
144	91/2227	3	6	1.8	-3.33333333	3.3	7.7	2.5	-3.52	64.7	80.1	15.1	-2.0397351
145	91/2206	2.5	4.3	1.8	-2	4.8	7.2	4.7	-1.021276596	48.9	48.3	12.6	0.0952381
146	91/2184	4.8	6	1.8	-1.33333333	4.9	7.7	2.5	-2.24	69.8	80.1	15.1	-1.3642384
147	91/2183	2.7	4.3	1.8	-1.77777778	4.8	7.2	4.7	-1.021276596	37.8	48.3	12.6	-1.6666667
148	92/1304	0.4	4.3	1.8	-4.33333333	0	7.2	4.7	-3.063829787	0	48.3	12.6	-7.6666667
149	92/2564	1.1	6	1.8	-5.44444444	1.8	7.7	2.5	-4.72	70.3	80.1	15.1	-1.2980132
150	92/2444	5.3	4.2	1	2.2	3.9	6.8	4.1	-1.414634146	29.9	77.5	13.3	-7.1578947
151	92/2408	2.4	4.3	1.8	-2.11111111	3.5	7.2	4.7	-1.574468085	29.4	48.3	12.6	-3
152	92/2404	1.6	4.3	1.8	-3	5	7.2	4.7	-0.936170213	51.2	48.3	12.6	0.46031746
153	92/2243	4.9	4.2	1	1.4	6.2	6.8	4.1	-0.292682927	49.6	77.5	13.3	-4.1954887
154	92/2219	0.2	4.3	1.8	-4.55555556	0.6	7.2	4.7	-2.808510638	0.5	48.3	12.6	-7.5873016
155	92/2146	2.9	4.3	1.8	-1.55555556	3.5	7.2	4.7	-1.574468085	45.7	48.3	12.6	-0.4126984
156	92/2030	7	6	1.8	1.11111111	4.9	7.7	2.5	-2.24	58.3	80.1	15.1	-2.8874172
157	92/2014	6.4	6	1.8	0.44444444	7	7.7	2.5	-0.56	60.1	80.1	15.1	-2.6490066
158	92/1958	1.5	4.3	1.8	-3.11111111	1.2	7.2	4.7	-2.553191489	36.5	48.3	12.6	-1.8730159
159	92/1554	3.9	4.3	1.8	-0.44444444	3.3	7.2	4.7	-1.659574468	27.6	48.3	12.6	-3.2857143

Appendix 1b

Histomorphometric data - Bone Appositional Rates in Trabecular, Cortical, Sub-Cortical & Periosteal Compartments (um/day)

	Trabecular compartment				Cortical compartment				Periosteal compartment			Sub-cortical compartment		
Biopsy	Raw valu	Mean	2SD	Z score	Raw value	Mean	2SD	Z score	Raw value	Mean	Z score	Raw val	Mean	Z score
94/1149	0.47	0.61	0.11	-2.54	0.51	0.75	0.28	-1.72	0.01	1.9	-2.52	2.63	3.7	-2.3778
94/1412	0	0.58	0.19	-6.1	0	0.73	0.21	-6.96	0	1.9	-2.5333	0	3.7	-8.2222
94/2156	0.38	0.6	0.2	-2.2	0.44	0.7	0.1	-5.2	1.85	1.9	-0.0667	2.75	3.7	-2.1111
94/2226	0	0.4	0.1	-8	0	0.6	0.2	-6	1.9	1.9	0	3.7	3.7	0
94/2280	0	0.61	0.11	-11.1	0	0.75	0.28	-5.36	0	1.9	-2.5333	4.7	3.7	2.22222
94/1623	0	0.66	0.14	-9.42	0	0.73	0.43	-3.4	0.31	1.9	-2.12	0.58	3.7	-6.9333
94/1620	0.58	0.64	0.34	-0.36	0.62	0.76	0.21	-1.34	4.8	1.9	3.86667	1.5	3.7	-4.8889
94/1516	0	0.66	0.14	-9.42	0	0.73	0.43	-3.7	0	1.9	-2.5333	0.67	3.7	-6.7333
94/1655	0.27	0.57	0.23	-2.6	0.19	0.71	0.23	-4.52	0.01	1.9	-2.52	2.95	3.7	-1.6667
94/1687										1.9		3.2	3.7	
94/1756	0.54	0.65	0.15	-1.46	0.68	0.78	0.23	-0.86	0.01	1.9	-2.52	0.75	3.7	-6.5556
94/1916	0	0.6	0.2	-6	0	0.7	0.1	-14	0	1.9	-2.5333	0	3.7	-8.2222
94/1928	0	0.57	0.23	-4.96	0	0.71	0.23	-6.2	2.35	1.9	0.6	3.85	3.7	0.33333
94/1945	0.66	0.64	0.34	0.12	0.79	0.76	0.21	-0.28	1.55	1.9	-0.4667	1.2	3.7	-5.5556
94/2048	0	0.61	0.11	-11.08	0	0.75	0.28	-5.36	2.1	1.9	0.26667	3.9	3.7	0.44444
94/2050	0.72	0.65	0.15	0.94	0.81	0.78	0.23	0.26	5.6	1.9	4.93333	8.2	3.7	10
94/2105	0	0.6	0.2	-6	0	0.7	0.1	-14	4.6	1.9	3.6	4.3	3.7	1.33333
94/1255	0	0.66	0.14	-9.42	0	0.73	0.43	-3.4	0.01	1.9	-2.52	2.99	3.7	-1.5778
93/3112	0.03	0.58	0.19	-5.8	0.03	0.73	0.21	-6.66	0.66	1.9	-1.6533	1.15	3.7	-5.6667
94/0108	0.11	0.61	0.11	-9.1	0.2	0.75	0.28	-3.92	0.53	1.9	-1.8267	0.6	3.7	-6.8889
95/1302	0	0.57	0.23	-4.96	1.94	0.71	0.23	10.7	5.4	1.9	4.66667	7.3	3.7	8
94/0043	0.33	0.57	0.23	-2.08	0.33	0.71	0.23	-3.3	1.9	1.9	0	3.8	3.7	0.22222
94/0135	0	0.69	0.22	-6.28	0	0.79	0.21	-7.52	0	1.9	-2.5333	0	3.7	-8.2222
94/0169	0.3	0.58	0.19	-2.94	0.31	0.73	0.21	-4	0.01	1.9	-2.52	1.3	3.7	-5.3333
94/0187	0	0.6	0.1	-12	0	0.7	0.3	-4.6	0	1.9	-2.5333	0.05	3.7	-8.1111
94/0370				-4.86				-1.3	0.01	1.9	-2.52	2.8	3.7	-2
94/0309				-3.46				-1.42	1.85	1.9	-0.0667	1.4	3.7	-5.1111
94/0441	0	0.6	0.2	-6	0.85	0.7	0.2	3	1.79	1.9	-0.1467	3.62	3.7	-0.1778

	Trabecular compartment				Cortical compartment				Periosteal compartment			Sub-cortical compartment		
Biopsy	Raw valu	Mean	2SD	Z score	Raw value	Mean	2SD	Z score	Raw value	Mean	Z score	Raw val	Mean	Z score
94/0487	0.23	0.4	0.1	-1.4	0.49	0.6	0.2	-1.1	0.01	1.9	-2.52	2.5	3.7	-2.6667
94/0574	0	0.66	0.14	-9.42	0.13	0.73	0.43	-2.78	0.01	1.9	-2.52	0.92	3.7	-6.1778
94/0575	0.34	0.65	0.15	-4.14	0.35	0.78	0.23	-3.74	0.01	1.9	-2.52	0.08	3.7	-8.0444
94/0591	0.41	0.61	0.11	-3.64	0.48	0.75	0.28	-1.92	1.93	1.9	0.04	3.57	3.7	-0.2889
94/0602	0.43	0.57	0.23	-1.22	0.54	0.71	0.23	-1.48	2.11	1.9	0.28	3.6	3.7	-0.2222
94/0664	0	0.65	0.15	-8.66	0	0.78	0.23	-3.78	0	1.9	-2.5333	0.5	3.7	-7.1111
94/0783	0.65	0.61	0.11	0.72	0.68	0.75	0.28	-0.5	0.24	1.9	-2.2133	1	3.7	-6
94/0840	0	0.65	0.15	-8.66	0	0.78	0.23	-3.78	1.92	1.9	0.02667	0.03	3.7	-8.1556
94/1044	0.3	0.58	0.19	-2.94	0.31	0.73	0.21	-4	0.62	1.9	-1.7067	4.54	3.7	1.86667
94/1063	0.28	0.57	0.23	-2.52	0.47	0.71	0.23	-2.08	2	1.9	0.13333	3.8	3.7	0.22222
94/1096	0.35	0.58	0.19	-2.42	0.38	0.73	0.21	-3.34	0.01	1.9	-2.52	2.85	3.7	-1.8889
94/1342	0.44	0.61	0.11	-3.1	0.51	0.75	0.28	-1.72	0.5	1.9	-1.8667	3.1	3.7	-1.3333
95/0017	0	0.66	0.14	-9.42	0	0.73	0.43	-3.4	0.03	1.9	-2.4933	2.7	3.7	-2.2222
93/2999	0	0.66	0.14	-9.42	0	0.73	0.43	-3.4	0.28	1.9	-2.16	2.9	3.7	-1.7778
93/1431	0.24	0.6	0.2	-3.6	0.25	0.7	0.1	-9	2	1.9	0.13333	3.6	3.7	-0.2222
93/1524	0.11	0.4	0.1	-5.8	0.16	0.6	0.2	-0.4	0.01	1.9	-2.52	2.95	3.7	-1.6667
93/1845	0.23	0.58	0.19	-3.68	0.3	0.73	0.21	-4.1	1.8	1.9	-0.1333	3.2	3.7	-1.1111
93/1662	0.97	0.6	0.1	7.4	1.02	0.7	0.3	2.14	0.01	1.9	-2.52	5.2	3.7	3.33333
94/1067	0.4	0.6	0.1	-4	0.38	0.7	0.3	-2.14	0.01	1.9	-2.52	0.92	3.7	-6.1778
93/1670	0.22	0.4	0.1	-3.6	0.31	0.6	0.2	-2.9	0.01	1.9	-2.52	3.6	3.7	-0.2222
93/1841	0.4	0.6	0.1	-4	0.43	0.7	0.3	-1.8	0.83	1.9	-1.4267	2.1	3.7	-3.5556
93/1935	0.21	0.58	0.19	-3.9	0.19	0.73	0.21	-5.14	3.6	1.9	2.26667	0	3.7	-8.2222
94/1983	0.57	0.61	0.11	-0.72	0.49	0.75	0.28	-1.86	1.95	1.9	0.06667	3.95	3.7	0.55556
93/2193	0.32	0.61	0.11	-5.28	0.41	0.75	0.28	-2.42	0.36	1.9	-2.0533	3.1	3.7	-1.3333
93/2204	0.31	0.57	0.23	-2.26	0.38	0.71	0.23	-2.86	1.9	1.9	0	3.7	3.7	0
93/2217	0.4	0.57	0.23	-1.4	0.57	0.71	0.23	-1.22	2.2	1.9	0.4	4.2	3.7	1.11111
95/0771	0	0.61	0.11	-11.1	0.3	0.75	0.28	-5.14	0.36	1.9	-2.0533	1.43	3.7	-5.0444
93/2436	0.34	0.64	0.34	-1.76	0.45	0.76	0.21	-2.96	2.8	1.9	1.2	6.5	3.7	6.22222
93/2472	0.57	0.6	0.2	-0.3	0.56	0.7	0.1	-2.8	0.01	1.9	-2.52	0.01	3.7	-8.2
93/2483	0	0.63	0.34	-3.7	0	0.76	0.21	-7.24	3.8	1.9	2.53333	2.4	3.7	-2.8889
93/2866	0	0.6	0.2	-6	0	0.7	0.1	-14	0.01	1.9	-2.52	0.26	3.7	-7.6444
93/2962	0.32	0.6	0.1	-5.6	0.33	0.7	0.3	-2.46		1.9			3.7	
93/3032	0.54	0.58	0.19	-0.42	0.69	0.73	0.21	-0.38	2	1.9	0.13333	3.2	3.7	-1.1111
93/2946	0.23	0.61	0.11	-6.9	0.17	0.75	0.28	-4.14	0.34	1.9	-2.08	3.15	3.7	-1.2222

	Trabecular compartment				Cortical compartment				Periosteal compartment			Sub-cortical compartment		
Biopsy	Raw valu	Mean	2SD	Z score	Raw value	Mean	2SD	Z score	Raw value	Mean	Z score	Raw val	Mean	Z score
95/0042	0	0.66	0.14	-9.42	0	0.73	0.43	-3.38	0	1.9	-2.5333	0.05	3.7	-8.1111
95/0261	0	0.4	0.1	-8	0	0.6	0.2	-6	0.29	1.9	-2.1467	0.58	3.7	-6.9333
95/0257	0.12	0.61	0.11	-8.9	0.13	0.75	0.28	-4.42	0.31	1.9	-2.12	0.32	3.7	-7.5111
95/0317	0.29	0.57	0.23	-2.44	0.26	0.71	0.23	-4.92	0.42	1.9	-1.9733	1.36	3.7	-5.2
95/0460	0.41	0.64	0.34	-1.36	0.39	0.76	0.21	-3.52	2.15	1.9	0.33333	3.83	3.7	0.28889
95/0381	0	0.6	0.2	-6	0	0.7	0.1	-14	1.82	1.9	-0.1067	3.55	3.7	-0.3333
95/0418	0.22	0.58	0.19	-3.78	0.23	0.73	0.21	-4.76	0.53	1.9	-1.8267	5.16	3.7	3.24444
95/0490	0.63	0.57	0.23	0.52	0.88	0.71	0.23	1.48	2.02	1.9	0.16	1.85	3.7	-4.1111
95/0597	0	0.61	0.11	-11.1	0	0.75	0.28	-5.36	1.06	1.9	-1.12	1.83	3.7	-4.1556
95/0734	0.25	0.6	0.2	-3.5	0.3	0.7	0.1	-8	1.75	1.9	-0.2	2.9	3.7	-1.7778
95/0784	0.48	0.61	0.11	-2.36	0.44	0.75	0.28	-2.22	0.89	1.9	-1.3467	2.95	3.7	-1.6667
95/0856	0	0.6	0.2	-12	0	0.7	0.1	-4.66	0.93	1.9	-1.2933	1.45	3.7	-5
95/0825	0.61	0.58	0.19	0.32	0.66	0.73	0.21	-0.66	0.62	1.9	-1.7067	0.93	3.7	-6.1556
95/1108	0.11	0.58	0.19	-4.94	0.21	0.73	0.21	-4.96	0.01	1.9	-2.52	0.52	3.7	-7.0667
95/1175	0	0.6	0.2	-6	0	0.7	0.1	-7	0.03	1.9	-2.4933	0	3.7	-8.2222
95/1221	0	0.57	0.23	-4.96	0	0.71	0.23	-6.18	1.11	1.9	-1.0533	1.52	3.7	-4.8444
95/1409	0	0.6	0.2	-6	0.6	0.7	0.1	-2	0.01	1.9	-2.52	0.76	3.7	-6.5333
95/1539	0.34	0.57	0.23	-2	0.38	0.71	0.23	-2.86	2.12	1.9	0.29333	4.1	3.7	0.88889
95/0017	0	0.66	0.14	-9.42	0	0.73	0.43	-3.38	0.03	1.9	-2.4933	2.7	3.7	-2.2222
94/0308	0.61	0.66	0.14	-0.72	0.62	0.73	0.43	-0.52	1.85	1.9	-0.0667	1.4	3.7	-5.1111
91/2179	0.35	0.65	0.15	-4	0.39	0.78	0.23	-3.3913	2.1	1.9	0.26667	3.4	3.7	-0.6667
92/0028	0.25	0.6	0.2	-3.5	0.19	0.7	0.1	-10.2	1.9	1.9	0	3.6	3.7	-0.2222
91/2267	0	0.6	0.2	-6	0	0.7	0.1	-14	0	1.9	-2.5333	0	3.7	-8.2222
94/0051	0.1	0.6	0.2	-5	0.1	0.7	0.1	-12	0.01	1.9	-2.52	0.02	3.7	-8.1778
92/0125	0.3	0.6	0.2	-3	0.3	0.7	0.1	-8	0.01	1.9	-2.52	0	3.7	-8.2222
92/0403	0.38	0.57	0.23	-1.6522	0.38	0.71	0.23	-2.8696	0	1.9	-2.5333	2.95	3.7	-1.6667
92/0588	0.01	0.58	0.19	-6	0.01	0.73	0.21	-6.8571	0.01	1.9	-2.52	2.85	3.7	-1.8889
92/0550	0	0.66	0.14	-9.4286	0	0.73	0.43	-3.3953	0	1.9	-2.5333	0	3.7	-8.2222
92/0549	0.1	0.58	0.19	-5.0526	0.1	0.73	0.21	-6	0.01	1.9	-2.52	0	3.7	-8.2222
92/0685	0.43	0.65	0.15	-2.9333	0.49	0.78	0.23	-2.5217	1.95	1.9	0.06667	3.54	3.7	-0.3556
92/0690	0.8	0.4	0.1	8	0.9	0.6	0.2	3	3.8	1.9	2.53333	7.2	3.7	7.77778
92/0848	0.42	0.6	0.2	-1.8	0.54	0.7	0.1	-3.2	2.15	1.9	0.33333	4	3.7	0.66667
95/0540	0.23	0.6	0.2	-3.7	0.31	0.7	0.1	-7.8	1.9	1.9	0	1.2	3.7	-5.5556
92/1037	0.2	0.57	0.23	-3.2174	0.14	0.71	0.23	-4.9565	3.1	1.9	1.6	4.8	3.7	2.44444

	Trabecular compartment				Cortical compartment				Periosteal compartment			Sub-cortical compartment		
Biopsy	Raw valu	Mean	2SD	Z score	Raw value	Mean	2SD	Z score	Raw value	Mean	Z score	Raw val	Mean	Z score
92/1891	0.33	0.57	0.23	-2.087	0.34	0.71	0.23	-3.2174	1.6	1.9	-0.4	2.94	3.7	-1.6889
92/1903	0.51	0.4	0.1	2.2	0.5	0.6	0.2	-1	1.54	1.9	-0.48	3.8	3.7	0.22222
92/1970	0.31	0.63	0.34	-1.8824	0.33	0.76	0.21	-4.0952	3.12	1.9	1.62667	4.85	3.7	2.55556
92/2087	0.39	0.61	0.11	-4	0.51	0.75	0.28	-1.7143	2.13	1.9	0.30667	3.88	3.7	0.4
92/2120	0.33	0.57	0.23	-2.087	0.29	0.71	0.23	-3.6522	0.65	1.9	-1.6667	3.1	3.7	-1.3333
92/2178	0.27	0.6	0.2	-3.3	0.29	0.7	0.1	-8.2	1.65	1.9	-0.3333	3.9	3.7	0.44444
92/2266	0.3	0.57	0.23	-2.3478	0.29	0.71	0.23	-3.6522	0.36	1.9	-2.0533	3.1	3.7	-1.3333
92/2289	0.44	0.61	0.11	-3.0909	0.32	0.75	0.28	-3.0714	2.2	1.9	0.4	3.6	3.7	-0.2222
93/0199	0	0.6	0.2	-6	0	0.7	0.1	-14	0.01	1.9	-2.52	0.01	3.7	-8.2
93/0209	0.21	0.63	0.34	-2.4706	0.33	0.76	0.21	-4.0952	5.8	1.9	5.2	2.2	3.7	-3.3333
93/0389	0.27	0.65	0.15	-5.0667	0.33	0.78	0.23	-3.913	1.9	1.9	0	3.9	3.7	0.44444
93/0528	0.38	0.58	0.19	-2.1053	0.61	0.73	0.21	-1.1429	0.01	1.9	-2.52	1.05	3.7	-5.8889
93/0602	0.21	0.57	0.23	-3.1304	0.16	0.71	0.23	-4.7826	0.01	1.9	-2.52	3.3	3.7	-0.8889
93/0686	0.33	0.66	0.14	-4.7143	0.31	0.73	0.43	-1.9535	0.35	1.9	-2.0667	2.9	3.7	-1.7778
93/0848	0.59	0.66	0.14	-1	0.61	0.73	0.43	-0.5581	1.95	1.9	0.06667	3.65	3.7	-0.1111
93/0838	0.31	0.58	0.19	-2.8421	0.28	0.73	0.21	-4.2857	2.03	1.9	0.17333	3.8	3.7	0.22222
93/1136	0.09	0.64	0.34	-3.2353	0.08	0.76	0.21	-6.4762	0.93	1.9	-1.2933	1.25	3.7	-5.4444
93/1150	0.27	0.61	0.11	-6.1818	0.38	0.75	0.28	-2.6429	1.25	1.9	-0.8667	3.05	3.7	-1.4444
93/1411	0.1	0.61	0.11	-9.2727	0.1	0.75	0.28	-4.6429	0.31	1.9	-2.12	2.85	3.7	-1.8889
93/1788	0.11	0.6	0.2	-4.9	0.13	0.7	0.1	-11.4	0.25	1.9	-2.2	2.68	3.7	-2.2667
93/1568	0.111	0.6	0.2	-4.89	0.09	0.7	0.1	-12.2	0	1.9	-2.5333	3.6	3.7	-0.2222
93/1447	0.27	0.6	0.2	-3.3	0.33	0.7	0.1	-7.4	2	1.9	0.13333	3.8	3.7	0.22222
93/1279	0	0.57	0.23	-4.9565	0	0.71	0.23	-6.1739	0.88	1.9	-1.36	2.95	3.7	-1.6667
93/1232	0.3	0.6	0.2	-3	0.28	0.7	0.1	-8.4	0.03	1.9	-2.4933	0.16	3.7	-7.8667
93/0659	0.3	0.57	0.23	-2.3478	0.41	0.71	0.23	-2.6087	1.2	1.9	-0.9333	0.55	3.7	-7
93/0450	0.47	0.61	0.11	-2.5455	0.53	0.75	0.28	-1.5714	3.6	1.9	2.26667	4.4	3.7	1.55556
93/0183	0.32	0.4	0.1	-1.6	0.49	0.6	0.2	-1.1	0.65	1.9	-1.6667	2.99	3.7	-1.5778
93/0049	0.28	0.6	0.2	-3.2	0.39	0.7	0.1	-6.2	0.01	1.9	-2.52	2.55	3.7	-2.5556
92/1170	0.09	0.57	0.23	-4.1739	0.17	0.71	0.23	-4.6957	0.39	1.9	-2.0133	4.3	3.7	1.33333
92/1169	0.11	0.6	0.2	-4.9	0.15	0.7	0.1	-11	0.25	1.9	-2.2	1.95	3.7	-3.8889
92/1101	0.38	0.6	0.2	-2.2	0.36	0.7	0.1	-6.8	1.9	1.9	0	3.7	3.7	0
92/1039	0.18	0.57	0.23	-3.3913	0.19	0.71	0.23	-4.5217	0.01	1.9	-2.52	2.95	3.7	-1.6667
92/0982	0.19	0.57	0.23	-3.3043	0.3	0.771	0.23	-4.0957	0	1.9	-2.5333	2.9	3.7	-1.7778
92/0905	0.3	0.57	0.23	-2.3478	0.44	0.71	0.23	-2.3478	1.6	1.9	-0.4	3.2	3.7	-1.1111

	Trabecular compartment				Cortical compartment				Periosteal compartment			Sub-cortical compartment		
Biopsy	Raw valu	Mean	2SD	Z score	Raw value	Mean	2SD	Z score	Raw value	Mean	Z score	Raw val	Mean	Z score
92/0791	0.29	0.4	0.1	-2.2	0.37	0.6	0.2	-2.3	0.01	1.9	-2.52	2.81	3.7	-1.9778
92/0771	0.27	0.6	0.2	-3.3	0.25	0.7	0.1	-9	3.6	1.9	2.26667	4.1	3.7	0.88889
92/0752	0.29	0.6	0.2	-3.1	0.38	0.7	0.1	-6.4	1.95	1.9	0.06667	3.43	3.7	-0.6
92/0715	0	0.6	0.2	-6	0	0.7	0.1	-14	0	1.9	-2.5333	0.5	3.7	-7.1111
92/0504	0.36	0.6	0.2	-2.4	0.35	0.7	0.1	-7	1.95	1.9	0.06667	3.75	3.7	0.11111
92/0461	0.41	0.6	0.2	-1.9	0.41	0.7	0.1	-5.8	2	1.9	0.13333	4	3.7	0.66667
92/0347	0	0.4	0.1	-8	0	0.6	0.2	-6	0.01	1.9	-2.52	1	3.7	-6
92/0149	0	0.61	0.11	-11.091	0	0.75	0.28	-5.3571	3.2	1.9	1.73333	8.5	3.7	10.6667
92/0127	0.44	0.61	0.11	-3.0909	0.46	0.75	0.28	-2.0714	1.85	1.9	-0.0667	3.5	3.7	-0.4444
91/2227	0.3	0.57	0.23	-2.3478	0.41	0.71	0.23	-2.6087	0.18	1.9	-2.2933	2.75	3.7	-2.1111
91/2206	0.37	0.6	0.2	-2.3	0.42	0.7	0.1	-5.6	0.25	1.9	-2.2	2.9	3.7	-1.7778
91/2184	0.34	0.57	0.23	-2	0.29	0.71	0.23	-3.6522	1.54	1.9	-0.48	2.75	3.7	-2.1111
91/2183	0.32	0.6	0.2	-2.8	0.41	0.7	0.1	-5.8	1.75	1.9	-0.2	3.65	3.7	-0.1111
92/1304	0	0.6	0.2	-6	0.36	0.7	0.1	-6.8	1.8	1.9	-0.1333	3.6	3.7	-0.2222
92/2564	0.21	0.57	0.23	-3.1304	0.19	0.71	0.23	-4.5217	0.01	1.9	-2.52	0.01	3.7	-8.2
92/2444	0.26	0.4	0.1	-2.8	0.41	0.6	0.2	-1.9	4.2	1.9	3.06667	4.1	3.7	0.88889
92/2408	0.38	0.6	0.2	-2.2	0.39	0.7	0.1	-6.2	1.8	1.9	-0.1333	2.9	3.7	-1.7778
92/2404	0.31	0.6	0.2	-2.9	0.4	0.7	0.1	-6	0.12	1.9	-2.3733	0.65	3.7	-6.7778
92/2243	0.24	0.4	0.1	-3.2	0.26	0.6	0.2	-3.4	3.05	1.9	1.53333	4.8	3.7	2.44444
92/2219	0	0.6	0.2	-6	0	0.7	0.1	-14	4.8	1.9	3.86667	7.6	3.7	8.66667
92/2146	0.32	0.6	0.2	-2.8	0.19	0.7	0.1	-10.2	0.15	1.9	-2.3333	3.25	3.7	-1
92/2030	0.31	0.57	0.23	-2.2609	0.31	0.71	0.23	-3.4783	2.8	1.9	1.2	4.1	3.7	0.88889
92/2014	0.3	0.57	0.23	-2.3478	0.3	0.71	0.23	-3.5652	1.55	1.9	-0.4667	2.96	3.7	-1.6444
92/1958	0.41	0.6	0.2	-1.9	0.37	0.7	0.1	-6.6	0.36	1.9	-2.0533	3.05	3.7	-1.4444
92/1554	0.4	0.6	0.2	-2	0.39	0.7	0.1	-6.2	2.1	1.9	0.26667	3.8	3.7	0.22222

Appendix 1c - Sample Cases Histomorphometric Data (1)

General Details					Static Histomorphometric Measurements							
					Trabecular Bone Measurements							
No.	Biopsy No.	Slide No.	Sex	Age	Trab Bone Vol	Wall Thk	Ostd Surf	Ostd Vol	Ostd Thk	Ostb Surf	Mnrl Surf 1	Mnrl Surf 2
1	C9758	91/0283	M	5	8.10	43.60	9.50	1.70	8.60	3.70	6.80	71.30
2	D0069	94/1091	F	5	9.10	26.70	6.90	0.80	4.90	2.40	3.10	51.20
3	D0603	93/2471	F	6	8.70	24.80	1.30	0.10	3.00	0.10	0.00	0.00
4	D0733	92/2049	F	6	9.80	40.60	9.10	1.20	7.20	2.70	6.00	39.10
5	D1156	N/A	M	5	15.10	33.80	8.60	1.80	4.20	1.10	0.00	0.00
6	D1364	95/0726	M	5	5.30	26.00	28.90	2.50	5.70	5.20	6.00	58.10
7	D1417	91/0820	M	6	8.30	39.60	11.90	2.60	6.60	2.80	4.20	48.90
8	D1418	91/0691	M	5	10.20	29.50	14.20	1.50	7.00	2.70	8.60	87.30
9	D1637	92/1768	F	2	18.10	43.80	6.60	1.60	10.40	6.20	6.00	94.10
10	D1860	94/0193	F	6	8.90	29.90	7.80	0.50	5.00	2.90	1.20	15.90
11	D2060	92/2197	F	6	9.00	39.10	10.10	0.90	6.90	2.90	6.20	40.50
12	D2075	90/2046	M	4	16.00	48.60	18.60	3.90	9.85	5.84	12.60	91.60
13	D2086	92/2179	F	2	11.70	31.00	7.90	1.00	5.95	2.01	4.85	69.90
14	D2147	92/0664	F	0	5.60	11.20	3.80	0.70	7.70	1.90	1.90	51.20
15	D2197	90/2303	F	5	12.00	19.60	26.80	3.10	10.60	2.30	21.30	88.90
16	D2274	90/2446	F	11	9.10	28.10	7.20	1.30	6.40	3.80	5.10	76.40
17	D2337	92/2518	F	7	4.00	17.50	4.40	0.40	3.50	1.30	0.00	0.00
18	D2338	92/2517	F	6	9.00	21.80	0.70	0.20	1.00	0.10	0.60	90.20
19	D2375	90/2631	F	11	16.00	33.30	15.70	2.70	8.10	6.40	7.50	73.40
20	D2450	91/0123	M	5	8.60	39.80	15.40	3.20	7.50	3.50	8.00	75.60
21	D2532	91/0173	F	2	8.40	26.30	12.60	2.20	7.10	3.15	4.75	61.20
22	D2547	91/1506	F	3	17.00	34.10	13.10	3.00	7.20	1.10	6.80	75.60
23	D2550	91/0200	F	6	9.10	46.50	11.40	1.00	8.40	2.00	6.40	81.40
24	D2621	91/1568	F	3	21.80	47.60	13.20	3.00	8.20	4.60	7.10	72.60
25	D2677	91/0422	M	11	2.90	25.40	16.50	3.20	6.10	2.00	6.90	69.90
26	D2685	91/0437	F	4	13.00	32.10	10.50	3.10	7.90	5.30	5.90	74.20
27	D2775	91/0587	M	4	14.60	37.80	11.00	2.40	7.21	4.13	5.81	76.10
28	D2791	91/0726	M	4	10.40	28.70	15.40	2.90	8.89	1.99	6.35	69.80
29	D2862	91/0772	M	5	12.10	29.80	18.50	3.60	9.10	3.80	8.40	42.30

Appendix 1c - Sample Cases Histomorphometric Data (1)

General Details					Static Histomorphometric Measurements							
					Trabecular Bone Measurements							
No.	Biopsy No.	Slide No.	Sex	Age	Trab Bone Vol	Wall Thk	Ostd Surf	Ostd Vol	Ostd Thk	Ostb Surf	Mnrl Surf 1	Mnrl Surf 2
30	D2935	91/0955	F	7	6.40	20.50	16.30	2.10	7.80	1.10	9.20	81.60
31	D2936	93/2049	M	6	9.30	20.80	10.00	0.80	2.90	1.10	6.10	63.50
32	D2963	91/0997	F	11	20.60	46.80	18.50	3.10	7.94	3.67	7.40	69.90
33	D2964	91/0998	F	5	12.00	34.70	14.70	2.30	6.90	6.70	6.10	64.60
34	D2972	91/1099	M	6	13.20	34.70	6.10	0.50	6.00	1.30	5.30	84.20
35	D2981	91/1024	F	4	10.50	27.50	9.70	1.80	7.40	5.00	6.50	79.80
36	D3005	91/1069	M	4	8.60	21.40	9.70	1.30	7.60	4.70	5.90	65.10
37	D3068	91/1177	F	4	6.90	20.50	9.20	1.70	7.30	4.90	8.50	80.60
38	D3127	91/1332	F	11	10.30	34.00	15.20	2.20	7.50	7.20	8.00	82.50
39	D3191	91/1530	F	5	10.60	28.70	15.00	2.20	6.20	2.00	4.40	71.20
40	D3203	91/1751	F	5	10.00	34.30	8.70	0.70	4.50	1.90	6.40	84.00
41	D3216	91/1556	F	5	11.20	32.80	19.00	4.00	8.40	4.90	5.30	64.50
42	D3237	91/1539	M	7	9.40	41.50	8.80	2.00	7.20	2.60	3.90	59.70
43	D3339	91/1861	M	4	15.60	36.50	24.30	5.10	9.78	8.40	16.30	68.90
44	D3375	91/1974	F	5	11.40	34.80	15.60	2.80	8.40	3.20	5.10	41.30
45	D3445	91/1984	F	3	27.50	35.40	4.60	0.90	6.10	1.10	2.20	50.20
46	D3451	91/1993	M	5	7.90	17.80	3.70	0.90	3.60	1.20	2.00	54.10
47	D3502	91/2144	F	4	26.00	34.60	10.30	0.80	3.00	0.10	5.10	72.30
48	D3565	91/2179	F	3	14.60	40.10	7.80	1.40	7.12	2.43	4.30	63.80
49	D3606	91/2267	F	6	9.80	30.00	4.80	0.10	3.10	1.70	3.10	61.10
50	D3727	94/0051	F	6	4.40	16.00	1.00	0.10	3.00	0.10	0.50	50.00
51	D3882	92/0588	M	5	10.30	26.70	8.40	1.20	6.40	2.10	3.20	41.50
52	D4015	92/0685	F	3	14.20	31.50	14.20	2.90	7.40	3.10	6.00	69.10
53	D4038	92/0848	F	6	14.50	29.80	10.20	1.60	7.80	7.10	9.40	89.20
54	D4246	92/1098	M	2	7.40	28.90	19.40	0.50	4.80	2.90	6.80	59.90
55	D4581	92/1882	F	2	7.10	45.40	8.30	1.40	9.74	3.14	5.78	69.90
56	D4587	92/1903	F	7	3.60	37.50	9.40	1.10	11.50	7.30	7.10	85.10
57	D4615	92/1970	M	3	12.70	38.90	22.40	4.50	10.80	12.90	12.10	70.10

Appendix 1c - Sample Cases Histomorphometric Data (1)

General Details					Static Histomorphometric Measurements							
					Trabecular Bone Measurements							
No.	Biopsy No.	Slide No.	Sex	Age	Trab Bone Vol	Wall Thk	Ostd Surf	Ostd Vol	Ostd Thk	Ostb Surf	Mnrl Surf 1	Mnrl Surf 2
58	D4681	92/2087	F	4	17.10	44.70	13.80	3.10	8.20	5.30	7.10	68.70
59	D4695	92/2120	F	5	12.00	34.20	9.80	1.90	7.90	4.70	5.10	69.00
60	D4764	92/2266	F	5	11.70	33.60	9.30	1.40	6.10	4.40	4.10	58.20
61	D4768	92/2289	F	4	13.00	42.20	10.70	1.80	5.70	3.00	5.10	59.90
62	D4875	93/0199	M	6	4.20	21.60	2.20	0.20	3.00	0.50	0.00	0.00
63	D4935	93/0209	M	3	13.40	38.90	6.40	1.50	6.80	0.90	4.90	70.60
64	D4970	93/0389	F	2	8.90	46.20	13.20	2.80	10.32	7.50	7.40	70.80
65	D4996	93/0528	M	5	7.90	31.20	4.50	0.80	6.10	1.10	3.10	71.20
66	D5055	93/0602	F	5	9.40	32.10	3.00	0.70	8.00	2.30	1.70	73.10
67	D5085	93/0686	M	2	8.60	21.20	9.30	0.50	4.65	1.83	5.00	63.80
68	D5093	93/0848	M	4	10.00	41.20	5.30	0.50	8.65	2.83	2.18	81.90
69	D5094	93/0838	M	5	16.70	37.90	11.00	1.90	9.90	4.80	5.10	64.00
70	D5256	93/1136	M	3	14.90	30.40	10.00	1.90	6.90	3.70	3.90	65.00
71	D5262	93/1150	F	4	8.70	31.00	14.40	2.80	6.10	3.30	7.60	75.80
72	D5400	93/1431	F	6	9.10	28.90	8.00	1.00	8.30	3.00	5.20	61.00
73	D5451	93/1524	F	7	10.20	27.40	10.00	1.90	5.10	2.60	4.00	56.20
74	D5483	93/1845	M	5	8.70	30.50	9.20	1.50	7.80	3.00	6.00	69.90
75	D5505	93/1670	F	7	8.00	26.10	9.80	1.50	5.80	3.80	5.30	69.80
76	D5566	93/1841	M	6	9.90	29.40	6.10	1.20	6.70	2.00	4.70	70.00
77	D5768	93/2204	F	5	11.00	28.70	7.60	1.70	7.90	3.10	3.80	67.10
78	D5774	93/2217	F	5	17.90	38.20	13.40	2.90	9.20	7.10	6.80	76.40
79	D5888	93/2436	M	3	19.30	59.30	21.90	6.80	14.10	15.80	11.30	79.40
80	D5918	93/2483	M	3	14.40	38.90	2.40	0.50	5.80	0.90	0.00	0.00
81	D6025	93/2866	F	6	9.70	27.30	1.30	0.10	5.10	0.10	0.00	0.00
82	D6096	93/3032	M	5	11.10	36.90	15.60	3.10	9.10	7.90	8.20	86.10
83	D6105	93/2946	F	4	6.10	21.80	8.50	1.00	5.70	3.60	4.60	66.30
84	D6208	94/0043	F	5	8.30	24.30	6.80	0.90	4.10	1.80	4.30	65.70
85	D6211	94/0135	F	2	13.40	40.00	5.10	1.00	3.20	2.10	2.80	60.20

Appendix 1c - Sample Cases Histomorphometric Data (1)

General Details					Static Histomorphometric Measurements							
					Trabecular Bone Measurements							
No.	Biopsy No.	Slide No.	Sex	Age	Trab Bone Vol	Wall Thk	Ostd Surf	Ostd Vol	Ostd Thk	Ostb Surf	Mnrl Surf 1	Mnrl Surf 2
86	D6231	94/0169	M	5	7.40	29.70	4.20	0.20	3.00	1.00	2.70	62.00
87	D6309	94/0308	M	4	9.50	37.80	7.60	1.20	9.70	3.10	5.70	70.30
88	D6355	94/0370	M	4	13.80	40.10	4.70	0.30	3.10	1.90	3.30	60.70
89	D6374	94/0309	F	4	9.70	29.30	7.60	1.00	4.20	1.90	6.80	81.80
90	D6473	94/0487	F	7	9.10	25.10	8.30	1.20	5.30	2.90	3.80	50.50
91	D6507	94/0574	M	4	13.80	30.50	3.20	1.00	4.10	1.00	0.00	0.00
92	D6508	94/0575	F	3	17.60	43.50	4.10	1.30	8.60	1.20	2.60	60.70
93	D6557	94/0664	F	3	13.20	40.20	4.10	0.50	3.60	0.70	2.40	2.20
94	D6640	94/0783	F	4	11.00	31.70	5.80	1.00	4.00	2.10	3.00	80.20
95	D6671	94/0840	F	3	10.20	38.70	0.00	0.00	0.00	0.00	0.00	0.00
96	D6744	94/1096	M	5	12.50	34.30	8.10	1.30	5.00	2.90	2.50	46.10
97	D6780	94/1044	M	5	8.60	32.60	23.00	4.90	9.10	14.90	10.20	64.00
98	D6789	94/1063	F	5	9.60	30.40	11.20	2.80	7.90	5.80	4.50	51.00
99	D6834	94/1149	F	4	22.00	43.90	10.20	1.80	9.00	4.80	4.80	66.10
100	D6931	94/1342	F	4	15.90	45.10	13.80	2.10	7.50	3.40	5.60	51.80
101	D7006	94/1623	M	4	8.50	29.10	0.00	0.00	0.00	0.00	0.00	0.00
102	D7007	94/1620	M	3	6.70	33.30	3.00	0.10	5.90	1.20	1.60	63.20
103	D7022	94/1655	F	5	9.30	40.30	8.10	1.60	8.40	4.80	4.90	70.80
104	D7116	94/1756	F	3	9.40	39.10	7.10	1.00	6.00	1.80	3.70	70.50
105	D7215	94/1945	M	3	13.00	30.50	12.20	2.30	6.00	3.90	4.90	70.60
106	D7262	94/2048	F	4	9.50	31.60	12.00	2.60	8.70	4.10	4.40	40.10
107	D7264	94/2052	F	3	20.10	53.70	14.80	4.50	13.80	3.70	7.60	83.20
108	D7291	94/2105	F	6	11.60	36.00	10.40	0.50	4.80	2.10	6.80	57.20
109	D7321	94/2156	F	6	7.10	35.50	6.20	0.50	4.70	1.00	2.80	36.60
110	D7358	94/2226	F	7	9.10	27.60	7.90	1.00	6.10	1.50	1.20	18.30
111	D7451	95/0017	M	4	13.70	36.80	5.10	0.30	8.80	2.10	2.00	41.00
112	D7538	95/0261	F	7	12.10	21.60	0.60	0.00	0.00	0.10	0.00	0.00
113	D7578	95/0257	F	4	9.10	30.80	4.70	0.40	3.60	1.20	1.40	30.60

Appendix 1c - Sample Cases Histomorphometric Data (1)

General Details					Static Histomorphometric Measurements							
					Trabecular Bone Measurements							
No.	Biopsy No.	Slide No.	Sex	Age	Trab Bone Vol	Wall Thk	Ostd Surf	Ostd Vol	Ostd Thk	Ostb Surf	Mnrl Surf 1	Mnrl Surf 2
114	D7604	95/0317	F	5	5.90	20.80	7.30	1.10	4.80	1.90	1.00	24.80
115	D7618	95/0460	M	3	13.10	39.60	12.00	1.90	7.40	3.10	4.20	56.20
116	D7637	95/0381	F	6	3.80	32.00	0.00	0.00	0.00	0.00	0.00	0.00
117	D7663	95/0418	M	5	9.60	28.40	8.90	1.20	6.50	3.50	4.10	60.10
118	D7691	95/0490	F	5	19.70	35.50	9.00	2.50	13.20	5.60	8.20	93.00
119	D7755	95/0597	F	4	10.20	32.70	7.80	1.20	5.10	2.00	3.10	32.80
120	D7829	95/0734	F	6	8.20	20.50	7.90	0.90	6.10	2.90	4.60	51.20
121	D7850	95/0784	F	4	14.00	44.50	9.60	1.20	7.60	3.00	5.10	66.10
122	D7857	95/0856	M	6	14.50	36.70	4.70	0.80	6.10	2.00	3.50	50.20
123	D8025	95/1108	M	5	6.90	28.50	5.20	0.90	5.70	0.80	1.00	23.30
124	D8069	95/1175	F	6	4.60	25.70	1.10	0.10	3.90	0.00	0.00	0.00
125	D8089	95/1221	F	5	4.10	27.50	5.80	0.40	5.10	0.90	0.10	20.50
126	D8219	95/1409	F	6	0.30	20.20	0.00	0.00	0.00	0.00	0.00	0.00
127	D8300	95/1539	F	5	11.50	31.90	9.00	1.70	6.90	3.80	3.60	66.60
128	D8372	95/1657	M	5	12.60	31.00	0.60	0.10	0.50	0.00	0.00	0.00
129	D8409	95/1707	F	5	12.10	32.80	7.30	1.00	5.00	1.10	0.00	0.00
130	D8497	95/1840	F	4	13.70	51.70	9.70	2.70	10.80	4.60	7.10	73.40
131	D8531	95/1896	F	5	8.90	21.70	8.60	1.40	6.40	3.20	2.10	35.00
132	D8553	95/1936	M	4	10.00	30.70	5.10	1.00	5.90	2.60	2.60	56.80

Trab Bone Vol = Trabecular Bone Volume (BV/TVt) %
Wall Thk = Wall Thickness (um)
Ostd Surf = Osteoid Surface (OS/BS) %
Ostd Vol = Osteoid Volume (OV/BV) %
Ostd Thk = Osteoid Thickness (um)
Ostb Surf = Osteoblast Surface (ObS/BS) %
Mnrl Surf 1 = Mineralising Surface 1 (MS/BS) (dLS+1/2sLS) %
Mnrl Surf 2 = Mineralising Surface 2 (MS/OS) (sLS+dLS) %

Age:

Group	Year	Group	Year
0	1-10	5	51-60
1	11-20	6	61-70
2	21-30	7	71-80
3	31-40	11	40-70
4	41-50		

Appendix 1c - Sample Cases Histomorphometric Data (2)

General Details		Static Histomorphometric Measurements cont...											
		Trabecular Bone Measurements cont...							Cortical Bone Measurements				
No.	Biopsy No.	Mnlsing Surf	Erod Surf	Ostc Surf	Ostc No	Trab Thk	Trab No	Trab Sep	Cort Thk	Cort Vol	Cort Wll Thk	Ostc No	Subcort Ostc
1	C9758	21.60	4.80	0.20	0.20	118.50	1.90	799.60	442.00	78.60	42.40	0.80	1.30
2	D0069	20.60	2.50	0.10	1.00	114.80	0.50	882.80	897.20	92.10	40.00	1.00	0.60
3	D0603	0.00	2.90	0.10	0.10	89.00	0.30	2156.70	678.90	71.30	30.10	7.10	1.20
4	D0733	40.50	7.30	1.00	1.10	151.20	0.60	711.50	1008.00	84.10	41.20	1.30	1.50
5	D1156	0.00	7.30	0.00	0.00	120.20	1.80	645.00	1132.00	67.40	34.10	0.90	0.20
6	D1364	19.50	2.10	0.30	2.00	89.90	0.40	2011.40	1330.20	94.00	34.30	0.70	0.90
7	D1417	2.40	8.10	1.60	71.50	100.20	0.40	819.50	412.00	47.50	38.50	20.50	9.90
8	D1418	13.60	7.20	0.90	34.70	128.70	0.50	814.70	612.00	85.60	35.40	2.80	1.90
9	D1637	83.20	5.40	0.00	0.10	173.20	1.82	598.20	1325.00	90.70	53.10	0.10	0.10
10	D1860	0.00	2.30	0.10	0.30	111.40	0.80	934.10	1195.60	82.10	42.10	2.70	0.10
11	D2060	39.90	3.00	0.20	0.80	116.70	1.10	700.30	965.00	83.70	38.80	2.00	1.30
12	D2075	65.80	11.30	1.08	0.08	131.80	2.00	689.50	1486.00	79.40	61.60	0.87	0.03
13	D2086	15.90	2.70	0.31	1.70	107.20	0.32	913.50	1001.00	92.20	32.20	1.10	1.10
14	D2147	0.00	6.80	1.10	34.30	121.80	1.40	913.20	845.00	95.40	14.30	3.60	7.50
15	D2197	38.60	8.20	2.30	25.90	139.90	1.80	799.70	905.00	76.40	43.70	3.80	3.80
16	D2274	22.90	6.80	1.40	29.10	100.90	3.70	811.20	809.00	85.60	38.30	4.70	3.10
17	D2337	0.00	4.90	0.80	3.20	41.50	0.10	1299.80	1047.00	92.40	25.90	2.20	1.70
18	D2338	0.00	3.10	0.00	0.00	80.60	0.50	997.80	1216.00	87.90	29.80	0.00	0.00
19	D2375	26.50	5.90	0.60	6.50	149.90	1.70	429.80	1234.00	86.50	42.60	5.10	0.80
20	D2450	51.60	7.20	0.90	22.40	121.60	0.90	845.30	1388.00	92.20	53.10	1.50	1.40
21	D2532	8.60	5.90	0.75	20.00	85.30	1.51	756.20	935.00	81.40	69.30	4.50	1.10
22	D2547	60.30	3.10	0.02	0.01	143.90	1.00	675.00	1420.00	88.90	45.30	1.30	2.40
23	D2550	26.20	9.70	1.60	49.50	116.50	1.90	804.60	1362.00	76.00	44.50	12.80	3.50
24	D2621	60.30	5.00	0.62	0.10	158.50	1.51	486.20	1273.00	88.30	54.10	0.90	0.50
25	D2677	32.70	6.20	0.90	25.70	89.10	0.10	1876.90	745.00	90.30	35.70	3.30	2.90
26	D2685	21.20	7.00	1.20	24.30	148.30	1.40	546.70	1253.00	87.20	48.60	2.90	1.20
27	D2775	56.80	2.50	0.21	0.30	102.10	1.58	642.40	1652.00	92.00	41.60	6.10	0.80
28	D2791	24.60	7.00	0.93	18.90	119.60	1.23	887.60	885.00	89.90	31.10	1.90	0.50
29	D2862	4.60	5.70	1.10	34.30	127.80	1.80	599.60	1472.00	71.60	34.60	2.90	3.80

Appendix 1c - Sample Cases Histomorphometric Data (2)

General Details		Static Histomorphometric Measurements cont...											
		Trabecular Bone Measurements cont...							Cortical Bone Measurements				
No.	Biopsy No.	Mnlsing Surf	Erod Surf	Ostc Surf	Ostc No	Trab Thk	Trab No	Trab Sep	Cort Thk	Cort Vol	Cort Wll Thk	Ostc No	Subcort Ostc
30	D2935	37.60	5.90	1.50	31.70	67.20	0.70	811.60	587.00	67.10	21.90	4.00	3.20
31	D2936	46.60	2.70	0.10	0.70	90.40	0.40	1421.60	1378.90	91.90	48.00	1.40	0.80
32	D2963	41.60	7.50	0.99	28.60	164.50	1.43	489.90	1234.00	92.70	39.70	1.80	3.00
33	D2964	43.00	8.00	2.00	31.80	124.70	1.30	598.30	872.00	76.40	42.60	5.80	3.20
34	D2972	10.10	10.10	0.10	0.10	98.20	0.80	712.30	1436.00	92.90	45.50	0.40	0.90
35	D2981	42.70	3.90	0.50	0.90	86.30	1.00	745.20	946.00	71.70	42.10	6.30	3.00
36	D3005	9.30	2.80	0.20	0.20	83.80	0.47	811.40	933.00	86.70	31.20	1.10	0.30
37	D3068	10.60	7.80	2.40	48.20	110.40	0.60	847.60	146.90	79.80	40.60	9.70	6.40
38	D3127	36.10	6.90	0.90	27.60	113.70	1.20	783.90	893.00	79.40	39.60	5.80	0.00
39	D3191	48.70	8.30	0.20	0.70	115.30	1.80	666.30	1326.00	79.50	34.40	1.90	1.00
40	D3203	9.20	3.20	0.40	0.70	113.80	1.80	856.40	1002.00	80.20	41.10	1.40	0.80
41	D3216	44.60	10.20	1.60	46.10	99.10	1.30	618.70	1200.00	75.30	48.00	5.40	3.60
42	D3237	15.70	5.70	0.30	1.40	111.40	0.20	815.20	1086.00	79.50	38.90	6.90	0.90
43	D3339	29.70	5.70	0.70	12.70	156.80	1.45	673.50	1342.00	81.20	46.70	0.36	0.80
44	D3375	28.60	6.90	1.20	29.50	153.70	1.80	687.20	746.00	81.80	36.70	2.30	1.00
45	D3445	40.00	8.20	1.00	34.20	288.70	0.90	421.70	1403.00	90.20	40.00	3.30	1.10
46	D3451	63.20	2.00	0.20	0.20	122.10	0.50	998.40	1076.00	92.20	46.20	0.50	1.90
47	D3502	16.70	1.20	0.10	0.50	189.50	1.00	412.40	1423.00	84.50	43.10	7.10	0.70
48	D3565	36.70	2.90	0.42	3.80	132.60	1.30	612.30	1046.00	92.00	43.30	3.00	0.60
49	D3606	0.10	1.30	0.10	0.10	81.20	0.30	892.10	549.00	79.90	28.70	0.70	0.00
50	D3727	0.00	1.00	0.10	0.10	86.10	0.30	1329.50	440.20	84.50	20.50	0.20	0.10
51	D3882	0.00	5.90	0.90	25.40	95.60	0.70	711.10	1528.00	72.20	34.90	1.80	1.50
52	D4015	21.60	5.10	0.70	9.20	145.20	1.00	768.30	1334.00	91.00	49.80	2.10	1.30
53	D4038	28.60	6.60	1.20	29.90	154.60	1.20	562.70	1271.00	87.70	40.00	4.30	2.70
54	D4246	31.20	8.70	1.70	31.40	109.50	0.10	974.60	845.00	82.70	29.90	6.80	1.70
55	D4581	52.30	7.00	0.99	26.70	100.30	0.31	805.60	1104.00	64.50	46.80	4.00	1.80
56	D4587	76.90	7.90	1.10	23.80	89.10	0.10	999.80	1147.00	82.40	45.90	3.20	2.30
57	D4615	28.10	8.20	1.20	17.20	119.50	1.30	574.60	1145.00	89.70	49.90	1.80	0.80

Appendix 1c - Sample Cases Histomorphometric Data (2)

General Details		Static Histomorphometric Measurements cont...											
		Trabecular Bone Measurements cont...							Cortical Bone Measurements				
No.	Biopsy No.	Mnlsing Surf	Erod Surf	Ostc Surf	Ostc No	Trab Thk	Trab No	Trab Sep	Cort Thk	Cort Vol	Cort Wll Thk	Ostc No	Subcort Ostc
58	D4681	51.10	4.80	1.00	7.90	152.00	1.20	499.30	1341.00	83.10	39.30	2.30	1.90
59	D4695	28.40	6.30	0.90	17.40	178.30	1.40	713.20	1599.00	76.00	38.80	2.50	1.60
60	D4764	26.70	3.80	0.60	3.10	101.50	0.90	701.20	991.00	74.50	34.20	8.20	2.30
61	D4768	35.10	6.90	1.30	28.50	132.50	1.40	592.30	1734.00	92.00	45.20	1.50	1.20
62	D4875	0.00	2.00	0.10	1.00	74.70	0.20	992.60	884.90	86.60	32.80	1.40	0.10
63	D4935	31.20	7.70	1.20	17.90	109.50	0.90	774.60	1245.00	89.70	39.90	1.80	0.60
64	D4970	51.10	4.20	0.68	7.90	158.30	1.02	832.10	1241.00	91.20	44.40	0.90	0.80
65	D4996	52.60	2.80	0.10	0.50	104.50	0.40	829.10	711.20	91.20	34.20	5.90	0.50
66	D5055	48.10	5.20	0.10	0.10	84.50	0.70	773.20	1111.00	74.40	45.10	7.90	0.00
67	D5085	30.10	2.10	0.01	0.10	82.00	1.00	761.00	753.00	93.20	40.00	0.11	2.40
68	D5093	60.10	9.10	0.92	28.50	102.00	1.26	761.00	953.00	85.20	40.00	0.61	0.41
69	D5094	34.20	4.00	0.90	14.30	118.70	1.20	600.30	1513.00	94.00	54.10	1.30	1.50
70	D5256	9.10	6.20	0.90	6.50	101.80	0.90	699.80	887.50	92.80	36.50	5.90	0.60
71	D5262	50.20	4.10	0.70	14.80	94.60	0.60	1312.70	1136.00	95.20	34.40	19.20	11.20
72	D5400	19.10	6.10	0.50	3.10	133.30	0.80	867.30	1102.30	72.50	36.90	3.60	2.60
73	D5451	20.70	2.90	0.10	1.00	90.10	0.60	891.20	387.60	82.50	28.60	2.90	3.20
74	D5483	38.20	3.80	0.60	6.50	146.20	0.80	995.50	576.30	76.40	34.40	4.00	0.10
75	D5505	53.00	9.10	2.10	24.50	128.70	0.60	856.00	1126.40	76.80	32.20	2.90	4.70
76	D5566	41.20	1.90	0.10	0.20	89.10	0.40	1172.40	1312.40	94.00	34.40	1.20	0.90
77	D5768	40.00	6.00	1.10	9.90	139.50	1.10	971.80	871.40	78.50	33.30	5.00	1.40
78	D5774	44.50	5.00	0.80	3.90	153.80	1.40	599.20	1325.80	93.20	46.80	6.10	1.50
79	D5888	68.10	8.30	2.00	15.20	104.50	1.20	688.10	1182.40	91.90	61.20	4.90	3.10
80	D5918	0.00	3.70	0.10	0.10	109.50	0.90	974.60	1345.00	92.70	39.90	0.80	0.40
81	D6025	0.00	5.20	0.40	2.90	109.70	0.50	1437.60	598.80	83.00	30.20	2.10	2.00
82	D6096	69.10	7.10	1.20	10.80	111.30	0.60	836.40	1400.20	93.70	42.30	3.50	1.90
83	D6105	21.90	3.20	0.50	3.00	95.60	0.20	2378.40	786.70	89.90	38.10	1.50	1.20
84	D6208	45.10	2.00	0.20	0.40	106.70	0.40	1200.30	1102.60	89.50	40.10	1.80	1.20
85	D6211	0.00	5.40	0.10	0.10	159.60	1.20	691.40	899.30	94.30	43.00	0.00	0.00

Appendix 1c - Sample Cases Histomorphometric Data (2)

General Details		Static Histomorphometric Measurements cont...											
		Trabecular Bone Measurements cont...							Cortical Bone Measurements				
No.	Biopsy No.	Mnlsing Surf	Erod Surf	Ostc Surf	Ostc No	Trab Thk	Trab No	Trab Sep	Cort Thk	Cort Vol	Cort Wll Thk	Ostc No	Subcort Ostc
86	D6231	26.50	2.70	0.00	0.00	103.20	0.30	993.50	699.20	93.50	36.10	0.80	0.10
87	D6309	56.20	2.20	0.10	0.20	140.20	0.30	982.60	1117.40	90.20	41.60	0.05	0.30
88	D6355	40.40	3.00	0.40	0.50	114.20	0.90	603.40	1319.80	85.10	40.20	0.10	0.50
89	D6374	36.10	1.50	0.20	1.50	79.20	0.30	649.20	1056.30	68.20	40.20	2.10	0.80
90	D6473	28.70	3.00	0.50	5.20	80.40	0.70	949.00	1089.40	76.70	40.30	1.40	1.60
91	D6507	0.00	1.30	0.10	0.30	122.20	1.00	934.50	998.70	89.90	26.70	0.20	0.10
92	D6508	19.60	1.80	0.10	0.30	153.40	1.40	521.30	1320.20	95.50	54.80	1.20	1.10
93	D6557	0.00	1.30	0.10	0.10	132.50	0.90	895.10	1087.30	95.30	46.10	0.30	0.00
94	D6640	56.30	2.50	0.10	0.10	150.20	0.50	898.60	687.10	89.90	40.20	1.60	0.00
95	D6671	0.00	1.80	0.00	0.00	135.10	0.80	947.10	1214.30	91.30	42.90	4.80	0.10
96	D6744	26.10	4.70	1.50	8.30	126.10	0.90	897.30	979.20	86.70	44.40	1.00	2.50
97	D6780	40.80	8.10	0.80	3.90	121.50	0.70	896.70	801.20	92.40	46.70	2.60	0.50
98	D6789	27.60	8.30	1.40	17.40	132.70	0.60	1099.60	1212.10	88.50	43.90	7.80	2.30
99	D6834	45.50	3.90	1.10	12.90	153.70	1.60	497.30	1505.80	91.70	50.00	1.50	0.60
100	D6931	37.60	3.90	0.50	4.90	132.60	1.60	531.20	755.50	94.40	49.50	4.20	0.90
101	D7006	0.00	1.20	0.00	0.00	97.20	0.50	1843.60	1006.00	92.10	40.90	0.02	0.00
102	D7007	59.40	5.90	1.10	8.30	88.80	0.40	994.10	1310.50	93.30	40.20	1.10	0.20
103	D7022	17.90	5.10	0.60	7.00	116.50	0.60	1623.90	1098.50	93.60	43.60	3.60	1.50
104	D7116	54.00	5.00	0.20	1.00	80.30	0.50	1492.80	1538.20	93.00	48.00	1.80	1.60
105	D7215	64.30	5.10	0.80	4.30	108.60	0.60	1210.50	1087.70	92.00	49.80	2.10	1.20
106	D7262	0.00	6.80	1.00	11.80	121.70	0.80	897.60	1183.60	90.00	46.40	1.90	2.00
107	D7264	68.20	7.20	0.90	5.80	141.10	1.60	518.50	1171.10	94.80	49.30	4.30	1.70
108	D7291	0.00	3.70	0.45	1.30	99.60	1.10	921.40	1100.40	86.80	38.30	2.70	1.80
109	D7321	36.50	6.20	1.10	10.90	110.60	0.40	1749.30	899.50	87.80	30.30	2.60	2.20
110	D7358	0.00	7.80	1.50	13.70	142.80	1.00	810.40	914.50	83.80	39.60	2.50	1.90
111	D7451	0.00	7.80	1.00	7.20	101.20	0.60	883.30	1110.90	90.00	40.50	0.19	0.30
112	D7538	0.00	3.40	0.00	0.00	103.20	0.80	992.20	1011.50	83.20	23.40	0.00	0.00
113	D7578	1.90	7.60	1.40	16.20	136.30	0.60	1004.20	1314.70	92.50	35.30	5.60	2.50

Appendix 1c - Sample Cases Histomorphometric Data (2)

General Details		Static Histomorphometric Measurements cont...											
		Trabecular Bone Measurements cont...							Cortical Bone Measurements				
No.	Biopsy No.	Mnlsing Surf	Erod Surf	Ostc Surf	Ostc No	Trab Thk	Trab No	Trab Sep	Cort Thk	Cort Vol	Cort Wll Thk	Ostc No	Subcort Ostc
114	D7604	43.10	3.70	0.30	2.80	104.50	0.40	1328.60	1042.70	90.40	30.20	1.60	3.00
115	D7618	30.30	7.90	1.80	10.50	137.40	0.90	853.40	1293.40	93.90	38.60	2.90	1.70
116	D7637	0.00	2.80	0.00	0.00	74.80	0.10	2310.80	903.20	85.50	20.30	0.90	0.90
117	D7663	48.30	6.20	1.00	9.90	109.80	1.00	1500.30	1272.10	93.70	38.40	0.60	0.50
118	D7691	70.70	6.10	1.10	8.80	171.20	1.30	498.70	1302.10	94.00	51.20	1.90	1.00
119	D7755	0.00	7.10	1.00	12.80	99.40	0.80	1634.80	1333.10	93.10	50.50	1.90	3.90
120	D7829	20.10	9.50	3.80	12.70	99.40	0.60	1423.60	1205.30	83.00	38.20	4.80	3.00
121	D7850	45.50	4.10	0.50	5.80	151.10	1.40	777.30	1189.30	93.80	49.20	2.00	1.50
122	D7857	0.00	3.10	0.50	2.40	129.50	1.10	729.60	1219.60	94.00	34.30	1.40	0.90
123	D8025	30.00	4.90	1.30	9.40	90.40	0.30	1884.50	1112.10	86.40	30.30	1.80	1.80
124	D8069	0.00	2.90	0.20	1.00	80.50	0.10	2104.30	600.40	86.10	27.50	2.50	1.10
125	D8089	0.00	7.30	1.40	6.30	90.10	0.30	1846.30	1099.80	90.50	42.20	1.90	1.40
126	D8219	0.00	2.80	0.00	0.00	59.50	0.10	2319.50	1113.40	69.50	26.50	2.90	4.80
127	D8300	38.70	8.30	1.30	14.20	114.20	1.20	899.20	1006.70	92.30	42.80	4.90	2.60
128	D8372	0.00	2.80	0.00	0.10	100.20	1.20	992.80	1285.30	93.70	38.50	0.20	0.00
129	D8409	0.00	7.80	1.30	7.90	139.50	1.30	613.20	1200.70	90.00	43.70	1.20	1.10
130	D8497	36.10	7.90	2.20	19.70	141.70	1.50	557.90	1326.40	87.40	47.60	4.20	2.40
131	D8531	0.00	12.10	3.00	37.50	139.20	1.00	899.30	1007.60	92.20	40.70	3.00	1.50
132	D8553	40.10	7.30	0.90	19.30	140.40	0.80	998.90	1210.70	89.50	50.60	0.70	1.60

Key:

Mnlsing Surf = Mineralising Surface (dLS/sLS) %
Erod Surf = Eroded Surface (ES/BS) %
Ostc Surf = Osteoclast Surface (OcS/BS) %
Ostc No = Osteoclast Number (Noc/TVt) / mm2 x10-2
Trab Thk = Trabecular Thickness (um)
Trab No = Trabecular Number /mm3

Cort Thk = Cortical Thickness (um)
Cort Vol = Cortical Volume (CV/TVc) %
Cort Wll Thk = Cortical Wall Thickness (um)
Ostc No = Osteoclast Number (Noc/TVc) /mm2 x10-2
Subcort Ostc = Subcortical Osteoclasts (Noc/BSs) /mm x10-2

Appendix 1c - Sample Cases Histomorphometric Data (3)

General Details		Dynamic Histomorphometric Measuremtents		
No.	Biopsy No.	Trab App Rate	Cort App Rate	Bone Form Rate
1	C9758	0.23	0.31	10.00
2	D0069	0.27	0.35	9.40
3	D0603	0.00	1.45	0.00
4	D0733	0.35	0.42	9.30
5	D1156	0.00	0.00	0.00
6	D1364	0.11	0.21	8.80
7	D1417	0.04	0.02	2.10
8	D1418	0.11	0.11	8.50
9	D1637	0.68	0.69	14.90
10	D1860	0.00	3.10	0.00
11	D2060	0.33	0.41	10.90
12	D2075	0.70	0.78	34.60
13	D2086	0.23	0.18	7.80
14	D2147	0.10	0.10	0.10
15	D2197	0.40	0.40	6.80
16	D2274	0.21	0.27	8.20
17	D2337	0.00	0.00	0.00
18	D2338	0.10	0.10	0.10
19	D2375	0.32	0.55	11.30
20	D2450	0.75	0.88	20.20
21	D2532	0.13	0.94	8.90
22	D2547	0.58	0.60	18.90
23	D2550	0.40	0.40	9.40
24	D2621	0.58	0.54	20.40
25	D2677	0.34	0.45	12.60
26	D2685	0.24	0.36	9.40
27	D2775	0.43	0.45	15.90
28	D2791	0.41	0.39	13.70
29	D2862	0.05	0.10	2.60

Appendix 1c - Sample Cases Histomorphometric Data (3)

General Details		Dynamic Histomorphometric Measuremtents		
No.	Biopsy No.	Trab App Rate	Cort App Rate	Bone Form Rate
30	D2935	0.21	0.19	11.20
31	D2936	0.21	0.53	5.90
32	D2963	0.38	0.41	13.80
33	D2964	0.32	0.35	13.60
34	D2972	0.03	0.12	1.79
35	D2981	0.57	0.54	17.20
36	D3005	0.24	0.30	8.10
37	D3068	0.11	0.23	7.50
38	D3127	0.28	0.27	13.20
39	D3191	0.33	0.33	11.40
40	D3203	0.06	0.13	2.40
41	D3216	0.36	0.35	14.30
42	D3237	0.20	0.10	8.10
43	D3339	0.42	0.42	10.50
44	D3375	0.32	0.41	10.50
45	D3445	0.26	0.32	8.40
46	D3451	0.49	0.53	14.30
47	D3502	0.18	0.20	4.70
48	D3565	0.35	0.39	12.90
49	D3606	0.00	0.00	0.00
50	D3727	0.10	0.10	2.10
51	D3882	0.01	0.01	0.01
52	D4015	0.43	0.49	24.10
53	D4038	0.42	0.54	16.50
54	D4246	0.25	0.31	10.00
55	D4581	0.58	0.58	20.70
56	D4587	0.51	0.50	14.80
57	D4615	0.31	0.33	11.80

Appendix 1c - Sample Cases Histomorphometric Data (3)

General Details		Dynamic Histomorphometric Measuremtents		
No.	Biopsy No.	Trab App Rate	Cort App Rate	Bone Form Rate
58	D4681	0.39	0.51	11.70
59	D4695	0.33	0.29	12.70
60	D4764	0.30	0.29	10.10
61	D4768	0.44	0.32	15.20
62	D4875	0.00	0.00	0.00
63	D4935	0.21	0.33	9.40
64	D4970	0.27	0.33	11.50
65	D4996	0.38	0.61	14.20
66	D5055	0.21	0.16	8.60
67	D5085	0.33	0.31	9.50
68	D5093	0.59	0.61	12.00
69	D5094	0.31	0.28	11.10
70	D5256	0.09	0.08	2.60
71	D5262	0.27	0.38	14.90
72	D5400	0.24	0.25	7.90
73	D5451	0.11	0.16	7.10
74	D5483	0.23	0.30	10.60
75	D5505	0.22	0.31	10.10
76	D5566	0.40	0.43	11.50
77	D5768	0.31	0.38	12.10
78	D5774	0.40	0.57	13.60
79	D5888	0.34	0.45	42.10
80	D5918	0.00	0.00	0.00
81	D6025	0.00	0.00	0.00
82	D6096	0.54	0.69	20.80
83	D6105	0.23	0.17	8.20
84	D6208	0.33	0.33	15.00
85	D6211	0.00	0.00	1.00

Appendix 1c - Sample Cases Histomorphometric Data (3)

General Details		Dynamic Histomorphometric Measuremtents		
No.	Biopsy No.	Trab App Rate	Cort App Rate	Bone Form Rate
86	D6231	0.30	0.31	10.60
87	D6309	0.61	0.62	15.80
88	D6355	0.32	0.45	17.80
89	D6374	0.42	0.55	16.00
90	D6473	0.23	0.49	10.10
91	D6507	0.00	0.13	0.00
92	D6508	0.34	0.35	14.00
93	D6557	0.00	0.00	0.00
94	D6640	0.65	0.68	9.50
95	D6671	0.00	0.00	0.00
96	D6744	0.35	0.38	8.50
97	D6780	0.30	0.31	11.10
98	D6789	0.28	0.47	10.70
99	D6834	0.47	0.51	17.90
100	D6931	0.44	0.51	15.90
101	D7006	0.00	0.00	0.00
102	D7007	0.58	0.62	24.80
103	D7022	0.27	0.19	8.70
104	D7116	0.54	0.68	13.90
105	D7215	0.66	0.79	15.20
106	D7262	0.00	0.00	0.00
107	D7264	0.72	0.81	27.10
108	D7291	0.00	0.00	0.00
109	D7321	0.38	0.44	7.80
110	D7358	0.00	0.00	0.00
111	D7451	0.00	0.00	0.00
112	D7538	0.00	0.00	0.00
113	D7578	0.12	0.13	2.51

Appendix 1c - Sample Cases Histomorphometric Data (3)

General Details		Dynamic Histomorphometric Measuremtents		
No.	Biopsy No.	Trab App Rate	Cort App Rate	Bone Form Rate
114	D7604	0.29	0.26	8.40
115	D7618	0.41	0.39	14.20
116	D7637	0.00	0.00	0.00
117	D7663	0.22	0.23	9.10
118	D7691	0.63	0.88	26.10
119	D7755	0.00	0.00	0.00
120	D7829	0.25	0.30	7.10
121	D7850	0.48	0.44	16.20
122	D7857	0.00	0.00	0.00
123	D8025	0.11	0.21	4.70
124	D8069	0.00	0.00	0.00
125	D8089	0.00	0.00	0.00
126	D8219	0.00	0.60	0.00
127	D8300	0.34	0.38	13.00
128	D8372	0.00	0.00	0.00
129	D8409	0.00	0.00	0.00
130	D8497	0.34	0.40	8.80
131	D8531	0.00	0.00	0.00
132	D8553	0.28	0.33	9.90

Key: Trab App Rate = Trabecular Appositional Rate (um/day)
Cort App Rate = Cortical Appositional Rate (um/day)
Bone Form Rate = Bone Formation Rate (BFR/BVt [dLS+1/2sLS]) % /year

Appendix 1d - Normal Control Adipocytic / Haematopoietic Ratios

General Details			Raw Data						Ratios (Adipocytic/Total)			
			Meas. 1		Meas. 2		Meas. 3					
No.	Biopsy No.	Slide No.	Adip A	Tot A	Adip A	Tot A	Adip A	Tot A	Meas. 1	Meas. 2	Meas. 3	Av Ratio
			mm^2	mm^2	mm^2	mm^2	mm^2	mm^2				%
1	D1540	90/0887	0.19	0.88	0.18	0.78	0.20	0.81	0.22	0.23	0.25	23.12
2	D2602	91/0289	0.20	0.51	0.06	0.21	0.11	0.23	0.39	0.29	0.48	38.54
3	D3431	91/1958	0.24	0.76	0.19	0.65	0.23	0.71	0.32	0.29	0.32	31.07
4	D4476	92/1539	0.15	0.88	0.17	0.78	0.12	0.53	0.17	0.22	0.23	20.49
5	D5120	93/0779	0.23	0.70	0.21	0.64	0.11	0.38	0.33	0.33	0.29	31.54
6	D5139	93/0816	0.03	0.52	0.14	0.95	0.08	0.61	0.07	0.15	0.12	11.24
7	D5334	93/1291	0.04	0.49	0.07	0.34	0.05	0.37	0.08	0.21	0.13	14.08
8	D5490	93/1808	0.16	0.66	0.14	0.77	0.12	0.65	0.24	0.18	0.18	20.30
9	D8899	96/0403	0.06	0.67	0.08	0.71	0.11	0.82	0.10	0.11	0.13	11.41
										Overall Ratio		22.42

Key Adip A=Adipocytic Area; Tot A=Total Area; Meas. x=Measurement x; Av Ratio=Average Ratio

Appendix 1e - Sample Adipocytic / Haematopoietic Ratios

General Details			Raw Data						Ratios (Adipocytic/Total)			
			Meas. 1		Meas. 2		Meas. 3					
No.	Biopsy No.	Slide No.	Adip A	Tot A	Adip A	Tot A	Adip A	Tot A	Meas. 1	Meas. 2	Meas. 3	Av Ratio
			mm^2	mm^2	mm^2	mm^2	mm^2	mm^2				%
1	C9758	91/0283	0.35	1.33	0.45	0.95	0.26	1.11	0.26	0.47	0.23	32.37
2	D0069	94/1091	0.41	0.84	0.54	1.07	1.35	2.13	0.49	0.50	0.63	54.22
3	D0603	93/2471	0.91	1.36	1.37	2.10	1.27	1.79	0.67	0.65	0.71	67.70
4	D0733	92/2049	0.31	1.32	0.47	1.13	0.17	0.68	0.23	0.42	0.25	30.03
5	D1156	N/A	0.35	1.01	0.18	0.75	0.46	1.62	0.35	0.24	0.28	29.02
6	D1364	95/0726	0.92	1.70	0.91	1.77	1.85	3.20	0.54	0.51	0.58	54.45
7	D1417	91/0820	1.23	1.89	0.99	1.75	1.46	2.41	0.65	0.57	0.61	60.74
8	D1418	91/0691	1.16	2.25	1.13	1.88	1.73	2.69	0.52	0.60	0.64	58.66
9	D1637	92/1768	0.73	1.62	0.79	1.58	0.92	1.41	0.45	0.50	0.65	53.44
10	D1860	94/0193	1.56	2.33	0.84	1.77	1.39	2.50	0.67	0.47	0.56	56.67
11	D2060	92/2197	1.22	2.33	1.36	2.56	0.99	1.88	0.52	0.53	0.53	52.72
12	D2075	90/2046	0.94	2.61	0.95	2.02	1.04	2.38	0.36	0.47	0.44	42.25
13	D2086	92/2179	1.16	2.40	1.35	2.46	1.15	2.38	0.48	0.55	0.48	50.51
14	D2147	92/0664	0.56	1.20	0.90	1.63	1.13	2.06	0.47	0.55	0.55	52.25
15	D2197	90/2303	1.07	2.10	1.01	1.65	1.76	2.29	0.51	0.61	0.77	63.01
16	D2274	90/2446	1.03	2.53	0.99	1.50	1.74	2.22	0.41	0.66	0.78	61.70
17	D2337	92/2518	0.75	1.37	0.75	1.74	1.54	2.44	0.55	0.43	0.63	53.65
18	D2338	92/2517	0.81	1.58	1.05	2.03	0.93	1.52	0.51	0.52	0.61	54.72
19	D2375	90/2631	1.12	1.81	1.33	2.59	0.82	1.35	0.62	0.51	0.61	57.99
20	D2450	91/0123	0.95	1.84	0.55	2.41	0.57	1.24	0.52	0.23	0.46	40.14
21	D2532	91/0173	0.66	1.64	0.71	1.93	0.64	1.79	0.40	0.37	0.36	37.60
22	D2547	91/1506	0.25	1.59	0.23	1.30	0.41	2.56	0.16	0.18	0.16	16.48
23	D2550	91/0200	0.50	0.70	0.63	1.99	0.81	1.22	0.71	0.32	0.66	56.49
24	D2621	91/1568	0.51	1.45	0.39	1.38	0.49	1.10	0.35	0.28	0.45	35.99
25	D2677	91/0422	1.14	2.94	1.02	2.09	1.12	2.50	0.39	0.49	0.45	44.13
26	D2685	91/0437	0.53	2.39	0.70	1.53	0.66	2.04	0.22	0.46	0.32	33.43
27	D2775	91/0587	0.32	1.76	0.38	1.46	1.08	1.67	0.18	0.26	0.65	36.29

Appendix 1e - Sample Adipocytic / Haematopoietic Ratios

General Details			Raw Data						Ratios (Adipocytic/Total)			
			Meas. 1		Meas. 2		Meas. 3					
No.	Biopsy No.	Slide No.	Adip A mm^2	Tot A mm^2	Adip A mm^2	Tot A mm^2	Adip A mm^2	Tot A mm^2	Meas. 1	Meas. 2	Meas. 3	Av Ratio %
28	D2791	91/0726	1.19	2.13	0.67	1.86	1.30	2.65	0.56	0.36	0.49	46.98
29	D2862	91/0772	0.55	2.55	0.47	2.08	0.82	2.47	0.22	0.23	0.33	25.79
30	D2935	91/0955	0.84	1.62	0.74	0.97	0.79	1.04	0.52	0.76	0.76	68.03
31	D2936	93/2049	1.54	2.05	1.13	1.94	1.64	2.46	0.75	0.58	0.67	66.68
32	D2963	91/0997	0.71	1.52	0.54	2.04	0.96	2.47	0.47	0.26	0.39	37.35
33	D2964	91/0998	0.52	1.27	0.38	1.30	0.86	1.65	0.41	0.29	0.52	40.77
34	D2972	91/1099	1.03	2.24	0.99	2.17	1.47	2.48	0.46	0.46	0.59	50.29
35	D2981	91/1024	1.31	2.48	0.83	1.87	0.90	1.39	0.53	0.44	0.65	53.99
36	D3005	91/1069	0.55	1.60	0.61	1.71	0.52	1.48	0.34	0.36	0.35	35.06
37	D3068	91/1177	0.95	2.22	0.55	1.58	0.64	1.62	0.43	0.35	0.40	39.04
38	D3127	91/1332	0.56	2.09	0.73	1.96	0.50	1.99	0.27	0.37	0.25	29.72
39	D3191	91/1530	0.68	1.35	0.90	1.66	1.11	2.36	0.50	0.54	0.47	50.54
40	D3203	91/1751	0.86	2.02	0.84	2.31	0.85	2.05	0.43	0.36	0.41	40.13
41	D3216	91/1556	1.61	3.07	0.57	1.37	1.55	2.83	0.52	0.42	0.55	49.61
42	D3237	91/1539	0.64	2.46	0.64	2.09	1.00	2.69	0.26	0.31	0.37	31.27
43	D3339	91/1861	0.43	2.50	0.45	2.32	0.30	1.99	0.17	0.19	0.15	17.22
44	D3375	91/1974	0.61	1.42	0.57	1.26	0.91	1.71	0.43	0.45	0.53	47.14
45	D3445	91/1984	0.41	2.28	0.37	1.99	0.65	1.87	0.18	0.19	0.35	23.78
46	D3451	91/1993	1.25	2.54	0.77	1.79	1.36	2.26	0.49	0.43	0.60	50.80
47	D3502	91/2144	0.41	2.26	0.27	1.00	0.32	1.74	0.18	0.27	0.18	21.18
48	D3565	91/2179	0.54	2.17	1.01	2.44	0.84	2.72	0.25	0.41	0.31	32.39
49	D3606	91/2267	0.58	1.14	1.45	2.45	0.69	1.08	0.51	0.59	0.64	57.98
50	D3727	94/0051	1.05	2.07	1.38	2.32	1.81	2.71	0.51	0.59	0.67	59.00
51	D3882	92/0588	0.48	0.61	0.40	0.80	0.67	0.94	0.79	0.50	0.71	66.66
52	D4015	92/0685	0.09	0.47	0.29	0.54	0.23	0.71	0.19	0.54	0.32	35.08
53	D4038	92/0848	0.33	0.90	0.66	1.32	0.48	1.13	0.37	0.50	0.42	43.05
54	D4246	92/1098	0.57	1.42	0.53	1.24	0.96	2.13	0.40	0.43	0.45	42.65

Appendix 1e - Sample Adipocytic / Haematopoietic Ratios

General Details			Raw Data						Ratios (Adipocytic/Total)			
			Meas. 1		Meas. 2		Meas. 3					
No.	Biopsy No.	Slide No.	Adip A mm^2	Tot A mm^2	Adip A mm^2	Tot A mm^2	Adip A mm^2	Tot A mm^2	Meas. 1	Meas. 2	Meas. 3	Av Ratio %
55	D4581	92/1882	0.12	0.60	0.20	0.64	0.24	0.73	0.20	0.31	0.33	28.04
56	D4587	92/1903	0.44	1.22	0.41	0.77	0.37	1.22	0.36	0.53	0.30	39.88
57	D4615	92/1970	0.27	1.11	0.34	0.87	0.47	1.64	0.24	0.39	0.29	30.69
58	D4681	92/2087	0.39	0.97	0.24	0.52	0.38	0.94	0.40	0.46	0.40	42.26
59	D4695	92/2120	0.19	1.05	0.18	0.71	0.12	0.50	0.18	0.25	0.24	22.48
60	D4764	92/2266	0.39	1.01	0.42	1.04	0.28	0.76	0.39	0.40	0.37	38.61
61	D4768	92/2289	0.20	0.57	0.21	0.60	1.03	1.17	0.35	0.35	0.88	52.71
62	D4875	93/0199	0.57	0.95	0.96	1.16	0.84	1.24	0.60	0.83	0.68	70.17
63	D4935	93/0209	0.46	1.26	0.19	0.85	0.30	0.74	0.37	0.22	0.41	33.13
64	D4970	93/0389	0.94	1.40	0.38	0.96	0.29	0.80	0.67	0.40	0.36	47.66
65	D4996	93/0528	0.39	1.09	0.38	0.76	0.32	0.77	0.36	0.50	0.42	42.45
66	D5055	93/0602	0.93	1.68	0.69	1.10	0.87	1.51	0.55	0.63	0.58	58.57
67	D5085	93/0686	0.41	0.99	0.32	0.66	0.52	0.97	0.41	0.48	0.54	47.84
68	D5093	93/0848	0.60	1.41	0.50	0.89	0.44	1.04	0.43	0.56	0.42	47.01
69	D5094	93/0838	0.23	0.55	0.39	1.14	0.23	0.66	0.42	0.34	0.35	36.96
70	D5256	93/1136	0.35	0.79	0.39	0.83	0.37	0.68	0.44	0.47	0.54	48.57
71	D5262	93/1150	0.23	1.14	0.29	1.06	0.65	1.26	0.20	0.27	0.52	33.04
72	D5400	93/1431	0.54	1.13	0.64	1.13	0.52	1.21	0.48	0.57	0.43	49.13
73	D5451	93/1524	0.71	1.29	0.59	1.24	0.57	1.13	0.55	0.48	0.50	51.02
74	D5483	93/1845	0.63	1.29	0.45	0.99	0.82	1.49	0.49	0.45	0.55	49.78
75	D5505	93/1670	0.44	1.33	0.39	0.82	0.77	1.17	0.33	0.48	0.66	48.82
76	D5566	93/1841	0.34	0.68	0.54	1.21	0.37	0.90	0.50	0.45	0.41	45.25
77	D5768	93/2204	0.57	1.11	0.57	1.19	0.69	1.48	0.51	0.48	0.47	48.62
78	D5774	93/2217	0.23	0.69	0.36	0.95	0.31	0.83	0.33	0.38	0.37	36.19
79	D5888	93/2436	0.39	1.41	0.35	1.40	0.49	1.64	0.28	0.25	0.30	27.51
80	D5918	93/2483	0.21	0.74	0.27	1.03	0.25	0.72	0.28	0.26	0.35	29.77
81	D6025	93/2866	0.27	0.81	0.46	1.34	0.42	0.80	0.33	0.34	0.53	40.05

Appendix 1e - Sample Adipocytic / Haematopoietic Ratios

General Details			Raw Data						Ratios (Adipocytic/Total)			
			Meas. 1		Meas. 2		Meas. 3					
No.	Biopsy No.	Slide No.	Adip A mm^2	Tot A mm^2	Adip A mm^2	Tot A mm^2	Adip A mm^2	Tot A mm^2	Meas. 1	Meas. 2	Meas. 3	Av Ratio %
82	D6096	93/3032	0.77	1.17	0.17	0.46	0.36	0.65	0.66	0.37	0.55	52.72
83	D6105	93/2946	0.55	1.22	0.48	1.06	0.53	1.03	0.45	0.45	0.51	47.27
84	D6208	94/0043	0.41	0.85	0.51	1.25	0.84	1.19	0.48	0.41	0.71	53.21
85	D6211	94/0135	0.35	0.72	0.40	0.89	0.51	0.87	0.49	0.45	0.59	50.73
86	D6231	94/0169	0.33	1.14	0.35	0.70	0.96	1.70	0.29	0.50	0.56	45.14
87	D6309	94/0308	0.30	1.13	0.37	1.02	0.60	1.05	0.27	0.36	0.57	39.99
88	D6355	94/0370	0.40	1.04	0.56	1.30	0.36	0.72	0.38	0.43	0.50	43.85
89	D6374	94/0309	0.31	0.84	0.59	1.11	0.67	1.04	0.37	0.53	0.64	51.49
90	D6473	94/0487	0.19	0.79	0.59	1.22	0.33	0.87	0.24	0.48	0.38	36.78
91	D6507	94/0574	0.33	0.83	0.25	0.78	0.42	0.87	0.40	0.32	0.48	40.03
92	D6508	94/0575	0.23	0.78	0.34	0.92	0.35	0.91	0.29	0.37	0.38	34.97
93	D6557	94/0664	0.21	1.15	0.29	0.95	0.10	0.56	0.18	0.31	0.18	22.21
94	D6640	94/0783	0.22	1.23	0.38	1.79	0.20	0.87	0.18	0.21	0.23	20.70
95	D6671	94/0840	0.49	1.08	0.35	0.94	0.44	1.23	0.45	0.37	0.36	39.46
96	D6744	94/1096	0.25	0.75	0.27	0.42	0.30	0.80	0.33	0.64	0.38	45.04
97	D6780	94/1044	0.40	0.75	0.59	1.22	0.99	1.80	0.53	0.48	0.55	52.23
98	D6789	94/1063	0.27	0.75	0.24	0.73	0.69	1.14	0.36	0.33	0.61	43.13
99	D6834	94/1149	0.12	0.47	0.13	0.50	0.05	0.25	0.26	0.26	0.20	23.84
100	D6931	94/1342	0.09	0.40	0.39	1.07	0.29	0.91	0.23	0.36	0.32	30.27
101	D7006	94/1623	0.16	0.46	0.20	0.84	0.22	0.62	0.35	0.24	0.35	31.36
102	D7007	94/1620	0.42	0.82	0.36	0.67	0.20	0.74	0.51	0.54	0.27	43.99
103	D7022	94/1655	0.49	1.20	0.40	1.20	0.62	1.23	0.41	0.33	0.50	41.52
104	D7116	94/1756	0.08	0.81	0.29	0.87	0.42	1.36	0.10	0.33	0.31	24.70
105	D7215	94/1945	0.37	0.71	0.22	0.59	0.38	0.91	0.52	0.37	0.42	43.72
106	D7262	94/2048	0.23	1.37	0.20	0.65	0.44	1.17	0.17	0.31	0.38	28.39
107	D7264	94/2052	0.24	0.96	0.14	0.51	0.14	0.56	0.25	0.27	0.25	25.82
108	D7291	94/2105	0.27	0.51	0.17	0.56	0.84	1.70	0.53	0.30	0.49	44.24

Appendix 1e - Sample Adipocytic / Haematopoietic Ratios

General Details			Raw Data						Ratios (Adipocytic/Total)			
			Meas. 1		Meas. 2		Meas. 3					
No.	Biopsy No.	Slide No.	Adip A mm^2	Tot A mm^2	Adip A mm^2	Tot A mm^2	Adip A mm^2	Tot A mm^2	Meas. 1	Meas. 2	Meas. 3	Av Ratio %
109	D7321	94/2156	0.65	1.62	0.20	0.72	0.44	0.96	0.40	0.28	0.46	37.91
110	D7358	94/2226	0.13	0.76	0.25	1.23	0.21	0.58	0.17	0.20	0.36	24.55
111	D7451	95/0017	0.83	2.65	0.32	1.11	1.06	1.76	0.31	0.29	0.60	40.13
112	D7538	95/0261	0.54	0.82	0.32	0.60	0.37	0.73	0.66	0.53	0.51	56.62
113	D7578	95/0257	0.22	0.54	0.32	0.97	0.22	0.70	0.41	0.33	0.31	35.05
114	D7604	95/0317	0.50	0.95	0.85	1.26	0.83	1.31	0.53	0.67	0.63	61.15
115	D7618	95/0460	0.49	1.21	0.57	1.43	0.78	1.74	0.40	0.40	0.45	41.73
116	D7637	95/0381	0.86	1.33	0.64	1.73	1.12	1.29	0.65	0.37	0.87	62.83
117	D7663	95/0418	0.79	1.15	0.65	1.30	0.53	1.26	0.69	0.50	0.42	53.59
118	D7691	95/0490	0.11	0.51	0.19	0.87	0.16	0.44	0.22	0.22	0.36	26.59
119	D7755	95/0597	0.47	1.70	0.46	1.42	0.29	0.84	0.28	0.32	0.35	31.52
120	D7829	95/0734	0.95	2.09	0.83	1.62	0.43	1.09	0.45	0.51	0.39	45.38
121	D7850	95/0784	0.29	1.20	0.31	1.09	0.37	1.50	0.24	0.28	0.25	25.76
122	D7857	95/0856	0.35	1.27	0.60	1.11	0.82	1.37	0.28	0.54	0.60	47.16
123	D8025	95/1108	0.47	1.21	0.99	1.34	1.20	2.19	0.39	0.74	0.55	55.84
124	D8069	95/1175	1.07	1.87	0.64	1.54	1.50	2.32	0.57	0.42	0.65	54.48
125	D8089	95/1221	0.76	1.80	0.43	1.34	0.69	1.58	0.42	0.32	0.44	39.33
126	D8219	95/1409	0.44	1.95	0.15	1.31	0.55	2.52	0.23	0.11	0.22	18.61
127	D8300	95/1539	0.28	1.36	0.31	1.45	0.60	1.69	0.21	0.21	0.36	25.82
128	D8372	95/1657	1.06	1.26	1.07	1.21	0.94	1.12	0.84	0.88	0.84	85.50
129	D8409	95/1707	0.42	1.65	0.32	0.60	0.28	0.81	0.25	0.53	0.35	37.79
130	D8497	95/1840	0.37	1.60	0.35	1.54	1.02	2.25	0.23	0.23	0.45	30.40
131	D8531	95/1896	0.89	1.12	1.10	1.58	0.44	0.95	0.79	0.70	0.46	65.13
132	D8553	95/1936	0.61	2.01	0.59	1.78	0.66	2.58	0.30	0.33	0.26	29.69
										Overall Ratio		43.06

Key Adip A=Adipocytic Area; Tot A=Total Area; Meas. x=Measurement x; Av Ratio=Average Ratio

Appendix 1f - Inter-Observer Ratios

General Details Researcher			Raw Data						Ratios (Adipocytic/Total)			
			Meas. 1		Meas. 2		Meas. 3					
No.	iopsy N	Slide No	Adip A	Tot A	Adip A	Tot A	Adip A	Tot A	Meas. 1	Meas. 2	Meas. 3	Av Ratio
			mm^2	mm^2	mm^2	mm^2	mm^2	mm^2				%
1	C9758	91/0283	0.35	1.33	0.45	0.95	0.26	1.11	0.26	0.47	0.23	32.37
2	D0069	94/1091	0.41	0.84	0.54	1.07	1.35	2.13	0.49	0.50	0.63	54.22
3	D0603	93/2471	0.91	1.36	1.37	2.10	1.27	1.79	0.67	0.65	0.71	67.70
4	D0733	92/2049	0.31	1.32	0.47	1.13	0.17	0.68	0.23	0.42	0.25	30.03
5	D1156	N/A	0.35	1.01	0.18	0.75	0.46	1.62	0.35	0.24	0.28	29.02
6	D1364	95/0726	0.92	1.70	0.91	1.77	1.85	3.20	0.54	0.51	0.58	54.45
										Overall Average		44.63

General Details Validator 1			Raw Data						Ratios (Adipocytic/Total)			
			Meas. 1		Meas. 2		Meas. 3					
No.	iopsy N	Slide No	Adip A	Tot A	Adip A	Tot A	Adip A	Tot A	Meas. 1	Meas. 2	Meas. 3	Av Ratio
			mm^2	mm^2	mm^2	mm^2	mm^2	mm^2				%
1	C9758	91/0283	0.44	1.63	0.71	1.65	0.70	2.05	0.27	0.43	0.34	34.72
2	D0069	94/1091	0.80	2.42	1.12	2.17	1.12	1.89	0.33	0.52	0.59	47.98
3	D0603	93/2471	1.51	2.13	2.11	2.54	0.92	1.57	0.71	0.83	0.59	70.85
4	D0733	92/2049	0.38	1.87	0.35	1.70	0.51	1.34	0.20	0.21	0.38	26.32
5	D1156	N/A	0.75	1.31	0.50	1.24	0.20	0.79	0.57	0.40	0.25	40.96
6	D1364	95/0726	0.91	2.09	1.17	2.35	0.58	1.12	0.44	0.50	0.52	48.37
										Overall Average		44.37

Key: Adip A=Adipocytic Area; Tot A=Total Area; Meas. x=Measurement x; Av Ratio=Average Ratio

Appendix 1f - Inter-Observer Ratios

General Details Validator 2			Raw Data						Ratios (Adipocytic/Total)			
			Meas. 1		Meas. 2		Meas. 3					
No.	iopsy N	Slide No	Adip A mm^2	Tot A mm^2	Adip A mm^2	Tot A mm^2	Adip A mm^2	Tot A mm^2	Meas. 1	Meas. 2	Meas. 3	Av Ratio %
1	C9758	91/0283	0.19	0.68	0.07	0.20	0.38	0.85	0.28	0.35	0.45	35.88
2	D0069	94/1091	0.39	1.16	0.24	0.52	0.62	1.24	0.34	0.46	0.50	43.26
3	D0603	93/2471	0.49	0.84	1.63	2.60	0.37	0.75	0.58	0.63	0.49	56.79
4	D0733	92/2049	0.18	0.48	0.44	1.07	0.28	1.26	0.38	0.41	0.22	33.61
5	D1156	N/A	0.40	0.68	0.23	0.52	0.16	0.49	0.59	0.44	0.33	45.24
6	D1364	95/0726	0.07	0.20	0.97	1.91	0.94	2.11	0.37	0.51	0.45	44.11
										Overall Average		43.15

Key: Adip A=Adipocytic Area; Tot A=Total Area; Meas. x=Measurement x; Av Ratio=Average Ratio

Appendix 1g - Inter-Observer Ratios Correlation

Ratio Diff : (Researcher - Validator1)				Corr	Ratio Diff : (Researcher - Validator2)				Corr
Meas. 1	Meas. 2	Meas. 3	Ov Diff		Meas. 1	Meas. 2	Meas. 3	Ov Diff	
-0.01	0.04	-0.11	-0.02	0.84	-0.02	0.12	-0.21	-0.04	-0.20
0.16	-0.01	0.04	0.06	0.79	0.15	0.04	0.13	0.11	0.75
-0.04	-0.18	0.12	-0.03	-0.97	0.09	0.03	0.22	0.11	-1.00
0.03	0.21	-0.13	0.04	-0.42	-0.14	0.00	0.03	-0.04	0.59
-0.23	-0.16	0.03	-0.12	0.61	-0.24	-0.20	-0.04	-0.16	0.64
0.11	0.02	0.06	0.06	0.32	0.17	0.01	0.13	0.10	-0.37
			Ov Corr	0.90				Ov Corr	0.78

Key: Meas. x=Measurement x; Ov Diff=Overall Difference; Corr=Correlation; Ov Corr=Overall Correlation

Note: Overall Correlation indicates correlation of Researchers' Average Ratios with Validators Average Ratios.

Appendix 1h - Intra-Observer Ratios

General Details 1st Measurement			Raw Data						Ratios (Adipocytic/Total)			
			Meas. 1		Meas. 2		Meas. 3					
No.	Biopsy	Slide No	Adip A mm^2	Tot A mm^2	Adip A mm^2	Tot A mm^2	Adip A mm^2	Tot A mm^2	Meas. 1	Meas. 2	Meas. 3	Av Ratio %
1	C9758	91/0283	0.35	1.33	0.45	0.95	0.26	1.11	0.26	0.47	0.23	32.37
2	D0069	94/1091	0.41	0.84	0.54	1.07	1.35	2.13	0.49	0.50	0.63	54.22
3	D0603	93/2471	0.91	1.36	1.37	2.10	1.27	1.79	0.67	0.65	0.71	67.70
4	D0733	92/2049	0.31	1.32	0.47	1.13	0.17	0.68	0.23	0.42	0.25	30.03
5	D1156	N/A	0.35	1.01	0.18	0.75	0.46	1.62	0.35	0.24	0.28	29.02
6	D1364	95/0726	0.92	1.70	0.91	1.77	1.85	3.20	0.54	0.51	0.58	54.45
										Overall Average		44.63

General Details 2nd Measurement			Raw Data						Ratios (Adipocytic/Total)			
			Meas. 1		Meas. 2		Meas. 3					
No.	Biopsy	Slide No	Adip A mm^2	Tot A mm^2	Adip A mm^2	Tot A mm^2	Adip A mm^2	Tot A mm^2	Meas. 1	Meas. 2	Meas. 3	Av Ratio %
1	C9758	91/0283	0.44	1.26	0.39	0.89	0.31	1.19	0.35	0.44	0.26	34.93
2	D0069	94/1091	0.56	1.05	0.64	1.23	0.85	1.48	0.53	0.52	0.57	54.27
3	D0603	93/2471	0.99	1.84	1.40	2.03	1.36	1.68	0.54	0.69	0.81	67.91
4	D0733	92/2049	0.34	1.22	0.76	1.53	0.33	0.94	0.28	0.50	0.35	37.55
5	D1156	N/A	0.31	0.96	0.28	1.02	0.40	1.26	0.32	0.27	0.32	30.50
6	D1364	95/0726	0.61	1.19	0.80	1.37	1.63	2.38	0.51	0.58	0.68	59.38
										Overall Average		47.42

Key: Adip A=Adipocytic Area; Tot A=Total Area; Meas. *x*=Measurement *x*;

Appendix 1i - Intra-Observer Ratios Correlation

Ratio Diff : (1st Measurement - 2nd Measurement)				
Meas. 1	Meas. 2	Meas. 3	Ov Diff	Corr
-0.09	0.04	-0.03	-0.03	0.92
-0.05	-0.02	0.06	0.00	0.94
0.13	-0.04	-0.10	0.00	0.64
-0.04	-0.08	-0.10	-0.08	0.97
0.02	-0.03	-0.03	-0.01	0.87
0.03	-0.07	-0.11	-0.05	0.65
			Ov Corr	0.99

Key: Meas. *x*=Measurement *x*; Ov Diff=Overall Difference; Corr=Correlation; Ov Corr=Overall Correlation

Note: Overall Correlation indicates correlation of Researchers' Average Ratios for the 1st Measurements with Average Ratios for the 2nd Measurement.

Appendix 2 Sequence data and gene homology alignments

For each of the genes isolated the nucleotide sequences derived by sequencing are shown below. The overlaps of the derived nucleotide sequences with those of the homologous sequences in the BLAST nucleotide database are given, together with the length of, and the percentage homology over, the overlaps. The points at which the derived sequences differ from those in the BLAST database are underlined in bold. The accession number for each gene is underlined in plain text.

Forward and reverse sequences were derived for alpha enolase, 14-3-3 zeta, and aldolase A, and only reverse sequences for non-skeletal tropomyosin, KIAA0081, CGI-120 protein.

Alpha enolase

Forward sequence

81% overlap in 131 base pair segment

gb AF035286

CCAACAGGNGACCAAAGGGTTTGCNTNCCCACAGTTTATTTGGCCAAGGG
GTTTCTGAAGTTCNTGCCGGAAAATTNANCNTTGTNGCCCAGNTCNTTAAAT
TTTGAGGAGCTGGTTGTNCTTGGCCAA

Reverse sequence

100% overlap in 516 base pair segment

emb M14328

ACGGAGATCTCGCCGGCTTTACGTTCACCTCGGTGTCTGCAGCACCCTCCGCTT
CCTCTCCTAGGCGACGAGACCCAGTGGCTAGAAGTTCACCATGTCTATTCTCAA
GATCCATGCCAGGGAGATCTTTGACTCTCGCGGGAATCCCACTGTTGAGGTTG
ATCTCTTCACCTCAAAAGGTCTCTTCAGAGCTGCTGTGCCCAGTGGTGCTTCAA
CTGGTATCTATGAGGCCCTAGAGCTCCGGGACAATGATAAGACTCGCTATATG
GGGAAGGGTGTCTCAAAGGCTGTTGAGCACATCAATAAAACTATTGCGCCTGC
CCTGGTTAGCAAGAACTGAACGTCACAGAACAAGAGAAGATTGACAAACTGA
TGATCGAGATGGATGGAACAGAAAATAAATCTAAGTTTGGTGCGAACGCCATT
CTGGGGGTGTCCCTTGCCGTCGTCAAAGCTGGTGCCG

14-3-3 zeta

Forward sequence

97% overlap in 537 base pair segment

embl U28964

GGCCCCCCCTCGAGACATCCAGGGACAGAGTCTCAGCCTCGCCGCTGCTGCC
GCCGCCGCCGCCCAGAGACTGCTGAGCCCGTCCGTCCGCCGCCACCACCCACT
CCGGACACAGAGCATCCAGTCATGGATAAAAATGAGCTGGTTCAGAAGGCCA
AACTGGCCGAGCAGGCTGAGCGATATGATGACATGGCAGCCTGCATGAAGTCT
GTAACTGAGCAAGGAGCTGAATTATCCAATGAGGAGAGGAATCTTCTCTCAGT
TGCTTATAAAAATGTTGTAGGAGCCCGTAGGTCATCTTGGAGGGTCGTCTCAA
GTATTGAACAAAAGACGGAAGGTGCTGAGAAAAAACAGCAGATGGCTCGAGA
ATACAGAGAGAAAATTGAGACGGGAGCTAAGAGATATCTGCAATGATGTACT
GTCTCTTTTGGAAAAGTTCTTGGATCCCCAATGCTTCACAAGCAGAGAGCAAA
GNCTTCTATTTTGAAAATGAAAGGAGATTACTACCCGTACTTNGCTGAGGTTG
CCG_TGGTGATGACAANAANGGGAT

Reverse sequence

96% overlap in 516 base pair segment

emb U28964

ACCACCCACTCCGGACACAGAACATCCAGTCATGGATAAAAATGAGCTGGTTC
AGAAGGCCAAACTGGCCGAGCAGGCTGAGCGATATGATGACATGGCAGCCTG
CATGAAGTCTGTAACTGAGCAAGGAGCTGAATTATCCAATGAGGAGAGGAATC
TTCTCTCAGTTGCTTATAAAAATGTTGTAGGAGCCCGTAGGTCATCTTGGAGGG
TCGTCTCAAGTATTGAACAAAAGACGGAAGGTGCTGAGAAAAAACAGCAGAT
GGCTCGAGAATACAGAGAGAAAATTGAGACGGAGCTAAGAGATATCTGCAAT
GATGTACTGTCTCTTTTNGGAAAAGTTCTGATCCCCAATGCTTCACAAGCAGAG
AGCAAAGTCTTCTATTTGAAAATGAAANGAGATTACTACCCGTACTTGGCTGA
_GTTGCCCGTGGTGATGACCAAGAAAAGGGATTGGCG_TCAGTCACAACAAG_
GTACCCAGAAGTTTTTGAAATCAGCNAAAAAGGGAATGCAACCAACACATCCT
ATCAGACTGGGTCTGGCCCTTAACTTCTCTGTGTTCTAT

Non-skeletal tropomyosin

Reverse sequence

99% over 548 base pair segment

emb X04588

GAGGAGGCAGGAACCGGAGCGCGAGCAGTAGCTGGGTGGGCACCATGGCTGG
GATCACCACCATCGAGGCGGTGAAGCGCAAGATCCAGGTTCTGCAGCAGCAG
GCAGATGATGCAGAGGAGCGAGCTGAGCGCCTCCAGCGAGAAGTTGAGGGAG
AAAGGCGGGCCCGGGAACAGGCTGAGGCTGAGGTGGCCTCCTTGAACCGTAG
GATCCAGCTGGTTGAAGAAGAGCTGGACCGTGCTCAGGAGCGCCTGGCCACTG
CCCTGCAAAAGCTGGAAGAAGCTGAAAAAGCTGCTGATGAGAGTGAGAGAGG
TATGAAGGTTATTGAAAACCGGGCCTTAAAAGATGAAGAAAAGATGGAACTC
CAGGAAATCCAACTCAAAGAAGCTAAGCACATTGCAGAAGAGGCAGATAGGA
AGTATGAAGAGGTGGCTCGTAAGTTGG
TGAT**T**ATTGAAGGAGACTTGGAACGCACAGAGGAACGAGCTGAGCTGGC
AGAGTCCCGTTG**C**CCGAGAGATGGATGAGCAGATTAGACTGATGGACCAGAA
CCT

KIAA0081

Reverse sequence

99% over 601 base pair segment

dbj D42039

GACGAGTCTACCCCACCTCCCCGGAAGAAGAAGAAGGATATTCGCGATTACAA
TGATGCAACATGGCGCGTCTTCTGGAGCAATGGGAGAAAGATGATGACATTGA
AGAAGGAGATCTTCCAGAGCACAAGAGACCTTCAGCACCTGTCGACTTCTCAA
AGATAGACCAAGCAAGCCTGAAAGCATATTGAAAATGACGAAAAAAGGGAAG
ACTCTCATGATGTTTGTCACTGTATCAGGAAGCCCTACTGAGAAGGAGACAGA
GGAAATTACGAGCCTCTGGCAGGGCAGCCTTTTCAATGCCAACTATGACGTCC
AGAGGTTCATTGTGGGATCAGACCGTGCTATTTCATGCTTCGCGATGGGAGCT
ACGCCTGGGAGATCAAGGACTTTTTGGTCG
GTCAAGCAGGTGTGCTGATGTAACTCTGGAGGGCCTGGTGTACCCCGG
AAGGAGGAGGAAGCNAAGAGAAAAAT

Aldolase A

Forward sequence

83% over 149 base pair segment

emb X05236

AGGCNTTNAGGGCAAAGGCCTCCAGGGCTCGGCCCNANNAAAAGGTNAGGGC
CCAGGGNTTAANAGGGGGCNCTTGTTAATGGCATTGNGGTTGANGGC-
CCTCCTCCTTANTTTGGCCTCCAAANAGGAAGGGGATCCCAGGGACA

Reverse sequence

96% over 656 base pair segment

emb X05236

CTTGAATCCACTCGCCAGCCCGCCGCCCTCTGCCGCCGCACCCTGCACACCCGC
CCCTCTCCTGTGCCAGGAACTTGCTACTACCAGCACCATGCCCTACCAATATCC
AGCACTGACCCCGGAGCAGAAGAAGGAGCTGTCTGACATCGCTCACCGCATCG
TGGCACCTGGCAAGGGCATCCTGGCTGCAGATGAGTCCACTGGGAGCATTGCC
AAGCGGCTGCAGTCCATTGGCACCGAGAACACCGAGGAGAACCGGCGCTTCT
ACCGCCAGCTGCTGCTGACAGCTGACGACCGCGTGAACCCCTGCATTGGGGGT
GTCATCCTCTTCCATGAGACACTCTACCAGAAGGCGGATGATGGGCGTCCCTTC
CCCAAGTTATCAAATCCAAGGGCGGTGTTGTGGGCATCAAGGTAGACAAGGGC
GTGGTCCCCCTGGCAGGGACAAATGGCGAGACTACCACCCAAGGGTTGGATGG
GCTGTCTGAGCGCTGTGCCCAGTACAAGAANGACNGAGCTGACTTTNCCAAGT
GGCGGTGTGTGCTTGAAAATTGGGGAACAACCCCCTTAAGCCTTGGCATCATG
GNAAAAGCCAATGTTCTGGNCCGTTATGCCATATTCTGCCACAAAANGGCATT
GTGNCCATCGNGGAG

CGI-120 protein

Reverse sequence

97% over 666 base pair segment

gb AF151878

GGCAAGATGGAGGCGCTGATTTTGGAACCTTCCCTGTATACTGTCAAAGCCAT
CCTGATTCTGGACAATGATGGAGATCGACTTTTTGCCAAGTACTATGACGACA
CCTACCCCAGTGTCAAGGAGCAAAAGGCCTTTGAGAAGAACCTTTTCACAAGA
CCCATCGGACTGACAGTGAAATTGCCCTCTTGGAAGGCCTGACAGTGGTATAC
AAAAGCAGTATAGATCTCTATTTCTATGTGATTGGCAGCTCCTATGAAAATGA
GCTGATGCTTATGGCTGTTCTGAACTGTCTCTTCGACTCATTGAGCCAGATGCT
GAGGAAAAATGTAGAAAAGCGAGCACTGCTGGAGAACATGGAGGGGCTGTTC
TTGGCTGTGGATGAAATTGTAGATGGAGGGGTGATCCTAGAGAGTGATCCCCA
GCAAGTGGTACACCGGGTGGCATTAAGGGGTGAAGATGTCCCCCTTACGGAGC
AGACCGTGTCTCAAGTGTGCAGTCAGCCAAAGAACAGATCAAGTGGTCACTCC
TTCGATGAAGACCTCACTGGTCCTGGCTCTTCATCCTCTTCAAAAAATTTGCAT
GTCTGCTGNGAATTTTATCTAATTCCCCAATCGATGCTNTTAAGGGCATCTCGG
GGATCACAAGGATCCNTAAATCTCC