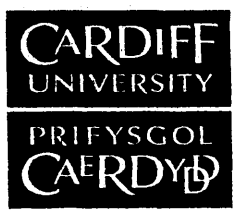

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Bone Grafting:

Tissue Treatment and Osseointegration

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Submitted for

Philosophiae Doctor Degree

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To my husband Dan, who supported me through this project.

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Summary

Bone grafts fill skeletal defects and provide a structure upon which new bone can be deposited. There is no standard method of storing bone prior to grafting, the three main storage regimes being stored fresh frozen at –80°C, gamma irradiated or freeze dried. The initial aim of this project was to determine how osteoblastic cells behaved when exposed to bone treated in the above ways. It was found that sterilisation of bone with gamma irradiation caused cell death in a number of the cells that came into contact with it. Therefore the use of gamma irradiation for grafting is contraindicated, a similar observation was observed for freeze-dried bone whereas cells grew and differentiated on fresh frozen tissue.

The second aim of this study was to develop a system whereby bone marrow cells could be expanded in culture and retain their osteogenic potential so that they would be suitable for either coating a bone graft (thus increasing the rate of osseointegration of the graft) or used alone to treat small bone defects. Rodent bone marrow was used in a variety of cultures and bone formation was induced by either BGJ-b medium or ECCM (Endothelial cell conditioned medium). Control cultures were grown in alpha modification minimum essential medium.

ECCM was overall found to produce a greater number of cells at the end of the incubation periods studied than BGJ-b medium. BGJ-b medium preferentially selected mineralization over cell proliferation under all of the culture conditions studied (monolayers, collagen gels and organ cultures). This medium would be best suited to forming small pieces of bone rapidly from bone marrow, to fill small bone defects such as those seen in the dental field. ECCM produced large numbers of osteogenic cells, which could potentially be used to coat large bone grafts.

Abbreviations

ALP	Alkaline phosphatase
α-MEM	Alpha modification minimum essential medium
ATP	Adenosine triphosphate
BGJ-b	Bigger's medium
β-GP	β–glycerophosphate
BMP	Bone morphogenic proteins
BMPR	Bone morphogenic protein receptor
BMSC	Bone marrow stromal stem cell
BSP/BSPII	Bone sialoprotein
cAMP	Cyclic adenosine monophosphate
CD105	Endoglin
cDNA	Copy deoxyribonucleic acid
COL	Collagen
DBM	Demineralised bone matrix
DCN	Decorin
DNA	Deoxyribonucleic acid
ECCM	Endothelial cell conditioned medium
EDTA	Ethylenediaminetetraacetic acid
FGF	Fibroblast growth factor
FN	Fibronectin
GPI	Glycosyl-phosphatisylinositol
H & E	Haemotoxylin and eosin
HBGF	Heparin binding growth factor

HIV	Human immuno deficiency virus
HLA	Human lymphocyte antigen
HSC	Hematopoietic stem cell
IGF	Insulin-like growth factors
IGFBP	Insulin like growth factor binding protein
IL	Interleukin
LTBMC	Long term bone marrow culture
LTBP	Latent TGF-β binding protein
M-CSF	Macrophage colony stimulating factor
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
ON	Osteonectin
OPG	Osteoprotegerin
OPN	osteopontin
PBS	Phosphate buffered saline
PG	Prostaglandin
PPARγ2	Peroxisome proliferator activated receptor γ2
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related peptide
RANK	Receptor activator of NF-kB
RANK-L	Receptor activator of NF-kB ligand
RGD	Arginine-Glycine-Aspartate
SCS	Single cell suspension
SDS	Sodium dodecylsulphate (SDS)

SEM	Scanning electron microscope
SPARC	Secreted protein, which is acid and rich in cysteine (also called osteonectin)
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TRAF	TNF receptor-associated factor
VDR	Vitamin D receptor
vWF	von Willebrad factor
WPBs	Weibel-Palade bodies

Chapter 1

General Introduction

1.1 Bone Remodelling

Bone is generally considered a very hard and rigid material, however the tissue needs to be constantly renewed in order to maintain its mechanical strength. Two cell types perform this remodelling; osteoclasts that resorb the old calcified matrix and osteoblasts that synthesise new bone matrix. Both cell types interact during this process enabling a continuous turnover of the bone matrix.

In the adult skeleton bone resorption and formation are balanced so that there is no net change in bone mass but a continuous turnover of substance. The remodelling cycle lasts approximately three months; during which time groups of osteoclasts (known as a cutting cone) take about two to three weeks to create a tunnel (up to 1mm in diameter). Osteoblasts then invade the tunnel, filling it in with new bone for the remainder of the period. About one million bone remodelling units are present in the human skeleton at any moment and approximately 3-4 million are initiated per year.

The remodelling sequence begins with osteoclast differentiation; this is ensued by resorption of mature matrix by osteoclasts. The reversal phase then commences; with osteoblast precursors filling the resorption cavity. New osteoblasts assemble only at sites where osteoclasts have recently completed resorption, a phenomenon known as coupling, and bone formation begins to occur while resorption advances. This coupling process is dependent on the release of local factors that are able to stimulate proliferation and/or differentiation of osteoblasts, from the aged matrix, by osteoclasts. Osteoblasts will then synthesise and mineralise new bone matrix. The end result of this process is the replacement of old bone with a new packet of bone in the form of either a cylindrical Haversian system (figure 1.1), or a plate-like hemiosteon [1].

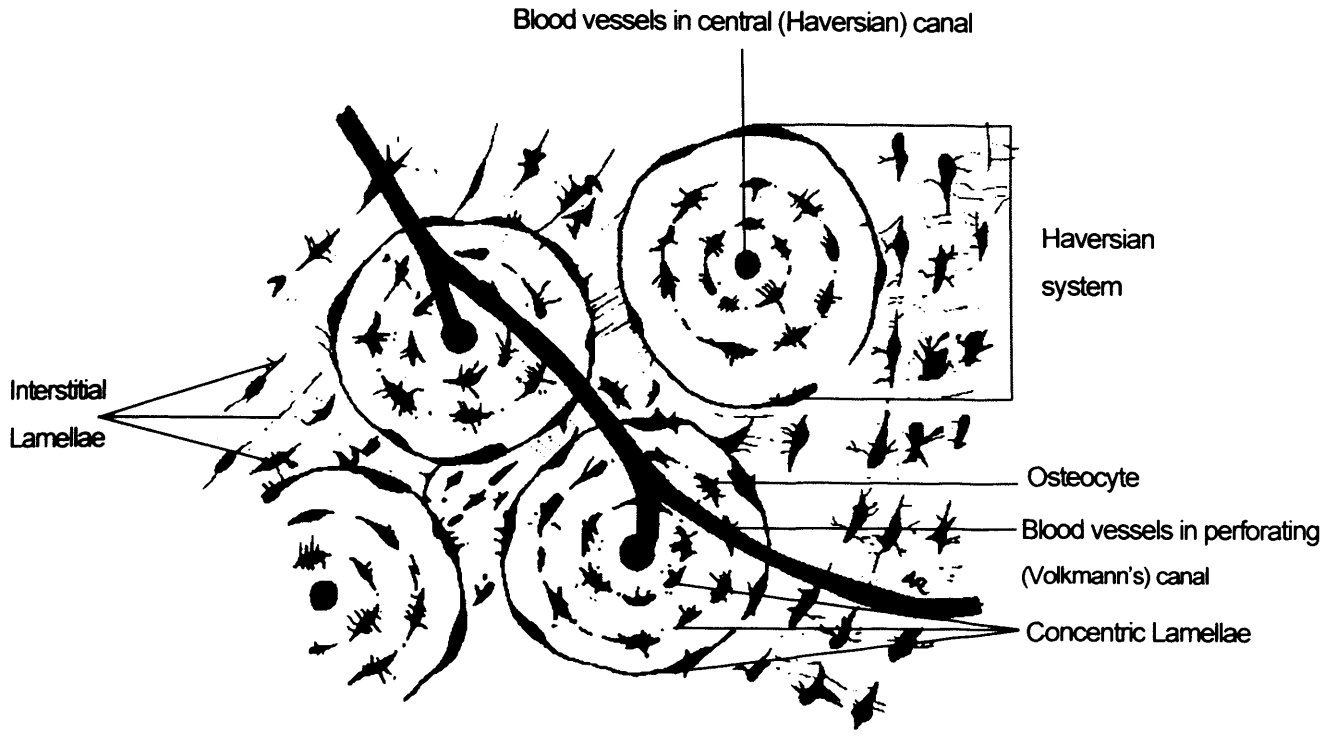


Figure 1.1: Diagram of a cross section of bone showing Haversian systems. Several concentric plates (lamellae) form a cylindrical unit, the Haversian system. A Haversian canal at its middle contains blood vessels and nerves that are fed from the periosteum through Volkmann's canals. Around the central canal are numerous small spaces between the lamellae called lacunae, which contain osteocytes. Interstitial lamellae are fragments of older Haversian systems that have been partially destroyed during bone remodelling. A long bone consists of numerous Haversian systems running parallel to bone length.

The continuous supply of osteoclasts and osteoblasts from their respective cells in the bone marrow is essential for the origination of remodelling units and their progression on the bone surface. Both osteoblasts and osteoclasts are derived from precursors originating in the bone marrow. Osteoblast and osteoclast precursor proliferation and differentiation appear to occur concurrently in remodelling units. The involvement of osteoblasts in osteoclast development has been demonstrated in several culture and co-culture models. In a culture system with bone rudiment, living bone (containing osteoblasts) was found to be essential for the development of osteoclasts [2].

1.1.1 Osteoclast Differentiation

The origin of osteoclasts is haematopoietic, they share a common precursor with the monocyte-macrophage, and macrophage colony stimulating factor (M-CSF) plays an essential role in their differentiation. This was discovered when it was found that osteopetrotic, op/op, mice not only have depleted osteoclast numbers, but also a reduced quantity of macrophages. Osteopetrosis in these mice was not cured by marrow transplantation and was therefore secondary to a defect in the local bone microenvironment and not to a defect in haematopoietic osteoclast precursors. The op/op mice it evolved, failed to release M-CSF due to a mutation in the M-CSF gene [3]; the osteopetrosis being cured by an injection of M-CSF.

The function of M-CSF in osteoclast differentiation has been further demonstrated in *in vitro* experiments, however in this instance stromal cells were required in addition to M-CSF to cause monocytes and splenocytes to differentiate into osteoclasts. This meant that other osteoblastic factors were also involved in osteoclast differentiation [2].

The molecular mechanism of the dependency of osteoclast differentiation on cells of the mesenchymal lineage was elucidated with the discovery of three proteins involved in the tumour necrosis factor (TNF) signalling pathway. The first of these proteins to be discovered was osteoclast differentiating factor, also called RANK ligand (RANK-L). RANK-L, a cytokine released by osteoblasts, is necessary for osteoclast differentiation, in conjunction with M-CSF [3].

Simonet *et al.* [4] discovered that the effect of RANK-L on osteoclast differentiation could be blocked by a secreted disulfide-linked dimeric glycoprotein, called osteoprotegerin (OPG). Consistent with an important role in the regulation of osteoclast formation, administration of OPG to mice or the over expression of its cDNA as seen in OPG transgenic mice, causes osteopetrosis. The protein is also able to prevent ovariectomy-induced osteoporosis [5].

Following on from this, two independent groups discovered that RANK-L was a membrane-bound protein identical to a factor known to enhance the growth of T cells. The ligand expressed in several osteoblastic and stromal cell lines *in vitro*, and in committed preosteoblastic cells *in vivo,* bound to a third protein, the membrane bound receptor RANK, with high affinity. This interaction is essential, in the presence of M-CSF, for complete osteoclastic differentiation *in vitro* [6, 7].

The antiosteoclastogenic property of OPG is due to its ability to act as a decoy by binding to RANK-L and blocking the RANK-ligand/RANK interaction. RANK-L is not only necessary for osteoclast development but it is also responsible for the activation of mature osteoclasts and a reduction in osteoclast apoptosis. The fact that RANK-L is also responsible for lymphocyte differentiation suggests it may have a role in the loss of bone during inflammation, as activated T lymphocyte secrete RANK-L [8].

RANK-L is a member of the tumour necrosis factor family (TNF) while RANK is a member of the TNF receptor super family [9]. This lead to the suggestion that

signalling through RANK should involve the TNF receptor-associated factor TRAF6. The concept was confirmed when mice deficient in TRAF6 were found to have osteopetrosis [10].

As outlined in figure 1.2, RANK-L and OPG production by osteoblasts are regulated by many factors [11]. 1,25- dihydroxyvitamin D, PGE_2 and PTH all elevate the ratio of RANK-L/OPG [12] whereas transforming growth factor β (TGF-β) has the opposite effect [13].

1.1.2 Osteoclast Function

Osteoclasts are highly specialised cells found on the endosteal surfaces of bone, where they function in bone resorption, which is important in the development, growth, maintenance, and repair of bone. Osteoclasts have important functions in repairing fractures, remodelling new bone and integrating bone grafts.

Mature osteoclasts are usually large (50 to 100 μm diameter) cells with abundant mitochondria, numerous lysosomes, and free ribosomes. They are also multinucleated cells, with an average of 10-20 nuclei. Relative to other bone cells, osteoclasts are quite uncommon, with only 2-3 cells found per mm^3 on average.

The osteoclasts' most remarkable morphological feature is the ruffled border, a complex system of finger-shaped projections of the membrane, the function of which is to mediate the resorption of the calcified bone matrix. This structure is completely surrounded by another specialised area, called the clear zone. The clear zone delineates the area of attachment of the osteoclast to the bone surface and seals off a distinct area of bone surface that lies immediately underneath the osteoclast. This region beneath the osteoclast is eventually excavated, and is known as the microcompartment. In order for resorption of the mineralised bone matrix to occur, osteoclasts release into the microcompartment hydrogen ions to solubilise the mineral phase and proteolytic enzymes to degrade the organic matrix [14].

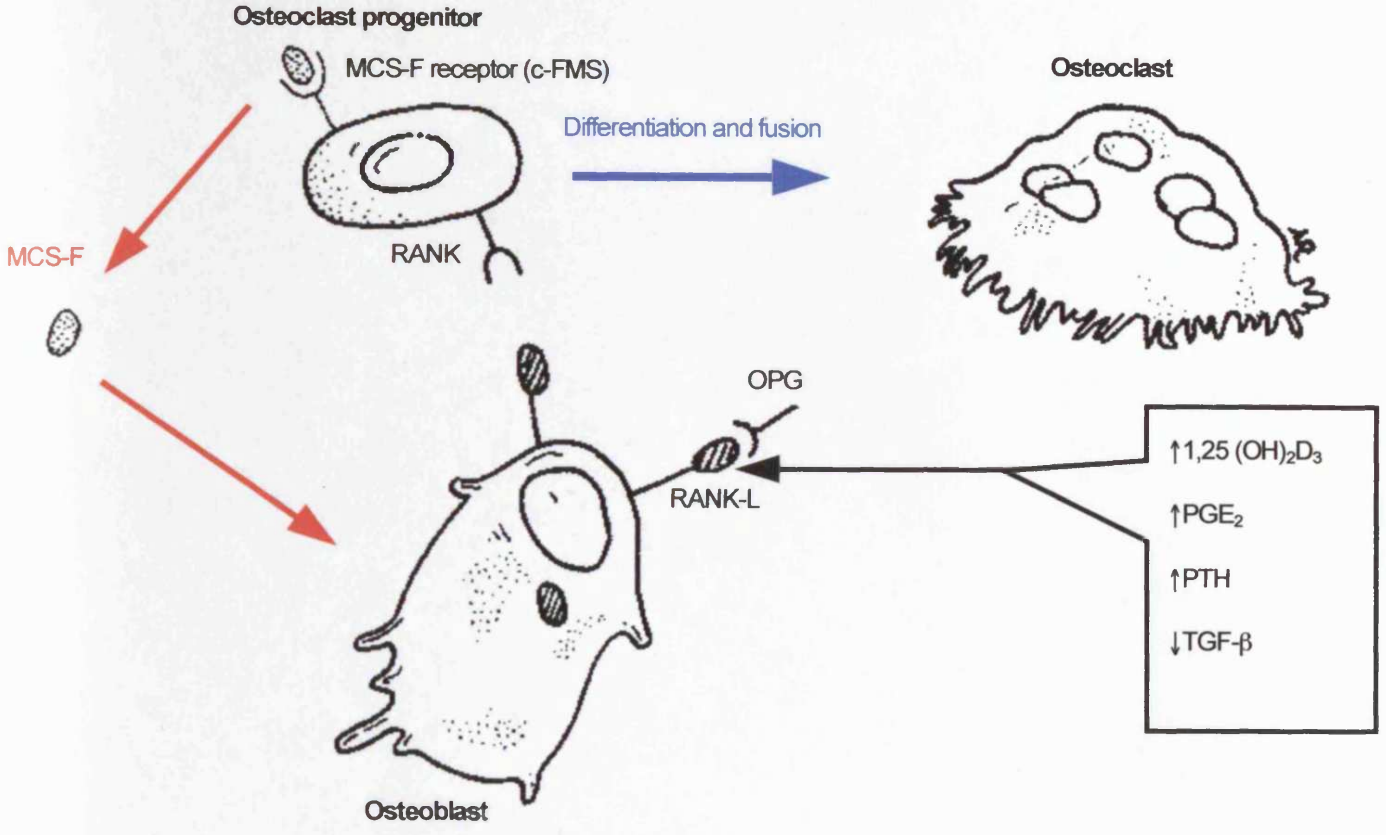


Figure 1.2: The osteoclast differentiation pathway. Rank-L = Rank ligand; OPG = Osteoprotegerin; M-CSF = Macrophage colony stimulating factor.

The mineral component of the matrix is dissolved in the acidic environment of the microcompartment. The low pH in the microcompartment is dependent on proton production by carbonic anhydrase II, whose deficiency induces lack of bone resorption and osteopetrosis [15]; and proton excretion into the compartment by an ATP-driven proton pump (vacuolar H^+ - ATPase). This proton pump located in the ruffled border membrane is also found in all mammalian cells where it is responsible for the acidification of various intracellular compartments [16].

The protein components of the matrix, mainly collagen, are degraded by cathepsins. Ubiquitous cathepsin B, D and L are able to degrade collagen at the low pH present in the microcompartment, and it has been demonstrated that a fourth cathepsin, K, is also responsible for degradation of the bone matrix [17]. Cathepsin K knock out mice have been found to have inhibition of bone resorption and osteopetrosis [18].

Osteoclasts also produce matrix metalloproteinase (MMP) -1 and –9, however their role in bone resorption is unknown since they are inactive at the low pH present in the microcompartment. In bone MMP–9 is localised exclusively in osteoclasts, it is thought that maybe it plays a role in osteoclast migration [4]. Another feature of osteoclasts is the presence of high amounts of the phosphohydrolase enzyme, tartrate-resistant acid phosphatase, released into the microcompartment. Its role in bone resorption is unclear, however the enzyme is known to be a quite specific to osteoclasts, and hence its presence is commonly used for the detection of osteoclasts in bone specimens.

Highly polarised osteoclasts attach to bone during resorption at the sealing zone. The most important integrin responsible for osteoclast attachment in this zone is the vitronectin receptor ($\alpha_v\beta_3$). It has been shown that inhibiting this integrin impairs bone resorption, thus highlighting the importance of osteoclastic attachment in bone resorption [19].

1.1.3 Osteoblast Differentiation

Following bone resorption, for bone formation to take place stem cells (cells that can replicate themselves and generate more specialised cell types as they multiply) residing within the bone marrow stroma are required to first differentiate into osteoprogenitor cells. Eventually these osteoprogenitors differentiate into mature, functional osteoblasts.

There are two major cellular systems associated with bone marrow, the haematopoietic and stromal systems. The stromal tissue forms a network of cells and extracellular matrix that physically supports the haematopoietic cells and influences their differentiation [20], it comprises of osteoblasts and preosteoblasts, fibroblasts, reticular cells and marrow adipocytes.

The concept of stromal stem cells populating the bone marrow stroma, was first proposed by Owen in 1978 [21]. As a working hypothesis for differentiation in the marrow stromal system Owen introduced a scheme analogous to that in the haematopoietic system, where stromal stem cells were believed to yield committed progenitors, each giving rise to a different stromal cell line (Figure 1.3) [21, 22]. Therefore organ-specific stem cells and committed progenitors are expected to exist for each organ, and these give rise to specialised stromal cell lines for the organ concerned.

Confirmation of the presence of stem cells within the marrow came from early *in vivo* studies, which demonstrated that bone marrow cells or fibroblastic cells harvested from confluent *in vitro* cultures of marrow cells produce bone tissue when closed in diffusion chambers and then transplanted intraperitoneally. This was first demonstrated by Friedenstein [23, 24] and later confirmed and extended by other workers. *In vivo* studies have also shown bone marrow cells to produce osteogenic tissue when transplanted under the renal capsule [25].

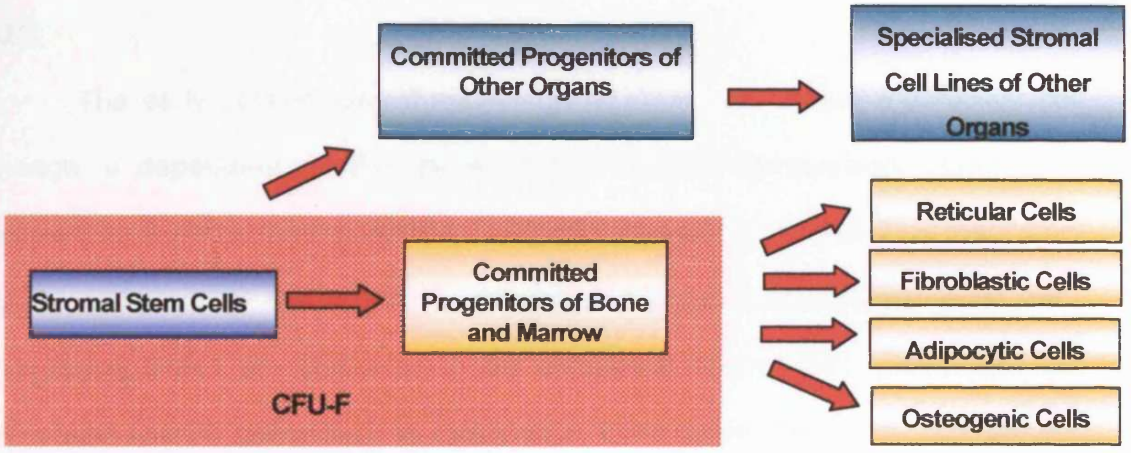


Figure 1.3: Hypothetical diagram for the marrow stromal system. It is proposed that stromal stem cells generate progenitors committed to one or more cell lines. The proposed stromal fibroblastic lines in marrow have been designated fibroblastic, reticular, adipocytic and osteogenic. The single fibroblastic colony forming cell (FCFC) or colony forming unit fibroblast (CFU-F), which form fibroblastic colonies *in vitro* are components of the stem and progenitor cell population.

Further evidence for the existence of marrow stem cells came from Maniatopoulos *et al.* [26] who were the first to create a bone-like structure *in vitro* using bone marrow cells from rats. They showed that bone marrow cells from the femora of adult rats would form nodules of mineralised tissue with the characteristics of bone in the presence of 10^{-8} M dexamethasone and 10mM β-glycerophosphate [26].

The early commitment of mesenchymal stem cells toward a particular cell lineage is dependent on the expression of specific transcription factors. The expression of peroxisome proliferator activated receptor γ2 (PPARγ2) is mandatory for commitment to the adipocytic lineage whereas mesenchymal stem cells expressing Cbfa1 are dedicated to the osteoblast lineage [27]. Cbfa1 activates osteoblast-specific genes such as osteopontin, bone sialoprotein, type I collagen and osteocalcin [28, 29]. The importance of Cbfa1 for osteoblasts has been highlighted by the evidence that knockout of the Cbfa1 gene in mice prevents osteoblast development [30]. In addition, *in vitro* studies showed that Cbfa1 is a crucial transcription factor required for postnatal osteoblast differentiation and for the maintenance of osteoblast differentiation [31]. It has been shown that osteoblast differentiation is mediated by multiple effects on Cbfa1 expression, DNA binding, or degradation [31], which makes this transcription factor an important modulator of the osteoblast lineage.

1.1.4 Osteoblast Function

Once stem cells are committed to the osteoblast lineage, proliferating osteoprogenitors become pre-osteoblasts, cell growth declines, and there is a progressive expression of markers of differentiation by post-mitotic osteoblasts [32-34]. The sequence of osteogenic differentiation is characterized by the expression of

alkaline phosphatase (ALP) an early marker of osteoblast phenotype, followed by the synthesis and deposition of type I collagen, bone matrix proteins, and proteoglycans, and an increased expression of osteocalcin and bone sialoprotein at the onset of mineralization. Bone matrix proteins play an essential role in the mechanical properties of bone, and some of these molecules and proteoglycans can bind growth factors released by osteoblasts (thus protecting them from enzymatic degradation). RGD sequences present in type I collagen chains, osteopontin, and bone sialoprotein are also recognized by integrins present on osteoclasts [35], whilst osteocalcin localized in the matrix increases osteoclast function *in vitro* [36] and *in vivo* [37].

Mature osteoblasts, in addition to being the cells that produce the osteoid matrix, are essential for its mineralization (the process of deposition of hydroxyapatite). Osteoblasts regulate the local concentrations of calcium and phosphate in such a way as to promote the formation of hydroxyapatite. Calcium at the mineralization site is ensured by its passive diffusion and its active transcellular transport by calcium pumps from the extracellular fluid of osteoblasts to the mineralization sites [38]. Meanwhile, phosphate ions can also be passively transferred or actively transported in osteoblasts by a sodium-dependent phosphate transport system [39]. Phosphate might come from the hydrolysis of phosphate esters by ALP anchored to the external surface of the plasma membrane. Bone matrix proteins such as osteopontin and bone sialoprotein may also contribute to the initiation of bone mineralization through their calcium-binding properties [40].

Once the bone matrix is deposited and calcified, the fate of osteoblasts is variable. Most osteoblasts show decreased matrix synthesis activity and become lining cells. Lining cells, are flat elongated cells that form a layer on top of the 1-2 μm thick coating of unmineralized collagen matrix that covers the surface of normal quiescent bone (i.e. bone that is not undergoing remodelling).

Alternatively, about 10% of osteoblasts are buried within lacunae of the matrix they synthesised (figure 1.4). These cells are termed osteocytes and are characterised by striking stellate morphology, reminiscent of the dendritic network of the nervous system. Osteocytes, the most abundant cell type in bone, are regularly spaced throughout the mineralised matrix and communicate with each other and with osteoblasts on the bone surface via multiple extensions of their plasma membrane that run along canaliculi. Osteoblasts in turn, communicate with cells of the bone marrow stroma, which extend cellular projections onto endothelial cells inside large blood vessels (sinusoids). This syncytium extends the entombed osteocytes all the way to the vessel wall.

The strategic location of osteocytes makes them excellent candidates for mechanosensory cells; able to detect the need for bone augmentation or reduction during functional adaptation of the skeleton, or the need for repair at a graft site. In both instances osteocytes are able to transmit signals leading to the appropriate response. These signals may be transferred to the necessary cells by changes in integrins and the cytoskeletal network [41].

Osteocytes sense changes in interstitial fluid flow (produced by mechanical forces) through canaliculi. They detect changes in the levels of hormones, such as oestrogen and glucocorticoids (that influences their survival) circulating in the interstitial fluid also. The capacity of bone to respond to local or hormonal stimuli may depend in part on osteoblast and osteocyte viability. About half of the osteoblasts appear to undergo apoptosis at the end of bone formation and this fate may contribute to the control of bone formation [42, 43].

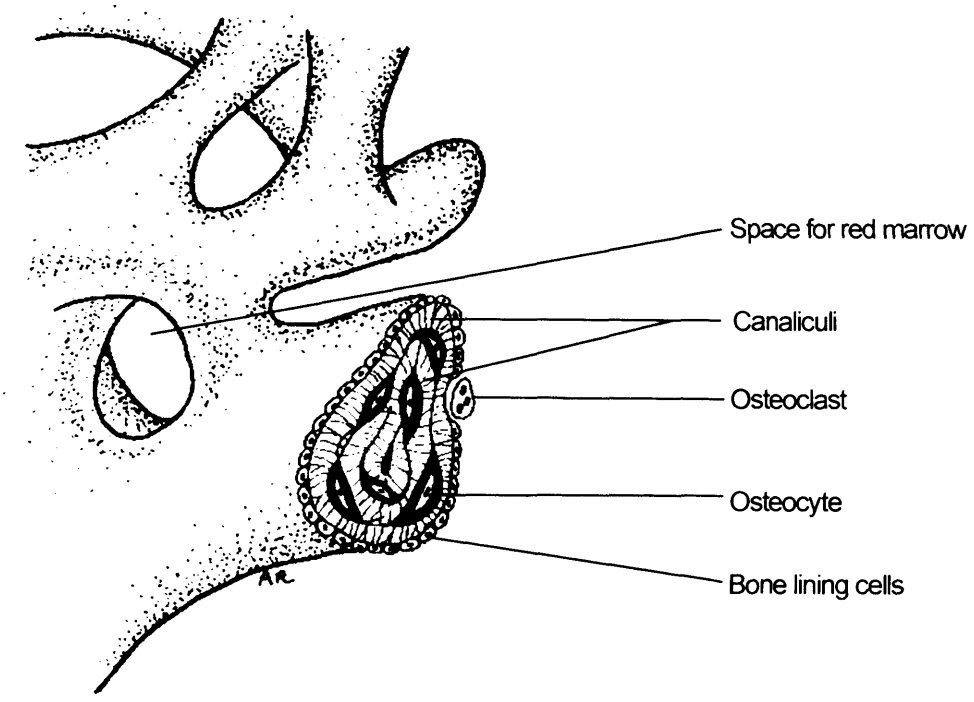


Figure 1.4: Cross section of a cancellous bone trabecule. Osteocytes are evident residing in lacunae, interlinked by canaliculi. Bone lining cells are also visible, aligned on the surface of the bone.

1.1.5 Regulation of Bone Remodelling by Systemic Hormones

Systemic hormones and local factors responsible for the interaction between osteoclasts and osteoblasts direct the bone remodelling process. Bone formation is primarily dependent on the number, rather than the activity, of osteoblasts [44]. Most hormones, however, act on both osteoblastic cell number and activity. The most important hormones regulating bone formation include parathyroid hormone (PTH), sex hormones, glucocorticoids, and vitamin D. These hormones induce gene expression through transcriptional effects in osteoblasts. The intracellular mechanisms involved in the actions of these hormones have been in part identified [45]. They also act indirectly on the cells through changes in the synthesis of local factors.

In contrast to the direct action of hormones on osteoblasts in bone formation, the regulation of bone resorption is quite indirect. Most hormones or cytokines do not have specific receptors on mature osteoclasts, with the notable exception of calcitonin. Resorption is regulated at the level of osteoclastic differentiation through growth factors and cytokines produced by osteoblasts and other haematopoietic cells of the bone microenvironment [2].

1.1.5.1 Calcitonin

Calcitonin is a peptide hormone secreted by the parafollicular cells of the thyroid. It is a potent inhibitor of bone resorption, and hence an effective therapy for the management of osteoporosis and other diseases characteristic of bone loss. Calcitonin receptors are expressed by committed osteoclast precursors and by mature osteoclasts [46]. The main mechanism of the antiresorptive actions of calcitonin is decreased development of osteoclast progenitors, decreased osteoclast recruitment, and promotion of apoptosis of mature osteoclasts leading to a slow rate of bone remodelling. When exposed to calcitonin, the calcitonin receptor (coupled to

several G proteins) induces an increase in intracellular calcium and cyclic AMP [47]. This in turn causes the osteoclast to become immobilized and disembark from the bone surface.

1.1.5.2 1,25-dihydroxyvitamin D_3 and Parathyroid hormone

In contrast to the effects of calcitonin, PTH and 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] are potent stimulators of osteoclast formation. The ability of these hormones to stimulate osteoclast development and to regulate calcium absorption and excretion from the intestine and kidney, respectively, are key elements of extracellular calcium homeostasis.

1,25-$(OH)_2D_3$, the most active form of vitamin D, is a potent stimulator of bone resorption *in vitro* [48]. It induces bone lining cell retraction, through alterations of cytoskeletal proteins [49] (thereby allowing osteoclasts to bind to the bone surface). 1,25-$(OH)_2D_3$ also increases osteoclast differentiation, it is thought to do this by changing the balance between RANK-L and OPG in osteoclasts [11, 12]. However, in *in vivo* experiments in rats, as well as in osteoporotic patients, there has been no clear evidence of an increase in bone resorption after 1,25-$(OH)_2D_3$ administration. One of the reasons for this could be a 1,25-$(OH)_2D_3$-induced decrease in PTH secretion (see below).

1,25 $(OH)_2$ dihydroxyvitamin D3, in addition to its effects on osteoclasts, exerts many effects on osteoblasts. Osteoblasts express receptors for vitamin D (VDR), and upon binding to this receptor, 1,25-$(OH)_2D_3$ down regulates α1(I) collagen, and induces the transcription of ALP, osteopontin, and osteocalcin [50, 51].

Osteoblasts are also the primary target cells of PTH [52, 53]. The hormone binds to PTH/PTHrP receptors on osteoblasts, activating second messenger systems such as adenylyl cyclase and phospholipase C [54]. *In vitro*, PTH exerts multiple effects on cells of the osteoblastic lineage at different stages of differentiation. It stimulates

proliferation in osteoblastic precursors [55], a mitogenic effect that may be mediated by the induction of local growth factors such as insulin-like growth factors (IGFs) and TGF-β; and in mature osteoblasts it inhibits apoptosis. This increases the life span of osteoblasts, which in turn may contribute to the bone forming effect of PTH [56]. This is in agreement with i*n vivo* studies, which have shown increased bone formation in trabecular bone with intermittent treatment with PTH [54].

As well as possessing bone formation properties, PTH; to a lesser extent; also increases bone resorption *In vitro* and *in vivo.* Nevertheless, it is well established that PTH/PTHrP receptors are not present on osteoclasts. Two possible explanations for this phenomenon have been proposed. The first is that PTH has a direct action on the proliferation or differentiation of osteoclast precursors [2]; and the second is that it influences the secretion of cytokines, by osteoblasts or stromal cells, which increase osteoclast differentiation. In keeping with the second mechanism PTH activates the production of IL-6 and IL-11 by osteoblasts, and these cytokines may mediate in part the effect of PTH on osteoclasts [57]. PTH perhaps also increases bone resorption by stimulating the expression of OPG-ligand (an inducer of osteoclast differentiation) in osteoblasts and stromal cells [11].

The bone resorption caused by PTH is further increased by the hormones ability to modulate plasmin metalloproteinases. These enzymes may initiate bone matrix degradation prior to resorption by osteoclasts; and, as seen with 1,25-$(OH)_2D_3$, induce retraction of lining cells through alteration in cytoskeletal protein [49]. This effect again, may be important for osteoclast binding to the matrix surface.

Despite the multiple direct and indirect actions of PTH, the hormone overall, causes an increase in osteoblast number and life span, and activation of new bone resorption.

1.1.5.3 Oestrogens

At menopause the rate of bone remodelling increases precipitously, trabecular separation and osteoclast number increase and circulating markers of bone turnover rise [1, 58, 59]. Oestrogens are major inhibitors of osteoclast formation. Although the main action of oestrogens is on osteoclast differentiation, the existence of oestrogen receptors on mature osteoclasts is still a matter of debate. It is in fact likely that there are several target cells for oestrogens in bone, and that multiple cytokines and growth factors may be implicated in the increased osteoclast number following menopause.

One such growth factor is transforming growth factor-β (TGF-β). This growth factor mainly inhibits bone resorption and promotes osteoclast apoptosis [60]. Its synthesis is increased by an oestrogen, oestradiol, *in vivo* and *in vitro* [61]. Oestradiol has been indicated in modification of the synthesis of osteoclast regulators, RANK-L and OPG, by osteoblasts *in vitro* [11].

Other studies point to an effect of oestrogens on bone resorption through the production of cytokines by monocytes [58]. For example, the *ex vivo* synthesis of IL-1 and TGF-α by monocytes in post-menopausal women is increased [62], and inactivation of both types of cytokines decreases the bone loss induced by ovariectomy in rats [58]. The monocyte may also have a more direct role in inhibiting osteoclast formation, since the monocyte – an osteoclast precursor cell, has oestrogen receptors. It is thus possible that oestrogens act directly on this cell to decrease osteoclast differentiation.

Osteoblastic cells express α and β oestrogen receptors and the expression of these receptors depends on the stage of differentiation [63]. *In vitro*, oestrogens increase osteoblast precursor cell proliferation, an effect that is in part mediated by the stimulatory effect of oestrogens on the production of TGF-β, IGF, and IGF-I binding proteins by osteoblasts [64]. In more mature cells, oestrogens induce

transcriptional stimulation of type I collagen and ALP and reduce osteocalcin expression. Other effects of oestrogens on osteoblasts *in vitro* include modulation of their response to PTH and 1,25 $(OH)_2$ dihydroxyvitamin D_3.

Thus, oestrogens are important modulators of osteoblast and osteoclast recruitment, function, and fate, which is in line with the well-known major effects of oestrogen deficiency on the skeleton.

1.1.5.4 Glucocorticoids

Glucocorticoids exert multiple and complex effects on osteoblasts, depending on the stage of maturation [65]. For example, they promote the differentiation of early osteoblast precursors in the marrow stroma and increase the expression of marker genes such as ALP, osteopontin, and osteocalcin. However, dexamethasone reduces the replication of osteoblastic cells, which is consequently responsible for the decreased bone formation induced by long-term glucocorticoid administration *in vivo*. It also inhibits type I collagen synthesis in more differentiated osteoblasts [65]. Thus, glucocorticoids act by promoting early stages of osteoblast maturation but also reduce osteoblast number and life span.

1.1.6 Regulation of Bone Remodelling by Growth factors

Growth factors play important roles in the control of osteoblast function by acting through complex cellular and molecular mechanisms [66, 67]. The most prominent factors are those that are produced locally by osteoblasts and are present in the bone matrix. They include IGFs, fibroblast growth factors (FGFs), TGF-βs, and bone morphogenic proteins (BMPs). Some of the signaling mechanisms of these factors have been identified in osteoblastic cells [45].

1.1.6.1 Insulin-like growth factors

Insulin-like growth factors (IGFs) are produced by osteoblasts, and through their receptors activate both proliferation and differentiation [68]. *In vivo*, IGF-I stimulates bone formation by acting on osteoblast recruitment and function [69]. However *in vitro*, IGF-I increases collagen type I expression and synthesis [64], and promotes osteoblast survival [70].

The actions of IGFs are largely controlled by IGF binding proteins (IGFBPs), also produced by osteoblasts. IGFBPs can additionally be found in the bone matrix where they are regulated by local agents and specific proteases [68, 71]. Both IGFs and IGFBPs are controlled by 1,25-dihydroxyvitamin D, estrogens, and PTH; confirming that these factors are important local regulators of bone formation.

1.1.6.2 Transforming growth factor β

TGF-β is produced in latent forms by osteoblasts and stored in the bone matrix in association with latent TGF-β binding protein (LTBP). The latent complex is then released as mature biologically active TGF-β, following the action of plasmin and the low pH produced by osteoclasts during resorption [72]. Small proteoglycans, decorin and beta glycan, also contribute to this process [40].

In vivo, TGF-β markedly stimulates bone formation [73], whilst *in vitro*, it increases the proliferation of normal osteoblastic cells and stimulates the expression and production of bone matrix proteins such as type I collagen and osteopontin. Further to this, TGF-β was found to exert anti-apoptotic effects on osteoblasts, which could complement its anabolic effects on bone formation [43].

Besides its actions on bone formation, TGF-β has an inhibitory effect on bone resorption. In *in vivo* studies, local injection of TGF-β to ovariectomized rats reduced bone hyperresorption [74]. TGF-β decreases the rate of matrix degradation by acting on collagenase and metalloproteinase enzymatic activities [84]. It was also found to

decrease osteoclastic differentiation through a direct action on haematopoietic precursor cell proliferation [76], and through an indirect action (reducing RANK-L and increasing OPG expression by stromal/osteoblastic cells) [13]. Furthermore, TGF-β increases osteoclast apoptosis [76].

Since TGF-β released from the matrix during resorption may stimulate osteoblast recruitment, this factor may serve as a coupling agent linking bone resorption to the subsequent bone formation during the bone remodeling cycle. The synthesis of TGF-β by osteoblasts is increased by oestradiol and could, therefore also be one of the mediators of the inhibitory effect of oestradiol on bone resorption.

1.1.6.3 Bone morphogenic proteins (BMPs)

Bone morphogenic proteins (BMPs) are members of the TGF-β family. They are important in the control of skeletal development and postnatal osteogenesis [77, 78]. *In vivo*, BMPs activate bone formation when applied locally. Whilst *in vitro* BMPs (BMP-2, BMP-3, and BMP-7) produced by osteoblasts; induce the expression of osteoblastic markers, such as Cbfa1, in uncommitted mesenchymal cells. In addition, BMPs promote markers of osteoblast differentiation such as type I collagen, alkaline phosphatase, and osteocalcin in differentiating osteoblastic cells.

Osteoblast differentiation by BMP-2 occurs when the protein binds to a BMP type II receptor (BMPR-II), which dimerizes with a type I receptor (BMPR-I), leading to phosphorylation of Smad proteins and activation of gene transcription [88]. In osteoblasts; Smad 1, Smad 5, and Smad 8 are essential for induction of differentiation by BMP-2 [79]. In addition to this direct mechanism, BMPs may also act through the induction IGFBPs or TGF-β in osteoblasts, thus emphasizing their important role in osteoblast commitment and function [77].

1.1.6.4 Fibroblast growth factors

Fibroblast growth factors (FGFs) are essential for the regulation of bone formation [80, 81]. Their biological activity depends on their binding to and activation of high affinity FGF receptors (FGFRs). *In vitro,* FGF-1 and FGF-2 stimulate osteoblastic cell proliferation, inhibit ALP and type I collagen expression, and modulate osteocalcin expression [81]. Additionally, FGF-2 promotes osteoblast survival [70]. This factor is therefore likely to be important in the local control of bone formation.

1.1.7 Regulation of Bone Remodelling by Cytokines

1.1.7.1 Interleukin 1

Interleukin 1 (IL-1), produced by activated monocytes, was demonstrated by Gowen *et al.* to increases bone resorption *in vitro* at very low concentrations. IL-1 increases osteoclast differentiation rather than mature osteoclast activity. Infusion of the cytokine in mice was found to induce hypercalcaemia and local injection *in vivo* induced increased bone resorption [82]. IL-1 has been implicated in several conditions associated with increased bone resorption such as post-menopausal bone loss and local bone loss associated with inflammation. Its action on bone resorption is partly dependent of prostaglandins.

1.1.7.2 Tumor necrosis factor α

Tumour necrosis factor α (TNF-α) is also secreted by activated macrophages and has an action on bone resorption similar to the one of IL-1. In addition, secreted TNF-α actually induces IL-1 synthesis [7, 17, 82]. Both IL-1 and TNF-α are produced by osteoblasts, which also express receptors for these cytokines [83]. IL-1 and TNF-α

act as autocrine growth factors for osteoblasts [84], however they have also been found to induce apoptosis in osteoblasts [85].

1.1.7.3 Interleukin 6

Several cells of the bone microenvironment produce Interleukin 6 (IL-6), yet osteoblasts and their precursors release particularly large amounts. The secretion of IL-6 by osteoblasts is modulated by several systemic hormones. PTH increases its synthesis whereas estrogens have been shown to decrease its synthesis.

In vitro cultures demonstrated that IL-6 increases bone resorption, however for this to occur early osteoclast precursors had to be present [17]. IL-6's role in bone resorption is therefore not clear. Nevertheless, IL-6 has been implicated in post-menopausal bone loss [59], and high production of IL-6 has also been implicated in increased osteoclast activity of a number of other diseases characterized by high bone turnover [7].

1.1.7.4 Prostaglandins

Prostaglandins (PGs), primarily PGE_2, increase bone resorption in cultured bone [86]. The action of prostaglandins on resorption *in vitro* changes with the stage of cell differentiation. PGs decrease the resorbing activity of mature osteoclasts by increasing intracellular cAMP content and decreases osteoclastic differentiation of haematopoietic osteoclast precursors. However PGs are seen to increase bone resorption in mouse marrow cultures, by modulating the synthesis of cytokines or growth factors released from osteoblasts present in these cultures [87].

In contrast to their effect on bone resorption, PGs are furthermore known to increase bone formation *in vitro* through IGF-I synthesis in osteoblasts [65]. The overall effect of PGs on bone remodeling *in vivo* have not been clearly evaluated.

1.2 Osseointegration - The Remodeling of Bone Grafts

It is now apparent that the differentiation and function of bone cells are dependent both on the expression of specific transcription factors and local factors, and are controlled by cellular interactions mediated by cell surface and soluble molecules. These systems play major roles both in the remodeling of normal bone and grafted bone.

Bone formation on or about a graft can be either of graft origin (that is, from osteoblasts that survive the transfer) or from cells of host origin. Osteoblasts on the surface of bone grafts, that are properly handled, can survive and produce new bone. This early bone formation by graft osteoblasts is often critical in callus formation during the first few weeks following surgery [88].

Another way in which a bone graft may assist new bone formation is by being osteoinductive. Osteoinduction is the recruitment of mesenchymal cells from the area surrounding the graft, which then differentiate into osteoblasts. Osteoinduction is mediated by graft-derived factors. Bone matrix contains several BMPs, TGFβ, IGFs, FGFs, ILs and macrophage colony-stimulating factors. These moieties induce or influence the differentiation of mesenchymal cells into bone-forming cells (as previously described). The condition of the host bed is critical in the process of osteoinduction, because new osteoprogenitor cells are recruited by induction of residual mesenchymal cells in marrow reticulum, endosteum, periosteum, and connective tissue [88].

In addition to their ability to recruit host cells by osteoinduction, bone grafts function as a trellis or scaffold for the ingrowth of new host bone. The 3-dimensional process of ingrowth of sprouting capillaries, perivascular tissue, and osteoprogenitor cells from the recipient bed into the structure of a graft is termed osteoconduction. Osteoconduction may result from active bone formation and osteoinduction (for

example, in a fresh autograft) or it may occur passively, without the active participation of the graft, as would be the case with most cortical allografts. Osteoconduction is not random; indeed, it follows an ordered, predictable, spatial pattern, determined by the structure of the graft, the vascular supply from the surrounding soft tissue, and the mechanical environment of the graft and surrounding structures [89].

1.2.1 Remodeling of Different Graft Types

1.2.1.1 Autografts

Autogenous cancellous (trabecular) bone, due to it's 'honeycomb'-like structure is highly osteogenic, easily revascularized, and quickly integrated into the recipient site. It does not however provide structural support, but the rapidity with which autogenous cancellous bone both produces and stimulates new bone formation often contributes to the early stabilization of a fracture site. The primary source of this type of bone is the iliac crest. Bone grafts from this area are extremely useful, however significant morbidity accompanies the harvest procedure [90].

The biologic activity of autogenous cancellous bone results from its histocompatibility, large surface area covered with osteoblasts and their precursors, and trabecular architecture. The host response to cancellous autografts can be described in 5 stages, which overlap and form a continuum. The first two stages, hemorrhage and inflammation, occur rapidly after the surgical procedure. Many of the grafted cells die, particularly osteocytes in trabecular lacunae, but surface osteoblasts survive and produce early new bone. Host vessels, osteoblasts, and osteoblast precursors then infiltrate the graft, because it is quite porous, from the periphery toward the centre as early as 2 days after surgery. Osteoclast precursors are blood-borne (see 'Osteoclast Differentiation'); therefore, the ingrowth of vessels marks the beginning of graft resorption [90].

As the third stage, vascular invasion of the cancellous graft proceeds; osteoblasts line the edges of dead trabeculae and deposit a seam of osteoid, which eventually surrounds the central core of dead bone. Subsequently, the graft is remodeled; that is, the new host bone and entrapped cores of necrotic bone are gradually resorbed by osteoclasts and replaced with newly synthesized bone by host osteoblasts (the fourth stage) [90].

Nonvascularized dense cortical autografts, provide structural support and are somewhat osteogenic, however they revascularize slowly. The delay in revascularization may be attributed to the structure of cortical bone, the vascular penetration of the graft is primarily the result of peripheral osteoclastic resorption and vascular invasion of Volkmann's and Haversian canals (see figure 1.1). In contrast to cancellous grafts, which are initially strengthened with new host bone formation on dead, grafted trabeculae, cortical bone becomes significantly weaker as osteoclasts spearhead the invasion of vessels from the surrounding host bed. Also in contrast to cancellous bone grafts, large portions of the dead cortical autograft may remain for significant periods of time. The main source of nonvascularized cortical autograft is the fibula; although the ilium will provide corticocancellous bone, but the mass and strength of the cortex is less than that of the fibula [91].

Vascularized cortical autografts provide limited structural support and function relatively independently of the host bed. None the less, their turnover and remodeling resemble that of normal bone, and the ingrowth of vascular buds from the host bed is not necessary for its incorporation. Because vascularized cortical autografts are implanted with a functional blood supply, their incorporation differs markedly from that of nonvascularized cortical autografts. When anastomosis of the vessels is a success, the graft suffers only transient intraoperative ischemia, and over 90% of osteocytes survive the transplantation procedure. Graft-host union occurs quickly, as does resorption followed by osteoconduction and remodeling [92].

The three major sources of vascularized bone autografts are the fibula, iliac crest, and rib. The fibula may be isolated on its peroneal vessels. The iliac crest graft uses the deep circumflex iliac artery and vein, and the rib graft uses the posterior intercostal artery and vein. Although the graft will not be weakened by marked resorption, it must be supported with appropriate internal or external fixation until it can remodel in response to the mechanical loading of its new site [92].

1.2.1.2 Allografts

Grafts are remodeled in response to the same local mechanical stimuli as normal skeletal bone. Allografts of demineralized bone matrix (DBM) are quickly revascularized, and may be moderately osteoinductive, however they cannot provide structural support. Implantation of allogeneic DBM is followed by platelet aggregation, hematoma formation, and inflammation characterized by migration of polymorphonuclear leukocytes into implants within 18 hours. Thereafter, fibroblast-like mesenchymal cells are attracted to and establish close contact with the implanted matrix. Interactions between the DBM and mesenchymal cells results in cellular differentiation into chondrocytes around day 5 after implantation. Chondrocytes produce cartilage matrix, which is then mineralized. By days 10 to 12, vascular invasion accompanied by osteoblastic cells is observed, multinuclear cells also appear, and the chondrocytes begin to degenerate [93].

New bone is formed apposed to the surface of the mineralized cartilage. Remodeling and replacement of these composite structures with new host bone ensues. With time and continued remodeling, all of the implanted DBM is resorbed and replaced with host bone suitable for the environment in which it finds itself.

The source and processing of DBM has a direct effect on its osteoinductive capacity. For instance, sterilization by ethylene oxide under certain conditions and 2.5 mrad of gamma irradiation both substantially reduce osteoinductivity. An

1.4 Aims of Thesis

The aims of this thesis were to:

- Study the distribution of primary markers of bone and marrow tissue using immunohistochemical analysis.

- Investigate the effect of bone allografts, sterilized by different techniques, on the proliferation and differentiation of culture expanded bone marrow cells.

- To enhance the differentiation of bone marrow cells into osteoblastic cells using three different culture media, including a novel medium endothelium cell conditioned medium.

- To evaluate the genetic response of bone marrow cells, to endothelial cell conditioned medium.

- To establish the optimum method for culturing osteoblastic cells from bone marrow cells inside type I collagen gels, such that the gels could then be used to coat bone grafts, or used alone to fill small bone defects.

- To establish the best method for growing bone from marrow cells, in organ culture. Thus potentially creating autologous bone suitable for grafting back into the host.

Chapter 2

General Methodology

2.1 Harvesting Bone Marrow

MF1 adult mice were used in all experiments. Animals were killed humanely by cervical dislocation. The femora were carefully dissected from the mouse and excess tissue surrounding the bones removed using tweezers. Two cuts were made across each femur, one across each metaphysis (see figure 2.1). A 25G needle (Gibco BRL, Paisley, UK), attached to a 10ml syringe (Gibco BRL, Paisley, UK) containing approximately 10ml of culture medium, was inserted into the marrow cavity of the diaphysis and approximately 1-2ml of medium expelled from the syringe. The force of the medium passing into the marrow cavity pushed the marrow out in the form of a whole marrow plug. Marrow plugs were then utilised as described in the relevant chapters.

2.2 Endothelium Cell Conditioned Medium

Bovine aortas were collected from a local abattoir. Fatty tissue surrounding the blood vessel was removed and segments of aorta small enough to fit into a Petri dish cut from the central sections of the aortas. The aortas were then opened into flat sheets, by making an incision along the length the vessels. The inside surface of the aortas were washed with PBS to remove any blood and the surface of the vessels scraped very lightly with a scalpel to remove any additional debris. The surface was scraped once more, a little harder with a new scalpel to remove endothelial cells. Cells were placed in a universal containing PBS and centrifuged for 5 minutes at 1500 rpm. The pellet was re-suspended in 1ml alpha modification minimum essential medium eagle (α-MEM) (Gibco BRL, Paisley, UK) supplemented with 10% foetal calf serum, 2mM L-glutamine, penicillin (10, 000U/100ml), streptomycin (10mg/ml) and amphoterycin (25μg/ml); and made into a single cell suspension by repeatedly

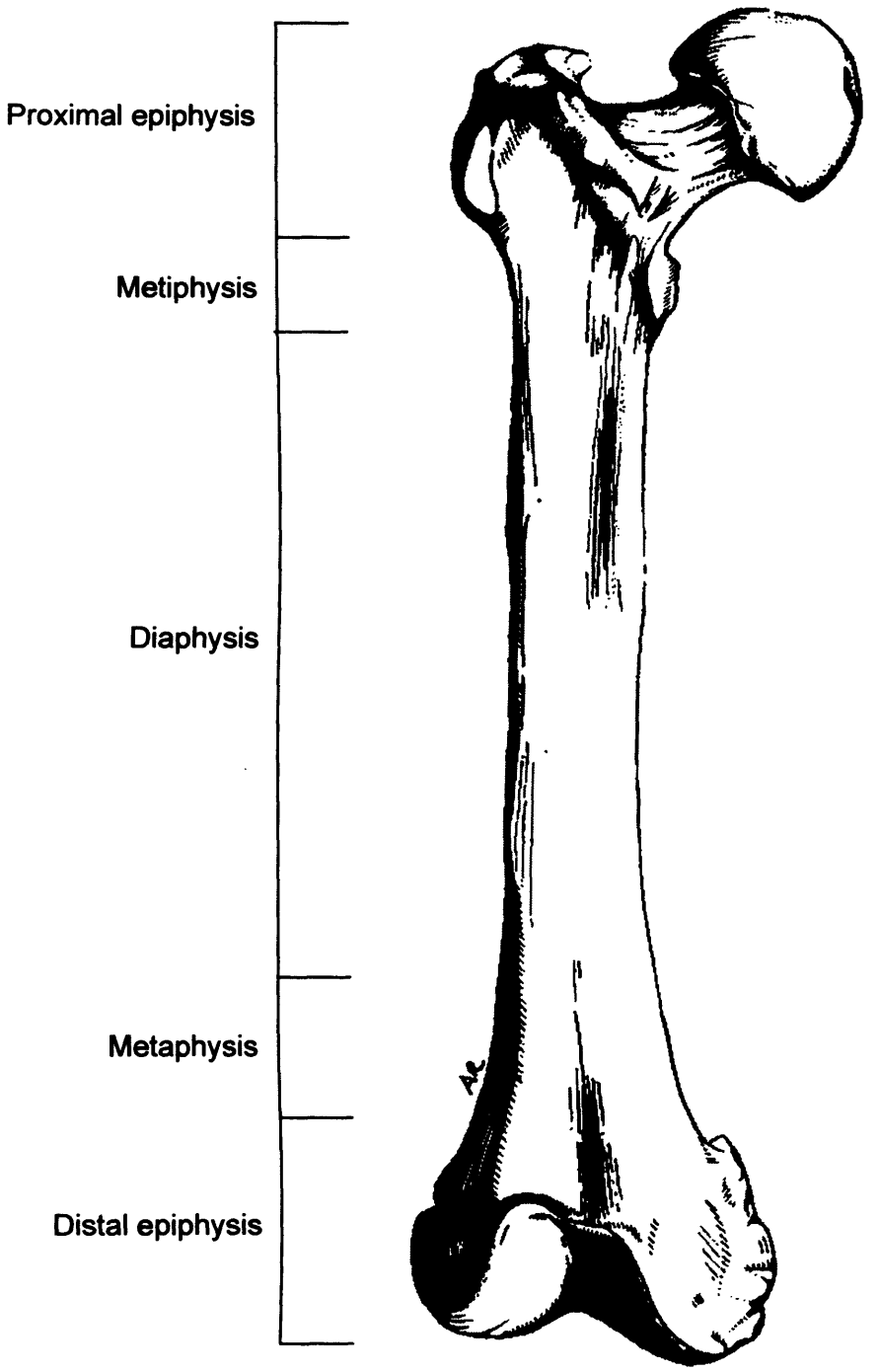


Figure 2.1: Diagram of the femur.

drawing the solution up into a syringe through a 25G needle (Gibco BRL, Paisley, UK) and then expelling it.

The resulting single cell suspension was plated out into a $75cm^2$ flask (Gibco BRL, Paisley, UK) containing 10 ml of supplemented α-MEM. The culture medium was changed every other day, which removed non-adherent cells, and the culture was incubated in a humidified atmosphere of 95% air and 5%CO_2.

Once confluent, the cells were passaged and then plated out in a 24-well plate (Gibco BRL, Paisley, UK) at a density of 1 cell per well. After a few days incubation in α-MEM, colonies of endothelial cells were confirmed using antibodies for endothelial cell markers - von Willebrand factor (AbCam, Cambridge, UK) and CD105 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa, USA). Endothelial cells were plated out into culture flasks and incubated with α-MEM. The medium was collected from confluent cultures (passages 2 through 10), filtered to remove any non-adherent cells in the solution and added directly to cultures requiring endothelial cell conditioned medium.

2.3 Immunohistochemistry

All cultures were fixed for immunohistochemistry in 1-2ml 10% formal-saline in phosphate buffer (pH7.4) for 24 hours and then dehydrated through increasing concentrations of ethanol. Collagen gel cultures and organ cultures were embedded in paraffin wax, and cut into 5μm sections. Sections were mounted on glass slides and heated in an oven overnight to 60°C. The sections were then deparaffinized and hydrated though xylenes and graded alcohol series to water. Monolayer cultures were also rehydrated to water and all samples were then washed twice (5 minutes per wash) with PBS.

A protease mediated retrieval was required, which entailed treating the sample with hyaluronidase (1mg/ml) for 1 hour at room temperature. This was followed by 3, 10 minute washes with PBS. Primary antibody (100 μl) diluted in buffer containing 1.5% blocking serum (Vector Laboratories Ltd, Peterborough, UK), 0.1% Tween-20 and 0.1% bovine serum albumin (BSA) in PBS; was added per sample. Samples were incubated with the primary antibody for 1 hour in a humid atmosphere at room temperature.

Following incubation, the primary antibody was washed off the samples by 3, 10 minute, washes with PBS. The secondary antibody, prediluted biotinylated panspecific universal secondary antibody (Vector Laboratories Ltd, Peterborough, UK) was added for a further 10 minutes before the sections were washed twice in 0.1% tween-20 buffer and finally in PBS (5 minutes/wash).

Ready-to-use streptavidin/peroxidase complex reagent (Vector Laboratories Ltd, Peterborough, UK) was added for 5 minutes to the samples, and the samples received a duplicate wash in 0.1% tween-20 buffer and a single wash in PBS (each for 5 minutes). To visualise the bound antibody, peroxidase substrate solution (Vector Laboratories Ltd, Peterborough, UK) was added until the desired stain intensity developed. Sections were rapidly washed in tap water once the desired intensity of staining was observed, and then counterstained with haemotoxylin, which was again followed by a rinse with tap water.

2.4 Von Kossa Method

Cultures were fixed, wax embedded and sectioned (if required) and rehydrated to water as described for immunohistochemistry. The samples were then washed several times in double distilled water before 1% silver nitrate solution was added. The samples were left in brightlight for 10-20minutes before being washed 3 times in

double distilled water. Samples were treated with 2.5% sodium thiosulphate for 5 minutes, to enhance the silver nitrate staining, and washed in tap water.

2.5 Oil Red O Staining

Cultures were fixed and rehydrated to water as described for immunohistochemistry, and allowed to air dry. A stock solution of oil red o (0.5% oil red o in 99% isopropranol) was prepared at the beginning of this study, and a 0.3% working solution of oil red o was made up immediately prior to use (by diluting the stock solution with double distilled water and then filtering it through a No.46 Whatman filter paper and a 0.2μm syringe filter). Cells were immersed in the working solution for 15-20 minutes and then washed 3 times in distilled water. Under the light microscope lipid vacuoles appeared bright red relative to other organelles, thus allowing adipocytes to be identified.

2.6 Autoradiography

2.6.1 Sample preparation

A final concentration of 1μCi/μl of L-[5-^{3}H]-Proline (Amersham Biosciences UK Limited, Bucks, UK) in culture medium was added to cultures and left overnight to incubate. Media from the cultures was collected the following day, and the cells rinsed with PBS. The PBS rinse was also collected and combined with the culture medium. Protease inhibitors were added to the culture medium-PBS removed from the cells. A solution (10 μl) containing the protease inhibitors phenylmethanesulfonyl fluoride (3.5%) and N-ethylmaleimide (12.5%) in ethanol was added to each ml of medium-PBS removed from the cells. EDTA solution (100μl of 9.3%) was then added to each ml of medium-PBS. Following the addition of the protease inhibitors 3/7 of the total volume of a 10-15% solution of ammonium

sulphate was added. The sample, containing medium-PBS, protease inhibitors and ammonium sulphate; was then distributed between eppendorff tubes (Gibco BRL, Paisley, UK) at volumes of approximately 1ml/tube. The eppendorff tubes were then centrifuged for 10 minutes at 125rpm.

The supernatants were removed from the tubes and pipetted into pre-soaked dialysis tubing. 1ml of 0.5M acetic acid was then used to suspend the precipitates, which were then added to another dialysis tube. Both dialysis tubes were added to a bucket containing 5L of 0.2M acetic acid which was refrigerated at 4°C. The acetic acid in the bucket was changed daily for 4 days.

After dialysis the solutions from each dialysis tube were pipetted into separate universals (Gibco BRL, Paisley, UK). The quantity of radioactivity in each sample was assessed using a scintillation counter, and aliquots each containing 20,000 counts per minute made from the samples. The aliquots were frozen for around 1 hour at –20°C, and then for a further hour at –70°C. The aliquots were placed in a freeze drier over night and once desiccated, the samples were placed in a freezer and kept at –20°C.

2.6.2 SDS-PAGE

Sample buffer (10-15μl), made up from a stock solution of 0.05% bromophenol blue and 10.9% sodium dodecylsulphate (SDS) in 6.25% 1M tris buffer (pH 6.8); was added to each eppendorff of desiccated dialysis sample. The rehydrated samples were then heated in a beaker containing boiling water for 5 minutes, before being loaded; alongside molecular weight markers; into a 15% polyacrylamide gel containing 0.09% bisacrylamide and 0.1% sodium dodecylsulphate. The gel was electrophoresed using Laemmli's continuous buffer system [95].

Gels were fixed in 30% methanol-10% acetic acid, and soaked in a solution of 2,5-diphenyloxazole (PPO) in dimethylsulphonate (Amersham Biosciences UK Limited, Bucks, UK). The polyacrylamide gels were then dried for approximately one hour in a vacuum drier in which the gel was supported on Whatman 3MM paper.

2.6.3 Detection of ^{3}H-labelled Collagens

To detect the radioactivity being released by the collagens in the dried polyacrylamide gels, RP Royal 'X-Omat" film (Amersham Biosciences UK Limited, Bucks, UK) was placed in contact with the gel and exposed at –70°C. The film was pre-exposed to a brief flash of light to increase its sensitivity before it was secured to the gel between clamped glass plates. Light was excluded by wrapping the film-gel sandwich in a black plastic bag.

After exposure, the film was allowed to warm up to room temperature, and then unwrapped in a dark room. The film was placed in a developer tank for 5 minutes containing Kodak GBX developer (Amersham Biosciences UK Limited, Bucks, UK), removed and washed in water for 30 seconds. The film was then placed in a fixer tank for 5-10 minutes, containing Kodak GBX fixer (Amersham Biosciences UK Limited, Bucks, UK). The film was washed once more in water for 5-10 minutes and then hung with a clip to dry.

Alpha particles from ^{3}H, incorporated into the collagen molecules, interact with the 2,5-diphenyloxazole emitting light which causes local blackening of an X-ray film. The approximate molecular weight of the bands produced on the film, by collagen molecules in the sample, were compared to the bands of the molecular weight markers in the original gel.

2.7 Isolation of total RNA from mouse bone marrow monolayer.

Cells were enzymatically released by trypsin (0.05%)-EDTA (0.02%) and counted. Cells ($4x10^6$) were transferred to RNase-free polypropylene centrifuge tubes (Quiagen Ltd, Sussex, UK) and the cells pelleted by centrifugation at 300 x g for 5 minutes. The supernatant was completely aspirated.

The cells were then treated using the RNeasy mini kit (Quiagen Ltd, Sussex, UK). Cells were disrupted by vortexing the cells with 350μl buffer RLT. The sample was then homogenised by centrifuging the lysate for 2 minutes at maximum speed in a QIAshredder spin column placed in a 2ml collection tube. One volume of 350μl of 70% ethanol was added to the lysate, and mixed well. Up to 700μl of the sample, including the precipitate formed after the addition of ethanol, was added to an RNeasy mini column placed in a 2ml collection tube. The tube was centrifuged for 15s at ≥8000 x g, and the flow through discarded.

The RNase-free DNase set (Quiagen Ltd, Sussex, UK) was then used, which entailed adding 350μl buffer RW1 into the RNeasy column, and centrifuging for 15s at ≥ 8000 x g to wash. The flow through was discarded and 80μl Dnase I incubation mix added directly onto the RNeasy membrane. The sample was allowed to incubate for 15 minutes with the incubation mix. 350μl of buffer RW1 was then added onto the RNeasy mini column, which was centrifuged for 15 seconds at 8000 x g. The flow through was then discarded.

The RNeasy column was transferred to a new 2ml collection tube and 500μl of buffer RPE (RNeasy mini kit) added onto the RNeasy column. The tube was then centrifuged for 15 seconds at ≥8000 x g to wash the column. The flow through was discarded and another 500μl buffer RPE added to the RNeasy column. The tube was centrifuged for 2 minutes at ≥8000 x g to dry the RNeasy membrane. The RNeasy

column was placed in a new 2ml collection tube, and the old collection tube with the flow-through discarded. The new collection tube was centrifuged at full speed in a microcentrifuge for 1 minute. To elute the RNA, the column was transferred to a new 1.5ml collection tube and 30-35μl RNase-free water added directly onto the RNeasy membrane. The tube was centrifuged for 1 minute at ≥8000 x g to elute. The eluted sample was then sent to the Microarray centre, Imperial College, London for microarray analysis.

2.8 Preparation of rat tail type I collagen

Tails were collected from approximately 10 rat cadavers. The tail skin was cut from base to tip and the tendon and bone removed. The tip of the tail was then clamped in a pair of strong tweezers and at every ~2-3cm along the tail the bone was broken and the strands of tendon removed. The tendon pieces were placed in 100ml PBS at 4°C for 15 minutes, and blotted dry on filter paper. The tendon was weighed, suspended in 0.5M acetic acid (2mg tendon/ml) and allowed to stir for 24 hours. The tendon-acetic acid solution was centrifuged at 2000rpm for 15 minutes, the supernatant was removed and an equal volume of 20% NaCl solution added. The solution was left overnight at 4°C to stir and then centrifuge at 2000rpm. The supernatant was removed and the remaining pellet weighed. The pellet was re-suspended in 0.5M acetic acid (4mg/ml) and allowed to stir overnight at 4°C. The solution was divided between 3 dialysis tubes which were placed in double distilled water to dialysed for two days. The contents of the dialysis tubes were then frozen in a –20°C freezer overnight and then freeze dried. The dessicated sample (type I collagen) was weighed and re-suspended in filter sterilised 0.1M acetic acid (1mg/ml). The resultant collagen solution was then stored at 4°C in preparation for making collagen gels.

2.9 Statistics

All data was expressed as mean ± standard deviation from the mean (SD) unless otherwise stated. Cultures grown in Biggers medium and endothelium cell conditioned medium were compared to cultures grown in control medium (αMEM) using a one-way analysis of variance ANOVA test. The statistical analyses were carried out in Microsoft excel software. Significant differences were identified at the Bonferroni 95% confidence interval.

2.10 General Materials

All chemicals and reagents were obtained from Sigma, UK and dissolved in double distilled water immediately prior to use unless otherwise stated.

Chapter 3

Immunohistological Features of Mouse Bone and Marrow

3.1 Introduction

3.1.1 Stem Cells

The cells of most skeletal tissues, are derived from multipotent mesenchymal stem cells (MSCs). MSCs give rise to all of the skeletal elements during development, and remain present in low numbers in sites such as the bone marrow throughout life. It is these cells that can differentiate into bone, cartilage, and fibrous tissue following a bone graft or fracture and generate a reparative callus. Several markers putatively specific for MSCs have been reported, one such marker is STRO1.

3.1.1.1 STRO-1

STRO-1 has proven to be an extremely valuable reagent for the identification, isolation and functional characterisation of MSCs [95]. STRO-1 identifies a cell surface antigen expressed by MSCs in bone marrow [96]. When plated under long-term bone marrow culture (LTBMC) conditions, STRO-1(+) cells generate adherent cell layers containing multiple stromal cell types, including adipocytes, smooth muscle cells and fibroblastic elements [97]. Gronthos *et al* in 1994, demonstrated that STRO-1(+) cells from adult bone marrow were capable of differentiating into functional osteoblasts and that osteoprogenitors are also present in the STRO-1(+) population [97].

3.1.1.2 CD34

In addition to the MSCs, hematopoietic stem cells (HSC) reside in bone marrow. HSCs represent a small subset of haematopoietic cells within the bone marrow. They possess differentiation capacity, like mesenchymal stem cells, which allows a constant supply of the entire hematopoietic spectrum. CD34 is used as a

convenient marker for these cells. CD34(+) cells have been shown to possess colony-forming potential in short-term assays, maintain long-term colony-forming potential in *in vitro* cultures and allow the expression and differentiation of blood cells from different hematopoietic lineages in *in vitro* models [98]. This antigen will be used to distinguish hematopoietic stem cells from mesenchymal stem cells in *in vitro* cultures.

3.1.2 Osteogenesis

There are a number of phenotypic parameters that characterize the osteoblast. The extracellular matrix structural proteins are one set of phenotypic parameters that define the osteoblast. These bone matrix proteins include type I collagen; and a variety of noncollagenous proteins such as osteocalcin, osteopontin, osteonectin, and proteoglycans.

3.1.2.1 Type I collagen

The major matrix protein synthesised by osteoblastic cells, which comprises more than 90% of the organic matrix of bone, is type I collagen. Type I collagen is constructed in the form of a triple helix of two identical α1(I) chains and one unique α2(I) chain (genes designated COLIA1 and COLIA2) [99]. The chains are composed of approximately 1,000 amino acids each, and produce a fairly rigid linear collagen molecule 300nm long. Each molecule is aligned with the next in a parallel fashion in a quarter-staggered array to produce a collagen fibril. The collagen fibrils are then grouped in bundles to form the collagen fibre. Within the collagen fibril, gaps, called "hole zones" exist between the ends of the molecules. In addition, "pores" exist between the sides of parallel molecules. Noncollagenous proteins or mineral deposits can be found within the holes and pores. Mineralisation of the matrix is

thought to commence in the hole zones [100, 101].

Collagen synthesis is completed within the cell, and processing continues in the extracellular matrix, and involves both posttranslational and postsecretory processing. In the cell, almost half of the proline and 15% to 20% of the lysine residues are hydroxylated on the individual α chains, and these hydroxylations are followed by the glycosylation of the hydroxylysine residues in an intracellular, posttranslational process. This step leads to the formation of the triple helical procollagen molecule, which is the secreted form. Once outside the cell, the nontriple helical amino terminal and carboxy terminal propeptides are cleaved by proteinases to form the collagen molecule. The C-propeptide is cleaved by proteolytic activity of BMP1, a member of the bone morphogenetic family that lacks osteoinductive capacity but has some homology to the other members [100]. The C-propeptide and N-propeptide fragments can be detected in serum, and are indicative of bone formation rates. Once the terminal propeptides are cleaved, the collagen molecules spontaneously self-assemble into collagen fibrils. The collagen molecules are stabilised by cross-links formed between reactive aldehydes on different chains. The reactive aldehydes are formed by oxidative deamination of both lysine and hydroxylysine [100].

3.1.2.2 Decorin

Decorin (DCN), belongs to a family of structurally related small interstitial proteoglycans with wide distribution in the matrices of various connective tissues [102]. DCN contains a short protein core of approximately 30kd, and in bone, a dermatan sulfate side chain. The secreted proteoglycan has the ability to bind to different extracellular matrix constituents and growth factors. It binds specifically via

its core protein to the surface of fibrillar collagens [103, 104] and it appears to influence *in vitro* fibrillogenesis of types I and II collagens [105]. DCN also influences cell adhesion [106], and appears to have an important role in cell proliferation through its ability to bind TGF-β [107]. It is hypothesised that DCN on the surface of the matrix fibrils binds TGF-β and then releases this powerful bioactive molecule when the matrix is disrupted (e.g. when bone is resorbed or fractured). Cells sensing the increase or decrease in the levels of TGF-β may use the mechanism to monitor the health of the matrix within its purview.

Dyne *et al.* reported two osteogenesis imperfecta patients, both with the same gly-415/ser mutation in the α1(I) chain of collagen, in which the patient with the more severe phenotype had little or no decorin production in fibroblasts while fibroblasts from the other patient produced normal amounts of DCN [108]. This suggests that changes in the expression of decorin may have phenotypic consequences in some tissues.

3.1.2.3 Osteonectin

Termine and co-workers identified osteonectin in 1981 [109, 110]. Initial reports indicated that osteonectin was found in bone matrix in high relative abundance, and was virtually absent from other tissues. Osteonectin, also known as SPARC (secreted protein, which is acidic and rich in cysteine) [111] is a 32kd protein now known to be secreted by both osteoblasts and platelets [112, 113].

One of the intriguing features of osteonectin is its ability to bind tightly to hydroxyapatite. This was first demonstrated by Termine *et al* [109]. This work was extended, and it was found that native adult osteonectin bound Ca^{2+} [114]. The other unique feature of osteonectin concerns collagen binding [109, 114]. These two properties, collagen binding and hydroxyapatite binding, were brought together in a

series of experiments in which osteonectin was shown to stimulate the binding of apatite to collagen and, moreover, effect nucleation of hydroxyapatite crystals from metastable solutions of Ca^{2+} and PO_3^{2-}, onto the collagen surface [109]. Osteonectin is thought to bind collagen and apatite through different regions of the molecule, and may, *in situ*, act as a regulator of hydroxyapatite formation on collagen fibrils.

3.1.2.4 Bone Sialoprotein

Bone sialoprotein (BSP or BSPII), a small (~75000 Da) protein, is a member of a group of acidic, integrin-binding sialoproteins, which also includes osteopontin (see below). Immunolocalisation and *in situ* hybridisation studies have shown BSP to be made not only by osteoblasts but also by osteocytes and the osteoclasts [116]. BSP is the only non-collagenous protein, along with osteopontin, that may be expressed by osteoclasts during bone turnover *in vivo* [116].

BSP makes up around 8 to 12% of the total non-collagenous proteins in bone [117]. It is a highly glycosylated and sulphated phosphoprotein, and is found almost exclusively in mineralised connective tissues. Outside of bone, BSP has been found in three other mineralised tissues, dentin [117], cementum [118], and calcifying cartilage of the growth plate [116]. The areas richest in BSP are the 'cement lines' or collagen-poor matrix found between areas of new bone, whether that is between the cartilage anlage and bone in development or between old bone and new bone during turnover [119]. Because of its restricted expression, BSP provides a valuable marker for osteogenic differentiation and bone formation.

BSP has a high binding affinity for calcium and hydroxyapatite, and has been shown to nucleate hydroxyapatite crystals *in vitro* [120]. The nucleating activity of BSP resides in its polyglutamic acid segments, the first polyglutamic sequence being more effective than the second sequence [120]. The polyglutamic acid sequences in

BSP are predicted by several different algorithms to form an α-helical structure which could provide an appropriate spacing of the γ-carboxylate groups for binding Ca^{2+} ions in the dimensions of the hydroxyapatite crystal [121].

In addition to binding hydroxyapatite, BSP also binds with high affinity to collagen, preferably to the α_2(I) chain [122] and to cell surface receptors including integrins [123]. BSP and the other members of the family (e.g. osteopontin) support cell attachment *in vitro* through its integrin-binding tripeptide, arginine-glycine-aspartate (RGD). This region has been shown to be the likely binding site to the vitronectin receptor, the $\alpha_V\beta_3$ integrin [123]. BSP promotes osteoclast attachment *in vitro* [124] via the vitronectin receptor, which is present on the surfaces of the osteoclasts, in a dose-dependent manner. BSP also modulates the activity of osteoclasts by signalling through the vitronectin receptor [125], as well as through non-RGD receptors [117]. An alternative form of cell attachment is supported by BSP in an RGD-independent manner [126]. BSP has two RGD-independent cell attachment domains that have recently been shown to be two tyrosine-rich domains [127].

3.1.2.5 Osteopontin

Osteopontin, also known as sialoprotein I, was first identified by Franzen and Heinegard [128]. It is an acidic glycoprotein of about 41,500 daltons that has been isolated from rat [129], human [130], mouse [131], pig, chicken [132] and bovine bone [133]. Osteopontin (OPN) appears to be only one protein coded by a single gene [134] judging by the compositions, amino acid sequences deduced from cDNA and the NH_2-terminal analyses. It is also clear that bone sialoprotein is distinct from osteopontin (sialoprotein I) although there may be structural and functional relationships between the two [128, 130].

In bone OPN is secreted by preosteoblasts, osteoblasts, osteocytes, and osteoclasts [133]. Immunohistochemical studies have localised OPN to the mineralisation front in developing bone, which suggests that OPN is important in the process of matrix mineralisation more likely by influencing the rate of mineralisation rather than nucleation of hydroxyapatite crystal growth [134]. In bone sections, it was also found at high concentrations on the cement lines where bone formation follows resorption [135]. OPN is capable of binding to the hydroxyapatite matrix of bone, possibly via its aspartic acid-rich region. The level of synthesis of osteopontin by osteoblasts in culture is increased by treating these cells with 1, 25-dihydroxyvitamin D_3 and TGF-β.

The high concentrations of OPN in cement lines as discovered in studies by McKee *et al.* [134-6] suggested that OPN was important in the attachment of cells. The cDNA sequence indicates the presence of a Gly-Arg-Gly-Asp-Ser- (GRGDS) amino acid at residues 128-130, the sequence is recognised by several integrins and was shown *in vitro* to serve as an attachment substrate to several cell types [137], primarily via the $\alpha_v\beta_3$ integrin. This integrin is abundant in osteoclasts and has been implicated in osteoclast activation [138, 139]. There is general agreement that an OPN-$\alpha_v\beta_3$ integrin interaction is important in adherence of the osteoclast to bone [140].

In addition to bone cells, osteopontin is synthesised by extraosseous cells in the inner ear, brain, kidney, and placenta. It is also synthsised in odontoblasts, certain bone marrow cells and hypertrophic chondrocytes. However, osteopontin does not appear to be expressed by mesenchymal cells, fibroblasts, epidermal cells or by most epithelial cells *in vivo* [135].

3.1.2.6 Alkaline Phosphatase

Alkaline phosphatases (ALP) are a group of enzymes found primarily in the liver (isoenzyme ALP-1) and bone (isoenzyme ALP-2). There are also small amounts produced by cells lining the kidney (in the proximal convoluted tubules), intestinal mucosal epithelial cells, placenta and vascular endothelial cells. The alkaline phosphatase molecules produced by bone, liver and kidney are all formed from an identical gene product, and the three types differ only in their post-translational carbohydrate modifications [141, 142].

The bone-specific alkaline phosphatase is produced by osteoblasts. It is a glyco-protein and functions as an ectoenzyme attached to the osteoblast cell membrane by a glycosyl-phosphatidylinositol (GPI) anchor [143, 144]. As the name implies, the enzyme works best at alkaline pH (a pH of 10), it acts by splitting off phosphorus (an acidic mineral) creating an alkaline pH:

$$\text{Orthophosphoric monoester} + H_2O \rightarrow \text{alcohol} + H_3PO_4$$

Alkaline phosphatase is a member of a family of zinc metalloprotein enzymes. Zn, along with Mg, ions are required for minimal activity [145].

The relationship of alkalinity produced by alkaline phosphatase to bone mineralisation plays a major role in the prevention and reversal of osteoporosis. The reason that calcium deposits on bones during bone remodelling is because the solution in the surrounding area is alkaline, and calcium comes out of solution and crystallises in an alkaline environment. The reverse is also true, acidic solutions dissolve the calcium deposits (as seen in bone resorption by osteoclasts). It makes sense that osteoblasts, by creating a local environment of alkalinity via alkaline phosphatase, helps build up bone.

3.1.3 Angiogenesis

Angigenesis is essential for the incorporation of bone grafts; it also plays an important role in endochondral ossification. The vasculature within a graft brings both nutrients to the cells and also stem cells with the potential to settle and divide into cells of the osteogenic lineage at the site. However, it has also been proposed that the cells lining the blood vessels, the endothelial cells, themselves might release factors that promote bone formation. CD105 is a useful marker of endothelial cells [146].

3.1.3.1 CD105

CD105 (also called endoglin) is a cell membrane glycoprotein mainly expressed on cellular lineages within the vascular system, and over-expressed on proliferating endothelial cells [146], where it functions as an accessory component of the receptor complex of TGF-β [147, 148]. CD105 is also involved in vascular development and remodelling. The absence of CD105 in knockout mice leads to their death from defective vascular development, but the role of CD105 in the modulation of angiogenesis has not been elucidated [149]. CD105 can be used to identify new vasculature in *in vitro* cultures [146].

3.1.4 Aims

The aim of this Chapter was to:

- Characterise mouse bone and marrow using antibodies for specific markers of stem cells (both stromal and haematopoietic), osteogenesis and angiogenesis.

3.2 Methodology

3.2.1 Tissue Preparation

Femora were harvested from adult male MF1 mice. The soft tissues about the femora were removed, and the bones fixed in 10% formal-saline for 24 hours. The femora were decalcified for a further week in a solution of 5.5% EDTA disodium salt in 10% formal-saline, and then paraffin embedded. Sections from the tissue blocks were cut at 5μm thick sections and mounted on glass slides (Fisher Scientific UK Ltd, Loughborough, Leicestershire), and dehydrated for immunohistochemistry.

3.2.2 Immunohistochemistry

The slides were deparaffinized in xylene and rehydrated in four sequential ethanol baths from 100% to 50% (each for 5 minutes) and finally double distilled water, prior to use. Immunohistochemistry was carried out as described in the general methodology. Samples were treated with mouse monoclonal antibodies, to either type I collagen (7.4 μg/ml) (Southern Biotechnology Associates, Inc. Birmingham, AL, USA), STRO-1 (8.06 μg/ml), B4-78 to alkaline phosphatase (8.04 μg/ml), WVID1(9C5) to bone sialoprotein (22.45 μg/ml), MPIIIB10_1 to osteopontin (16.6 μg/ml), DS1 to decorin (35 μg/ml), AON-1 to osteonectin (7.75 μg/ml), MJ7/18 to CD105 (6.7 μg/ml) (all Developmental Studies Hybridoma Bank, University of Iowa, Iowa, USA) or rat monoclonal antibody MEC14.7 to CD34 (10.1 μg/ml) (Serotec Ltd, Kidlington, Oxford) for 60 minutes at room temperature.

Positive controls were set up looking at the staining in bone, of primary antibodies for type I collagen, alkaline phosphatase, bone sialoprotein, decorin and osteonectin. Alkaline phosphatase was also tested on liver sections. CD105 was

tested on endothelial cell cultures. Negative controls looking for staining in the absence of primary antibody were also evaluated.

3.3 Results

STRO-1 labelling for stromal stem cells (figure 3.1) was restricted to the marrow cavity where it was particularly associated with groups of cells in close proximity to bone, both the cortical bone surrounding the diaphyseal shaft and trabeculae of bone found throughout the marrow. A few cells next to blood vessels also stained positive for STRO-1, as did cells labelled for the haemopoietic stem cell marker CD34 (figure 3.2). CD34(+) cells were also distributed evenly in the marrow, including cell clusters that not associated with any particular marrow structure.

Type I collagen (figure 3.3) was expressed throughout the bone matrix and also, to a lesser extent throughout the marrow cavity. Intense staining was observed particularly along the surface of bone and cells in groups, randomly distribute throughout the marrow and close to blood vessels. This distribution was mirrored when femur sections were labelled with antibodies for decorin (figure 3.4), bone sialoprotein (figure 3.5), osteonectin (figure 3.6), osteopontin (figure 3.7) and alkaline phosphatase (figures 3.8).

CD105 (figure 3.9) was used as a marker of endoglin, found on the surface of endothelial cells. Endothelial cells were found distributed consistently in the marrow, surrounding both large (sinusoidal) and small (micro) blood vessels.

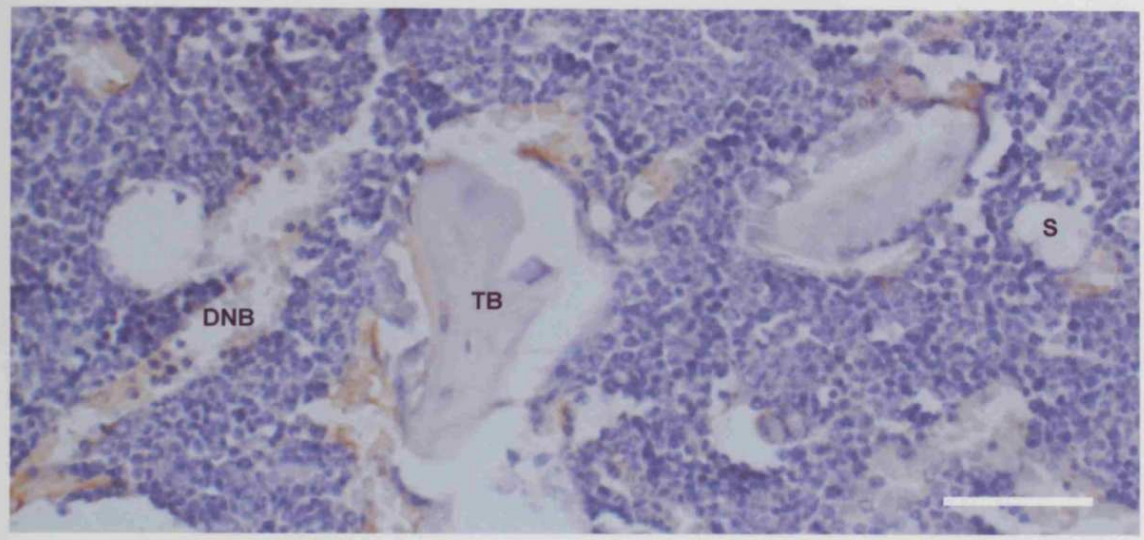


Figure 3.1: Distribution of STRO-1 in mouse bone and marrow. STRO-1, a marker of stromal stem cells is found distributed along the surface of trabecular bone (TB) and areas of de novo bone synthesis (DNB) in the marrow cavity and also in clusters distributed within marrow, particularly near sinusoid (S) blood vessels. Scale bar, 500μm.

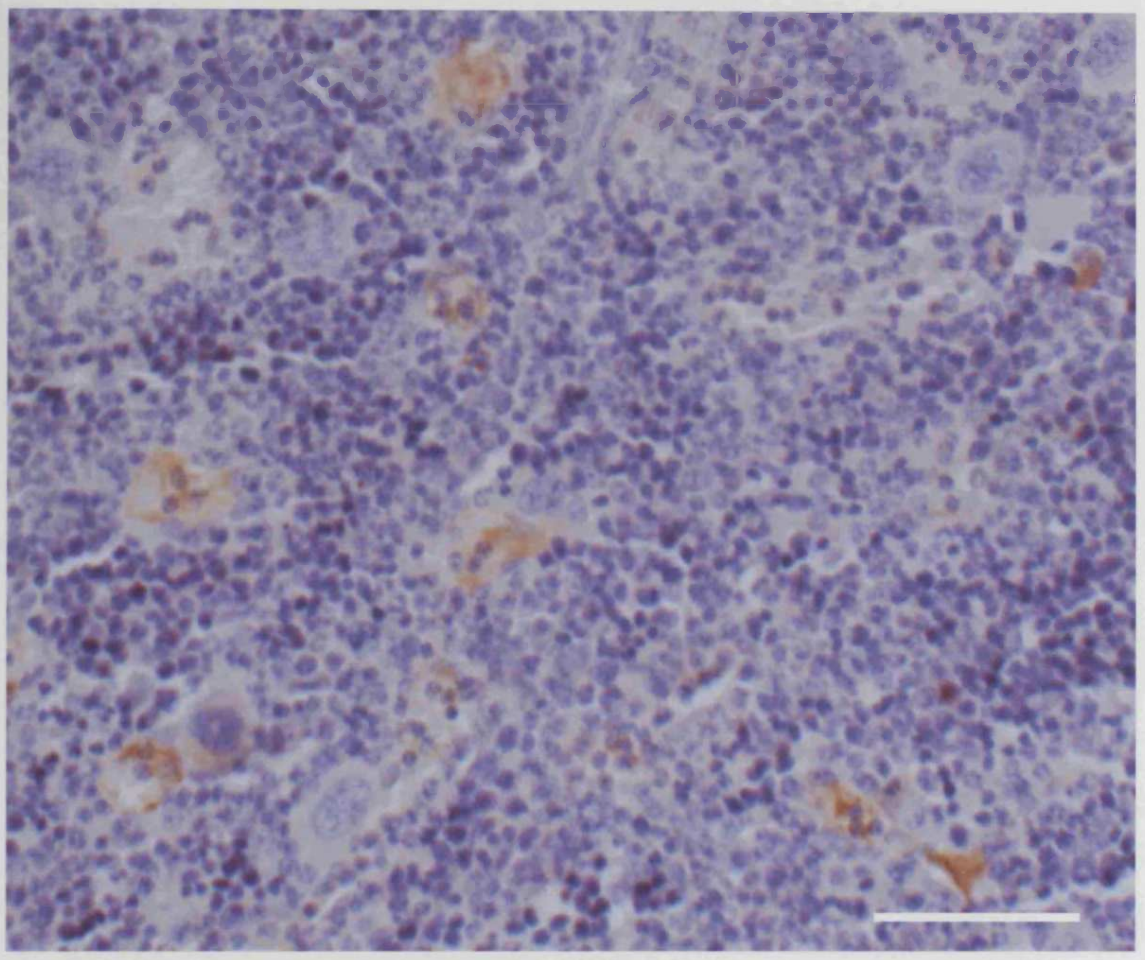


Figure 3.2: Haematopoietic stem cells in the marrow. Haematopoietic cells are labelled with CD34 antibody. Scale bar, 500μm.

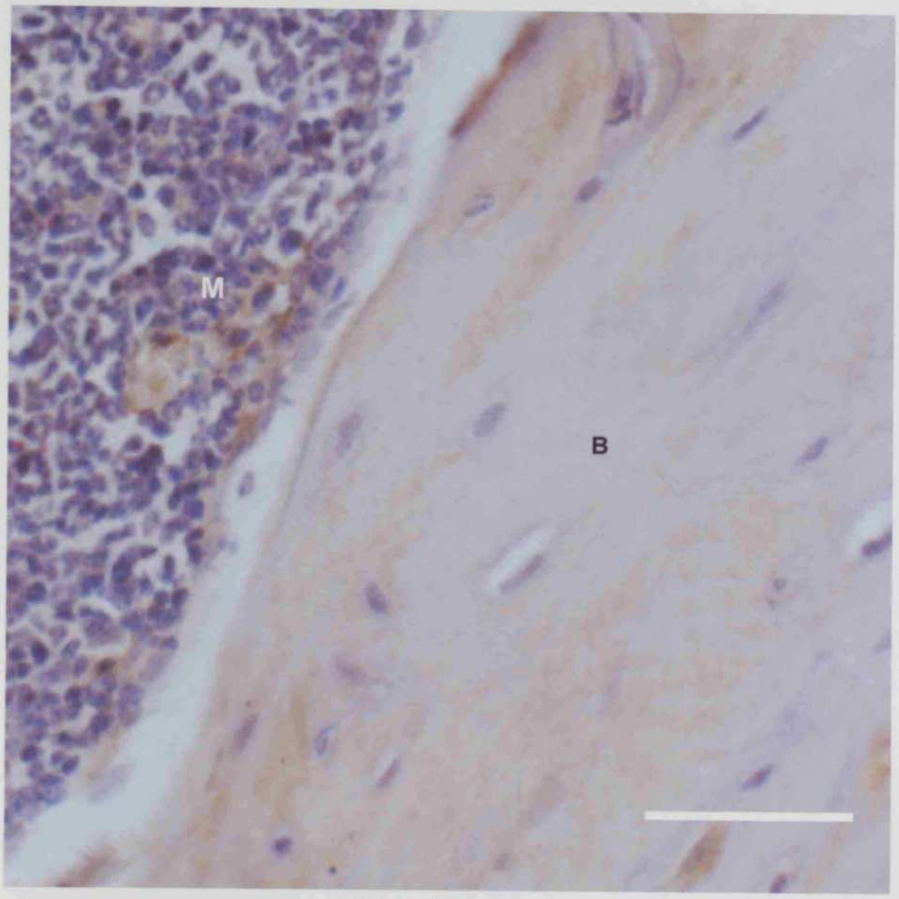


Figure 3.3: Collagen type I labelling in bone (B) and marrow (M). Scale bar, 500μm.

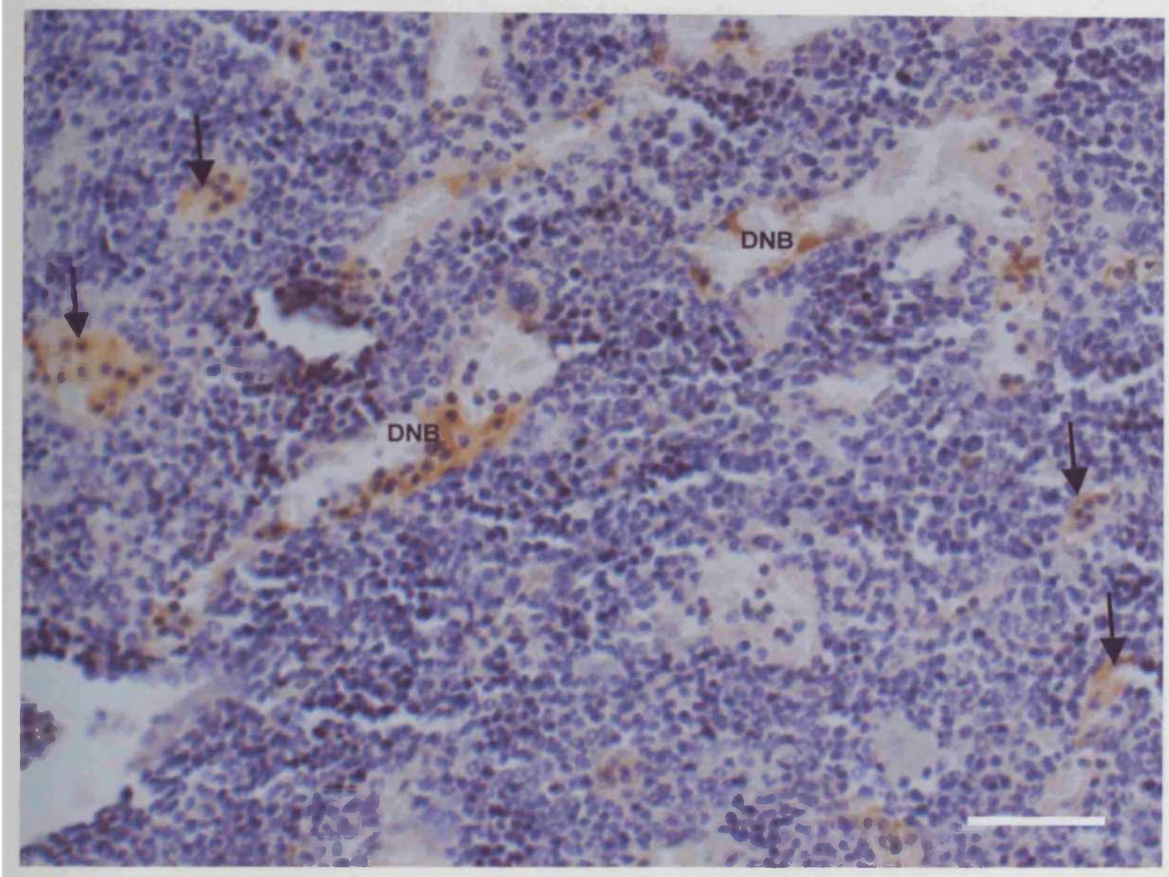
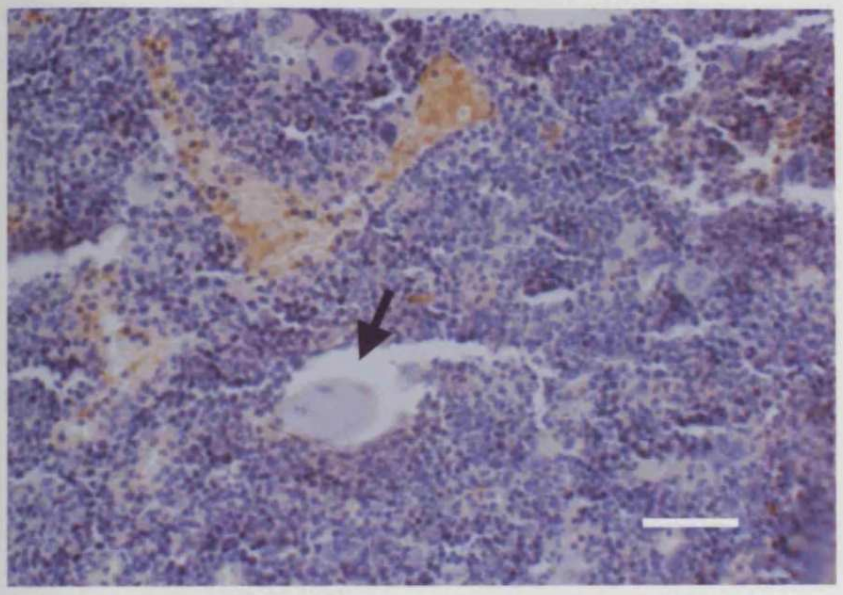


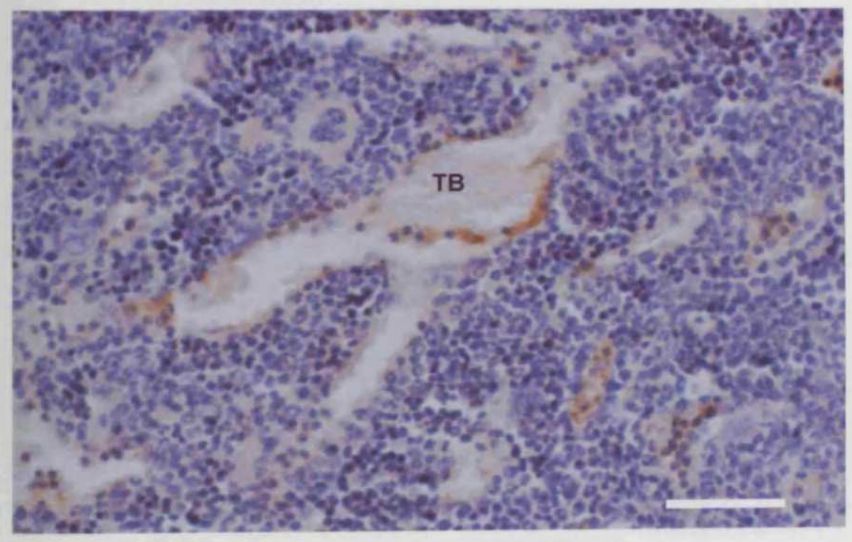
Figure 3.4: Distribution of decorin. Decorin expressed in areas of de novo bone (DNB) synthesis and produced by small groups of cells in the marrow (arrows). Scale bar, 500μm.

Figure 3.5 (A & B): Bone sialoprotein expression. Bone sialoprotein is produced by clusters of cells in the marrow (A), and also at the edge of bone (TB). The marrow shrinks away from the bone surface during processing (shrinkage indicated by arrow). Scale bar, 500μm.

A



B



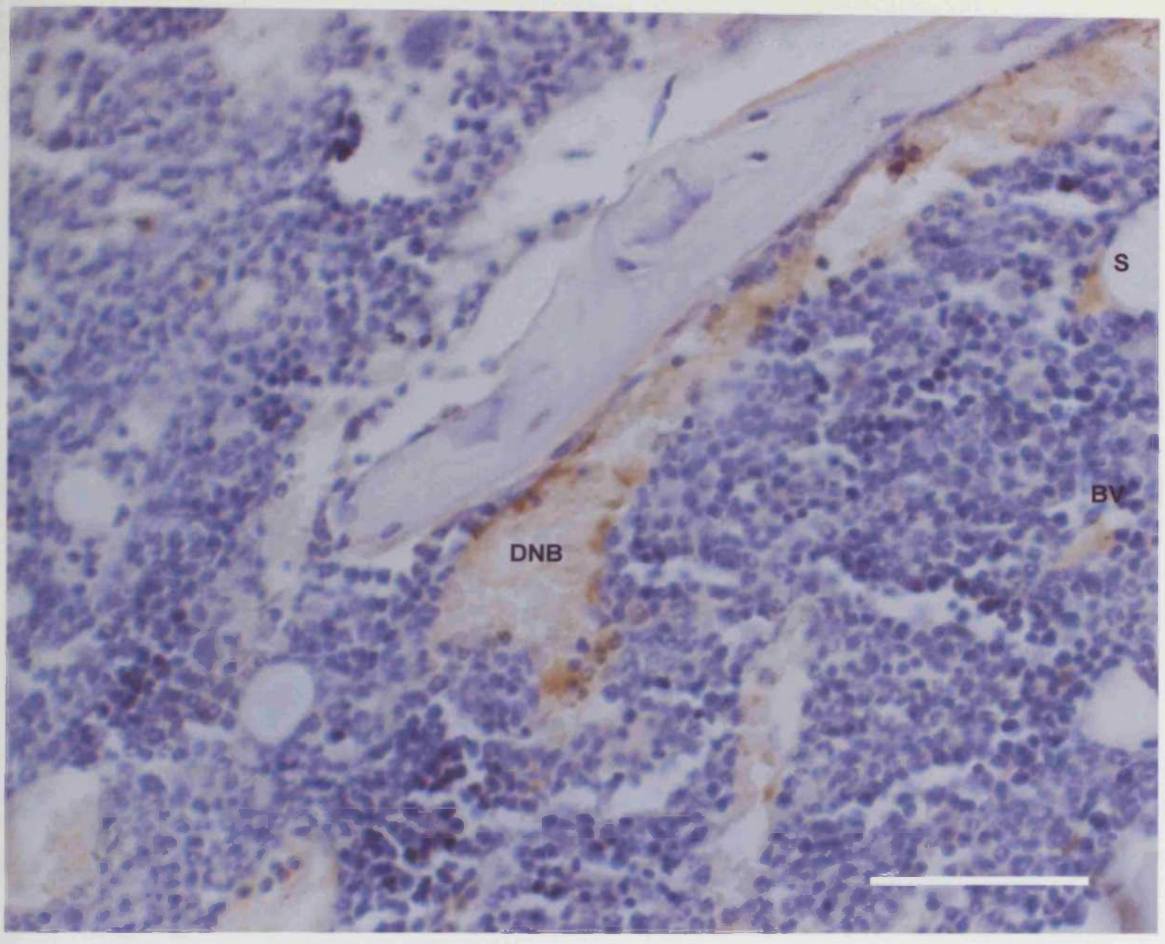


Figure 3.6: Ostenectin distribution. Osteonectin in an area of de novo bone (DNB) synthesis, near sinusoid (S) and micro blood vessels (BV). Scale bar, 500μm.

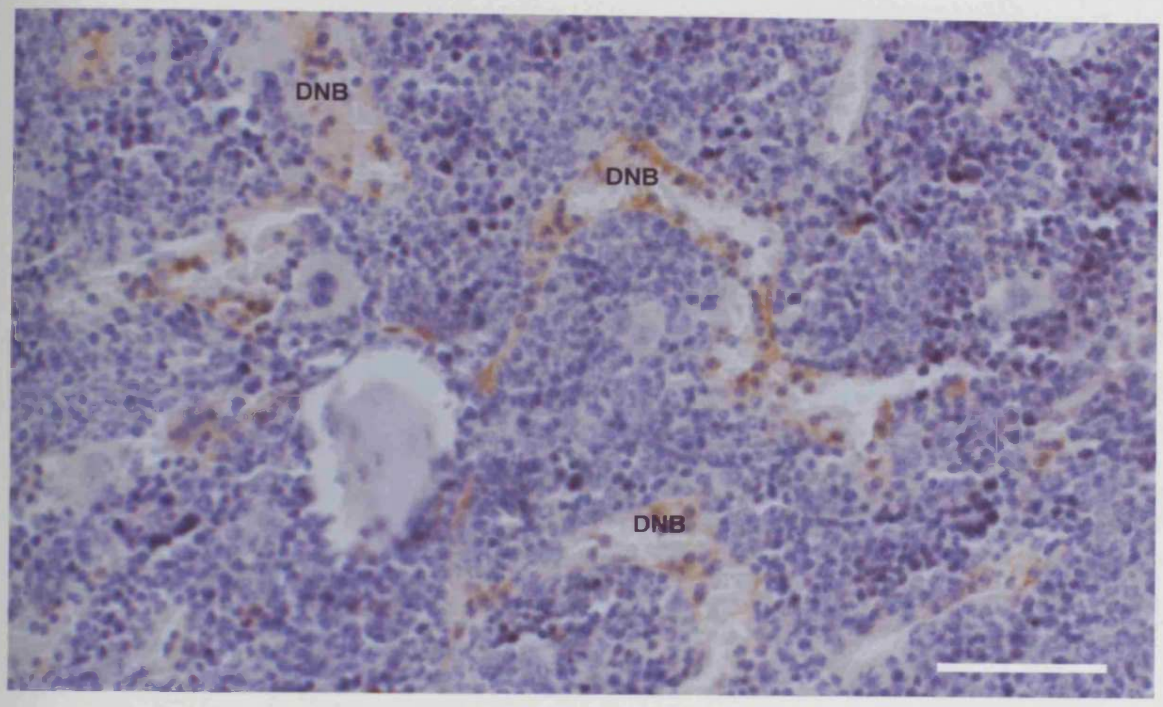


Figure 3.7: Distribution of osteopontin. Osteopontin in an area of de novo bone (DNB) synthesis. Scale bar, 500μm.

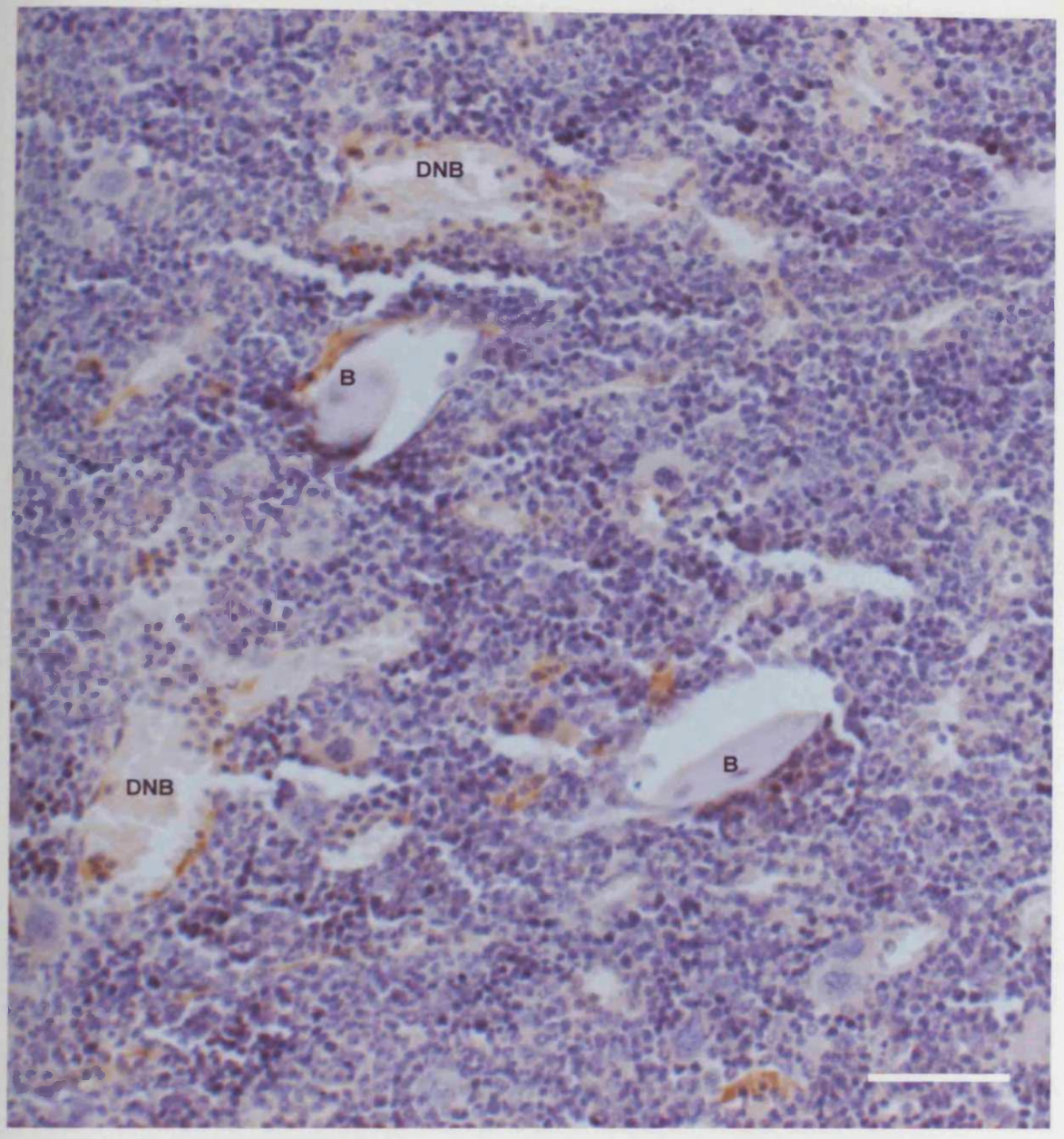


Figure 3.8: Distribution of alkaline phosphatase. Alkaline phosphatase from the surface of bone (B) and de novo bone (DNB). Scale bar, 500μm.

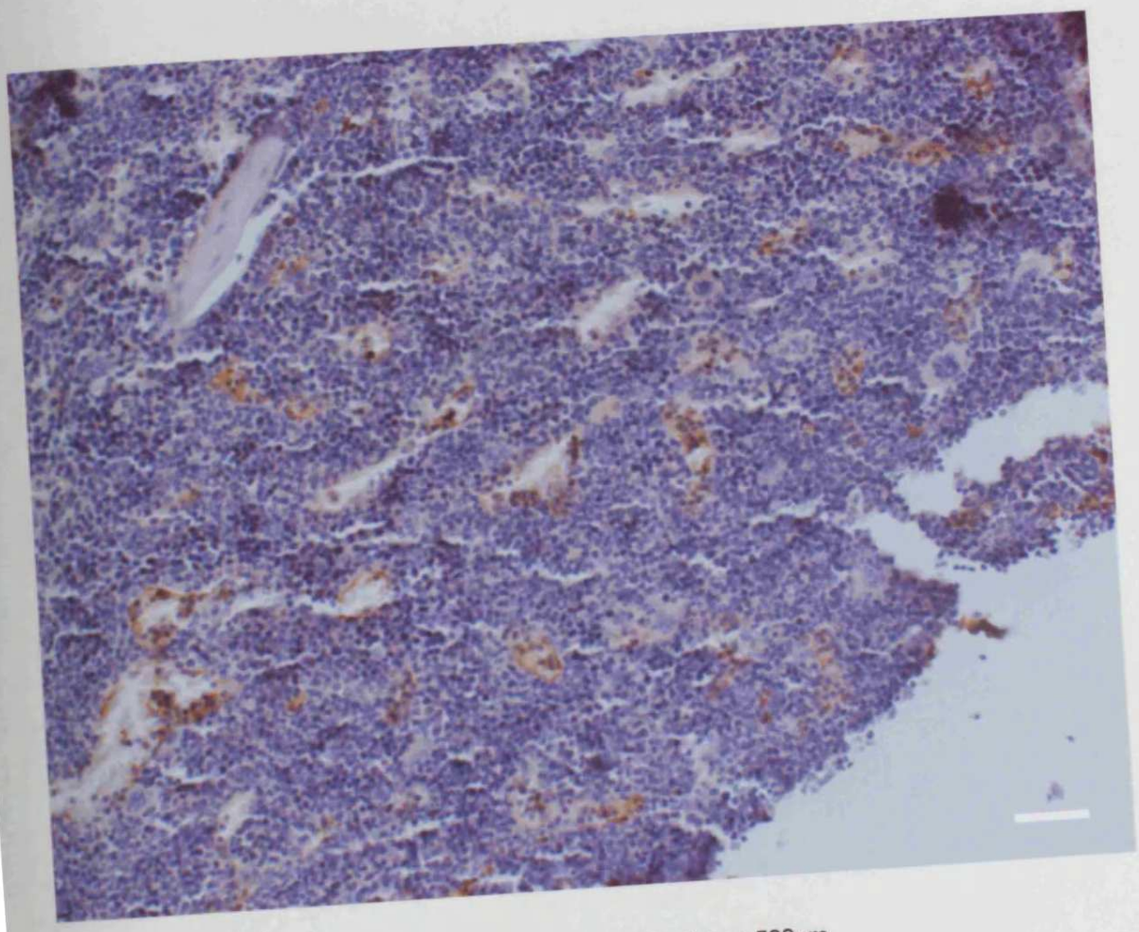


Figure 3.9: Distribution of CD105 throughout the marrow. Scale bar, 500μm.

3.3.1 Results Summary

- Stromal stem cell labelling with STRO-1 was associated with:
  - Bone lining cells
  - Cells surrounding blood vessels

- Cells expressing the haemopoetic stem cell marker CD34 were found:
  - Randomly distributed throughout the marrow cavity
  - Surrounding blood vessels

- Staining for type I collagen, decorin, BSP, osteonectin, osteopontin and alkaline phosphatase was found in:
  - Bone
  - Bone lining cells (intense staining)
  - Cell clusters throughout the marrow
  - Cells surrounding blood vessels

- Endothelial cell marker CD105 was expressed in:
  - Areas surrounding blood vessels

3.4 Discussion

The cells of the marrow are divided into haematopoietic cells and stromal cells. In adults stromal cells account for approximately 20% of all marrow cells. Stromal stem cells were identified in the marrow by the marker STRO-1 and haemopoietic stem cells by the antigen CD34. Immunohistochemistry demonstrated STRO-1 (+) cells to be unique to the bone marrow (no antibody binding was observed in bone) and distribution of this stromal stem cells marker within the marrow was restricted to just a few groups of cells neighbouring bone. The sparseness of the stromal stem cells, from which osteoprogenitors are produced, relative to the total number of marrow cells was low and thus a great deal of marrow would have to be collected to produce a reasonable quantity of preosteoblasts and consequently bone, unless the rate of proliferation in the stem cells was great. Therefore, one of the aims of future work within this study was to ensure the rate of stromal stem cell proliferation was sufficient to justify the quantity of marrow that would have to be removed to produce sufficient osteoblast numbers to either coat a graft or produce a new piece of autogenous bone. With many of the stromal stem cells being in such close contact with bone, it was also noted that it would have to be ensured in future work that marrow cells from the walls of the diaphyses were flushed out when marrow was harvested, to ensure the greatest number of stromal stem cells relative to the total number of cells obtained from the marrow.

Cells labelled with CD34 also accounted for a relatively small number of cells in the marrow cavity, since marrow cells are primarily mature haematopoietic cells or adipocytes in mature bone. The quantity of these cells present in future cultures will be monitored, as the bone marrow stromal cells may have a determinative role to play in hematopoietic cell growth and differentiation [150].

Type I collagen was found distributed throughout bone, as expected since it is the major component of this tissue [99]. The cells in the marrow expressing this collagen type may be osteoblasts or preosteoblasts, and the cells in close proximation to bone were almost certainly of this cell type. The cells more distant to bone, and also those near to microvessels and sinusoids may otherwise have been fibroblasts. Similar cellular distribution was observed for decorin, osteonectin, bone sialoprotein, osteopontin and alkaline phophatase; intense staining particularly observed again in the lining cells against the outer walls of the marrow cavity and around the marrow vasculature. This suggested that cells near blood vessels in the marrow may in fact be osteoblastic rather then fibroblastic in nature. The localisation of osteoblastic lining cells next to the inner bone surface in the cavity also confirms that to obtain the greatest osteogenic potential from a marrow sample we will have to ensure we obtain those cells closest to the bone surface.

The distribution of cells expressing osteogenic markers in close proximity to blood vessels (as confirmed by CD105 monoclonal antibody staining) suggests that perhaps cells of the vasculature may have a role in osteogenesis, perhaps by a direct influence on the bone forming cells.

Chapter 4

Effects of Freezing, Gamma Irradiation, and Ethylene Oxide Treatment of Bone Grafts on Bone Marrow Cultures.

4.1 Introduction

Over the last few decades bone grafts have proved to be a reliable method for the reconstruction of cortical and trabecular bone defects. Bone grafting is extensively used in bone tumour surgery, revision of total hip arthroplasty, bone loss due to traumatic injury, treatment of avascular necrosis and periodontal therapy [151-153]. Other substitutes of natural origin (e.g. coral and ivory) or prepared synthetically (e.g. hydroxylapatite and ceramics) have not yet been found to satisfy the demand for a mechanically resistant and osteoconductive material.

Although bone autograft is known to be the best material, it is necessarily limited in volume, requires additional surgery and is often associated with prolonged painful problems [154]. Xenogeneic bone had proved to satisfy good osteoconductive properties in orthopaedic and dental practice [155, 156]. However, xenografts have recently suffered from ethical controversies and their uses as a bone substitute have been drastically reduced [157]. The possibility that the prion protein associated with the bovine spongiform encephalopathy could be transmitted via bone grafts has led to a dramatic limitation of such xenografts. An important source of xenogenic bone became suspect during the last years albeit prion denaturing reagents can be used to prepare bone xenografts. The urgent need to use a hard substitute led to reconsideration of the use of human allogenic bone.

The increasing request for sufficient quantities of allogenic bone has led to the constitution of 'bone banks' for managing, storing and dispatching bone fragments or complete anatomical pieces obtained from live donors as well as from cadavers. Currently their most frequently used preparation technique for allografts, is cryopreservation in deep freeze (-40°C to -80°C) or liquid nitrogen (-196°C). Cryopreservation has been reported to reduce immunogenicity as the donor's cells are destroyed but it has no effect on bacterial and viral contamination [158, 159]. A

drastic selection of donors, the use of surgical theatre with aseptic conditions during harvesting and a serological survey are necessary [160].

However, an increasing number of bone banks are now sterilising bone allografts. A gamma irradiation dosage of 25,000 Gray is presently a widely accepted sterilisation procedure. Ethylene oxide has also been proposed, but the technique only appears useful for small bone samples due to the limited penetration of the gas [161].

Despite the use of these allograft treatment regimes worldwide, there is little data available on the cellular response of osteogenic precursor cells on contact with the grafted bone, a factor that would greatly influence the initial rate of osseointegration. In this chapter therefore, the effect of bone slices treated as outlined above, on osteogenic precursor cells was evaluated.

4.1.1 Aims

The aim of this chapter was to:

- Evaluate the cellular responses of osteoblastic cells when exposed to treated bone.
- Discover the treated bone most preferential for cell growth.

4.2 Methodology

4.2.1 Bone Marrow Cultures

Osteoblastic precursor cells were prepared by culturing mouse bone marrow single cell suspensions at a density of $1x10^7$ total marrow cells per $25cm^2$ tissue culture flask (Nalge NUNC International, Paisley, Scotland); in 5 ml α-modification minimal essential medium eagle (Gibco BRL Ltd., Paisley, Scotland) supplemented with 10% foetal calf serum, 2mM L-glutamine, penicillin (10,000U/100ml),

streptomycin (10mg/ml) and amphoterycin (25μg/ml). The medium was changed 2-3 times weekly and the cultures incubated in a humidified atmosphere of 95% air and 5% CO_2.

After 21 days, the cultures were confluent and the osteogenic precursor cells harvested by trypsin (0.05%)-EDTA (0.02%). The osteoblastic precursor cells were then plated in 35mm culture dishes (Nalge NUNC International, Paisley, Scotland), at a density of $3x10^5$ cells per dish, and incubated with 2ml supplemented culture medium (as above) for 24 hours in a humidified atmosphere of 95% air and 5% CO_2.

4.2.2 Bone allograft preparation

A human cancellous bone slice from the femoral neck, approximately 5mm x 5mm x 3mm, which had been either stored frozen at -80°C; sterilised by gamma irradiation (25,000Gy) and then frozen; or ethylene oxide treated and freeze dried; was then placed on top of the cells in the centre of each culture dish. Cultures were set up in replicate (tables 4.1 and 4.3) and additional culture medium added until the bone was submerged. Subsequent changes in media (2-3 times weekly) removed dead cells.

4.2.3 Cell Viability

The degree of cell survival was evaluated by counting the number of adherent cells in the culture, with a haemocytometer, that were enzymatically released after treatment with trypsin (0.05%)-EDTA (0.02%).

4.2.4 Cell Differentiation

To determine whether bone treatment impacted upon differentiation of the bone marrow stromal cells, the collagens released into the media by the cells were

analysed using autoradiography (see general methodology). L-[5 ^{3}H] Proline (Amersham Biosciences UK Limited, Bucks, UK) was added at a concentration of 1μCi/μl of culture medium to cultures following addition of the bone slices, or at day 6. The media along with PBS washes was then collected at days 1 and 7 of culture and the incorporation of the radiolabel into collagens was assessed.

4.3 Results

4.3.1 Cell Proliferation

Under the phase contrast microscope, during the 24 hour incubation period prior to addition of the bone slices, the osteogenic precursor cells exhibited a polyhedron or spindle appearance. On addition of bone slices, in less than a day, cells cultured under and around the gamma irradiated and ethylene oxide treated slices became rounded with considerable cell shrinkage. These cells readily detached from the culture surface upon washing with PBS.

The area devoid of cells, surrounding the central bone slices in the ethylene oxide and gamma irradiated cultures, varied greatly between cultures. Fresh frozen bone cultures were never associated with a surrounding area containing necrotic or dead cells. Slices treated with ethylene oxide induced a minimal amount of cell death around the bone slice. The thickness around the ethylene oxide sterilised slices was roughly the width of a cell. On the other hand, γ-irradiated slices were associated with a considerable amount of cell death at distance from the bone.

Cells at the edge of all of the dishes however, maintained their 'spindle' appearance. The peripheral cells proliferated in these cultures, and by 7 days they had grown towards the bone slices. Cells grown in the presence of fresh frozen bone maintained their 'spindle' shape throughout the culture period, with the cells appearing to grow both under and on the bone slice.

Figure 4.1: Number of adherent osteogenic precursor cells grown in the presence of fresh/frozen, gamma-irradiated/frozen or ethylene oxide/freeze dried bone slices. Values are given as means ± standard deviations of the number of adherent cells per culture.

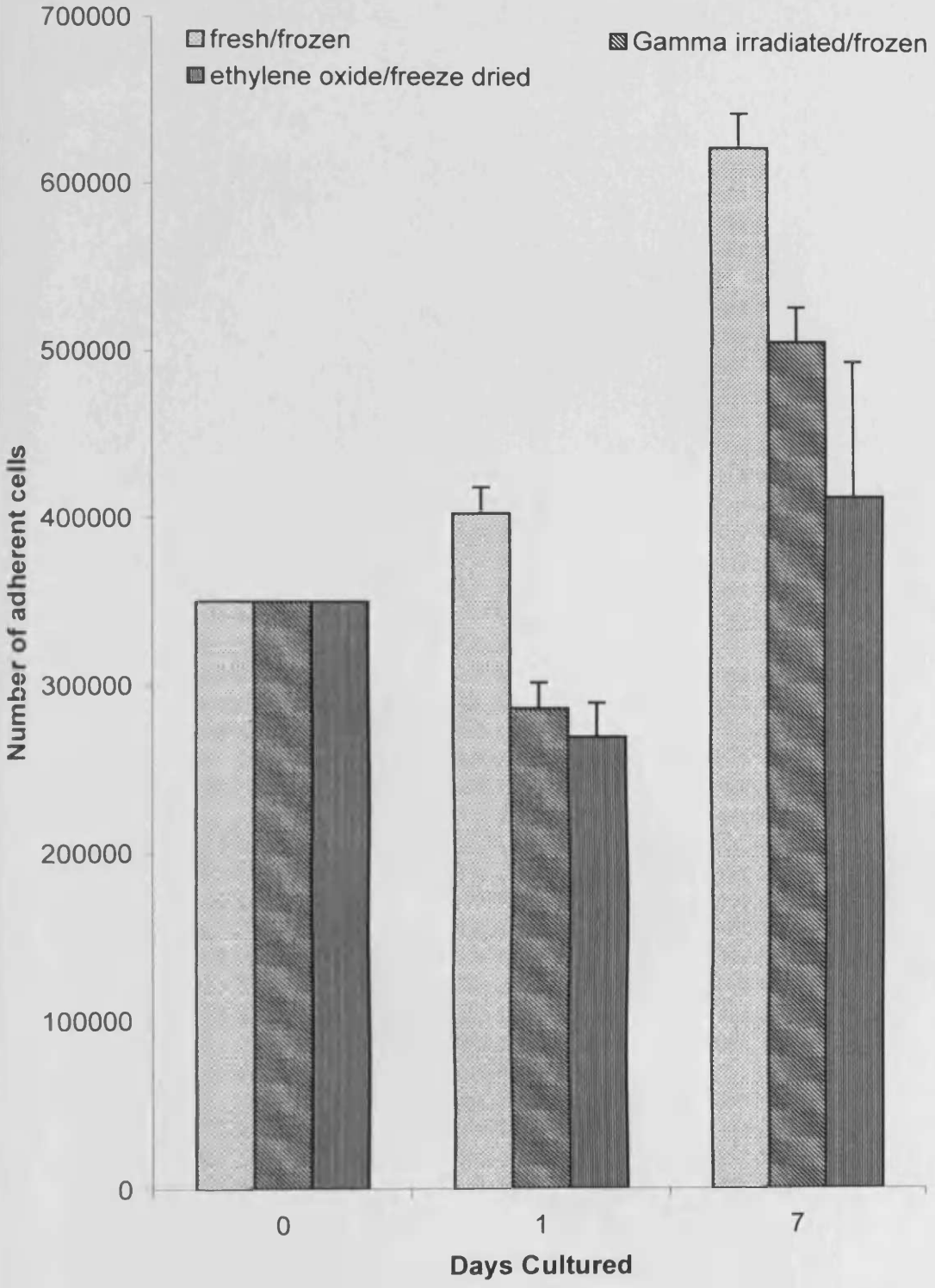


Table 4.1: Number of cultures counted for cell proliferation.

	Fresh/Frozen	Gamma irradiated/frozen	Ethylene oxide/freeze dried
Day 0	7	7	7
Day 1	9	7	7
Day 7	6	7	6

Table 4.2: ANOVA analyses on the number of adherent cells grown in the presence of gamma irradiated/frozen or ethylene oxide/frozen compared to cultures grown in the presence of fresh/frozen bone. * Indicates cultures in which cell numbers differed significantly from the control cultures.

	Fresh/Frozen & gamma irradiated/frozen	Fresh/frozen & ethylene oxide/freeze dried	ethylene oxide/freeze dried & gamma irradiated/frozen
Day 1	-227920.7 to –5412.7*	-244587.3 to –22079.3*	-94587.3 to 127920.7
Day 7	-227920.7 to –5412.7*	-321254.0 to –98746.0*	-17920.7 to 204587.3

Cell counts showed a drop in cell number over the first 24 hours in sterilised (ethylene oxide & gamma irradiated) bone cultures; whilst an increase in the number of cells was observed in non-sterilised (fresh/frozen) bone cultures (figure 4.1). The differences in cell number between sterilised and non-sterilised cultures were demonstrated to be of significant difference (table 4.2). After a week however cells in all three cultures proliferated, although the numbers of cells in the sterilised cultures were still significantly less than those of non-sterilised cultures.

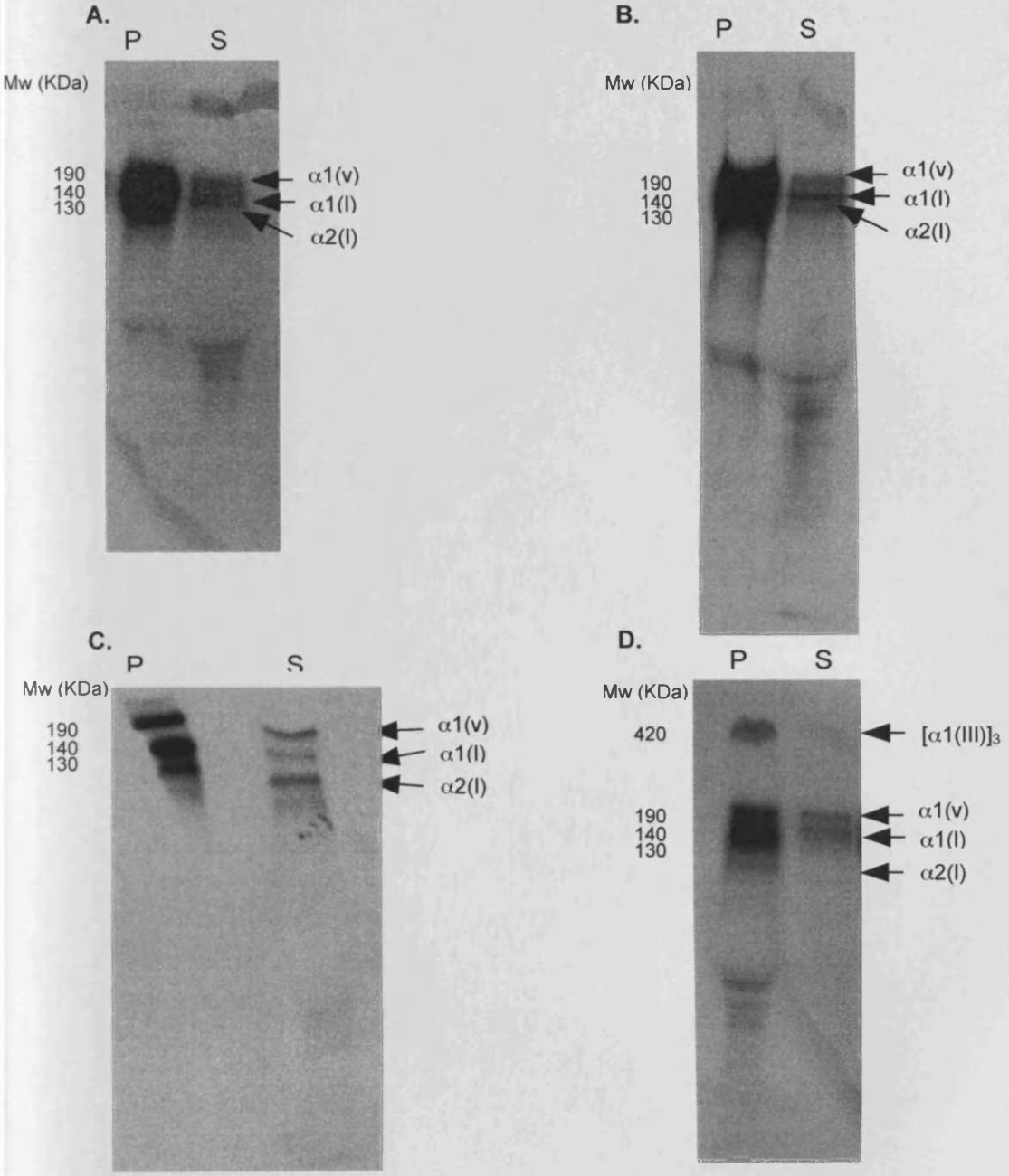
4.3.1 Cell Differentiation

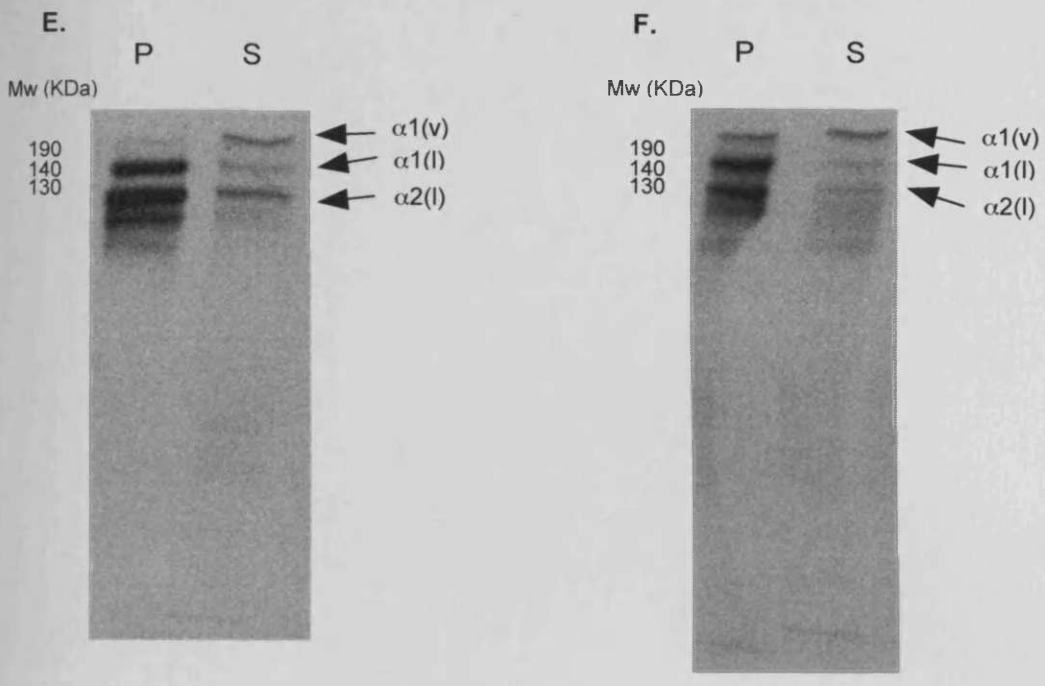
After a day in culture, few differences were observed between the collagens produced by cells cultured in the presence of gamma irradiated/frozen, fresh/frozen or ethylene oxide/freeze dried bone slices (figure 4.2). The collagens produced, were inferred to be mainly types I and V. Following the seventh day, autoradiography bands suggesting the presence of type I collagen (indicative of osteoblasts) was most prominent in the media surrounding the fresh-frozen bone (figure 4.2 B). Bands for this collagen were also present, although to a marginally lesser extent, in media from ethylene oxide treated bone cultures (figure 4.2 F). However, type III collagen was also believed to occur in the cultures grown with γ-irradiated slices (figure 4.2 D).

Table 4.3: Number of cultures analysed for autoradiography.

	Fresh/Frozen	**Gamma irradiated/frozen**	**Ethylene oxide/freeze dried**
Day 1	3	3	3
Day 7	3	3	3

Figure 4.2: Fluorography film, showing bands inferring the presence of type I, III and V collagens. Media taken from bone marrow cultures grown in the presence of Fresh/frozen bone at day 1 (A), and day 7 (B); Gamma irradiated/frozen bone at day 1 (C), and day 7 (D); and ethylene oxide/freeze dried bone (E) and (F). S = Supernatant and P = Precipitate obtained from the culture media (see general methodology).





4.3.3 *Results Summary*

- Necrosis of cells cultured under and around, gamma-irradiated and ethylene oxide treated bone was observed within 24 hours of culture.
- Cell death was greater in gamma-irradiated than ethylene oxide treated bone cultures.
- After 7 days, cells in sterilised cultures had proliferated, and the difference in cell number between the sterilised and non-sterilised bone cultures was no longer significant.
- Collagens produced by cells at 24 hours and after a week in culture appeared to be mainly of type I in the sterilised and non-sterilised bone cultures, with the exception of gamma-irradiated bone cultures which also seemed to produce type III collagen.

4.4 Discussion

Irradiation is a common way to sterilise bone allografts for storage in bone banks. Massive osteoarticular allografts and cancellous bone allografts are commonly treated in this manner. Irradiation is by far the preferred sterilisation technique for many physicians, since a γ-radiation dose of 15, 000 – 25, 000 gray has been shown to ensure full sterilisation of bacteria [169] and higher doses (>36, 000 gray) have been found to inactivate all but one in a million HIV-infected cells [170].

Deep freezing has no bactericidal activities and does not completely inactivate retroviruses [162-166]. Ethylene oxide has also been proposed but the technique appears useful for small bone samples due to the limited penetration of the gas [154]. In addition, the method suffers from several disadvantages: retention of breakdown products of gas in the tissue [167] and fatal allergic reaction to the ethylene oxide [168].

Undesirable consequences of high levels of γ-irradiation have been reported by several groups. Mechanical properties have been found to be altered in a dose-dependent way even after 20 000 gray irradiation [171]. Irradiation has also been found to alter proteins. This effect is favourable when it reduces immunogenicity (probably associated with residual HLA antigens) but appears undesirable when osteoinductive proteins are altered [172, 173].

From the results of this chapter it may be concluded that gamma irradiated bone may also have a direct impact on the cells surrounding the graft, in addition to the cells and proteins of the graft itself. Gamma irradiated, and to a lesser extent ethylene oxide treated, bone had detrimental effects on the osteogenic precursor cells within the first few days in culture. Cell death, and in the case of gamma

irradiated bone alterations in the lineage of preosteoblasts away from the osteoblastic route preferential for osseointegration, was observed.

In the present study it was found that γ-irradiation induced an area of cell death in the area surrounding the treated bone slice, in as little as 24hours after initial contact. This may have been down to free radicals leaching from the irradiated sample, since free radicals have been implicated in the pathogenesis of many diseases because they are known to injure cells and tissues [174]. Lipid peroxidation is a general mechanism of tissue damage by free radicals, and lipid molecules can be found in abundance in bone taken from the femoral head. Altered medullary lipids are known to be responsible for the brown tint on gamma irradiated samples [174].

It has previously been shown that free radicals released, could induce osteoblast alterations or necrosis [175]. Peroxidated lipids have often been reported to induce cell apoptosis rather than cell necrosis [176]. The exact cellular mechanism was not checked in the present study. However, the morphological appearance of dead cells indicates a necrotic process.

This necrotic effect diminishes with time, and is greatly reduced after the medium is changed. Therefore it is possible that cytotoxins leaching out of the sterilised bone slices within the initial few days of incubation are removed from the culture with a change of medium. The effects of gamma irradiated bone on preosteoblast lineage may also be a consequence of early exposure.

Sterilisation, by gamma irradiation and ethylene oxide, irradicates potentially harmful bacteria and viruses from bone allografts. A drastic selection of donors, the use of a surgical theatre with aseptic conditions during harvesting, and a serological survey are necessary in the collation of fresh/frozen bone allografts [152]. Therefore treating bone with gamma irradiation (in the case of large allografts) or ethylene oxide (for smaller allografts) is clearly advantageous. From the results of this chapter

it would appear that osseointegration could be made more rapid and reliable by pre soaking sterilised bone slices before use, to eliminate any cytotoxic effects, it would be a promising technique in the management of bone banks.

Chapter 5

Evaluation of the Effect of Different Cell Culture Media on Mouse Bone Marrow Stromal Cell Cultures

5.1 Introduction

As previously described (see *general introduction*), under physiological conditions bone is constantly turned over through waves of bone formation and bone resorption, a process called bone remodeling. However in pathological conditions, such as bone loss due to tumour removal, traumatic injuries, failed joint arthroplasties or avascular necrosis; the rate of normal turnover is insufficient for the amount of bone required. The requirement for bone can be treated through transplantation of bone from another individual (allograft) and xenotransplantation (i.e. the transplantation of bone into humans from other species). Presently allografting and xenotransplantation carry quantifiable risks of tissue rejection and disease transfer, which cast serious doubt on their use [1]. Autologous bone (the individuals own bone) would be the most desirable graft material, its source however is limited, and removal of donor bone is associated with patient morbidity. However, it may be possible to replace lost tissue by transplanting bone grown, in the laboratory, from autologous bone marrow stem cells. The genetic make up of these stem cells, from which the tissue is produced, would essentially be identical to the patient's cells. These cells may also be used to replace the loss of bone tissue seen in osteoporosis.

Maniatopoulos *et al.* were the first to create a bone-like structure *in vitro* using bone marrow cells from rats. The formation of mineralised nodules in Maniatopoulos' system however, required the addition of exogenous factors to the tissue culture media (dexamethasone and β-glycerophosphate) [26]. In this report, a major aim is to induce BMSC to form osteoblasts and consequently a mineralised matrix without these exogenous factors. There are many parameters that influence the expression of the osteoblastic phenotype in culture, i.e. culture medium, culture time and the presence of compounds that influence cell proliferation and differentiation, therefore, it is convenient to select suitable experimental conditions in order to obtain bone cell

5.2.4 Immunohistochemistry.

Immunohistochemistry was carried out on the fixed cultures as described in the general methodology. The cells were treated with mouse monoclonal antibodies, to either type I collagen (7.4 μg/ml) (Southern Biotechnology Associates, Inc. Birmingham, AL, USA), STRO-1 (8.06 μg/ml), B4-78 to alkaline phosphatase (8.04 μg/ml), WVID1(9C5) to bone sialoprotein (22.45 μg/ml), MPIIIB10$_1$ to osteopontin (16.6 μg/ml), DS1 to decorin (35 μg/ml), AON-1 to osteonectin (7.75 μg/ml), MJ7/18 to CD105 (6.7 μg/ml) (all Developmental Studies Hybridoma Bank, University of Iowa, Iowa, USA); rat monoclonal antibody MEC14.7 to CD34 (10.1 μg/ml) (Serotec Ltd, Kidlington, Oxford) or rabbit polyclonal antibody to von Willebrand factor (12.4 μg/ml) (AbCam, Cambridge, UK) for 60 minutes at room temperature.

5.2.5 Oil red o staining

Lipid vacuoles found within adipocytes in the BMSC cultured were stained bright red with 0.3% Oil red o solution in 99.5ml 99% isopropanol and 0.5ml ddH_2O.

5.2.6 Von Kossa Staining

Mineralisation within the cultures was confirmed using Von Kossa method (see general methodology), which stained calcium phosphate deposits black.

5.2.7 Scanning electron microscope (SEM) analysis

Cell cultures were fixed as described for immunohistochemical analysis. SEM analysis was carried out by an SEM technician (Mrs Wendy Rowe, Matrix Biology and Tissue Repair Research Unit, Dental School, University of Wales College of Medicine).

5.2.8 Statistical analysis

Each experiment in this chapter was repeated six times, therefore for cell counts, proliferation rates and colony counts each point represents the mean ± standard deviation of 6 different measurements for each culture condition at each time point. Statistical analysis was done by ANOVA test (one way analysis of variance). The statistical difference between the experimental culture media and the control media at each time point was determined. P values ≤ 0.05 were considered significant.

5.3 Results

Mouse bone marrow cells were cultured in control medium (alpha MEM), BGJ-b medium or ECCM for periods up to 21 days under selected experimental conditions. All the experiments were performed in the first subculture as previous studies showed that serial passage of bone marrow cells results in a progressive loss of the osteoblastic phenotype [177, 178].

5.3.1 Bone Marrow Stromal Cell microscopy (phase contrast and SEM) analysis and mineralization assay

Observation of the cultures by phase contrast microscopy during the first hours after plating showed the cell attachment to the standard plastic surface culture was similar in all the situations tested. Twenty four hours later, cells presented a fibroblastic morphology but, in 7 day cultures differences in cellular morphology between the cultures was distinct. At culture day 7, in control medium the cells maintained a few fibroblastic-like cells along with a predominantly rounded cell phenotype (figure 5.1), fibroblastic cells were also observed in ECCM cultures however an additional 'stellate' cellular phenotype prevailed along with a few rounded cells (figure 5.2). In the second experimental culture (BGJ-b medium

cultures) an assortment of cells were seen, including fibroblasts, polygonal cells and cells with long processes and enlarged nuclei at day 7 (figure 5.3). A few macrophage-like cells were randomly observed in all cultures.

The unique cellular make up of each culture condition persisted throughout the remainder of the period in culture. The distinct cells found in these cultures also grew with unique spatial arrangements on the culture dishes. Between days 7 and 14 in culture, cells grown in control medium formed rounded cell colonies of approximately equal size, whilst cells cultures in ECCM united to form colonies of many different sizes (figure 5.10). The 'stellate' cells which developed in endothelial cell condition medium created a lattice in certain areas on the surface of the culture dish (figure 5.6). One or two, or even twenty or more cells would form synaptic type junctions with neighbouring cells of the same stellate phenotype, or closely associated fibroblastic and rounded cells (figure 5.6). At day 21, cells grown in both ECCM and control medium were confluent and in some areas post confluent, and hence no colonies were observed (figures 5.7 and 5.9).

Cells cultured in BGJ-b medium did not form colonies (figure 5.10 and table 5.1), neither did the cells reach confluence at day 21; cells differentiating in this medium remained spatially distinct from other cells in the culture throughout the growth period (figure 5.5 and 5.8). Despite the lack of cell growth in this medium, when compared to the control and the BGJ-b medium, an abundance of matrix was produced by cells grown in this medium. The matrix secreted under culture in BGJ-b medium stained positively for calcium phosphate (von Kossa) confirming mineralization to be occurring in these wells (table 5.3). Mineralisation in these wells was analysed by electron microscopy, which showed the formation of crystals in these cultures (figure 5.11). Mineralised deposits were not formed in either the control medium, nor the ECCM (table 5.3).

Figure 5.1: Adherent cells of 7 day bone marrow cultures grown in α-MEM. Cells formed colonies such as that seen in the figure below, consisting of fibroblastic cells (arrows) and rounded cells (*), when grown in α-MEM. Scale bar, 150μm.

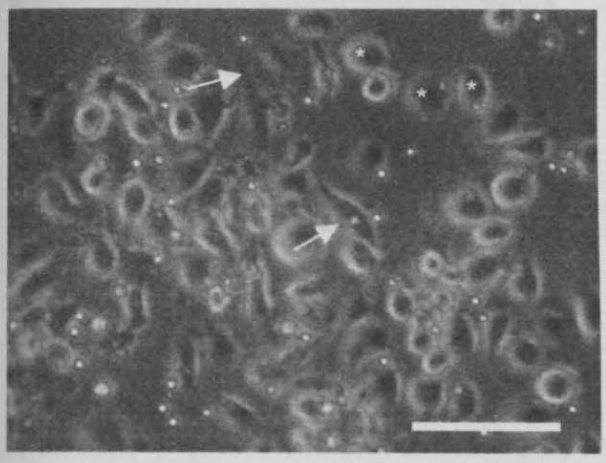


Figure 5.2: Adherent cells of bone marrow cultures grown for 7 days in BGJ-b. In addition to fibroblastic cells (arrows) and rounded cells (*), large nucleated cells (+) were visible in BGJ-b cultures. Scale bar, 150μm.

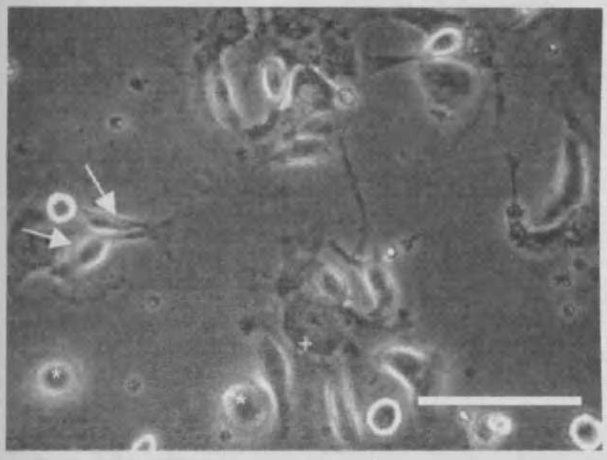


Figure 5.3: Adherent cells of bone marrow cultures grown for 7 days in ECCM. Cells grown in ECCM demonstrated the fibroblastic cells (arrows) and rounded cells (*) visible in other cultures at this time point. In addition 'stellate' cells (^) were also expressed in ECCM. Scale bar, 150μm.

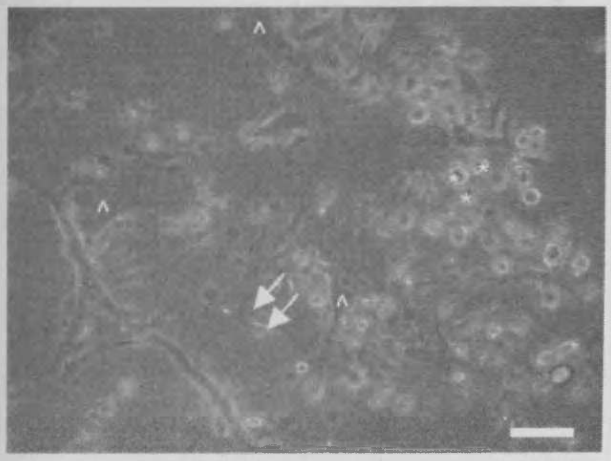


Figure 5.4: Adherent cells of bone marrow cultures grown for 14 days in α-MEM. Fibroblastic cells (arrows) and rounded cells (*) were still present in the now, confluent cultures. Scale bar, 150μm.

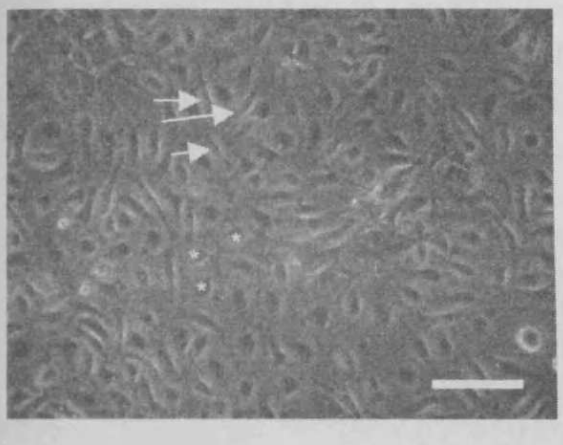


Figure 5.5: Adherent cells of bone marrow cultures grown for 14 days in BGJ-b. Fibroblastic cells (arrows) and rounded cells (*) also remained present in BGJ-b cultures. Scale bar, 150μm.

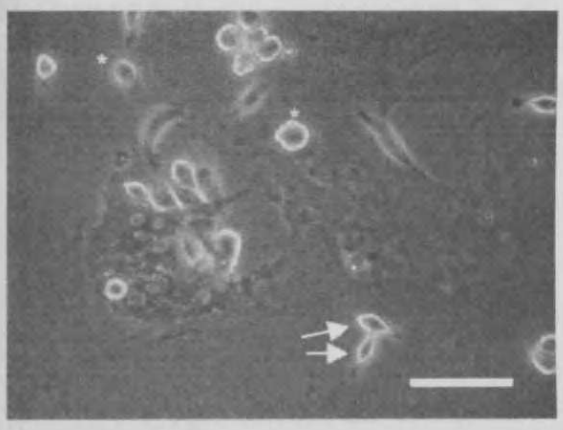


Figure 5.6: Adherent cells of bone marrow cultures grown for 14 days in ECCM . The 'stellate' cells (^) observed at day 7, had extended their processes by day 14, forming numerous junctions with neighbouring 'stellate' cells, fibroblastic cells (arrows) and rounded cells (*).Scale bar, 150μm.

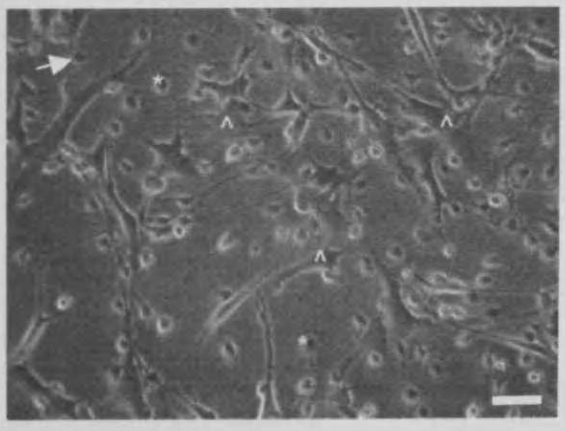


Figure 5.7: Adherent cells of bone marrow cultures grown for 21 days in α-MEM . Cultures grown in α-MEM were post-confluent by day 21, forming multiple cell layers. The two main cell types, fibroblastic cells (arrows) and rounded cells (*), were still observed. Scale bar, 150μm.

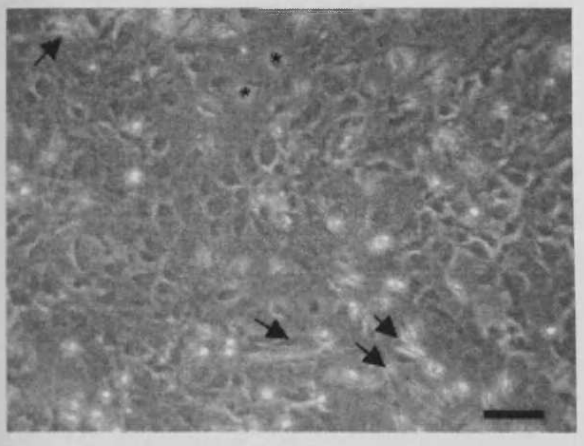


Figure 5.8: Adherent cells of bone marrow cultures grown for 21 days in BGJ-b. Cells grown in BGJ-b did not form confluent monolayers during the 21 day culture period studied. Fibroblastic cells (arrows) and rounded cells (*) were observed at the end of the study in BGJ-b cultures. Scale bar, 150μm.

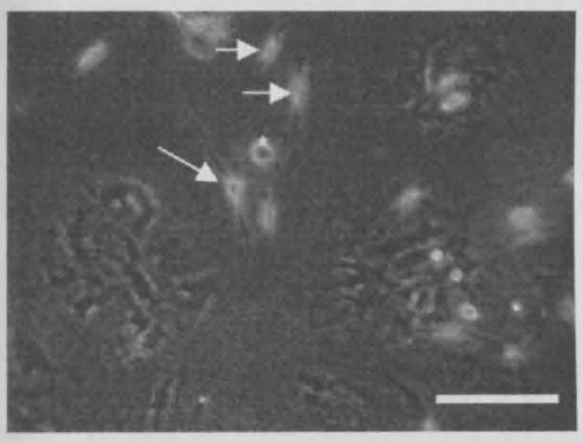


Figure 5.9: Adherent cells of bone marrow cultures grown for 21 days in ECCM. Post confluent ECCM cultures, at 21 days, formed multiple cellular layers consisting predominantly of rounded cells (*) and 'stellate' cells (^). Scale bar, 150μm.

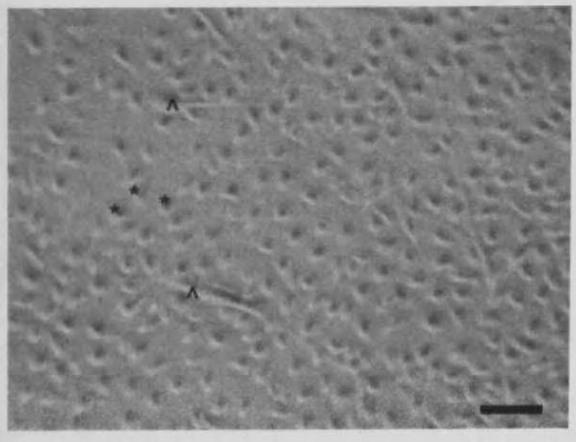


Figure 5.10: Mean number of cell colonies in bone marrow cultures grown in alpha MEM (Control), BGJ-b or ECCM.

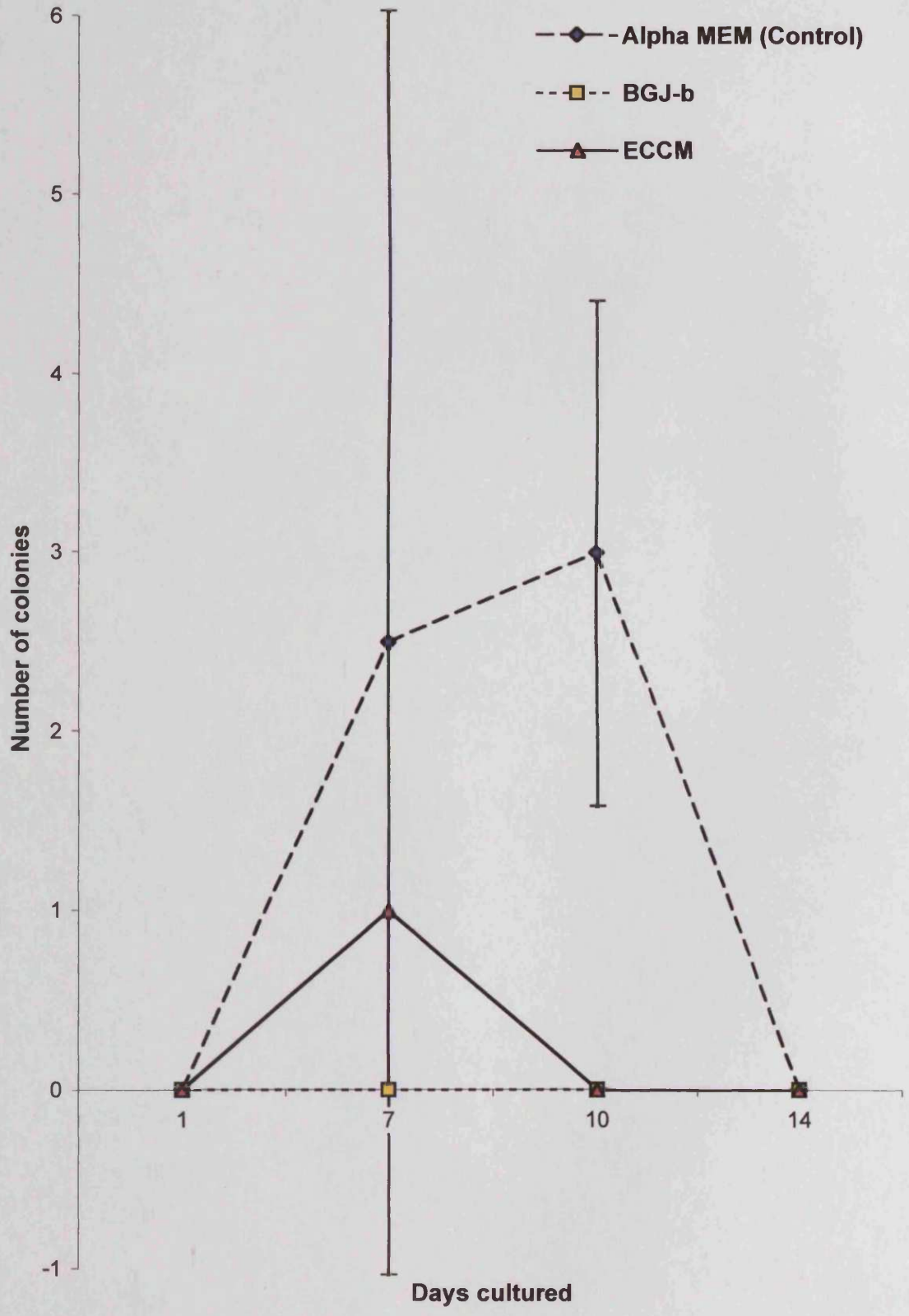


Table 5.1: Mean number of cell colonies in bone marrow cultures grown in alpha MEM (Control), BGJ-b or ECCM. Number of colonies at day 21 were not counted as cultures were confluent.

	α-MEM (Control)	BGJ-b	ECCM
Day 0	0	0	0
Day 1	2.5	0	1
Day 7	3	0	0
Day 10	0	0	0
Day 14	0	0	0

Table 5.2: Statistical analysis (ANOVA) of the mean number of cell colonies in bone marrow cultures grown in alpha MEM (Control), BGJ-b or ECCM. Cultures in which the mean number of cell colonies are significantly different are marked with an asterisk (*)

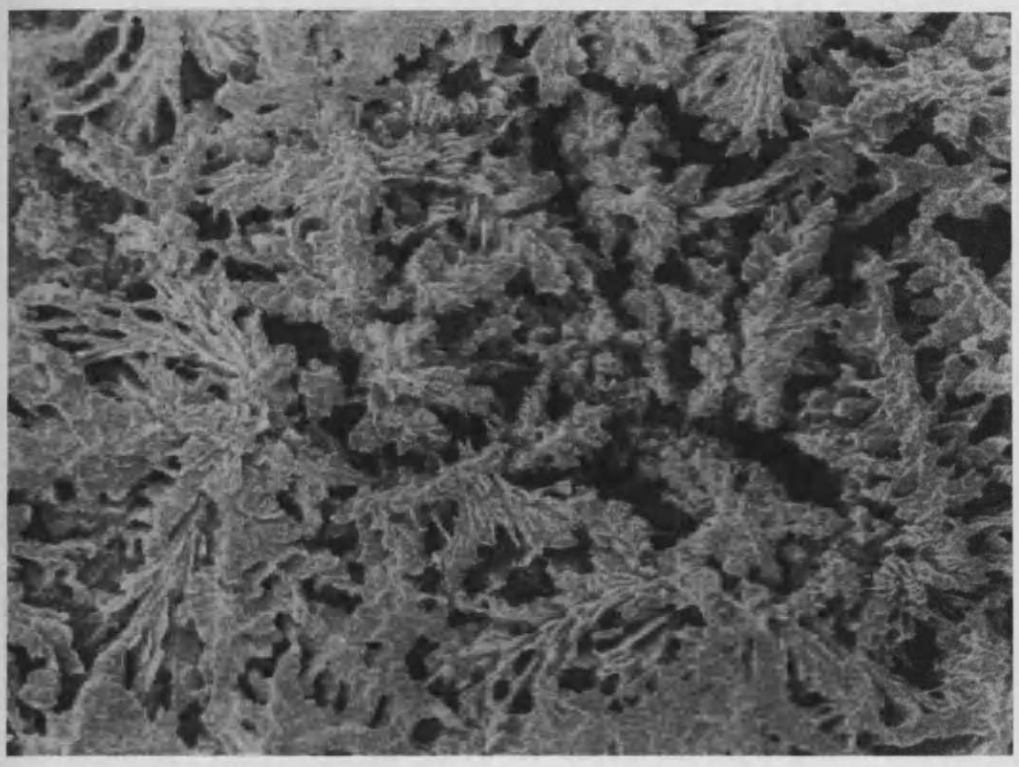
	α-MEM (Control)/BGJ-b	α-MEM (Control)/ECCM
Day 1	-5.2 to 5.2	-5.2 to 5.2
Day 7	-2.7 to 7.7	-3.7 to 6.7
Day 10	-2.2 to 8.2	-2.2 to 8.2
Day 14	-5.2 to 5.2	-5.2 to 5.2

Table 5.3: Summary of staining for calcium phosphate with von Kossa stain. Staining of 1 to 21 day bone marrow monolayers grown in alpha MEM (control medium), ECCM and BGJ-b medium. Intensity of staining was graded as follows: (-) negative staining; (+), definite staining but low intensity; (++), moderate staining; (+++), intense staining.

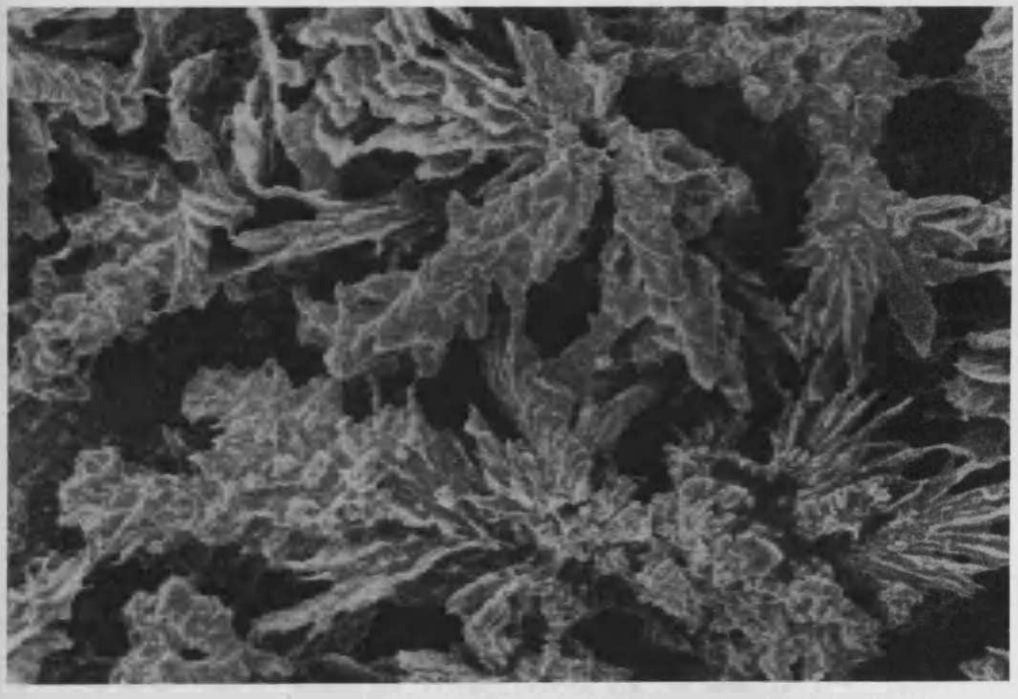
	α-MEM (Control)	BGJ-b	ECCM
Day 1	-	-	-
Day 7	-	-	-
Day 10	-	++	-
Day 14	-	+++	-

Figure 5.11: SEM images of mineralization crystals in BGJ-b cultures (**A**, and at higher magnification **B**).

A



B



5.3.2 Cell viability/proliferation

After a lag phase of approximately one week (figures 5.12 and 5.13), bone marrow cells entered a period of active proliferation up until around days 7 and 14 (figure 5.13). Cells cultured in control medium and in ECCM presented similar growth patterns, although maximal proliferation values occurred at around days 7 and 14 (figure 5.13 and table 5.5), cell proliferation and cell numbers decreased after this point (figure 5.12 and table 5.4)). Cells grown under experimental conditions that allowed the formation of mineralized calcium phosphate deposits (BGJ-b medium) attained maximal values for cell proliferation by day 21 (figure 5.13 and table 5.5).

Results presented in table 5.6 show that, when compared to cultures grown in control medium, ECCM did not produce significantly greater cell numbers. Following a burst of cell proliferation between days 14 and 21, cell numbers at day 21 in BGJ-b medium were also not significantly less than in control medium (table 5.6).

Figure 5.12: Number of adherent cells in bone marrow cultures grown in alpha MEM (Control), BGJ-b medium or ECCM.

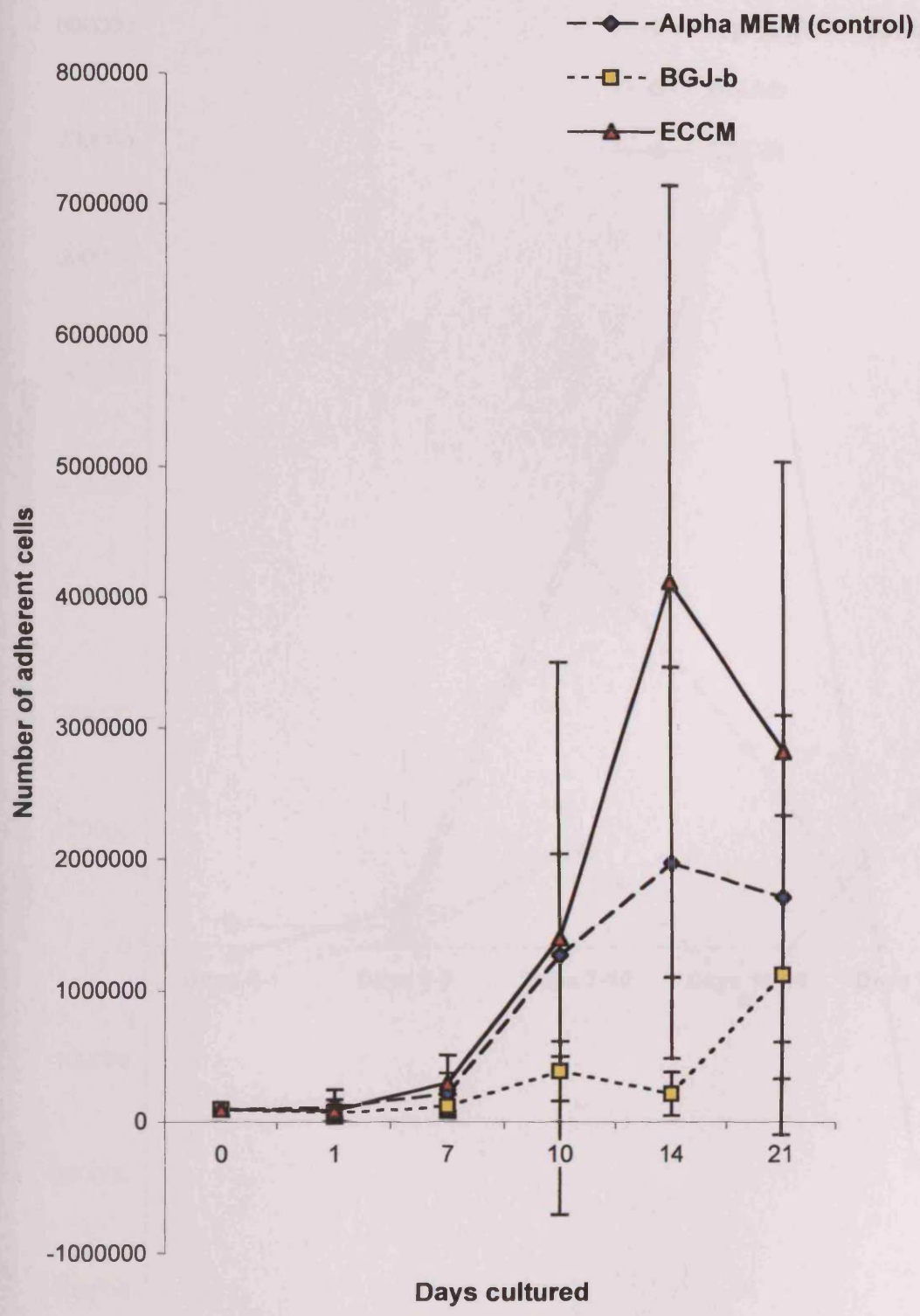


Figure 5.13: Proliferation rates of adherent cells in bone marrow cultures grown in alpha MEM (Control), BGJ-b medium or ECCM.

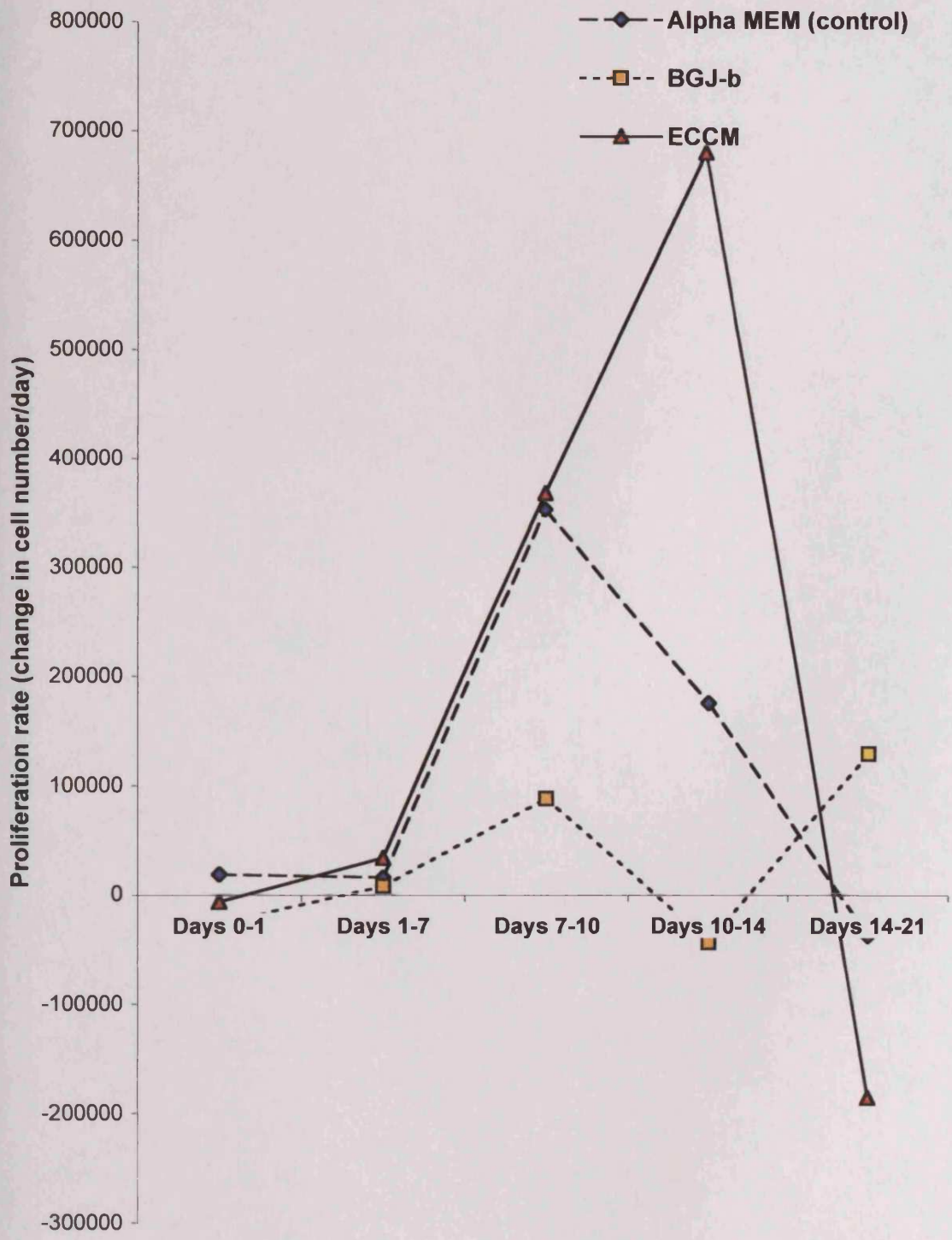


Table 5.4: Mean number of adherent cells in bone marrow cultures grown in alpha MEM (Control), BGJ-b medium or ECCM.

	α-MEM (Control)	BGJ-b	ECCM
Day 0	96000	96000	96000
Day 1	115146.1	70032.43	89818.71
Day 7	211493.6	120017	294718.1
Day 10	1272617	385718.9	1401189
Day 14	1977061	211652.9	4127625
Day 21	1716000	1119837	2827061

Table 5.5: Mean proliferation rates, as calculated from the mean number of adherent cells in bone marrow cultures grown in alpha MEM (Control), BGJ-b medium or ECCM.

	α-MEM (Control)	BGJ-b	ECCM
Day 0 - 1	19146.14	-25967.6	-6181.29
Day 1 - 7	16057.9	8330.762	34149.9
Day 7 - 10	353707.9	88567.29	368823.5
Day 10 - 14	176111	-43516.5	681609
Day 14 - 21	-37294.5	129740.5	-185795

Table 5.6: Statistical analysis (ANOVA) of the mean number of adherent cells in bone marrow cultures grown in alpha MEM (Control), BGJ-b medium or ECCM. Cultures in which the mean number of cells are significantly different are marked with an asterisk (*)

	α-MEM (Control)/BGJ-b	α-MEM (Control)/ECCM
Day 1	-2454993.5 to 2545221.0	-2474779.8 to 252543.7
Day 7	-2408630.7 to 2591583.8	-83224.6 to 2416882.7
Day 10	-1613209.0 to 3387005.5	-2628678.7 to 2371535.8
Day 14	-734699.0 to 4265515.5	-4650670.7 to 349543.9
Day 21	-1903944.0 to 3096270.5	-3611168.5 to 1389046.0

5.3.3 Stromal stem cell markers

Mesenchymal stromal stem cells were identified by STRO-1 antibody binding to a cell surface antigen, and haematopoietic stem cells by CD34 expression. Cultures grown in control medium presented only CD34 antigen during the first week of culture (figure 5.14 and table 5.7). Expression of the haemopatopoietic marker more than doubles within the culture during the first week of culture, suggesting that haematopoietic stem cells were increasing at a greater rate than other cells types cultured in this medium. By the second week of culture there were no haematopoietic stem cells left in any of the cultures grown in α-MEM (figure 5.14 and table 5.7).

The depletion in haematopoietic stem cells was accompanied by a large increase in the proportion of cells in α-MEM cultured populations expressing mesenchymal stem cells. At the final day in culture, however there were no stem cells expressed (figure 5.14 and table 5.7).

The mean number of cells expressing CD34 at day 1, for cells cultured in BGJ-b medium was identical to that in control cultures (table 5.7 and 5.8). The proportion of cells expressing STRO-1 however, differed significantly (table 5.10) with approximately 63% of the cells in BGJ-b medium expressing the stromal stem cell marker (table 5.7 and 5.8). Expression of the stromal stem cell marker depleted gradually to zero after a fortnight in culture, proportions of cells expressing the marker from the first through to the third week of culture, not being significantly different from control cultures (figure 5.15 and table 5.8).

Despite following similar expression patterns for the antigen CD34 during the first week of culture, as control cultures; the proportion of cells staining positive for the haematopoietic stem cell marker in the cultures grown in BGJ-b did not dwindle in the final fortnight in culture (figure 5.15 and table 5.8). Proportions of cells expressing CD34 in BGJ-b cultures fluctuated over the period in culture. As seen in control cultures maximum expression of the haematopoietic stem cell marker

was observed at culture day 7, with proportions of cells staining positive for the antigen decreasing and increasing over the remaining incubation period (table 5.7 and 5.8).

Cells grown in endothelial cell conditioned medium did not express either stem cell marker on any occasion during the incubation period and in any of the cultures set up in the medium (table 5.9). These observations were only considered to be significantly different from the control cultures during the first week of culture for CD34 and STRO-1 (table 5.11).

Figure 5.14: Percentage of adherent cells in bone marrow cultures grown in α-MEM (control medium) staining positive for stem cell markers CD34 and STRO-1.

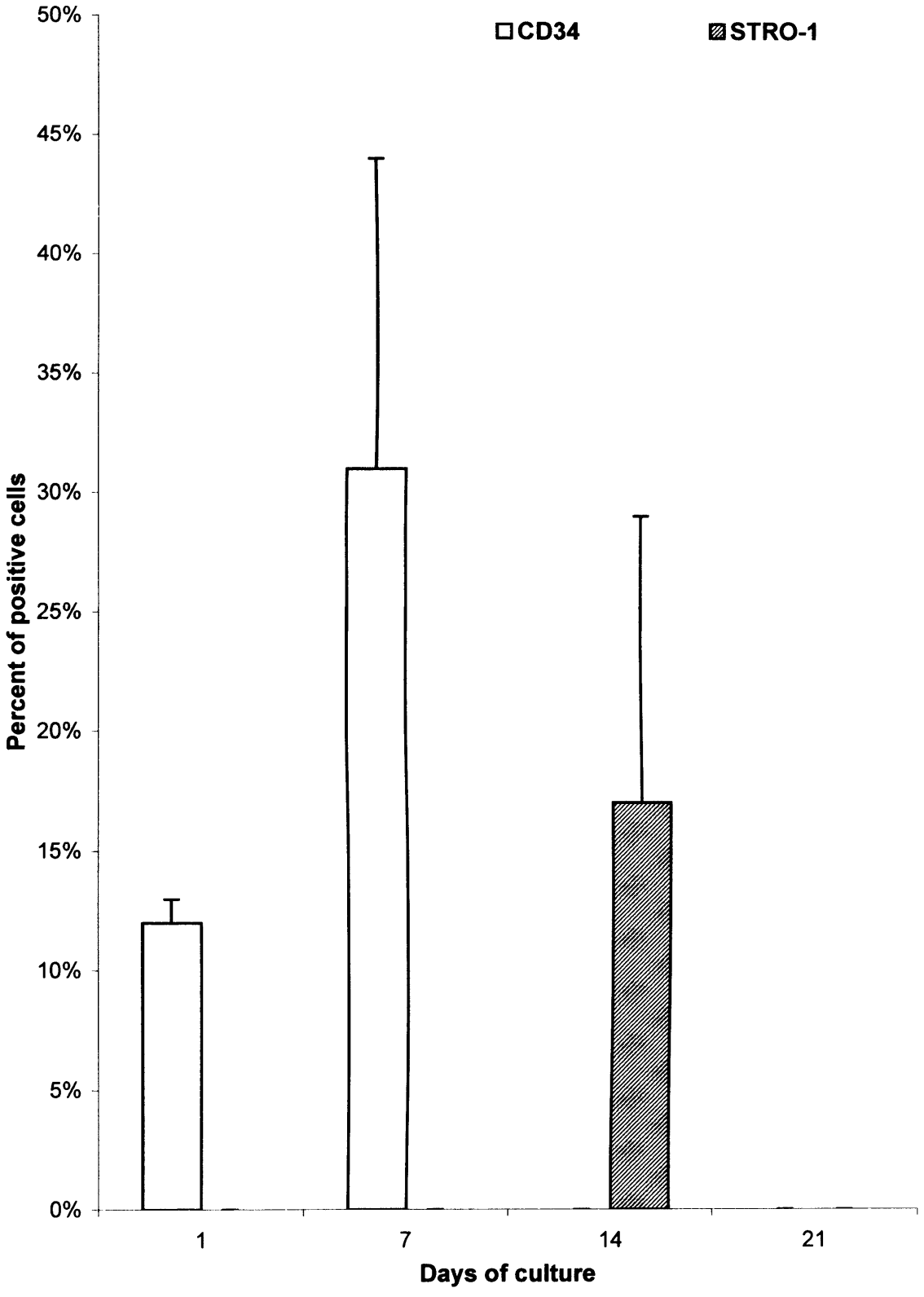


Figure 5.15: Percentage of adherent cells in bone marrow cultures grown in BGJ-b culture medium staining positive for stem cell markers.

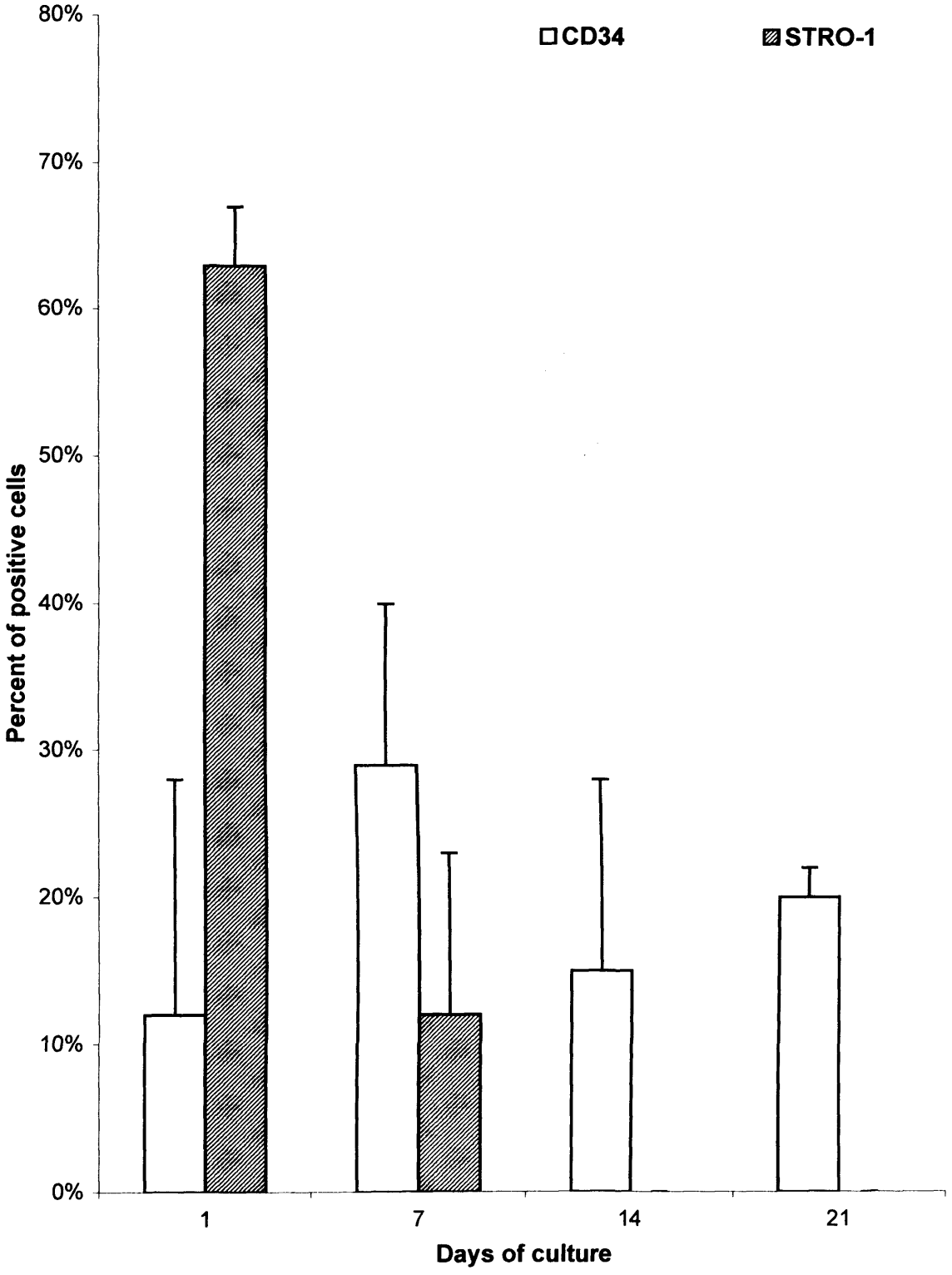


Table 5.7: Mean percentage of cells staining positive for stem cell markers in bone marrow cultures grown in α-MEM.

	Day 1	Day 7	Day 14	Day 21
CD34	12%	31%	0%	0%
STRO-1	0%	0%	17%	0%

Table 5.8: Mean percentage of cells staining positive for stem cell markers in bone marrow cultures grown in BGJ-b medium.

	Day 1	Day 7	Day 14	Day 21
CD34	12%	29%	15%	20%
STRO-1	63%	12%	0%	0%

Table 5.9: Mean percentage of cells staining positive for stem cell markers in bone marrow cultures grown in ECCM.

	Day 1	Day 7	Day 14	Day 21
CD34	0%	0%	0%	0%
STRO-1	0%	0%	0%	0%

Table 5.10: ANOVA for mean percentage of cells staining positive for stromal stem cell markers in bone marrow cultures grown in α-MEM (control medium) and BGJ-b medium. Cultures in which the mean number of positive cells is significantly different are marked with an asterisk (*)

	Day 1	Day 7	Day 14	Day 21
CD34	-27.7 to 28.7	-23.8 to 27.8	-40.8 to 10.8	-42.7 to 3.4
STRO-1	-78.2 to –47.8*	-10.3 to 20.0	-14.4 to 14.4	-13.9 to 13.9

Table 5.11: ANOVA test for mean percentage of cells staining positive for stromal stem cell markers in bone marrow cultures grown in α-MEM (control medium) and ECCM. Cultures in which the mean number of positive cells is significantly different are marked with an asterisk (*)

	Day 1	Day 7	Day 14	Day 21
CD34	-13.8 to 37.8	5.2 to 56.8*	-25.8 to 25.8	-23.1 to 23.1
STRO-1	-15.1 to 15.1	1.3 to 31.6*	-15.1 to 15.1	-15.1 to 15.1

5.3.4 Osteoblastic Markers

Alkaline phosphatase, bone sialoprotein II, collagen type I, Decorin, osteonectin and osteopontin are all routinely used in *in vitro* experiments as relative markers of osteoblastic differentiation.

5.3.4.1 α-MEM (Control) Cultures

Cultures grown in control medium, at 24 hours post plating, presented collagen type I staining in two thirds of the cultured cells (table 5.12 and figure 5.16). The proportion of cells staining for this collagen dropped to just under 50% at weeks one and two (table 5.12), but increased again at the final week in culture. Alkaline phosphatase expression also fluctuated very little overall, during the initial fortnight of culture, however expression of the enzyme decreased substantially at the final time point (figure 5.16 and table 5.12). The percentage of cells staining for alkaline phosphatase peaked at 14 days, and this was also true of bone sialoprotein II and osteopontin. Decorin and osteonectin exhibited their own unique expression patterns in α-MEM culture medium, with osteonectin being produced by the greatest proportion of cells at day one and decorin at week 1 (figure 5.16).

5.3.4.2 BGJ-b Medium Cultures

BGJ-b medium caused bone marrow derived cells to follow a different sequence of osteoblastic marker expression, to the control medium. For cells in BGJ-b medium, all but two of the markers showed maximum expression in the cultures at week 1 (table 5.13 and figure 5.17), and overall no less than 83% of the cells demonstrated staining for all 6 markers of the osteoblastic lineage at this time point. The number of cells expressing type I collagen increased throughout the 3 week duration in BGJ-b medium. Production of this core bone matrix constituent was more widespread in cells grown in BGJ-b medium than in control medium at days 7 and 14

(table 5.13 and figure 5.17). Alkaline phosphatase was not produced by a significantly greater number of cells in BGJ-b medium than in the control (table 5.15). The remaining bone matrix proteins, other than decorin, were all produced by a significantly greater proportion of the cultured cells in BGJ-b medium than in control medium at day 21. A significantly greater proportion of cells than in α-MEM, in BGJ-b medium produced osteopontin at day 7 and osteonectin at day 14 (table 5.15). Decorin followed a similar pattern of expression in the BGJ-b medium cultures as many of the other osteogenic markers, and also expression of decorin in control cultures, however it was not considered significantly different.

5.3.4.3 ECCM Cultures

Cells grown in ECCM demonstrated the greatest proportions of cells staining positive for osteogenic markers at days 14 and 21 (7 to 14 days after BGJ-b medium cultures) (figure 5.18 and table 5.14). The maximum proportion of cells staining for type I collagen was observed at day 14, however with the lowest mean proportion of stained cells being 68%, staining for this collagen did not drop to the levels seen in either BGJ-b (60%) or the control medium (46%) (table 5.12, 5.13 and 5.14).

Alkaline phosphatase production was not detected using immunohistochemistry at day 1. Cells in this culture began producing alkaline phosphatase at day 1 and the proportion of cells increased throughout the 21 day culture period (figure 5.18 and table 5.14). A significantly greater quantity of cells in ECCM were generating alkaline phosphatase at day 21 than in the controls (table 5.16). With regards to the other bone matrix proteins, bone sialoprotein demonstrated staining to significantly lesser extent than control cells after 24 hours in the medium. In contrast to this result staining for osteonectin at days 7 and 14 occurred in a significantly greater proportion of the ECCM cultures than the α-MEM cultures (table 5.16).

Figure 5.16: Percentage of adherent cells in bone marrow cultures grown in alpha MEM (Control) staining positive for Osteoblastic markers.

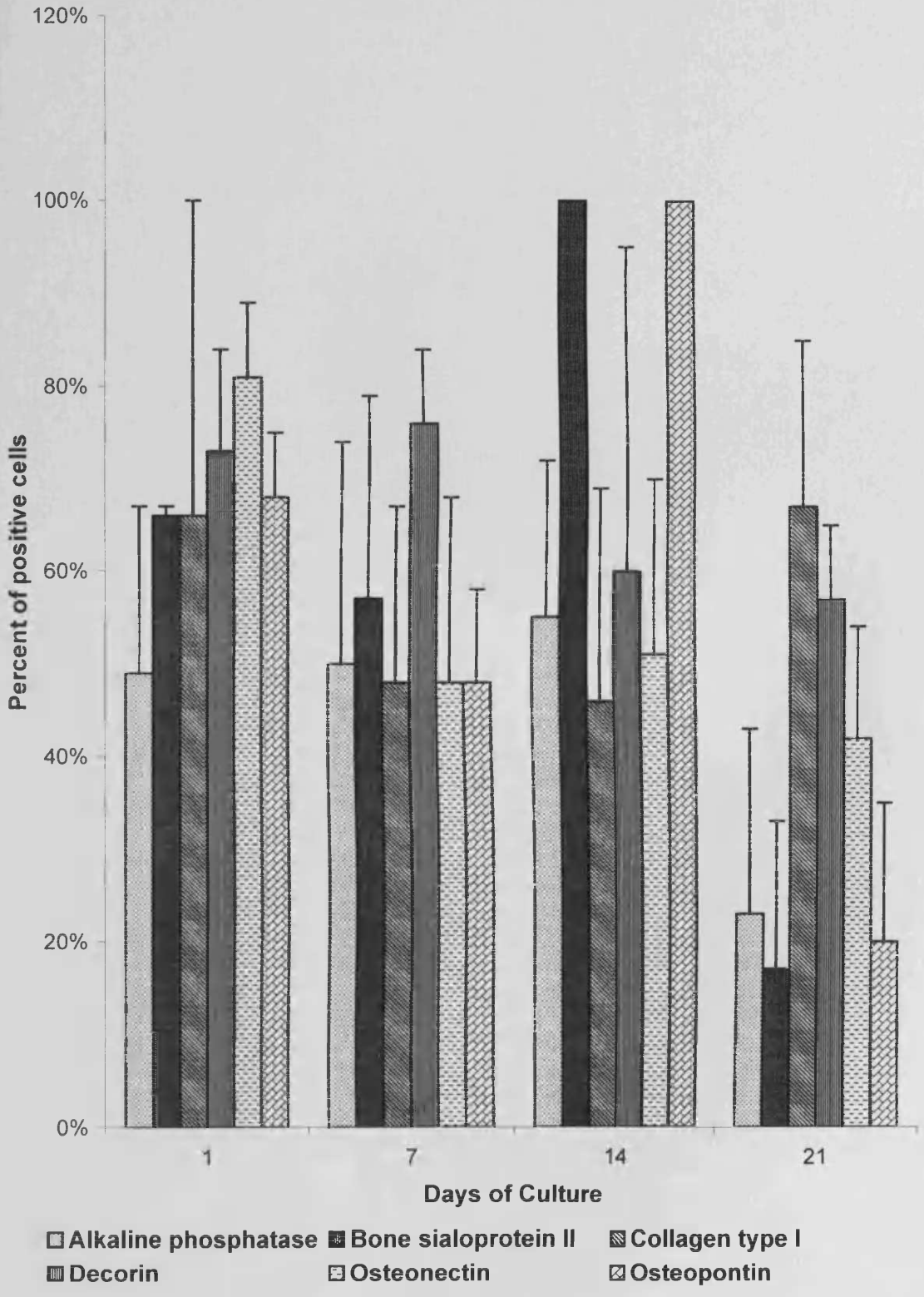


Figure 5.17: Percentage of adherent cells in bone marrow cultures grown in BGJ-b medium staining positive for Osteoblastic markers.

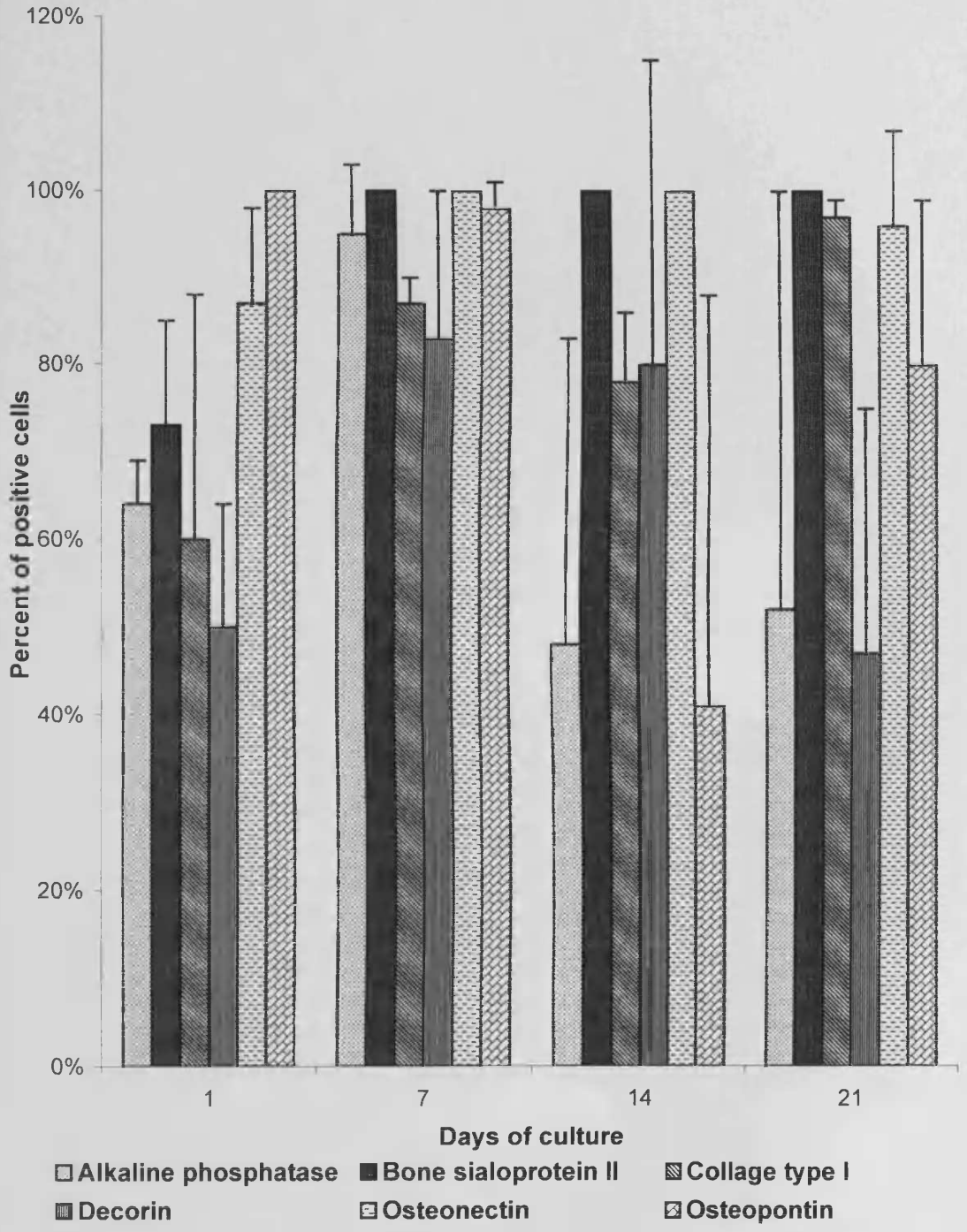


Figure 5.18: Percentage of adherent cells in bone marrow cultures grown in ECCM staining positive for Osteoblastic markers.

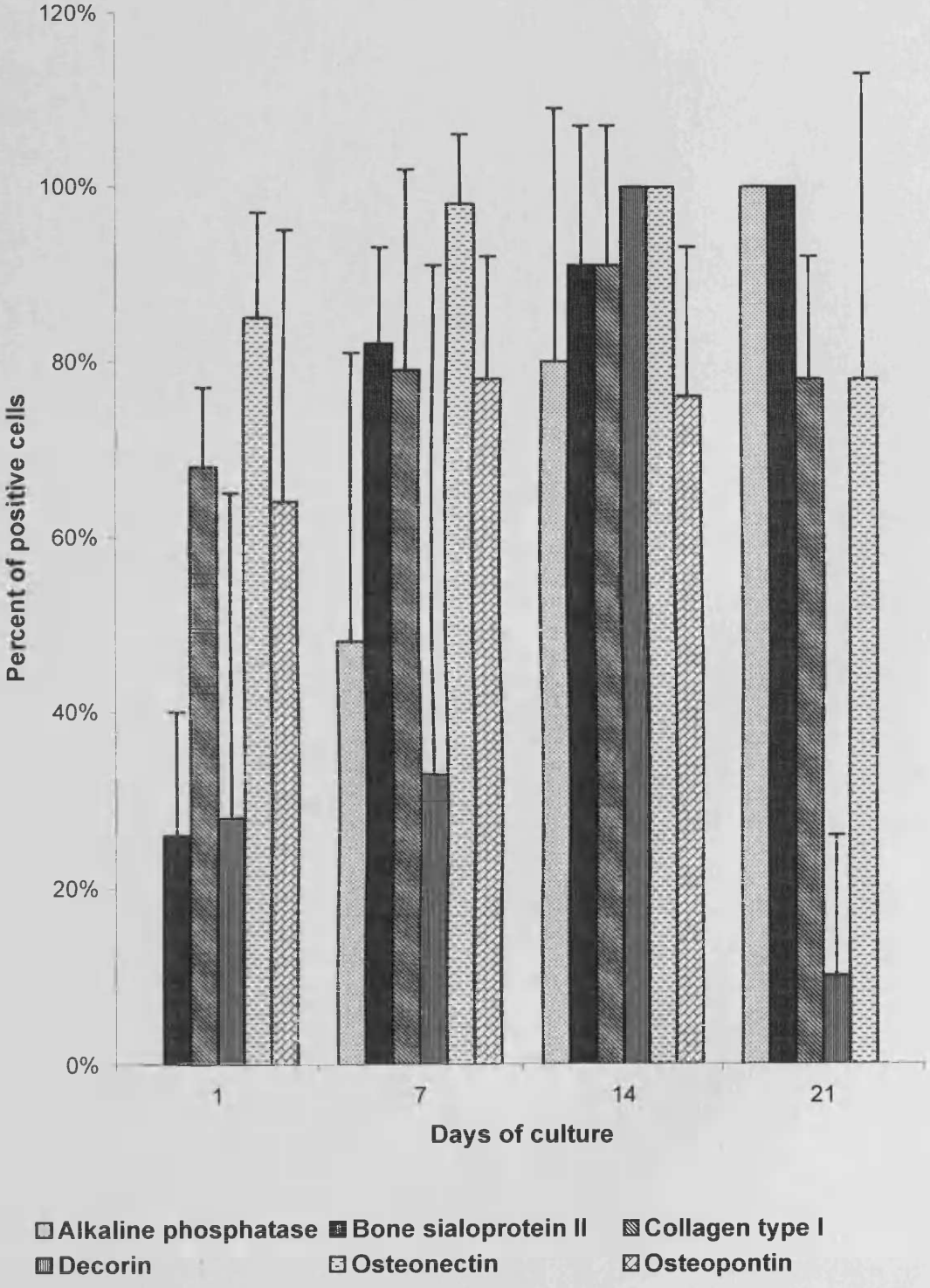


Table 5.12: Mean percentage of cells staining positive for osteogenic markers in bone marrow cultures grown in alpha MEM (Control).

	Day 1	Day 7	Day 14	Day 21
Alkaline phosphatase	49%	50%	55%	23%
Bone sialoprotein II	66%	57%	100%	17%
Collagen type I	66%	48%	46%	67%
Decorin	73%	76%	60%	57%
Osteonectin	81%	48%	51%	42%
Osteopontin	68%	48%	100%	20%

Table 5.13: Mean percentage of cells staining positive for osteogenic markers in bone marrow cultures grown in BJG-b medium.

	Day 1	Day 7	Day 14	Day 21
Alkaline phosphatase	64%	95%	48%	52%
Bone sialoprotein II	73%	100%	100%	100%
Collagen type I	60%	87%	78%	97%
Decorin	50%	83%	80%	47%
Osteonectin	87%	100%	100%	96%
Osteopontin	100%	98%	41%	80%

Table 5.14: Mean percentage of cells staining positive for osteogenic markers in bone marrow cultures grown in ECCM.

	Day 1	Day 7	Day 14	Day 21
Alkaline phosphatase	0%	48%	80%	100%
Bone sialoprotein II	26%	82%	91%	100%
Collagen type I	68%	79%	91%	78%
Decorin	28%	33%	100%	10%
Osteonectin	85%	98%	100%	78%
Osteopontin	64%	78%	76%	0%

Table 5.15: ANOVA test for mean percentage of cells staining positive for osteogenic markers in bone marrow cultures grown in alpha MEM (control medium) and BGJ-b medium. Cultures in which the mean number of positive cells are significantly different are marked with an asterisk (*)

	Day 1	Day 7	Day 14	Day 21
Alkaline phosphatase	-96.5 to 59.5	-110.3 to 20.1	-68.1 to 71.4	-106.7 to 23.7
Bone sialoprotein II	-41.6 to 33.2	-71.0 to 0.0	-37.4 to 37.4	-106.1 to –43.4*
Collagen type I	-74.9 to 45.9	-87.0 to 21.0	-81.1 to 33.6	-81.6 to 12.1
Decorin	-63.5 to 108.9	-92.5 to 79.9	-106.2 to 66.2	-75.5 to 96.9
Osteonectin	-46.5 to 32.5	-91.5 to –12.5*	-88.5 to –9.5*	-88.0 to –14.9*
Osteopontin	-100.8 to 36.8	-111.5 to 11.5	-24.7 to 105.7	-124.1 to –9.0*

Table 5.16: ANOVA test for mean percentage of cells staining positive for osteogenic markers in bone marrow cultures grown in alpha MEM (control medium) and ECCM. Cultures in which the mean number of positive cells are significantly different are marked with an asterisk (*)

	Day 1	Day 7	Day 14	Day 21
Alkaline phosphatase	-25.9 to 116.9	-59.6 to 70.9	-94.1 to 45.4	-130.8 to –16.2*
Bone sialoprotein II	6.2 to 74.8*	-50.8 to 16.1	-20.4 to 45.3	-100.4 to –49.0*
Collagen type I	-71.6 to 43.1	-75.3 to 16.1	-89.9 to 24.8	-62.6 to 38.6
Decorin	-41.9 to 130.5	-43.2 to 129.2	-126.5 to 45.9	-38.5 to 133.9
Osteonectin	-43.1 to 32.3	-85.2 to –12.1*	-86.7 to –11.2*	-71.2 to 7.7
Osteopontin	-57.4 to 63.4	-81.9 to 18.6	-29.6 to 84.3	-39.8 to 83.2

5.3.5 Endothelial Cell Markers

Endothelial cells were highlighted from the assortment of bone marrow cells using antibodies for endothelial cell markers CD105 (see chapter 3) and von willebrand factor. Von Willebrand factor (vWF) is a glycoprotein synthesized by endothelial cells and stored in specialized vesicles called Weibel-Palade bodies

(WPBs) [187]. vWF enables the platelet, via its surface glycoprotein receptors, to adhere to exposed subendothelium and to respond to shear stress in the blood. Via vWF released from the WPBs of the endothelial cells, the haemostatic system can respond locally to lesions in the vessel wall and can initiate the activation of platelets [188]. Not all vWF is released at one time; about one-third is released, and the remaining two-thirds is kept in reserve in the endothelial cells, thus vWF provides a means of identifying endothelial cells in the bone marrow cultures.

Only a small proportion of the cells in control and BGJ-b culture medium stained positively for endothelial cell markers. On average 3% of cells grown in control medium, at day 1, bound the antibody for vWF. There was no staining for vWF after this time point and there was no staining for the cell surface antigen CD105 throughout the culture period (figure 5.19 and table 5.17). In contrast, cells grown in BGJ-b medium did not produce vWF at any time point, however CD105 was found on the surface of a small number of the cells at days 7 and 21 (figure 5.20 and 5.18). The differences in expression of endothelial cell markers between cultures grown in control and BGJ-b medium was not considered significant at any of the time points (table 5.20).

Bone marrow samples grown in endothelial cell conditioned medium showed staining, to varying degrees, at every time point studied. It was observed that the percentage of cells staining for both endothelial cell markers in ECCM, were greatest at day 21 and least at day 7 (figure 5.21 and table 5.19). However, the proportion of cells staining for CD105 and vWF was not significantly greater in ECCM cultures than in control cultures (table 5.21).

Figure 5.19: Percentage of adherent cells in bone marrow cultures grown in α-MEM staining positive for endothelial cell markers.

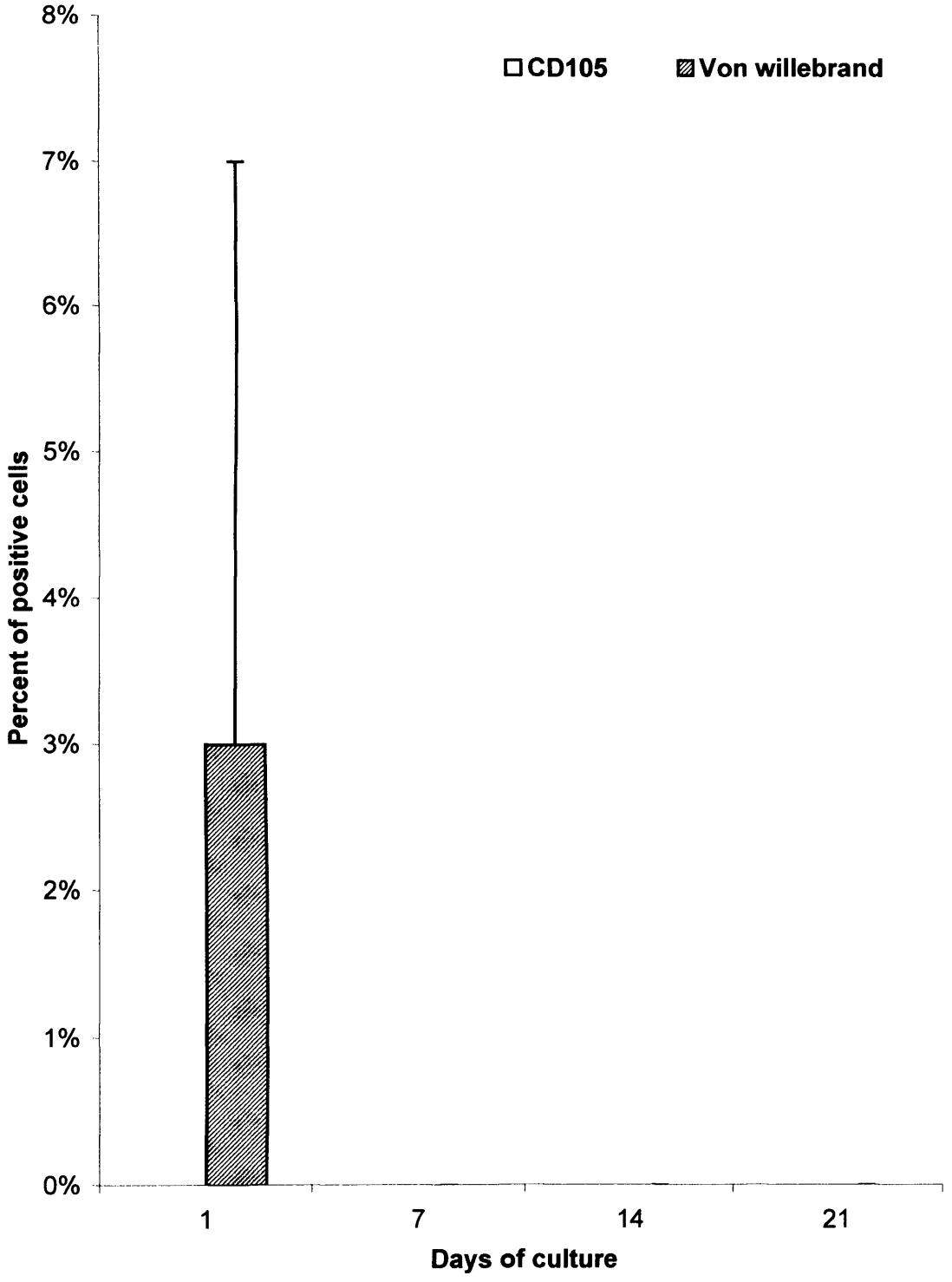


Figure 5.20: Percentage of adherent cells in bone marrow cultures grown in BGJ-b medium staining positive for endothelial cell markers.

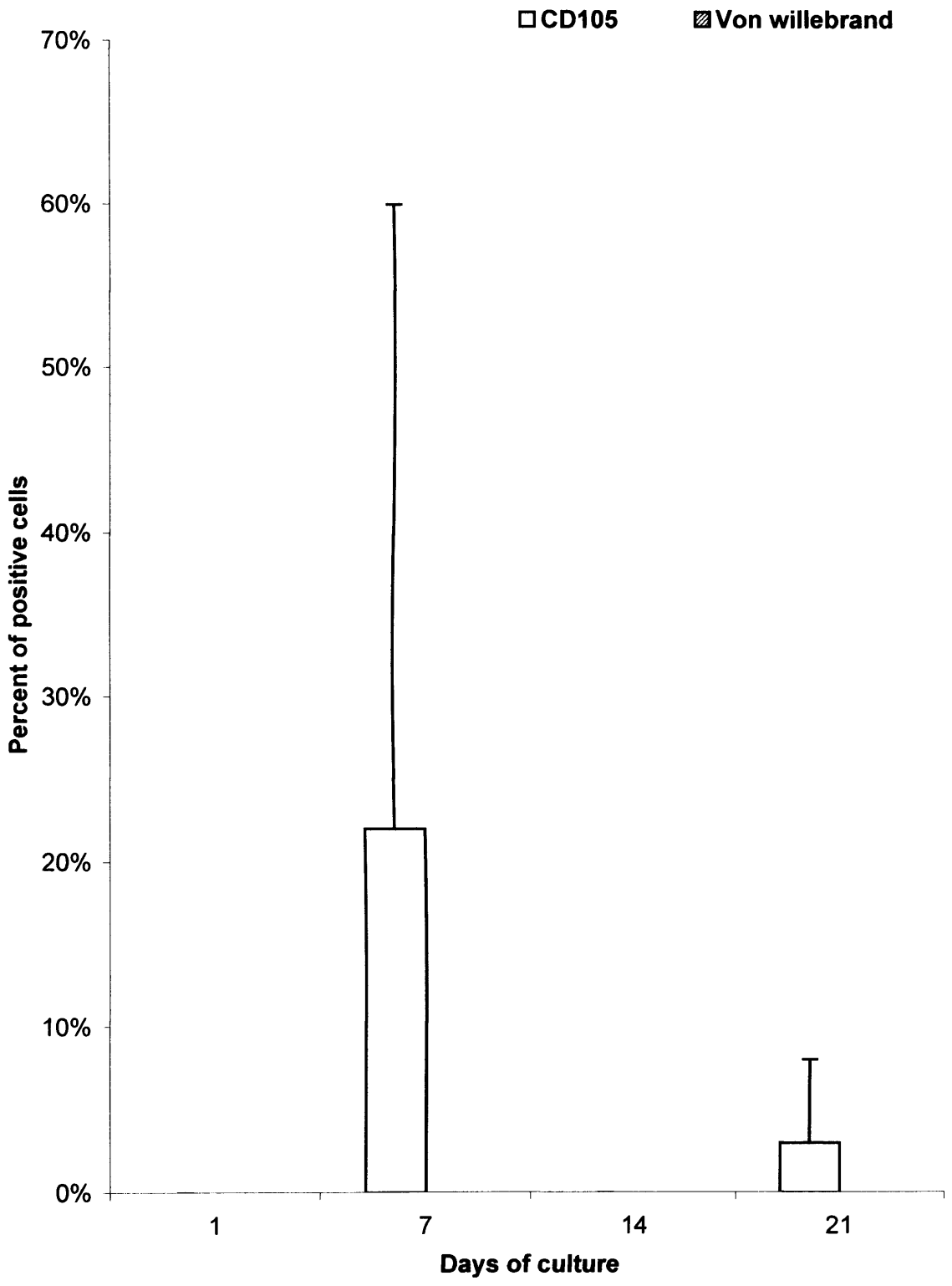


Figure 5.21: Percentage of adherent cells in bone marrow cultures grown in ECCM staining positive for endothelial cell markers.

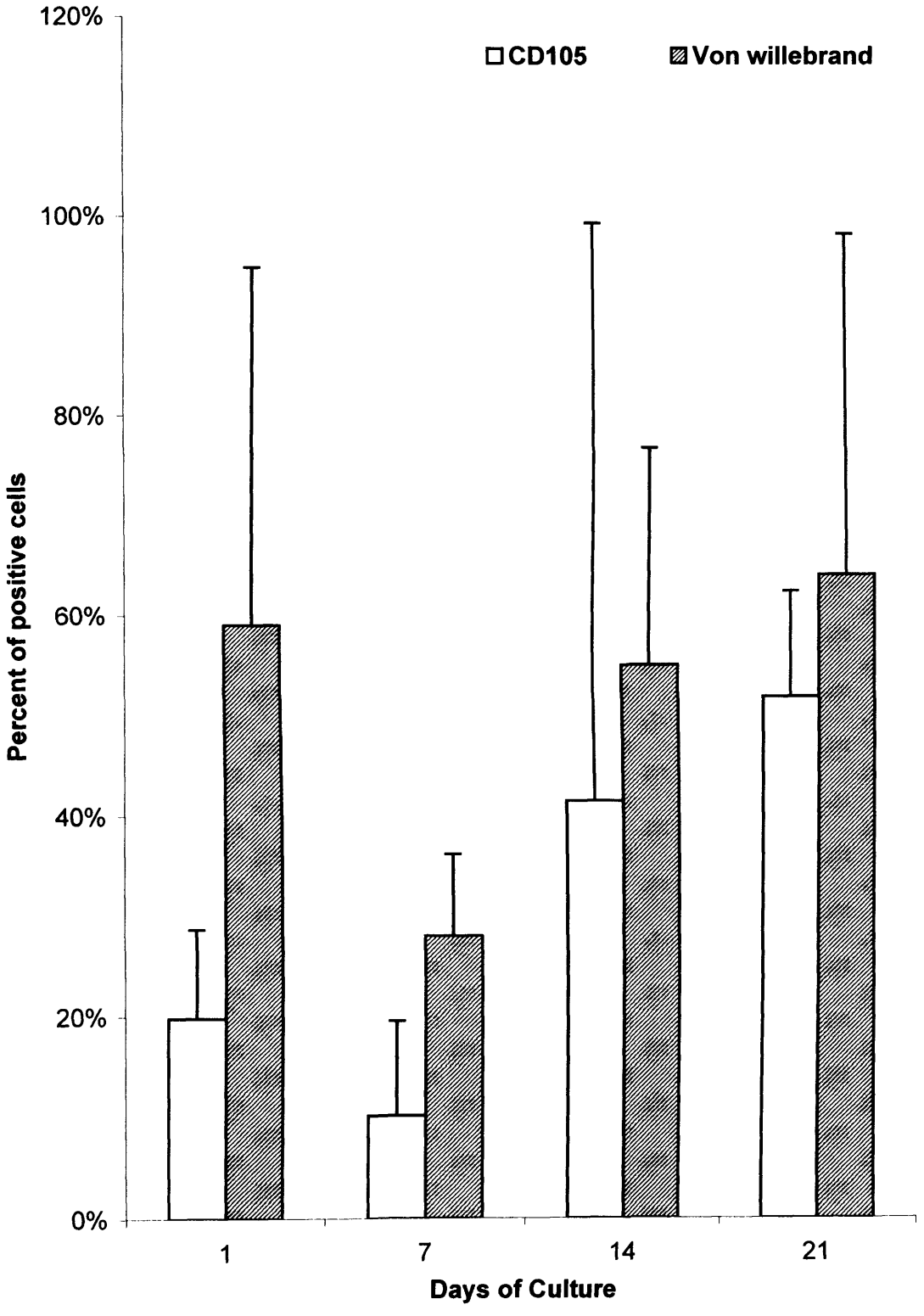


Table 5.17: Mean percentage of cells staining positive for endothelial cell markers in bone marrow cultures grown in α-MEM.

	Day 1	Day 7	Day 14	Day 21
CD105	0%	0%	0%	0%
Von willebrand	3%	0%	0%	0%

Table 5.18: Mean percentage of cells staining positive for endothelial cell markers in bone marrow cultures grown in BGJ-b medium.

	Day 1	Day 7	Day 14	Day 21
CD105	0%	22%	0%	3%
Von willebrand	0%	0%	0%	0%

Table 5.19: Mean percentage of cells staining positive for endothelial cell markers in bone marrow cultures grown in ECCM.

	Day 1	Day 7	Day 14	Day 21
CD105	20%	10%	41%	52%
Von willebrand	59%	28%	55%	64%

Table 5.20: ANOVA test for mean percentage of cells staining positive for endothelial cell markers in bone marrow cultures grown in α-MEM (control medium) and BGJ-b medium. Cultures in which the mean number of positive cells are significantly different are marked with an asterisk (*)

	Day 1	Day 7	Day 14	Day 21
CD105	-12.8 to 12.8	-12.4 to 10.4	-11.4 to 11.4	-18.4 to 4.4
Von willebrand	-0.6 to 5.6	-3.1 to 3.1	-2.7 to 2.7	-2.7 to 2.7

Table 5.21: ANOVA for mean percentage of cells staining positive for endothelial cell markers in bone marrow cultures grown in α-MEM (control medium) and ECCM. Cultures in which the mean number of positive cells are significantly different are marked with an asterisk (*)

	Day 1	Day 7	Day 14	Day 21
CD105	-11.4 to 11.4	-11.4 to 11.4	-11.4 to 11.4	-11.4 to 11.4
Von willebrand	-0.4 to 5.4	-2.9 to 2.9	-2.9 to 2.9	-2.9 to 2.9

5.3.6 Oil red o staining for adipocytes

Adipocytes were detected in the bone marrow cell cultures using oil red o stain. Control cultures exhibited the greatest proportion of cells staining positive with oil red o, with the exception of day 14 (figure 5.22 and table 5.22). The greatest proportion of adipocytes in α-MEM cultures was observed at day 7, when there was a greater percentage of adipocytes in this medium than in cultures grown in either of the investigative media. After day 7 in culture the ratio of adipocytes relative to other cell types dropped steadily over the following week in culture, numbers rose again towards the final time point (figure 5.22 and table 5.22).

Cultures grown in BGJ-b medium caused a slight increase in the percentage of adipocytes in the bone marrow culture between the first and seventh day of culture. At day 7 through to day 14 approximately one quarter of the bone marrow cells stained positive with oil red o, though by the final time point around one third of the cells were adipocytes (figure 5.22 and table 5.22).

ECCM produced the opposite effect to BGJ-b on adipocytes, it brought about a reduction in the proportion of adipocytes in the culture. Initially, at day 1, almost one third of the bone marrow cells being cultured in ECCM were adipocytes, nevertheless by day 7 only around 5% of the culture stained positive with oil red o, and no staining was detected during at the last two time points (figure 5.22 and table 5.22). The decline in adipocytic cells in samples grown in ECCM was significantly less than observed in control samples at day 7. (table 5.23)

Figure 5.22: Percentage of adipocytes in bone marrow cultures grown in α-MEM (control medium), BGJ-b medium or ECCM.

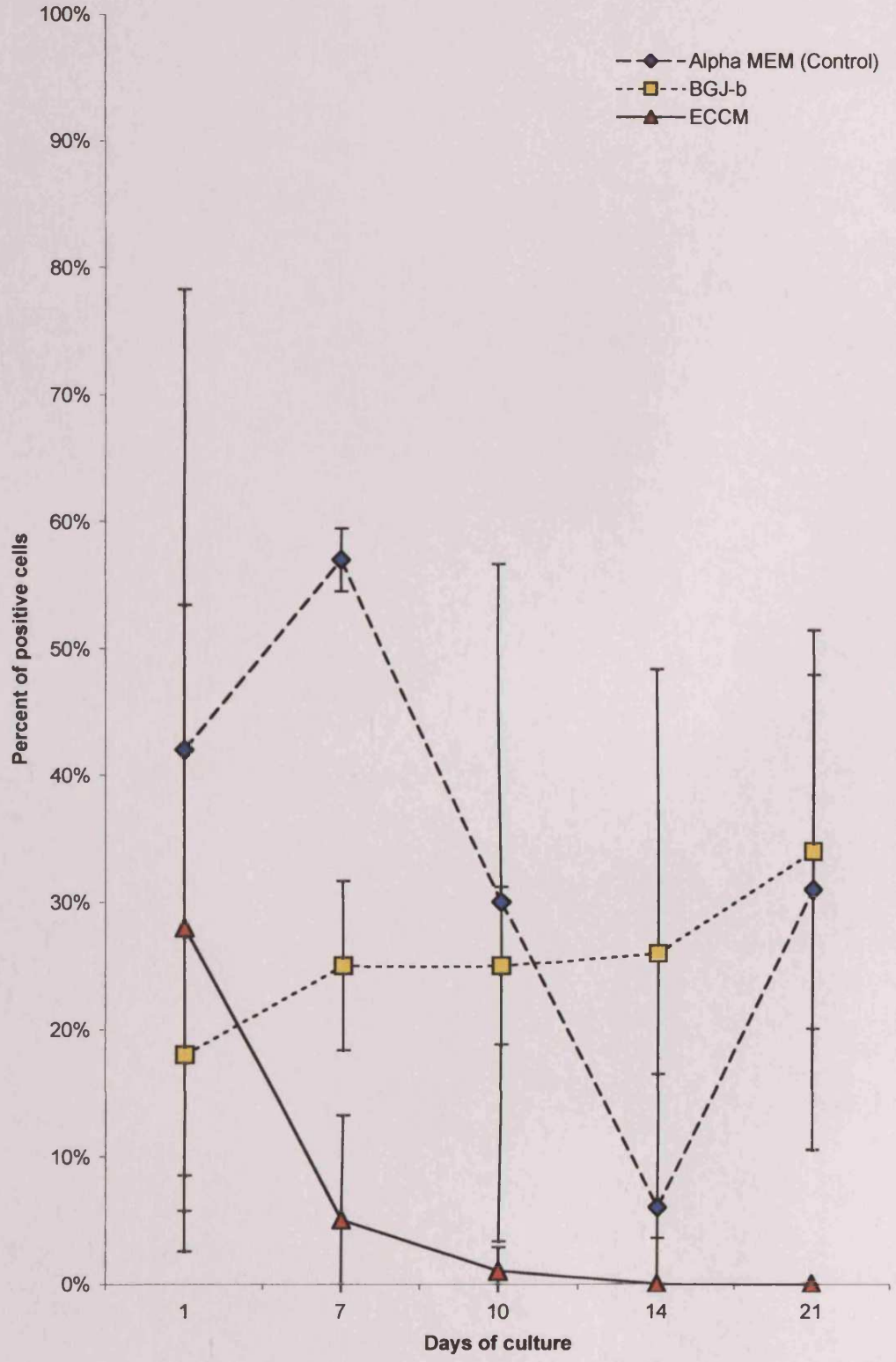


Table 5.22: Mean percentage of adipocytes in bone marrow cultures grown in alpha MEM, BGJ-b medium or ECCM; as determined by positive oil red o staining.

	Day 1	Day 7	Day 10	Day 14	Day 21
Alpha MEM (Control)	42%	57%	30%	6%	31%
BGJ-b Medium	18%	25%	25%	26%	34%
ECCM	28%	5%	1%	0%	0%

Table 5.23: ANOVA test for mean percentage of adipocytes in bone marrow cultures grown in α-MEM (control medium)/ECCM and α-MEM (control medium)/BGJ-b medium. Cultures in which the mean number of positive cells are significantly different are marked with an asterisk (*)

	Day 1	Day 7	Day 10	Day 14	Day 21
α-MEM/BGJ-b	-64.3 to 34.9	-78.3 to 14.6	-55.0 to 44.2	-29.6 to 69.7	-46.7 to 52.5
α-MEM/ECCM	-44.5 to 54.8	2.1 to 101.4*	-20.3 to 79.0	-43.6 to 55.7	-18.6 to 80.7

5.3.7 Results Summary

Alpha MEM cultures:

- Consisted primarily of rounded cells and a few fibroblasts, which formed rounded colonies during the first week of culture
- Expressed haemopoietic stem cell marker, CD34, from the beginning of the culture until day 7
- Produced stromal stem cell marker, STRO-1, at day 14
- Maximum staining for osteoblastic markers was observed at days 1 and 14, with the proportion of cells staining for osteogenic markers decreasing at day 21
- Stained for vWF, at day 1, in a small proportion of the cell population
- Overall produced the greatest proportion of adipocytes of all the cultures studied

BGJ-b cultures:

- Consisted of fibroblasts, rounded cells and cells with enlarged nuclei
- Were the only cultures to produce a mineralised matrix
- Expressed haemopoietic stem cell marker, CD34, throughout the culture period, and produced STRO-1 during the first fortnight in culture
- Osteoblastic markers reached maximum levels at day 7 and remained high throughout the remaining culture period
- Significantly greater proportions of cells stained for osteonectin at days 7 to 21, and for BSPII and osteopontin at day 21 than in control medium
- Staining for endothelial cell marker CD105, was observed in a small population of cells at days 7 and 21
- The proportion of adipocytes in the culture decreased over time

ECCM cultures:

- Consisted of fibroblasts, rounded cells and 'stellate' cells, which formed colonies during the first week of culture
- 'Stellate' cells extended processes onto neighbouring cells
- No stem cell markers were expressed in these cultures
- Expression of bone cell markers increased with incubation time, with osteonectin expression significantly greater than in control cultures at days 7 to 21; and BSPII and osteopontin expression greater at day 21
- Cells staining for both endothelial cell markers CD105 and vWF was observed at all time points
- Contained the fewest adipocytes of all cultures studied, with adipocyte numbers decreasing with incubation time (cultures at days 14 and 21 contained no adipocytes)

5.4 Discussion

Bone marrow tissue is known to contain multipotent mesenchymal stem cells (MSCs) [21, 22], that can differentiate into bone, cartilage and fibrous tissue following a fracture or a bone graft and generate a reparative callus. MSCs can be isolated from bone marrow, and under the correct culture conditions can be induced to differentiate into adipocytes, fibroblasts, osteoblasts and chondrocytes (figure 1.3). The number of MSCs declines with age, as does their responsiveness to growth factors; hence, ability to regenerate various mesenchymal tissues declines as a function of aging. With many of the patients requiring bone grafts being elderly (e.g. for failed joint arthroplasty), it is crucial that the technique of creating osteoblasts, to coat grafts with, from bone marrow is optimized. With increasingly limited starting material over time, the ideal method would create the maximum number of

osteoblasts from a given quantity of bone marrow, losing none of the valuable MSCs through differentiation pathways other than the osteoblastic lineage.

Studies concerning the development of the osteoblast phenotype, from the osteoprogenitor proliferative cell to the osteocyte embedded in the extracellular matrix, suggest a temporal sequence of differentiation involving active cell proliferation, expression of osteoblastic markers (figure 5.23), synthesis, deposition and maturation of a collagenous extracellular matrix, and matrix mineralization [177]. Expression of the osteoblastic phenotype in culture depends on the composition of the culture medium, namely, the presence of compounds that influence cell proliferation and differentiation [178-180]. In this work, several *in vitro* culturing conditions were compared in order to maximize the induction and expression of osteoblastic markers in mouse bone marrow cell cultures. Cells were cultured in control medium (α-MEM), BGJ-b medium or ECCM. Cultures growing in the various experimental situations were compared concerning cell growth (cell numbers, proliferation rates and morphology), stem cell, osteoblast, endothelial cell and adipocyte markers and also ability to form mineralized calcium phosphate deposits.

Control medium caused bone marrow cells to differentiate primarily into cells with rounded or fibroblastic morphology. After an initial lag phase, the cell population in this medium formed roughly equal sized rounded colonies and proliferated until the colonies merged, and cultures became confluent (around day 14). After confluence was reached cell layers started to dislodge from the culture dish causing a drop in the cell numbers recorded.

A small proportion of the rounded and fibroblastic cells presented the haematopoietic stem cell marker CD34 on their surface at day 1 and these cells increased in number by day 7. As the cells began to rapidly proliferate staining for this marker disappeared, and as cells reached confluence the MSC marker STRO-1 was detected. Post confluence, neither stem cell markers were observed.

The increase in the haematopoietic marker was also associated with an overall depletion in the numbers of cells staining for markers of the osteoblastic lineage. As with the MSC marker, the proportion of cells staining for osteoblastic markers increased in the absence of the haemopoietic stem cell marker and depleted once the cells surpassed confluence.

The pattern of osteogenic marker staining over time demonstrated in α-MEM cultures was the exact reverse of the staining observed for adipocytes, suggesting perhaps a change in cellular differentiation over time.

The high proportion of osteogenic cells after the first 24 hours in α-MEM may be due to osteoblastic lining cells in the culture, in addition to the bone marrow cells. By the first week in culture the proportion of osteogenic cells has dropped, perhaps due to bone marrow cells preferentially differentiating into adipocytes, and haemopoietic cells being counted along with the stromal cells of interest. Once confluence was attained, cells chose to differentiate along the osteoblastic lineage rather than the adipocytic lineage. Post confluence, however, the culture favors the adipocytic lineage once again.

Cells grown in BGJ-b medium differentiated into a variety of cell types, that adhered randomly across the plastic culture surface, without forming colonies. Adipocytes accounted for approximately 20-30% of the culture, and haemopoietic stem cells between 10 and 30%. The greatest proportion of STRO-1 positive cells was seen in this medium. At day 1 over 60% of the cell population expressed the STRO-1 MSC marker. As the proportion of cells staining for the MSC marker decreased, so the numbers of cells staining for osteoblastic markers increased.

Cells in BGJ-b medium proliferated to a lesser extent than in the other two media, however, at day 7 80-100% of the cells had differentiated into osteoblastic cells. Almost all of the cells in this medium continued to stain positive for markers of

the osteoblastic lineage, as the cells slowly proliferated, until roughly day 14 when cell proliferation ceased and the cultures began to mineralize.

BGJ-b medium did not cause cells to proliferate to the same extent as those grown in the control medium, since cells appeared to differentiate rather than proliferate. BGJ-b medium caused fresh bone marrow cells to preferentially differentiate along the osteoblastic lineage. Proliferation of osteoblastic cells is functionally related to the synthesis of a bone extracellular matrix and its accumulation and maturation, essential to the mineralization process, contributes to the shut down of proliferation (figure 5.22). In the presence of BGJ-b, mineralization does occur when an appropriate relationship between the amount of the extracellular matrix and its maturation is achieved; as a result, cell proliferation ceases with the osteoblasts being trapped and embedded in the mineralizing matrix. Unfortunately, the cells mature and become engulfed in matrix at such an early stage in BGJ-b medium, they have little time to proliferate and final cell numbers are low relative to cells cultured in control medium or ECCM. The early differentiation and mineralization observed in this medium therefore, hinders it's potential use for growing large numbers of osteoblastic cells to assist in increasing the rate of bone graft remodeling.

ECCM medium induced the formation of unique cells from bone marrow, with a morphology similar to that of stellate cells. During the first week in the medium cells proliferated slowly, forming colonies made up of a few elongated cells with dendrites surrounded by fibroblasts and rounded cells that stained positive for STRO-1. An increase in the rate of cell proliferation between days 7 to 14 was also associated with increasing numbers of the stellate cells. The majority of cells in ECCM stained positive for various osteoblastic markers at the beginning of the culture period, however the number of cells staining increased as the cells proliferated. The stellate cells along with fibroblasts and rounded cells stained positive for osteogenic markers,

thus as a consequence of proliferation and differentiation simultaneously occurring up to day 14, a vast number of cells (greater than in either of the two other culture media) were formed, nearly all of which presented an osteoblastic characteristics. It was also discovered, however, that all of the stellate cells grown in ECCM stained positive for endothelial cell markers as well as osteogenic markers.

The stellate morphology of cells also resembled the typical endothelial morphology of BBE-1 cells (an endothelial cell clone from bovine parathyroid tissue). BBE-1 form extensive intercellular connections and branching and occasional sprouting (or 'tubular' structures) in monolayer cultures [183]. If ECCM selects for endothelial cells in the bone marrow culture, as the immunohistochemistry data suggests then the medium would be an extremely valuable tool for investigating the vasculature of bone, as it is extremely difficult to obtain pure cultures of bone endothelium.

Staining for both osteogenic and endothelial cell markers within a single cell type suggests an overlap in the differentiation pathway between the osteogenic and endothelial lineages. The intimacy of endothelial cells and osteoblasts has led to the theory that the endothelial cell itself is the osteoblast precursor [184].

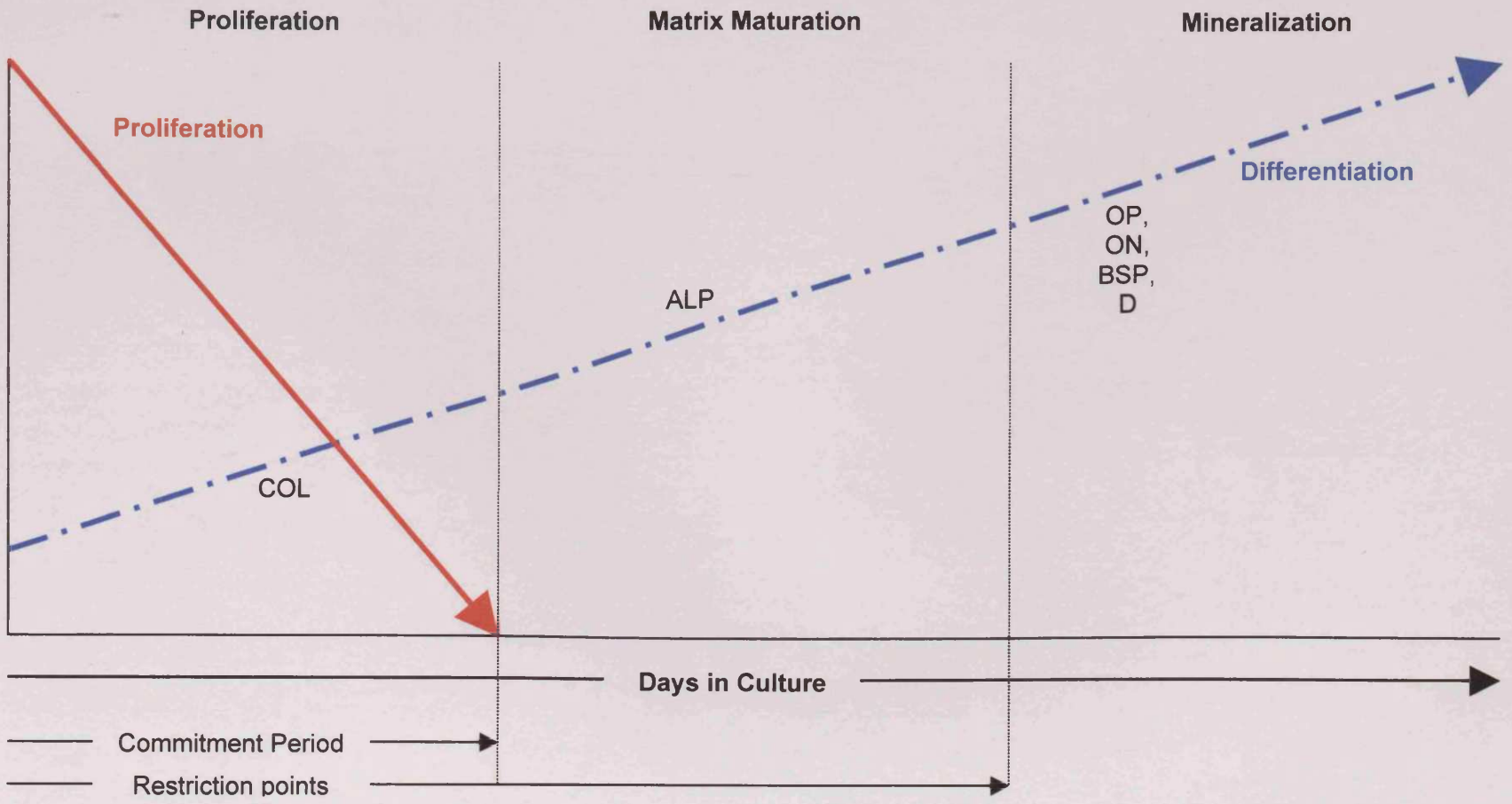
Endothelial cell markers were only found in a small number of cells in both the control medium and BGJ-b medium. Cells staining positive for endothelial markers might have included microvasculature cells or sinusoidal cells, such as those observed in the characterization of the bone marrow in chapter 3.

In conclusion BGJ-b medium produces only a small number of cells from the initial quantity of bone marrow, however all of the cells differentiated into osteoblasts rapidly (within 7 days). BGJ-b medium would, therefore, be useful for the rapid formation of small quantities of bone. However, large amount of bone marrow would be required to produce sufficient quantities of osteoblast like cells to coat a graft for large bone defects.

ECCM produced a large quantity of cells from a small amount of bone marrow, all of which expressed osteoblastic markers. Therefore, for sufficient cells to coat, and improve the ossoeintegration of large bone grafts cells bone marrow cells should be cultured in ECCM before implantation onto the graft. ECCM also had the advantage of preventing cells differentiating into unwanted adipocytes.

Cells grown in ECCM may have further applications in the formation of an autogenous bone graft. From the high osteoblastic cell numbers produced from bone marrow in ECCM media, comes the potential for forming a larger piece of mineralized bone, than with BGJ-b medium. Therefore, in the following chapters we will investigate the three dimensional growth of cells in the two investigative media, and compare the size of the mineralized matrix produced in each medium.

Figure 5.22: Reciprocal relationship between genes associated with proliferation and differentiation in an osteoblast culture model. [182] During early culture, genes associated with proliferation are down regulated, while matrix genes such as type I collagen (COL) begin to increase. This continues throughout matrix maturation, when proliferation ceases and onset of expression of alkaline phosphatase (ALP) begins. During the final phases of differentiation, the matrix mineralizes, and osteopontin (OP), osteonectin (ON), bone sialoprotein (BSP) and decorin (D) are expressed.



Chapter 6

Gene Expression Profile of Mouse Bone Marrow Stromal Cells Grown in Endothelial Cell Conditioned Medium Determined by cDNA Microarray Analysis.

6.1 Introduction

There is little understanding of the role of endothelial cells in bone formation and their interaction with stromal and hematopoietic systems of bone tissue. Vascularisation is required before osteogenesis will occur, yet the reason for this dependence have not been fully elucidated. After vascular invasion, the hypertrophied cartilage is degraded and replaced by bone marrow and later bone tissue. Mineral formation is observed in the immediate vicinity of vessels and seems to correlate with the onset of cartilage vascularisation [189]. Endothelial cells have been shown to synthesise bone cell active mitogen(s) and, therefore, the endothelium may represent an important element in the formation of bone [190].

A schematic of the different locations of bone microvascular cells can be seen in figure 6.1. The functional connections among endothelial cells, osteoblasts and osteoclasts are evident. In osteon formation, the gradual filling-in process that converts cancellous bone into compact bone (see chapter 1) creates a number of narrow canals that are lined with osteogenic cells. These canals enclose vessels that were formerly present in soft tissue spaces in the cancellous network (figure 6.1.A). Also, on other bone surfaces (the deeper layers of the periosteum and endosteum that line the internal surfaces of all cavities within bone), the osteogenic cells are intimately associated with blood vessels (figure 6.1.B). Endochondral and intramembranous ossification proceed in association with capillaries (figure 6.1.B & C). In the formation of ossification centers in long bones, mineralization occurs only when arterioles penetrate the periosteum, invading cartilage (figure 6.1.B). In the epiphyseal plate, ossification occurs when metaphyseal capillary sprouts appear (figure 6.1.D). In the intramembranous ossification model, the center of osteogenesis develops in association with capillaries that grow into the mesenchyme (figure 6.1.C). The mesenchymal cells, characterized by a stellate appearance and pale cytoplasm, pass imperceptibly through the osteogenic cell stage, becoming more rounded and

basophilic, with thicker interconnecting processes. An intimate functional relationship also exists between the sprouting capillaries invading the epiphyseal plate and osteoclastic and osteoblastic cells (figure 6.1.D) In fact, osteoclasts are typically located on the tip of the sprouting capillary, in a strategic position to resorb calcified cartilage, while the osteoblasts line the terminal wall of the capillary, like the rearguard ready to regenerate the matrix of bone (figure 6.1.E). A frequent paratrabecular continuity of the sinusoid endothelium and the osteoblastic seams, together with the possible direct endosteal production of some sinusoidal compartment [191] represent convincing evidence of a relationship between endothelial cells and osteoblasts (figure 6.1. F).

With strong evidence for a close functional relationship between endothelial cells and bone marrow, along with the findings in chapter 5, that ECCM induces osteogenic markers in bone marrow cells after only 24 hours in culture; it was decided to investigate the genetic effect that substances released by endothelial cells were having on bone marrow cells.

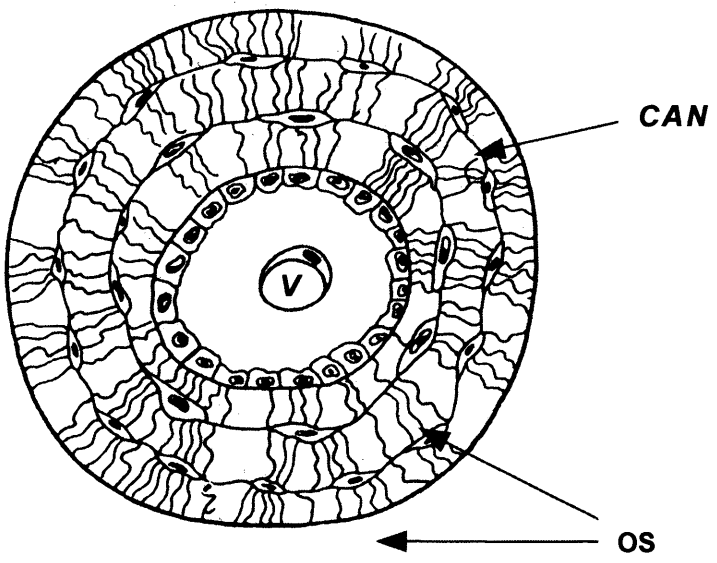
6.1.1 Aim

The aim of this chapter was to:

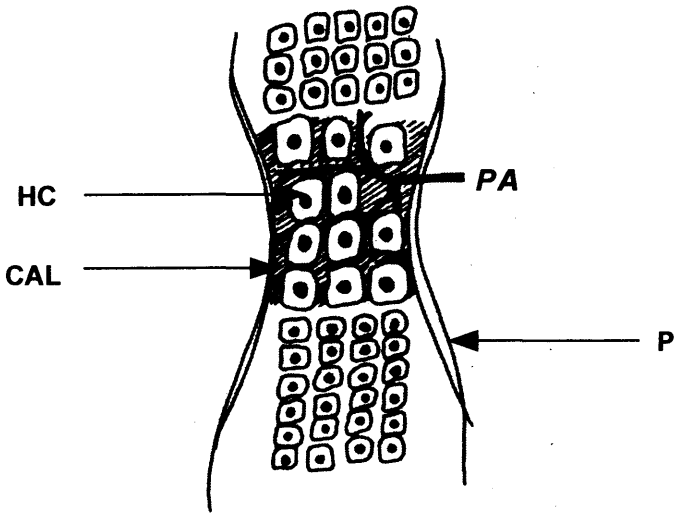
- Investigate the effects of factors released by endothelial cells on bone marrow stromal cells at a genetic level.

Figure 6.1: Schematic of the microvasculature of bone. OB, osteoblast; V, vessel; OS, osteocyte; CAN, canaliculi; CAP, capillary; M, mesenchyme; HC, hypertrophied chondrocytes; CAL, calcified matrix; PA, periosteal arteriole; P, periosteum; EP, epiphyseal plate; RC, resting cartilage; PC, proliferating cartilage; MC, mature cartilage; OC, osteoclast.

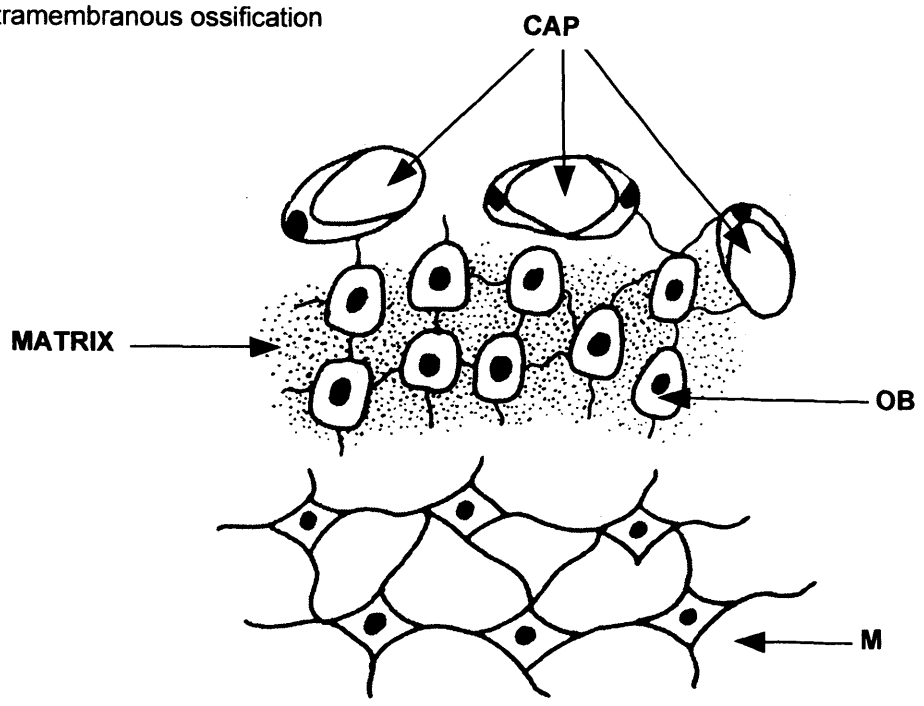
A. Osteon of compact bone



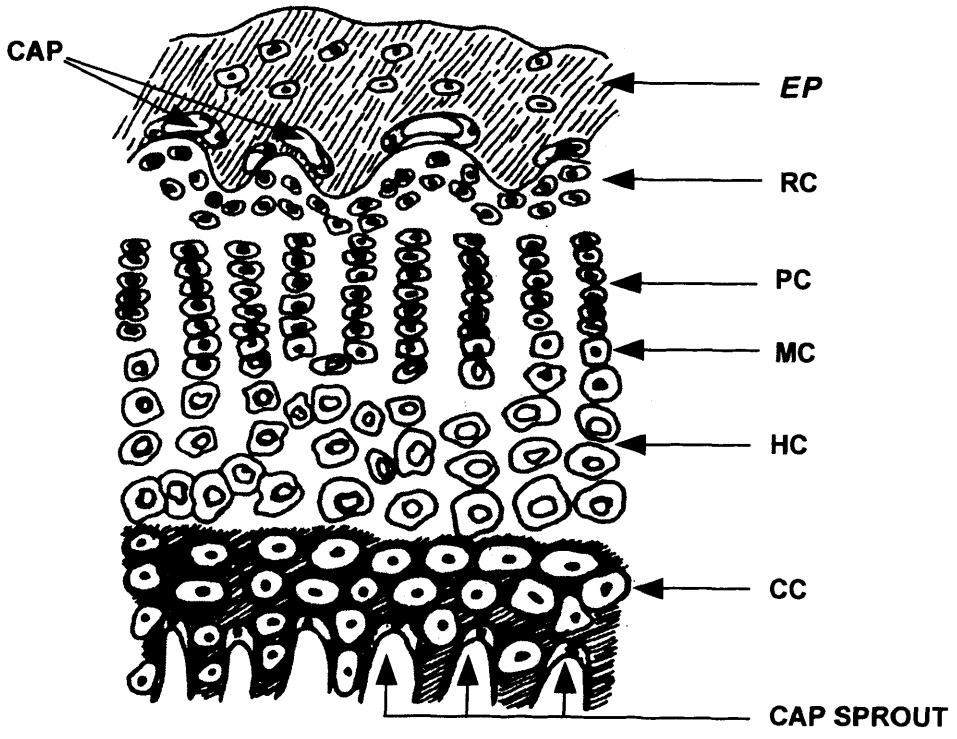
B. Endochondral ossification



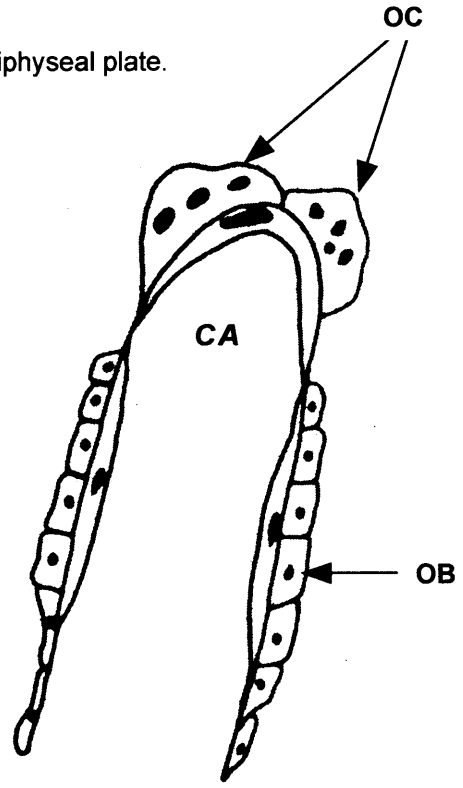
C. Intramembranous ossification



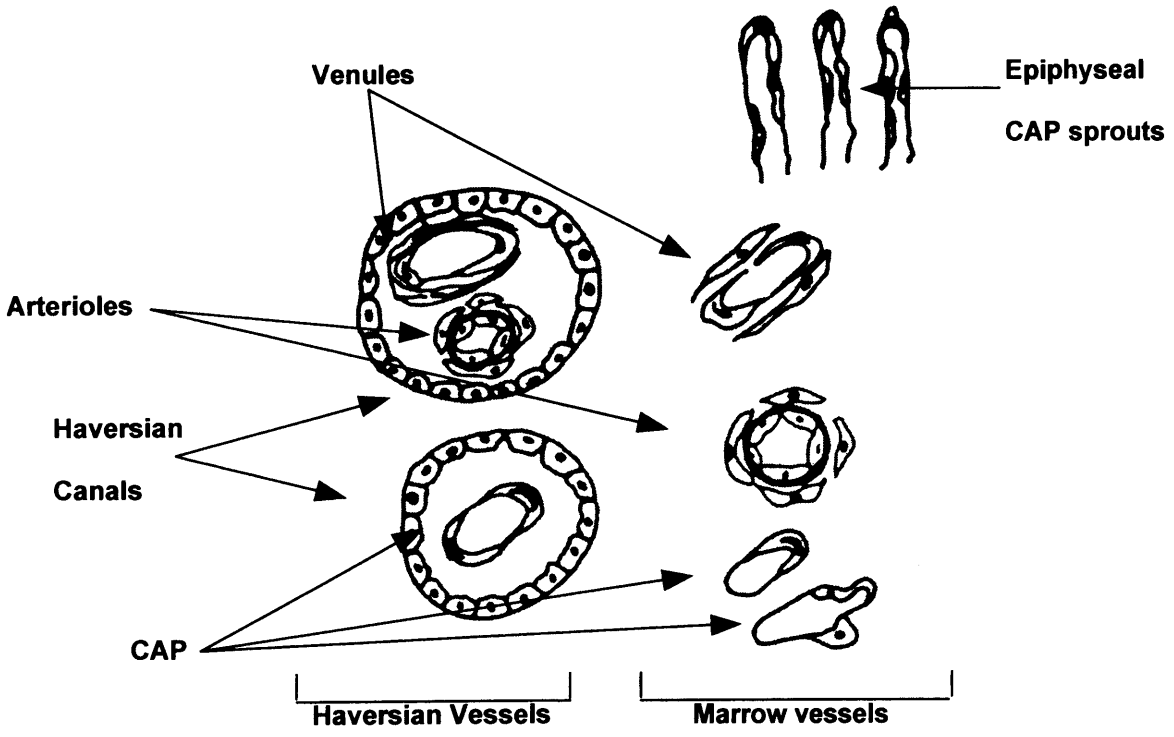
D. Epiphyseal plate



E. Sprouting capillary invading epiphyseal plate.



F. The microvascular system of bone and marrow.



6.2 Methodology

6.2.1 Culture of mouse bone marrow cells

Bone marrow cells were harvested from the mid-shafts of MF1 mice femurs with a syringe containing culture medium. The culture media used was α-modification minimal essential medium eagle (α-MEM) (Gibco BRL Ltd., Paisley, Scotland), or ECCM (α-MEM collected after 2-3 days from confluent bovine aortic endothelial cells). Media were supplemented with 10% foetal calf serum, 2mM L-glutamine, penicillin (10,000U/100ml), streptomycin (10mg/ml) and amphoterycin (25μg/ml).

Cells were incubated for 24 hours in a humidified atmosphere of 95% air and 5% CO_2 . Fresh bone marrow cells (2.5×10^{10}) were added per $25cm^2$ tissue culture flask (Nalge NUNC International, Paisley, Scotland), and 3 flasks were set up per culture medium. Cultures were washed thrice at 24 hours with PBS to remove non-adherent cells. The stromal cells of the adherent layer were released by trypsin (0.05%)-EDTA (0.02%) (Sigma, Poole, Dorset) for RNA collection.

6.2.2 RNA Collection

For each culture medium, control medium (α-MEM) and ECCM, total RNA was prepared from 2 million cells from the adherent layer of cultures grown. Total RNA was extracted as described in the general methodology.

6.2.3 Microarray

Experimental procedures for Affymetrix GeneChip microarray, on the RNA from cells cultured in ECCM and control medium, was performed by the Microarray centre, Imperial College, London.

6.3 Results

6.3.1 Gene expression profile of mouse bone marrow cells

Genes expressed in mouse bone marrow cell cultures were identified by cDNA microarray analysis. The results of this study were then compared with the results of two comprehensive gene expression studies of human bone marrow stromal cells. Tremain et al. (2001) [192] looked at the 50 most abundant transcripts, whereas in the publication by Jia et al (2002) [193], the 30 most highly expressed genes are shown. A comparison of these two sets of highly expressed genes with our study can be seen in table 6.1.

In the datasets for ECCM, genes characteristic for very different cell types were found. Among the highly expressed genes in ECCM, neuronal transcripts such as synapsin were present. In addition collagen type IX, an interfibrillar network-bonding agent was discovered. Mutations of collagen type IX are believed to affect cartilage matrix integrity and produce a phenotype similar to early-onset osteoarthritis [194]. Another gene expressed in ECCM cultured bone marrow cells was the tumor necrosis factor alpha gene (TNF). Tumor necrosis factor-alpha (TNF) is one member of a large family of inflammatory cytokines. TNF has a central role in bone pathophysiology. TNF is necessary for stimulation of osteoclastogenesis along with the receptor activator of Nf-kappa B ligand (RANKL). TNF also stimulates osteoblasts in a manner that hinders their bone-formative action. TNF suppresses recruitment of osteoblasts from progenitor cells, inhibits the expression of matrix protein genes, and stimulates expression of genes that amplify osteoclastogenesis [195].

In addition to the genes found in ECCM cultured cells, a gene for bone morphogenic protein 4 was discovered both in control medium cultures and the experimental cultures. Bone morphogenic protein-4 (BMP-4) is one of nine structurally related BMPs belonging to the transforming growth factor-beta (TGF-

Tremain *et al.*	Jia *et al.*	Microarray analysis		Cluster Inclusion
		Control Medium	ECCM	
-	Human normal keratinocyte substraction library mRNA, clone h22a, complete sequence	-	-	-
Vimentin	Vimentin	-	-	-
-	Tubulin, alpha, ubiquitous	-	Tubulin alpha 1	M28729
-	Human aortic-type smooth muscle a-actin gene, exon 9	-	Actin, gamma 2, smooth muscle enteric	U20365
Insulin-like growth factor binding protein 3	Insulin-like growth factor-binding protein 4	-	Insulin-like growth factor I receptor mRNA	AF056187
-	Plasminogen activator inhibitor, type I	-	Plasminogen activator inhibitor, type II	X16490
-	Decorin	-	-	-
-	*H.sapiens*opa-interactig protein oip3 mRNA, partial cds	-	-	-
Collagen, type III, alpha 1	Collagen, type III, alpha 1	-		-
-	Calumenin	-		-
-	*H.sapiens*clone 24703 b-tubulin mRNA	-	tubulin, beta 2	M13444
-	Tissue inhibitor of metalloproteinase 1	-	Tissue inhibitor of metalloproteinases-3	U26437

Tremain *et al.*	Jia *et al.*	Microarray analysis		Cluster Inclusion
		Control Medium	ECCM	
-	Thrombospondin 1	-	Thrombospondin 1	M62470
Ferritin, light polypeptide	Ferritin, light polypeptide	-	Ferritin light chain 1	L39879
-	Collagen, type V, alpha 2	-		-
23kd highly basic protein	*H. sapiens* mRNA for 23kd highly basic protein	-		-
-	Human beta-1d integrin mRNA, cytoplasmic domain	-	Integrin alpha 4	X53176 & X53177
Osteoblast specific factor 2	-	-		-
Collagen, type III, alpha 1	-	-		-
Eef2	-	-		-
Collagen, type I, alpha 1	-	-		-
Cathepsin b	-	-	Cathepsin b gene	M65270
Thymosin, beta-4 Chr X	-	-	Prothymosin beta 4	U38967
Matrix metalloproteinase 2	-	-	Matrix metalloproteinase 1	X66437
-	-	-	Matrix metalloproteinase 9	X72795
Beta-2 microglobulin	-	-	Beta-2 microglobulin	X01838
Tpt1	-	-		-

Tremain *et al.*	Jia *et al.*	Microarray analysis		Cluster Inclusion
		Control Medium	ECCM	
Glutamate receptor, nmda2c	-	-		-
Collagen, type VI, alpha 3	-	-		-
Keratin 8	-	-		-
Glia-derived nexin1, alpha	-	-		-
Heart nRNA for hsp90	-	-		-
-	-	-	Procollagen, type IX, alpha 2	Z22923
-	-	-	Tumor necrosis factor alpha gene	D84196
-	-	mRNA for bone morphogenetic protein 4	mRNA for bone morphogenetic protein 4	X56848

6.3.2 Results Summary

- Genes highly expressed in ECCM cultures include:
  - Neuronal transcripts e.g. synapsin
  - Collagen type IX
  - TNF-α
  - BMP4

6.4 Discussion

It was established in chapter 5 that mouse bone marrow cultures are heterogeneous. Morphologically distinct cell types were observed in endothelial cell conditioned medium cultures, in particular rounded cells, fibroblast-shaped and stellate cells. The regulation of gene expression *in vivo* is influenced by the tissue environment of cells, and although the profile was obtained under *in vitro* conditions, it provides new insights into the possible role of endothelial cell factors on the biology of BMSC.

In line with the previous report by Tremain et al. (2001), the high plasticity of BMSC is mirrored by the expression of genes characteristic for very different cell types. The variety of cell type-specific genes indicates a kind of intermediate state of these cells, which might explain their broad differentiation potential. In this study, the identifiable genes were used to ascertain the effect of factors produced by endothelial cells on the differentiation of BMSC. Upon searching the data produced in this study for highly expressed genes that encode proteins of the extracellular matrix, cell adhesion proteins, cytoskeletal proteins and cytokines/cytokine receptors; it was revealed that the expression pattern in ECCM cultured BMSCs suggests a close association with vascular cells. Insufficient quantities of cDNA was obtained from cells cultured in the control medium.

The stroma represents the supporting connective tissue within the bone marrow and consists of cellular elements (connective tissue cells and vascular cells) and extracellular matrix. The presence of a cell-cell adhesion molecule transcript of cadherin 1 (E-cadherin) indicates that cells grown in ECCM form adherens-type intercellular junctions. An additional cell surface molecule of interest produced by these cells is PETA-3 (CD151, platelet endothelial tetraspan antigen 3), which has been described on endothelial cells, periarteriolar smooth muscle cells, hepatic sinusoids and basolaterally close to the basement membrane on polarized epithelia [198, 1199]. Moreover, integrin beta-1, which can be associated with PETA-3 and is known to mediate cell-matrix interactions via binding to fibronectin, is highly expressed.

Pericytes are contractile cells, expressing smooth muscle-specific actin and myosin isoforms and tropomyosin [200]. A high similarity of BMSC with vascular smooth muscle cells has previously been reported [201, 202]. In this study a gene specific for smooth muscle cells (gamma-2 actin) was revealed, in addition to other genes encoding contractile cytoskeletal components, such as beta-actin and gamma-actin.

Besides the overlap of expressed genes, both BMSC and pericytes have a striking differentiation potential [203]. Like BMSC, pericytes have been described as being capable of differentiating into osteblasts [204]. Although proposed previously, no conclusive evidence for the identity of BMSC and pericytes has been provided so far [205]. Following the 24 hour incubation period in ECCM, osteoblastic genes were not highly expressed by the BMSC in this instance. The TNF gene however, which is known to suppress the recruitment of osteoblasts from progenitor cells, was highly expressed.

This identified expression pattern of BMSC grown in ECCM in this study, suggests that they are similar to pericytes. Pericytes lack a specific marker that is common to

all species [206, 207] and are to date only defined by their position adjacent to endothelial cells within the basement membrane of vessels. Their relationship with pericyte-like perivascular cells, which are not completely surrounded by the basement membrane but are situated on its surface is as yet unclear [208]. They might be the same cell type or two different types derived from each other.

In conclusion, the present expression profile of BMSC grown in ECCM suggests that BMSC are vascular-associated cells resembling pericytes.

Chapter 7

The Effect of Type I Collagen Gels on Differentiation of Bone Marrow Stromal Cells

7.1 Introduction

To succeed in effective bone reconstruction, systematic consideration must be given to elucidating the biochemical principles of bone formation. It could be proposed that four important factors are involved in bone formation:

1. The cells directly involved in bone formation
2. Matrices produced by the cells
3. Mineral ions from body fluids
4. Regulators of general cellular activities as well as of the calcification process [209].

While not definitive, these 4 factors are at least strategically, very important.

In this study, a stable source of the first factor, osteogenic cells, was attempted. This was combined with the second factor, the matrix, to try and construct bone *in vitro*. As for the third and fourth factors, mineral ions are usually well supplied in a culture medium, and essential growth and differentiation factors are believed to be available in calf serum, which is also added to the culture medium.

Culturing cells on collagen gel has already been shown to produce remarkable effects on the differentiation and growth of various types of cells *in vitro* [210, 211]. Collagen beads have also proved to be good carriers for large-scale cell-culture due to their enormous surface areas [212].

The inducible nature of stromal cells was noted in earlier chapters, and has also been reported by Maniatopopolous *et al.* [213]. In chapter 5 BGJ-b medium or ECCM was substituted for dexamethasone and β-glycerophosphate. In this chapter, the same experimental media was used, however the bone marrow cells were cultured on collagen gel in attempt to produce a bone-like structure.

7.1.1 Aim

The aim of this chapter was to:

- Produce a bone-like structure from fresh bone marrow, by culturing the cells in type I collagen matrix.

7.2 Methodology

7.2.1 Collagen gels

Type I collagen gel (1mg/ml) were prepared from acid digestion of rat-tail tendon collagen (see general methodology). The gel solution consisted of 28% collagen solution, 42.4% D-modification minimal essential medium (Gibco BRL, Paisley, UK), 25.4% sodium bicarbonate solution and 4.2% HEPES buffer. 500μl of gel solution was poured into wells of a 4 well plate (Nalge NUNC international, Paisley, Scotland). Fresh bone marrow cells ($1x10^7$) were evenly distributed inside gels by drawing the cells and gel solution for each well into a syringe. Gels were allowed to set for 10 minutes at 37°C and 500μl medium (α-MEM, ECCM or BGJ-b medium) was added.

7.2.2 Cell counts/Proliferation rates

At time point up to 14 days, cells were enzymatically released for counting by collagenase digestion of the gels with collagenase from *Clostridium histolyticum* (Sigma, Poole, Dorset). The proliferation rates (change in cell number/24hours) were calculated using the formula:

$$\text{Proliferation (change in cell number/24hours)} = \frac{\text{Number of cells}}{\text{Number of days in culture.}}$$

7.2.3 Immunohistochemistry and staining of collagen gels

Gel cultures were fixed in 10% formal saline at 7 days, embedded in paraffin and sectioned serially. Sections were stained with H&E or by von Kossa method. Alternatively sections were treated with mouse monoclonal antibodies, to either STRO-1 (8.06μg/ml), B4-78 to alkaline phosphatase (8.04μg/ml), WVID1(9C5) to bone sialoprotein (22.45μg/ml), MPIIIB10$_1$ to osteopontin (16.6μg/ml), DS1 to decorin (35μg/ml), AON-1 to osteonectin (7.75μg/ml), MJ7/18 to CD105 (6.7μg/ml) (all Developmental Studies Hybridoma Bank, University of Iowa, Iowa, USA); rat monoclonal antibody MEC14.7 to CD34 (10.1μg/ml) (Serotec Ltd, Kidlington, Oxford) or rabbit polyclonal antibody to von Willebrand factor (12.4μg/ml) (AbCam, Cambridge, UK) for 60 minutes at room temperature. The antigen-antibody complex was visualised using R.T.U vectastain kit and peroxidase substrate DAB (both Vector Laboratories, Inc. Burlingham, CA, USA). Nuclei were counterstained with haematoxylin nuclear counterstain (Gill's formula) (Vector Laboratories, Inc. Burlingham, CA, USA).

7.2.4 Statistical analysis

Data presented in this work are the results of experiments repeated six times, performed in cell cultures from adult male mouse bone marrow. For cell counts, proliferation rates and colony counts each point represents the mean ± standard deviation of 6 different measurements for each culture condition at each time point. Statistical analysis was done by ANOVA test (one way analysis of variance). The statistical difference between the experimental culture media and the control media at each time point was determined. P values ≤ 0.05 were considered significant.

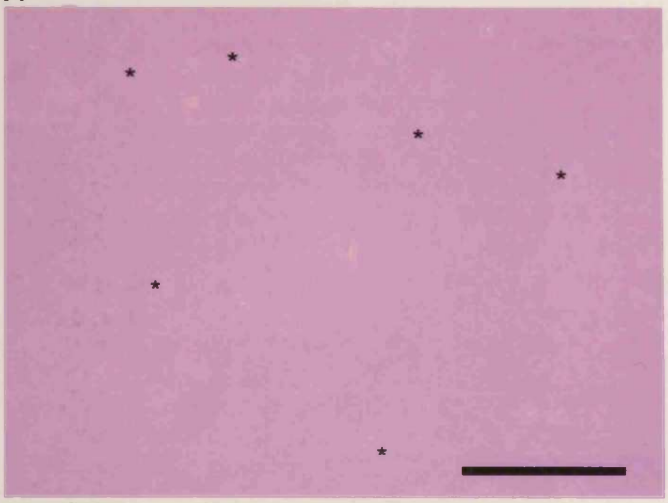
7.3 Results

7.3.1 Morphological changes and mineralization

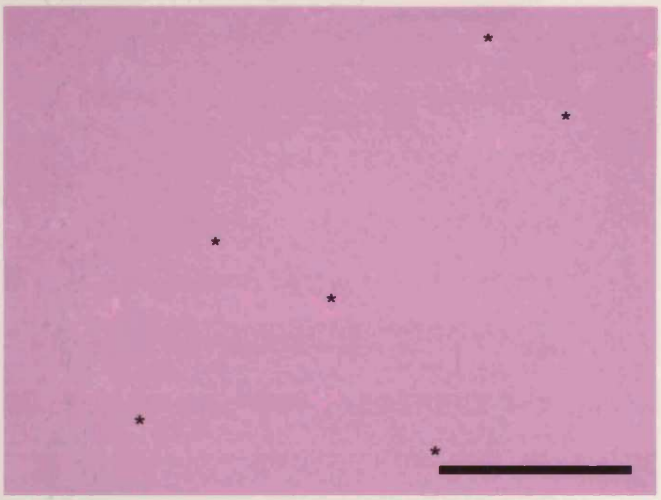
Bone marrow cells from mouse femora were primarily rounded throughout the first fortnight in collagen gel culture, regardless of the medium in which they were growing (figures 7.1). After 2 weeks of culturing, large numbers of mineral nodules had been formed by the cells inside the gels in BGJ-b medium and ECCM a (figure 7.2). In contrast, those cells in control medium remained rounded and showed neither cellular aggregation nor nodule formation (figure 7.2). By the third week in culture, the nodules in ECCM and BGJ-b medium cultures had fused together to form larger nodules as shown in figure 7.3. As seen in figure 7.4, and table 7.1; the nodules in both cultures stained well with Von Kossa reagent (BGJ-b medium cultures to a greater extent than those grown in ECCM) used to demonstrate mineralization. In BGJ-b medium the mineralised nodules fused to form large quantities of dense mineralised tissue (figure 7.3). Cells grown in endothelial cell conditioned medium formed needle-shaped mineral crystals along the collagen fibrils, which is similar to the structures characteristic of the early phase of mineralization in bone tissues (figures 7.2 and 7.3).

Figure 7.1: Collagen gels at day 14, in various culture media. Cells grown in Control medium (alpha MEM) (A), ECCM (B), and BGJ-b (C) appeared rounded (*). Scale bar, 500μm.

A



B



C

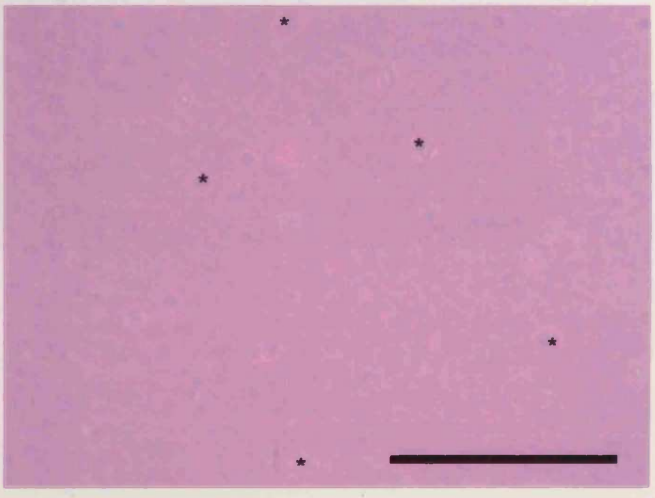
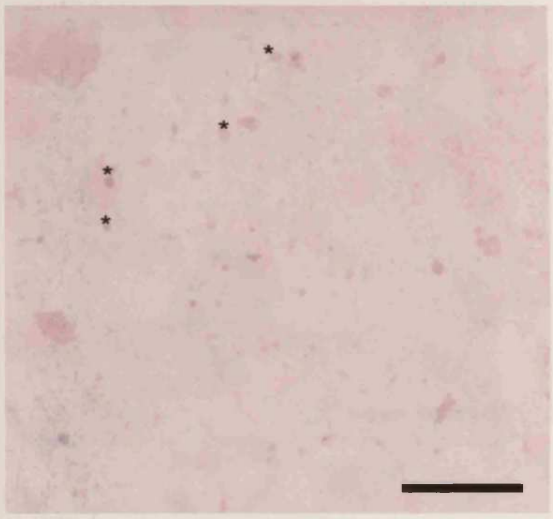


Figure 7.2: Cells cultured for 14 days inside collagen gels. The sections presented were stained with haemotoxylin and eosin. Cells cultured for 14 days in alpha MEM (A) and ECCM exhibited rounded cells (*) and mineral crystals forming along the collagen fibrils (X) (ECCM only). Scale bar, 500μm.

A



B

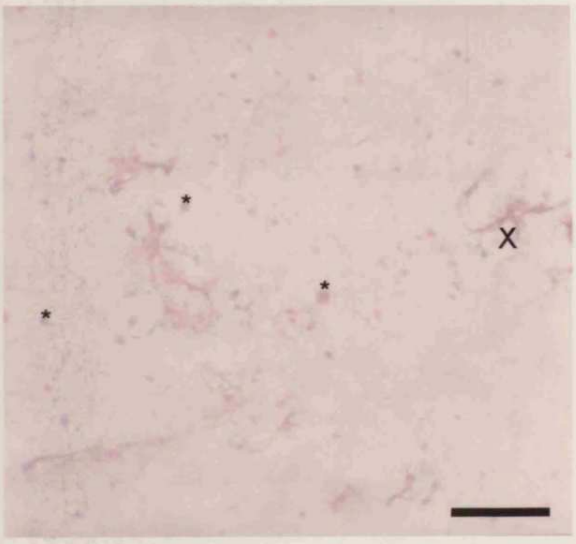
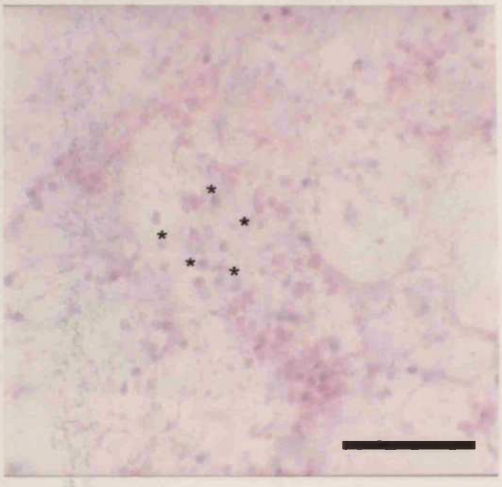
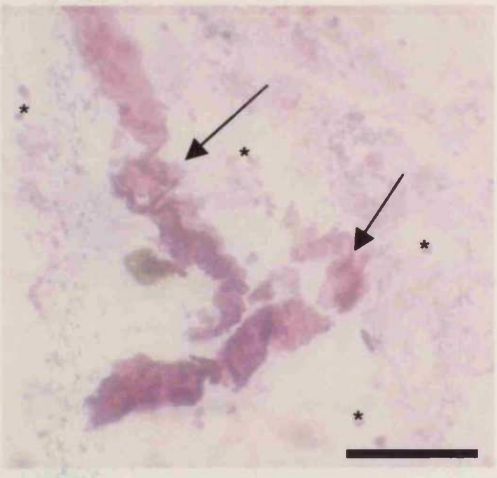


Figure 7.3: Cells cultured for 21 days inside collagen gels. The sections are stained with haemotoxylin and eosin. Rounded cells (*) are visible in cultures grown in alpha MEM (A), BGJ-b (B), and ECCM (C). BGJ-b cultures exhibited large mineralised nodules (arrows) and ECCM cultures formed needle shaped crystals (X). Scale bar, 500μm.

A



B



C

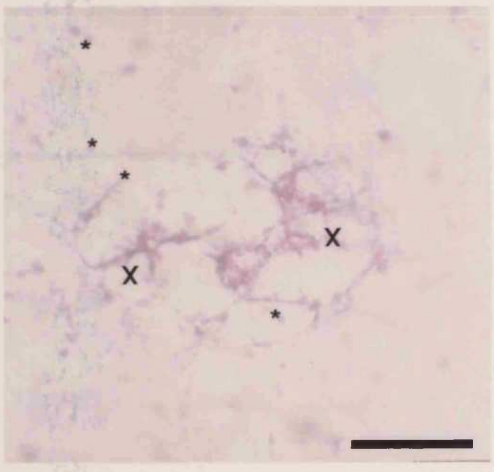
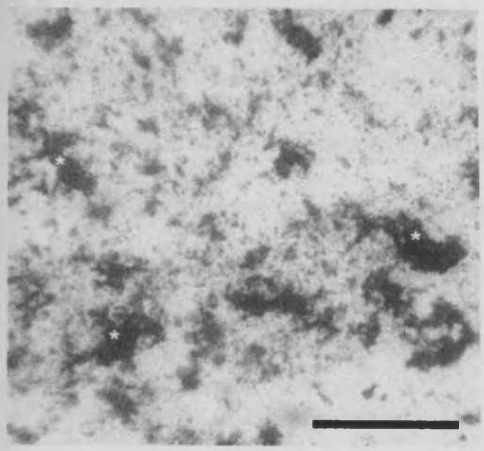


Figure 7.4: Cells cultured inside collagen gels, for 21 days. The section were stained using von Kossa method. Mineralization (*) was evident in BGJ-b cultures (A) and ECCM cultures (B). Scale bar, 500μm.

A



B

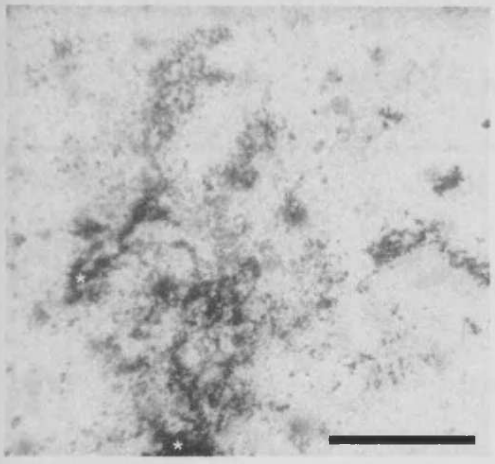


Table 7.1: Von Kossa staining of 7 to 21 day cell cultures grown in collagen gels. Intensity of staining was graded as follows: (-), negative staining; (+), definite staining but of low intensity; (++), moderate staining; (+++), intense staining.

	α-MEM (Control)	BGJ-b	ECCM
Day 7	-	+	+
Day 14	-	++	++
Day 21	-	+++	++

7.3.2 Cell counts and proliferation rates

All cell cultures demonstrated an increase in cell numbers during the first 24 hours in culture, this was followed by a decease in cell numbers until week 1 (figure 7.5 and table 7.2). For cultures in both the ECCM and BGJ-b medium, the cells proliferated until the final time point (figure 7.6 and table 7.4). Proliferation during the final weeks in culture was accompanied by increased mineral deposition (table 7.1) and also increased staining for bone formation markers (see below). At each of the time points studied, ECCM cultures contained a greater number of cells than in the control cultures. BGJ-b cultures also exhibited a significantly greater number of cells at the final time point than control cultures (table 7.3).

Table 7.2: Mean number of cells counted per culture at each time point.

	α-MEM (Control)	BGJ-b	ECCM
Day 0	10000000	10000000	10000000
Day 1	20500000	34000000	60864750
Day 7	696000	678000	33050000
Day 14	46400000	20600000	76400000
Day 21	33850000	37875000	94500000

Figure 7.5: Cell counts for cultures grown inside collagen gels in alpha MEM (control medium), ECCM and BGJ-b medium.

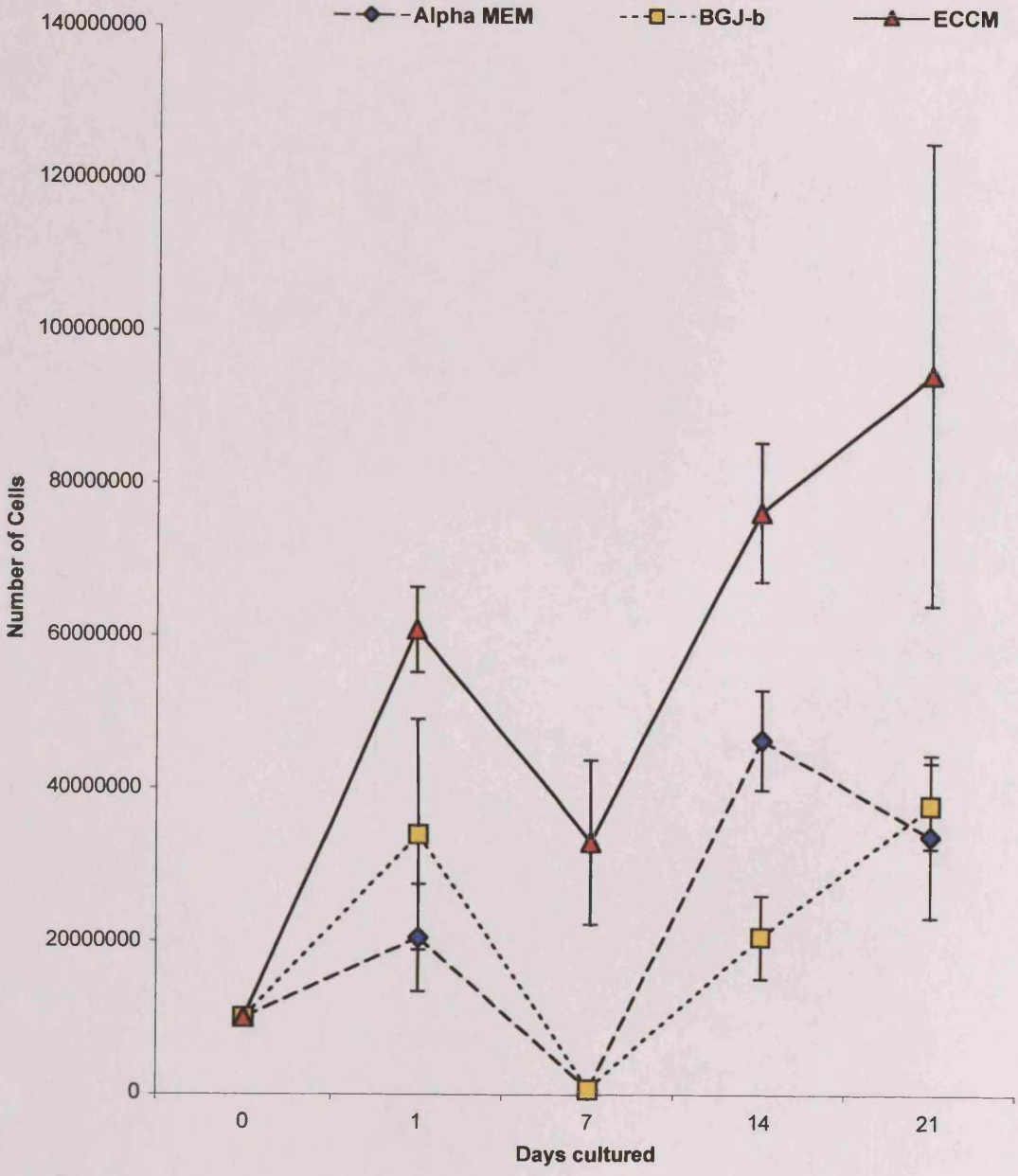


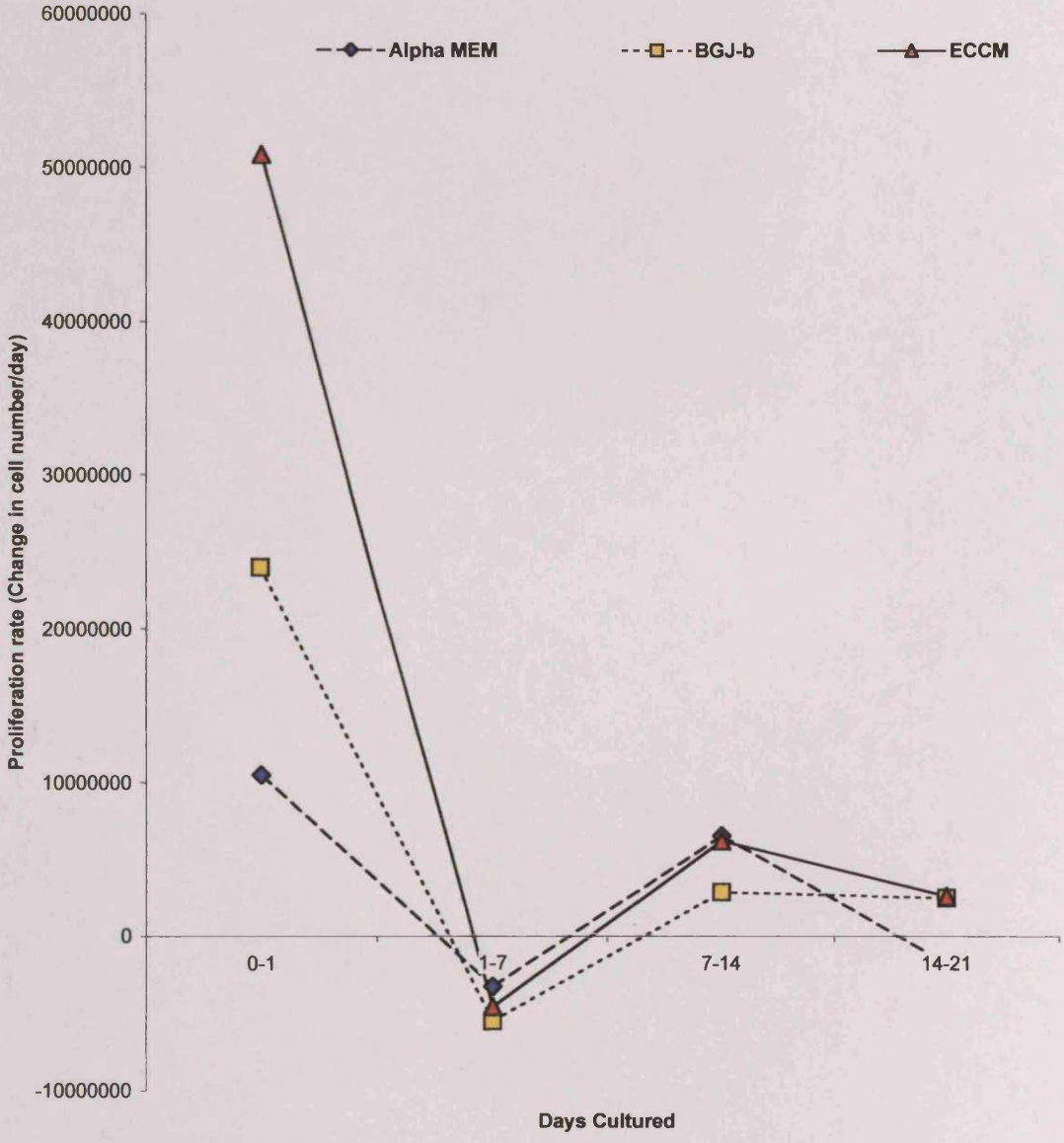
Table 7.3: Statistical analysis (ANOVA) of the mean number of cells counted in collagen gel cultures grown in alpha MEM (Control), BGJ-b or ECCM. Cultures in which the mean number of cells are significantly different are marked with an asterisk (*)

	α-MEM (Control)/BGJ-b	α-MEM (Control)/ECCM
Day 1	-55131813.6 to 28131813.6	-8199653.6 to 1267063.6
Day 7	-41613813.6 to 469813.6	-73985813.6 to 9277813.6
Day 14	-15831813.6 to 67431813.6	-71631813.6 to 11631813.6
Day 21	-68800566.8 to –816099.8*	-13000566.8 to 54983900.2

Table 7.4: Mean proliferation rates, as calculated from the mean number of cells cultured inside collagen gels in the presence of alpha MEM (control medium), BGJ-b and ECCM.

	α-MEM (Control)	BGJ-b	ECCM
Day 0-1	10500000	24000000	50864750
Day 1-7	-3300667	-5553667	-4635792
Day 7-14	6529143	2846000	6192857
Day 14-21	-1792857	2467857	2585714

Figure 7.6: Proliferation rates of cells cultured inside collagen gels in the presence of alpha MEM (control medium), BGJ-b and ECCM.



7.3.3 Markers of stromal and haemopoietic stem cells and endothelial cells

Markers of endothelial cells (table 7.6), and also stromal and haematopoietic stem cells (table 7.5), followed a similar pattern of labelling. Control and ECCM cultures demonstrated greater staining for these markers at the beginning of the culture period than at the end (table 7.5 and 7.6). In both control medium cultures and also ECCM cultures, colonies of erythrocytes were visible during the first week of culture, however these disappeared by the second week. BGJ-b cultures did not exhibit staining for stem cell markers, and only exhibited staining for endothelial marker, CD105, at day 1. These findings coincide with the results of haemopoietic stem cell markers.

Table 7.5: Staining of 1 to 21 day cell cultures for stromal, STRO-1 (A) and haematopoietic, CD34 (B) stem cell markers grown in collagen gels. Intensity of staining was graded as follows: (-), negative staining; (+), definite staining but of low intensity; (++), moderate staining; (+++), intense staining.

A. STRO-1

	Day 1	Day 7	Day 14	Day 21
α-MEM (Control)	+	+	-	-
BGJ-b Medium	-	-	-	-
ECCM	++	-	-	-

B. CD34

	Day 1	Day 7	Day 14	Day 21
α-MEM (Control)	++	+	-	-
BGJ-b Medium	-	-	-	-
ECCM	++	+	-	-

Table 7.6: Staining of 1 to 21 day collagen gel cell cultures; for endothelial cell markers, CD105 (A) and von willebrand factor (B). Intensity of staining was graded as follows: (-), negative staining; (+), definite staining but of low intensity; (++), moderate staining; (+++), intense staining.

A. CD105

	Day 1	Day 7	Day 14	Day 21
α-MEM (Control)	+	-	-	-
BGJ-b Medium	+	-	-	-
ECCM	++	-	-	-

B. Von Willebrand factor

	Day 1	Day 7	Day 14	Day 21
α-MEM (Control)	++	-	-	-
BGJ-b Medium	-	-	-	-
ECCM	++	+	-	-

7.3.4 Markers of bone formation

Intense staining for bone formation markers was observed in ECCM and BGJ-b culture media, towards the end of the culture period (days 14 to 21) (table 7.7). Collagen gel cultures incubated in BGJ-b medium demonstrated intense staining for osteoblastic markers early on in the culture period, which was accompanied by the formation of mineral deposits throughout the gel (tables 7.1 and 7.7). Cultures grown in ECCM showed similar intensities of staining for bone formation markers, but at a later time point; whilst cultures in control medium produced the markers later still (table 7.7).

Table 7.7: Staining for bone formation markers in 1 to 21 day collagen gel cell cultures grown in α-MEM (A), BGJ-b medium (B) and ECCM (C). Intensity of staining was graded as follows: (-), negative staining; (+), definite staining but of low intensity; (++), moderate staining; (+++), intense staining.

A. α-MEM (control)

	Day 1	Day 7	Day 14	Day 21
Alkaline phosphatase	+	+	+	++
Bone sialoprotein II	-	+	+	++
Decorin	-	++	+	+
Osteonectin	+	+	+	++
Osteopontin	+	+	++	++

B. BGJ-b medium

	Day 1	Day 7	Day 14	Day 21
Alkaline phosphatase	+	+++	+++	+++
Bone sialoprotein II	++	+++	+++	+++
Decorin	+	++	+++	+++
Osteonectin	++	+++	+++	++
Osteopontin	+++	+++	+++	+++

C. ECCM

	Day 1	Day 7	Day 14	Day 21
Alkaline phosphatase	+	++	+++	+++
Bone sialoprotein II	++	++	+++	+++
Decorin	+	++	+++	+++
Osteonectin	-	++	+++	++
Osteopontin	+	+++	+++	++

7.3.5 Results Summary

Alpha MEM cultures:

- Cells remained rounded throughout the culture period
- No mineralization was observed during the time points studied
- Stromal and haemopoietic markers were expressed initially in cultures
- Staining for osteoblastic markers was not as intense as in cultures grown in BGJ-b or ECCM
- Greatest staining for osteoblastic markers was observed at day 21

BGJ-b cultures:

- Cells appeared rounded during the 21 day culture period
- Mineralization was observed at day 14, mineral nodules increasing in size through to day 21
- Staining for osteoblastic markers was intense from day 7 onwards
- Cell numbers within BGJ-b cultures were significantly greater than in control cultures at day 21

ECCM cultures:

- Cells remained primarily rounded throughout the 21 day culture period
- Cell numbers were greatest in 21 day cultures
- Mineralization was evident at day onwards
- Staining for osteoblastic markers was most intense at day 14
- Stromal and haemopoietic stem cell markers, along with endothelial cell markers were expressed up to day 7

7.4 Discussion

In this study, bone-like tissue was produced in a culture of mouse bone marrow cells, using collagen instead of adding dexamethasone. Maniatopoulos *et al.* [213] reported that cultures of bone marrow cells produced bone-like tissue only in the presence of dexamethasone and β-glycerophosphate (β-GP). The effects of collagen gel in this system are considered to be critical and specific, since bone marrow cultured on ordinary plastic (see chapter 5) showed little or no mineralization.

Calcified nodules in this culture system were not simply the products of elevated concentrations of phosphate that might be caused by the addition of β-GP. Rather, mineral deposits in these calcified nodules were regarded as products similar to those found in bone tissue. What is more, in ECCM cultures there was evidence for hydroxyapatite deposits in collagen fibrils, which are well-documented characteristics of the early stage of physiological mineralization in bone.

There have been many reports concerning calcified nodules formed in cultures of osteoblastic cells cultured only in the presence of β-GP, while only a relatively small number of studies reported calcified nodules formed by cells cultured without adding β-GP [214, 215]. Maniatopoulos et al. [213] reported that in the absence of β-GP, nodules of bone matrix will still form but will not mineralise until a source of organic phosphate is provided. In BGJ-b culture medium and ECCM

collagen gel cultures, bone marrow cells produced a calcified bone-like tissue, even in the absence of β-GP. This may be because the matrix produced by the bone marrow cells on collagen gel was more calcifiable than the matrix produced by the cells on the plastic dish (chapter 5). One of the reasons why β-GP induces calcification seems to be that the hydrolysis of organic phosphate concentration enhances the local phosphate concentration that leads to mineralization in the matrix. Thus, the local phosphate concentration might regulate calcified nodule formation. On the other hand, if the calcifiability of the matrix is high enough, such as might be the case on collagen gel, elevation of the phosphate-ion concentration by the addition of β-GP is not essential for calcified nodule formation.

There are several possible mechanisms by which collagen gel facilitates differentiation and forms a calcifiable matrix when bone marrow cells are cultured on collagen gel:

7.4.1 Condensation of cell populations

For cell differentiation and matrix formation that is calcifiable, the cell density must be higher than a certain level [216, 217]. The collagen gel permitted cells to penetrate it, so that large numbers of the cells assembled within the gel, resulting in higher cell density than when cells were cultured on the plastic dishes. It is reasonable to assume that this is how collagen gel helps cells attain a higher density.

7.4.2 Effects of collagen gel on cell activation

Studies on the effect of collagen gel on cell growth and differentiation revealed that the adhesion of cells to the gel results in a sequence of events starting from the collagen receptor interaction. One of the remarkable events is the reorganisation of actin fibers, which may activate cells for differentiation [218] and

consequently mineralisation. These reactions on the gel may fundamentally differ from those in cultures on plastic dishes.

7.4.3 Collagen fibrils as a cofactor for growth factors

Another possibility is that collagen gels help growth factors to bind with receptors on the cell surface. It is known that heparan sulphate proteoglycan helps heparin binding growth factors (HBGF) to bind with the receptor of the target cell [219].

7.4.4 Growth factor accumulation and concentration by the matrix

Additionally, collagen gel may work to accumulate growth factors which are secreted by the cell itself [219]. Growth factors deposited in a matrix result in a high concentration in a local area that reacts with cells with greater efficiency, and the collagen gel is the best candidate for such a matrix. The accumulation and concentrated expression of growth factors by the matrix are now considered to be a general mechanism in growth-factor function for anchorage-dependent cells.

In this study an easily available source of osteoblastic cells was achieved, by using collagen gel and bone marrow cells. Hereafter all kinds of cytokines could be added to this system, either to the medium or the gel, and thereby approach the mechanism in which matrix-bound cytokines react to osteoblastic cells. From a clinical aspect however, the goal was to develop a system to reconstruct *in vitro* bone-like tissue from the patient's bone marrow that will be transplantable back to the donor.

Chapter 8

In vitro Bone Marrow Organ Cultures

8.1 Introduction

The presence of osteogenic precursors in bone marrow has been demonstrated in transplantation experiments. Marrow fragments form bone and cartilage in diffusion chambers *in vivo* [220] and, when grafted under the kidney capsule, develop into a heterotopic bone-marrow organ in which the bone tissue and osteogenic precursor cells were of donor origin [221, 222]. Osteogenic precursors are also present in suspensions of single cells prepared from bone marrow, as seen in chapter 5. When bone marrow cells are implanted in diffusion chambers *in vivo*, bone, cartilage and fibrous tissues are formed [223], and when grafted under the kidney capsule within porous sponges, they are able to form new bone-marrow organ [224]. Fibroblastic cells harvested from *in vitro* cultures of marrow cells also retain their osteogenic potential when transplanted *in vivo* [223, 225].

There have been many attempts to investigate osteogenesis *in vitro* from marrow-derived osteogenic precursors. Localised regions of mineralised collagenous tissue were found in the cell layer formed in monolayer cultures of bone marrow cells (Chapter 5) suggesting that some osteogenic differentiation had occurred [226]. However, the tissue formed was not well organised. Organ cultures *in vitro* of intact marrow fragments were used in this study, in an attempt to form well-organised trabecular tissue that was morphologically similar to bone.

8.1.1 Aims

The aim of this chapter was to:

- Form mineralised tissue from bone marrow cells, that was morphologically similar to bone.

8.2 Methodology

8.2.1 Bone marrow organ cultures

Whole marrow plugs were grown on Millipore filters (pore size 0.22μm, thickness 25μm) plated on individual stainless steel gauze platforms housed in 35mm culture dishes (Nalge NUNC International, Paisley, Scotland) with culture medium. The marrow plug (the whole contents of the femur) was placed on the filter surface, which was in contact with 5% CO_2 in air, the lower surface being in contact with the medium. The bone marrow plugs were grown in either alpha MEM control medium, endothelial cell conditioned medium or BGJ-b medium. The cultures together with the filters were fixed in 10% formal saline at intervals from 7-21 days, embedded in paraffin and sectioned at 5μm. Serial sections were stained with haematoxylin and eosin (H&E), or von Kossa method (which stained calcium phosphate deposits black) counterstained with haematoxylin. Alternatively sections were treated with a range of antibodies, as outlined below.

8.2.2 Immunohistochemistry

Sections were treated with mouse monoclonal antibodies, to either type I collagen (7.4μg/ml) (Southern Biotechnology Associates, Inc. Birmingham, AL, USA), STRO-1 (8.06μg/ml), B4-78 to alkaline phosphatase (8.04μg/ml), WVID1(9C5) to bone sialoprotein (22.45μg/ml), MPIIIB10_1 to osteopontin (16.6μg/ml), DS1 to decorin (35μg/ml), AON-1 to osteonectin (7.75μg/ml), MJ7/18 to CD105 (6.7μg/ml) (all Developmental Studies Hybridoma Bank, University of Iowa, Iowa, USA); rat monoclonal antibody MEC14.7 to CD34 (10.1μg/ml) (Serotec Ltd, Kidlington, Oxford)

or rabbit polyclonal antibody to von Willebrand factor (12.4μg/ml) (AbCam, Cambridge, UK) for 60 minutes at room temperature. The antigen-antibody complex was visualised using R.T.U vectastain kit and peroxidase substrate DAB (both Vector Laboratories, Inc. Burlingham, CA, USA). Nuclei were counterstained with haematoxylin nuclear counterstain (Gill's formula) (Vector Laboratories, Inc. Burlingham, CA, USA). Sections were graded according to the intensity of the staining with peroxidase substrate DAB and average result from 6 samples for each time point recorded.

8.3 Results

8.3.1 Organ culture morphology and mineralization

The size of the bone marrow fragment explanted did not alter the sequence of events in any of the organ cultures investigated. During the first week after cultivation the filters became covered with macrophage-like and fibroblastic cells in the control medium, and primarily fibroblasts in ECCM and BGJ-b medium (figure 8.1). During the second week myeloid cells and a few macrophages were restricted to the surface of the explanted fragments grown in control medium (not shown), and a multicellular layer of fibroblasts that stained intensely for alkaline-phosphatase covered the surface of the filter (figure 8.2). During the third week, the number of myeloid cells decreased and tiny areas staining positive for calcium phosphate (table 8.1) began to develop among the fibroblasts and matrix, which stained intensely for bone formation markers (figure 8.3 and table 8.4).

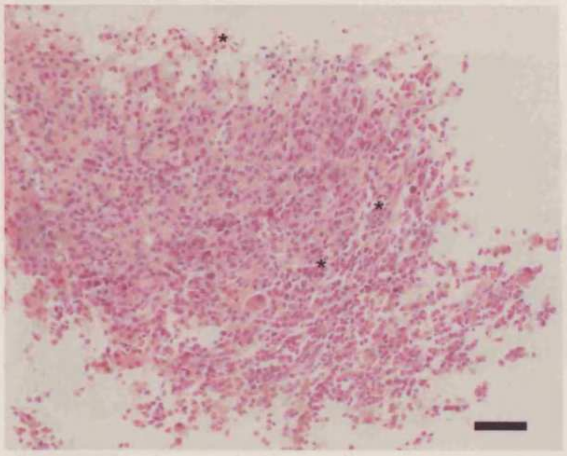
The appearance of cultures grown in BGJ-b and ECCM medium began to differ from control cultures at 14 days, when regions of mineralized matrix began to

appear in the cultures (figure 8.2 and tables 8.2 – 8.3). Mineralization was greater in BGJ-b cultures than ECCM cultures (figure 8.2). By the third week in culture, much of the cellular areas of BGJ-b and ECCM cultures had been replaced by mineral (figure 8.3 and tables 8.2 – 8.3).

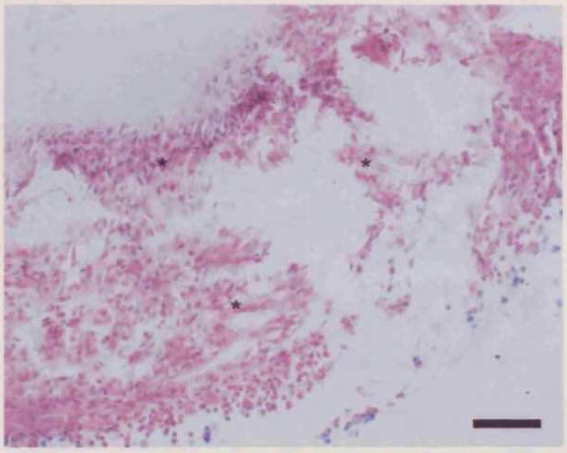
Mineralization initially was greatest on the filter side of the explants in BGJ-b and ECCM cultures. Towards the upper surface of the culture the mineralization was less than lower down in the culture, many fibroblasts and un-mineralized osteoid were also observed in this region.

Figure 8.1: Organ cultures cultivated for 7 days. Cultures were grown in alpha MEM (A), BGJ-b (B) and ECCM (C). Cells presented in all cultures were predominantly fibroblastic (*). Scale bar, 500μm.

A



B



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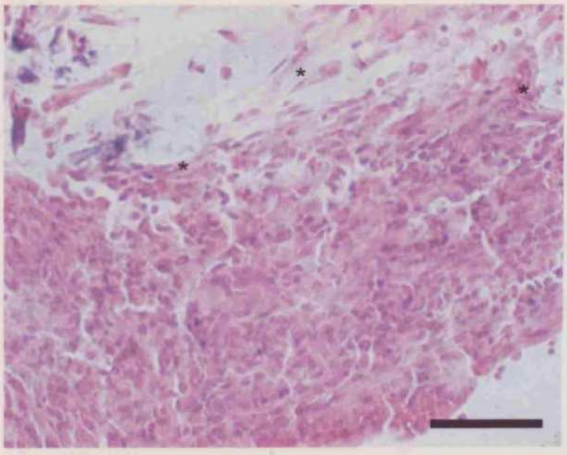
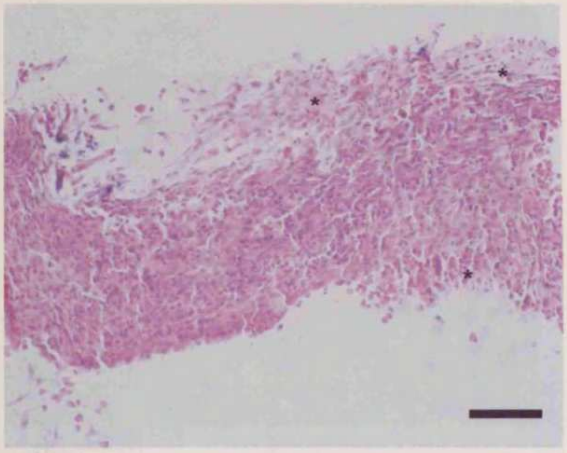


Figure 8.2: Organ cultures cultivated for 14 days. Cells remained predominantly fibroblastic (*) in alpha MEM cultures (A). Small areas of mineral (M) were visible in ECCM cultures (C). Larger areas of mineralization were seen in BGJ-b cultures (B). Scale bar, 500μm.

A



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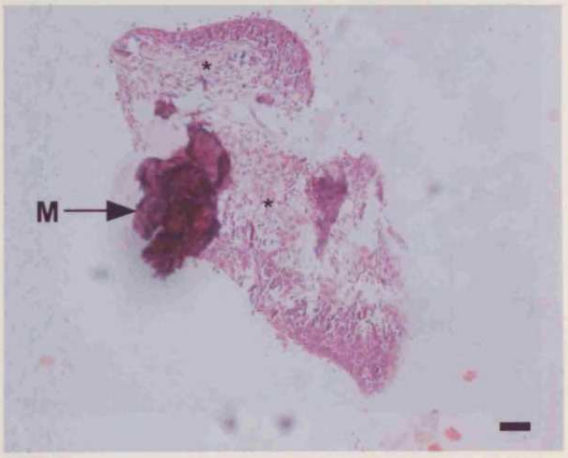


C

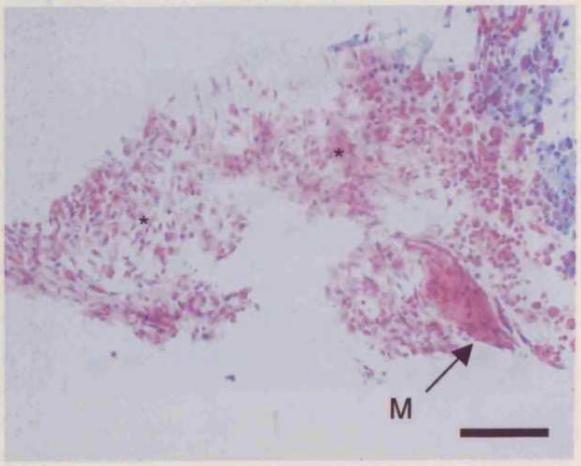
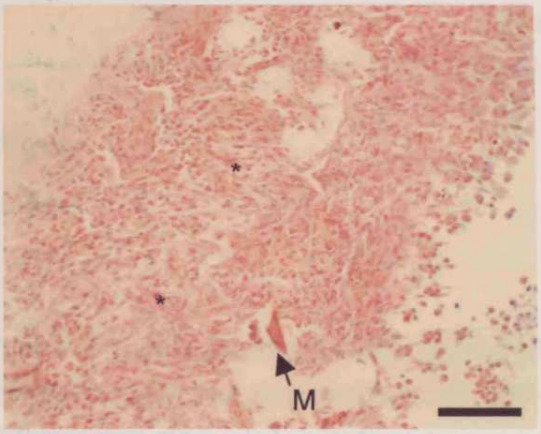
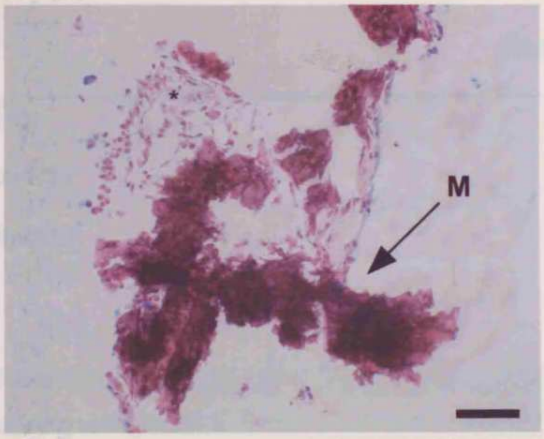


Figure 8.3: Organ cultures cultivated for 21 days. Small areas of mineral (M) formed among fibroblastic cells in alpha MEM cultures (A). BGJ-b cultures (B) were predominantly mineral at day 21. Areas of fibroblastic cells were also largely replaced by bone mineral (M) in ECCM cultures (C). Scale bar, 500μm.

A



B



C

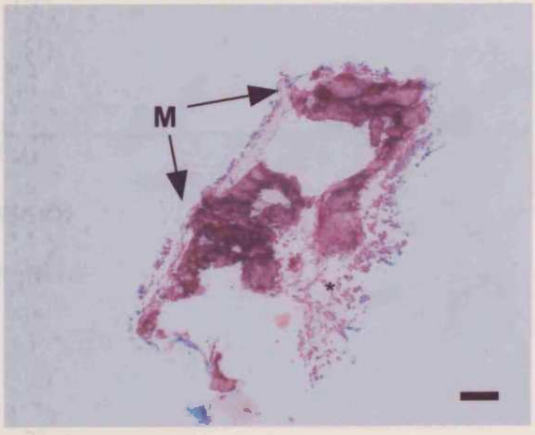


Table 8.1: Staining of sections of organ cultures grown in alpha MEM (control medium), with Von Kossa method. Intensity of staining was graded as follows: (-), negative staining; (+), definite staining but of low intensity; (++), moderate staining; (+++), intense staining.

	Day 7	Day 14	Day 21
Top	-	-	-
Middle	-	-	+
Bottom	-	-	+

Table 8.2: Staining of sections of organ cultures grown in BGJ-b medium, with Von Kossa method. Intensity of staining was graded as follows: (-), negative staining; (+), definite staining but of low intensity; (++), moderate staining; (+++), intense staining.

	Day 7	Day 14	Day 21
Top	-	+	++
Middle	-	+	++
Bottom	+	++	++

Table 8.3: Staining of sections of organ cultures grown in ECCM, with Von Kossa method. Intensity of staining was graded as follows: (-), negative staining; (+), definite staining but of low intensity; (++), moderate staining; (+++), intense staining.

	Day 7	Day 14	Day 21
Top	-	-	++
Middle	-	-	++
Bottom	-	+	++

8.3.2 Markers of stromal and haemopoietic stem cells, and endothelial cells

Staining for stromal stem cell markers was observed to varying degrees in all cultures at day 7 (tables 8.4 - 8.6). Control cultures exhibited low level staining for the markers for the remainder of the culture period (table 8.4), however CD34 staining was not present in either BGJ-b or ECCM cultures by day 14 and STRO-1 staining was observed only at the top of 14 day BGJ-b cultures (table 8.5) and at the bottom of 14 day ECCM cultures (table 8.6).

Endothelial cell markers were also present in low levels in control cultures throughout the culture period (table 8.4). BGJ-b cultures stained positively for CD105 and vWF at day 7, after which the markers were not expressed (table 8.5). CD105 and vWF staining was greatest in 7 day ECCM cultures, however the staining depleted with time and eventually at day 21, no staining was observed (table 8.6). vWF demonstrated a similar expression pattern with time in ECCM cultures, with intensity of staining for the marker diminishing to minimal levels at day 21, rather than disappearing altogether (table 8.6).

8.3.3 Markers of bone formation

Control cultures grown in alpha MEM, expressed only low levels of osteoblastic markers at day 7 (table 8.4), and staining was predominantly at the bottom of the culture. Staining intensity for markers of bone formation increased at the filter surface at days 14 to 21 (table 8.4) before small amounts of mineral were observed at day 21 (table 8.1).

BGJ-b and ECCM followed similar patterns of osteoblastic marker expression. Both cultures exhibited greater staining for alkaline phosphatase, BSP and collagen type I than any other marker at day 7.

At days 14 to 21, coinciding with the emergence of mineral in the cultures (tables 8.2 – 8.3), staining for bone markers increased (tables 8.5-8.6). As observed in control cultures, the greatest expression of osteoblastic markers at day 7 was at the bottom of ECCM and BGJ-b cultures, in the areas later replaced with calcium phosphate.

Table 8.4: Immunohistochemical staining of sections of organ cultures grown in alpha MEM (control medium). Intensity of staining was graded as follows: (-), negative staining; (+), definite staining but of low intensity; (++), moderate staining; (+++), intense staining.

A. 7 day alpha MEM cultures

	STRO-1	CD34	CD105	Von Willebrand factor	Alkaline Phosphatase	Bone sialoprotein	Decorin	Osteonectin	osteopontin	Collagen Type I
Top	++	++	+	+	+	-	-	-	+	+
Middle	++	++	+	+	+	-	-	-	-	+
Bottom	+	+	_	+	+	+	+	-	+	+

B. 14 day alpha MEM cultures

	STRO-1	CD34	CD105	Von Willebrand factor	Alkaline Phosphatase	Bone sialoprotein	Decorin	Osteonectin	osteopontin	Collagen Type I
Top	+	+	-	-	+	+	+	+	+	++
Middle	+	+	+	+	++	++	+	+	+	++
Bottom	+	+	-	-	++	++	+	+	++	++

C. 21 day alpha MEM cultures

	STRO-1	CD34	CD105	Von Willebrand factor	Alkaline Phosphatase	Bone sialoprotein	Decorin	Osteonectin	osteopontin	Collagen Type I
Top	+	+	-	-	+	+	+	+	+	++
Middle	+	+	+	+	++	++	+	+	+	++
Bottom	+	+	-	-	++	++	+	++	++	++

Table 8.5: Immunohistochemical staining of sections of organ cultures grown in BGJ-b medium. Intensity of staining was graded as follows: (-), negative staining; (+), definite staining but of low intensity; (++), moderate staining; (+++), intense staining.

A. 7 day BGJ-b medium cultures

	STRO-1	CD34	CD105	Von Willebrand factor	Alkaline Phosphatase	Bone sialoprotein	Decorin	Osteonectin	osteopontin	Collagen Type I
Top	+	+	+	+	++	+	+	+	+	++
Middle	-	-	+	-	++	+	+	+	+	++
Bottom	+	-	-	+	++	++	+	+	+	++

B. 14 day BGJ-b medium cultures

	STRO-1	CD34	CD105	Von Willebrand factor	Alkaline Phosphatase	Bone sialoprotein	Decorin	Osteonectin	osteopontin	Collagen Type I
Top	+	-	-	-	++	++	++	++	++	++
Middle	-	-	-	-	++	++	++	++	++	++
Bottom	-	-	-	-	++	++	++	++	++	++

C. 21 day BGJ-b medium cultures

	STRO-1	CD34	CD105	Von Willebrand factor	Alkaline Phosphatase	Bone sialoprotein	Decorin	Osteonectin	osteopontin	Collagen Type I
Top	-	-	-	-	++	++	++	++	++	++
Middle	-	-	-	-	++	++	++	++	++	++
Bottom	-	-	-	-	++	++	++	++	++	++

Table 8.6: Immunohistochemical staining of sections of organ cultures grown in endothelial cell conditioned medium. Intensity of staining was graded as follows: (-), negative staining; (+), definite staining but of low intensity; (++), moderate staining; (+++), intense staining.

A. 7 day ECCM medium cultures

	STRO-1	CD34	CD105	Von Willebrand factor	Alkaline Phosphatase	Bone sialoprotein	Decorin	Osteonectin	osteopontin	Collagen Type I
Top	+	+	++	++	+	+	+	+	+	+
Middle	++	+	+	+	++	+	+	+	+	+
Bottom	+	+	+	++	++	++	+	+	++	++

B. 14 day ECCM medium cultures

	STRO-1	CD34	CD105	Von Willebrand factor	Alkaline Phosphatase	Bone sialoprotein	Decorin	Osteonectin	osteopontin	Collagen Type I
Top	-	-	+	+	++	++	++	++	++	++
Middle	+	-	-	+	++	++	+	+	++	++
Bottom	+	-	+	++	++	++	++	++	++	++

C. 21 day ECCM medium cultures

	STRO-1	CD34	CD105	Von Willebrand factor	Alkaline Phosphatase	Bone sialoprotein	Decorin	Osteonectin	osteopontin	Collagen Type I
Top	-	-	-	+	++	++	++	++	++	++
Middle	-	-	-	+	++	++	++	++	++	++
Bottom	-	-	-	-	++	++	++	++	++	++

8.3.4 Results summary

All cultures

- Staining for osteoblastic markers was greater at the bottom of the cultures initially, with staining in the middle and eventually the top layers reaching an equivalent intensity with time
- Mineralization followed the same distribution pattern within the cultures over time, initially forming at the filter side (bottom) of the explant and expanding into the middle and upper layers (ECCM and BGJ-b) of the organ cultures with time

Alpha MEM cultures:

- Demonstrated the lowest levels of staining for osteoblastic markers at all time points
- Produced the least amount of mineral of all the cultures, and exhibited low levels of staining with von Kossa at the final time point
- Haemopoietic and steomal stem cell markers, along with endothelial cell markers were expressed throughout the culture period

BGJ-b cultures:

- Low level staining for haemopoietic and stromal stem cell markers, as well as endothelial cell markers were observed at day 7 and also STRO-1 at day 14
- Staining for all osteoblastic markers became equally distributed and intense, throughout the organ cultures at days 14 and 21
- Low level von Kossa staining was observed at day 7 at the bottom of the culture, throughout the culture at day 14 and more intensely throughout the culture at day 21

ECCM cultures:

- Staining for stromal and haemopoietic stem cell markers was observed at day 7, and also 14 for stromal stem cells
- Staining for endothelial cell marker vWF was observed until day 21, and CD105 until day 14
- Osteoblastic marker expression increased with duration in culture, as did mineralization
- Mineralization was observed at days 14 and 21

8.4 Discussion

In vitro organ cultures of marrow pieces followed a sequence of events and tissue organisation similar to that occurring after transplantation of marrow to an ectopic site *in vivo*; namely disappearance of haematopoietic cells within one to two weeks and proliferation and differentiation of fibroblastic cells to form bone tissue [221].

In this study organ cultures of marrow explants, were also found to be capable *in vitro,* of following a similar differentiation and mineralization pathway to *in vivo* bone formation from marrow [182]. It was observed that cells that stain positively for bone formation markers synthesise extracellular matrix that later mineralizes.

Unlike Maniatopoulos' cultures [213] that required β-glycerophosphate to mineralize; mineralization of the organ cultures did not require the presence of β-glycerophosphate in the medium. The rationale behind addition of an organic phosphate substrate is that it provides, through enzymatic hydrolysis, a source of inorganic phosphate ions at specific localised sites, which may be important to mineralization [226]. Inorganic phosphate ions are already present in BGJ-b medium,

however addition of β-glycerophosphate to ECCM cultures may have resulted in mineralization at an earlier time point.

The quantity of bone mineral produced in ECCM and BGJ-b medium was an impressive feature of the present culture system. This contrasted with previous experiments on osteogenesis in *in vitro* cultures of mouse bone marrow cells (chapters 5 and 7). Differentiation in the osteogenic direction occurred in monolayer cultures, and small amounts of mineralised collagenous tissue was formed in type I collagen gels. However, culture of intact bone marrow pieces may have advantages where formation of bone tissue *in vitro* is the objective. This culture method may also prove to be applicable to the study of human bone formation in both normal and pathological conditions.

Chapter 9

General Discussion

9.1 Summary of Results

Lack of osseointegration has been identified as a major independent risk factor for the failure of bone grafts [88]. It is therefore important to elucidate a method of increasing osseointegration at the bone-graft interface. Although mechanisms vary between species, it is not always possible to gain full knowledge of new bone formation (and hence osseointegration) solely through human studies. Several experimental models have been used to investigate factors involved in the formation of new bone [220-225]. The experiments presented in this thesis have been carried out using mouse bone marrow, extracted the femur. The models used in this thesis were simple and reproducible techniques that were low-cost and produced large numbers of osteogenic cells from a minimal number of starting bone marrow cells.

The first aim of this thesis was to establish the preferential treatment regime for bone allografts, the scaffold upon which new bone is deposited during osseointegration. Host-derived osteogenic precursor cells migrate towards the graft, osteoblastic cells adhere to the graft and osteoid is deposited at the beginning of osseointegration [88]. Fresh frozen bone maintained a greater number of cells in culture than other treatment regimes. Cells also grew in closer proximity to bone treated by this method, as well as producing primarily type I collagen, the main component of osteoid. This technique is ideal for treating bone prior to osseointegration, however with the threat of disease transfer such as HIV [159], pre-soaking bone treated by gamma irradiation or ethylene oxide is an alternative.

Initial characterisation of bone marrow suggested that approximately 20% of the cells in this organ were stromal stem cells. However, this figure varied between animals causing large differences in the final quantities of osteogenic cells produced in culture [177]. However, in all the techniques looked at the proportion of osteogenic cells

produced in ECCM and BGJ-b medium, was always far greater than the initial quantity of stromal stem cells and osteogenic cells in the bone marrow.

The stromal stem cells in the bone marrow are known to differentiate into various tissues (figure 1.3) [22], in this thesis techniques were devised that caused stromal cells to preferentially differentiate into osteoblasts and osteoblast precursors. Two culture media were studied. BGJ-b medium [185] caused mineralization under all culture conditions tested, probably due to its high phosphate content. ECCM also caused mineralization in the three dimensional techniques studied (the collagen gel and *in vitro* cultures) and produced the highest number of osteogenic cells of all the media studied.

Vascularisation appears to be required for endochondral and intramembranous ossification, and it has been found that endothelial cells synthesise potent bone cell active mitogens [190]. Endothelial cells synthesise and secrete an array of regulatory growth factors and cytokines such as fibroblast growth factor (FGF), interleukin-1, interleukin-6, colony stimulating factors, arachadonic acid metabolites like prostacyclin, small peptides like endothelin-1, and gaseous and free radical messangers like nitric oxide and superoxide anions [183, 190]. These molecules have been found to control the recruitment, proliferation, differentiation, function, and/or survival of various cells including bone-forming osteoblasts and bone resorbing osteoclasts [184].

Owing to the close proximity of endothelial cells to developing and maturing osteoblasts and osteoclasts, and their production of such known bone-regulating substances vascular endothelial cells may be a prime source for the local modulation of bone cell development and activity [184]. Endothelial cells implanted in diffusion chambers with fetal calvarial cells greatly enhance their bone-forming-ability [182], and FGFs released by endothelial cells have also been shown to elicit mitogenic responses in calvarial organ cultures and bone nodule formation associated with osteoblast-like cells in bone marrow cultures [183]. In addition another product of endothelial cells,

endothelin-1 has been shown to stimulate cell proliferation in osteoblast-like cells and decrease osteoclast bone resorption through a reduction in osteoclast cellular motility and functions as a catabolic (resorption) and anabolic (collagen synthesis) agent in bone organ cultures [189].

The effects of ECCM on bone marrow cells was further evaluated by gene chip microarray analysis. Results from this study showed that bone marrow cells grown in ECCM express genes that may be considered to be characteristic of the pericyte. Pericytes, like bone marrow stromal cells, have been described as being capable of differentiating into osteoblasts [204]. The pericyte could form an intermediate in the differentiation of bone marrow stromal cells to osteoblasts, when cells are cultured in ECCM. This theory was further supported when bone marrow cells grown in ECCM were found to form cells of a stellate morphology, similar to that seen in pericyte cultures.

In this thesis a range of culture techniques were looked at, which could all be utilised to increase osseointegration *in vivo*. The first technique studied was bone marrow monolayers. Culture medium conditioned by bovine aortic endothelial cells, which are known to synthesise multiple factors *in vitro* [190] that can act on a variety of cells in culture, was added to the monolayer cultures. From the studies in this thesis it was observed that bone marrow cells proliferated initially to create vast number of relatively undifferentiated cells. Following this phase of rapid cell growth, cell numbers decreased as they differentiated, cells of the osteogenic phenotype appearing to remain adhered to the culture dishes (as confirmed by immunological studies) whilst cells differentiating into other phenotypes were released into the medium. In this way, it would seem that ECCM in this culture system selects for cells of the osteogenic phenotype.

By comparison to α-MEM and BGJ-b it appears that, despite the rapid decline in cell numbers as the osteogenic phenotype is expressed, ECCM is capable of producing a greater number of osteogenic cells (although at a later stage in culture) from an

equivalent number of starting cells. It therefore follows that ECCM would be the culture media of choice for creating osteogenic cells from marrow.

However, should a small quantity of bone be required in a shorter period of time than the 21 days required by ECCM to form bone, then BGJ-b could be of use. BGJ-b caused cells to differentiate rather than proliferate (this relationship is well known in osteogenic research and is one of the major problems in obtaining sufficient osteogenic cells with the potential to form bone), producing osteogenic cells that laid down bone matrix (osteoid), from bone marrow in less than 7 days of culture. Cells grown in BGJ-b could therefore be used for the rapid formation of bone to fill small bone defects.

Cultures grown in the control medium (α-MEM) formed cells that expressed osteogenic markers, however these cells were not created as rapidly as those seen in BGJ-b or in the abundance seen in ECCM cultures, and cells cultured in this media as an organ culture system were unable to lay down osteoid. Consequently α-MEM is not recommended for the culturing of bone from bone marrow.

Further evidence for the selectivity of ECCM for osteogenic cells within bone marrow was seen in the bone marrow organ cultures. Under these conditions osteoid was deposited within the first week of culture and bone was formed by the third week. In this instance BGJ-b produced mineralised tissue within two weeks of culture, with almost the entire bone marrow plug converted to bone by the third week. Should a small bone plug be required quickly BGJ-b medium would be should be used. With bone marrow being such a malleable medium to work with, this culture system also provides the potential to grow bone into a specific shape with the use of moulds.

Cell growth and differentiation are known to depend in part on initial cell attachment [123]. Morphological changes occurring during the attachment and spreading of the cells correspond to the reorganisation of the cytoskeleton, the structure that plays a role in cell shape and behaviour [177]. During the 21 day culture period, bone marrow

cells differentiated to different extents depending on the culture media. Of the three culture media used in this study, the single cell suspensions inside gels exhibited mineralisation with both ECCM and BGJ-b medium. However,cells grown in control medium, as with the other culture set ups, did not mineralise even though new osteoid was laid down. The collagen gels contain both osteogenic cells and areas of mineralization could be used to fill small defects, or could be easily used to coat bone graft to increase osseointegration.

What we may conclude from this thesis is that ECCM was overall found to produce a greater number of cells at the end of the incubation periods studied than BGJ-b medium. BGJ-b medium preferentially selected mineralization over cell proliferation under all of the culture conditions studied (monolayers, collagen gels and organ cultures). This medium would be best suited to forming small pieces of bone rapidly from bone marrow, to fill small bone defects such as those seen in the dental field e.g. alveolar augmentation after periodontal disease and mandibular augmentation after tooth loss [151-153]. ECCM produced large numbers of osteogenic cells, which could potentially be used to coat large bone grafts. The small quantities of mineralized matrix observed in these cultures could also be used to fill minor bone defects.

9.2 Further Studies

Considering the results of the cultures studied, it may be possible to grow a larger quantity of bone or a greater number of osteogenic cells in a shorter duration than those seen in this thesis, by adding beta-glycerophosphate, dexamethasone and ascorbic acid [213] to the culture systems. Beta-glycerophosphate would provide additional phosphate for mineralization, ascorbic acid (which functions as a co-factor in the hydroxylation of lysine and proline residues in collagen and is essential for its normal synthesis and secretion) would increase the secretion of type I collagen and dexamethasone has been found to increase osteogenic differentiation in many culture systems [213].

ECCM cultures did not express mineralization to the same extent as cells grown in BGJ-b medium, and alpha MEM caused no mineralization. This may have been a result of the incubation period being studied; therefore longer incubation studies should be carried out to evaluate what occurs in the cultures at time points greater than 21 days. ECCM cultures were only partially mineralised at 21 days, it would be interesting to see how long it would take for the cultures to become completely mineralised and whether the quantity of mineralised tissue produced is equal or greater in size than those produced in BGJ-b medium.

A greater range of time points should also be extended to the microarray data, to fully establish the effects ECCM had on bone marrow cells. Cells cultured in alpha-MEM should also be studied as a control.

The next stage of this work would be to look at the success of new bone formation when cells cultured using the techniques described in this thesis were added to treated bone. Finally the cultured cells alone and in combination with treated bone should be investigated *in vivo* to see whether they elicit an angiogenic response when

grafted onto the chorioallantoic membrane of chick embryo. The degree of osteogenic cell attachment, differentiation and osteoid/bone deposition could also be evaluated when cultured bone marrow cells are inoculated onto bone slices and transplanted, within a diffusion chamber [223], intraperitoneally into the host.

This thesis has focused on the treatment of bone and bone marrow for grafting *in vitro*. Studies into the role of bone remodelling cells in osseointegration could help in the understanding of this process. Furthermore, exploration into the role of osteoblastic cells and bone formation *in vivo* may prove valuable.

Components	Molarity (mM)	
Vitamins	**MEM**	**BGJ-b**
Ascorbic Acid	0.284	0.284
Biotin	0.000410	0.000820
Choline chloride	0.00714	0.357
D-Calcium pantothenate	0.00210	0.000419
DL-alpha Tocopherol phosphate	-	0.00142
Folic Acid	0.00227	0.000454
i-Inositol	0.0111	0.00111
Niacinamide	0.00820	0.164
Para-Aminobenzoic Acid	-	0.0146
Pyridoxal phosphate	0.00490	0.000810
Riboflavin	0.000266	0.000352
Thiamine hydrochloride	0.00297	0.0119
Vitamin B12	0.00100	0.0000295

Components	Molarity (mM)	
Inorganic Salts	**MEM**	**BGJ-b**
Calcium Chloride ($CaCl_2$) (anhyd.)	1.8	-
Magnesium Sulphate ($MgSO_4$) (anhyd.)	0.814	0.817
Potassium Chloride (KCl)	5.33	5.33
Potassium Phosphate monobasic (KH_2PO_4)	-	1.18
Sodium Bicarbonate ($NaHCO_3$)	26.19	41.67
Sodium Chloride (NaCl)	117.24	91.38
Sodium Phosphate monobasic (NaH_2PO_4-H_2O)	1.01	0.652

Components	Molarity (mM)	
Other Components	**MEM**	**BGJ-b**
Calcium Lactate	-	2.55
D-Glucose (Dextrose)	5.56	55.56
Lipoic Acid	0.000971	-
Phenol Red	0.0266	0.531
Sodium Pyruvate	1.000	-
Sodium Acetate	-	0.610

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Publications Resulting from Thesis

- *Endothelial Cell Products Inhibit Cartilage Differentiation.* Rees AA, Hayes F & Rooney P. Int. J. Exp. Med. 81(5) 2000, p32.

- *Bone Grafting are all Storage Regimes the Same?* Rooney P & Rees AA. Journal of Dental Research 79(5) 2000, p1181.

alternative, freeze dried mineralized allogeneic grafts, provide some mechanical support (mostly resistance to compression). However, they are osteoconductive only [93].

Mineralized bone grafts may be derived from either cancellous or cortical bone. Mineralized cancellous or cortical bone may be processed to yield chips ranging from 0.5 to 3 mm in diameter or shapes up to 1 cm in diameter. Morcellized and cancellous mineralized bone allografts are characterized by an open, porous, almost lattice-like physical structure so there is no physical impairment to the ingrowth of vessels. The same stages of incorporation occur in morcellized/cancellous allografts as in autografts, but the allografts are osteoconductive only, as they have no living cells. They are not osteoinductive because the matrix is mineralized [92].

The mineralized matrix gives these allografts some inherent mechanical strength. Thus, mineralized bone chips may serve as weight bearing structures during the process of graft incorporation. Because resorption is not necessary in order that they be revascularized, these allografts do not suffer the transient loss in mechanical strength that is generally seen during the incorporation of large mineralized cortical bone grafts. Corticocancellous and cortical bone chip allografts provide structural support and are osteoconductive to a limited degree. Corticocancellous grafts may be prepared from the ilium, distal femur, and proximal tibia [92].

The early phase of inflammation following implantation of a mineralized allograft is similar to that following the implantation of all grafts. Vascular invasion begins, and inflammatory cells migrate into the area. It is during this time that the host encounters graft-derived cellular antigens, if present, and becomes sensitized to them. Massive cortical allografts are penetrated by vessels and are substituted with host bone very slowly, superficially, and to a limited degree. This may account for the

incidence of fracture in these grafts, which is reported to range from 16% to 50%. When biopsies of the fracture site were obtained at the time of surgical treatment of the fracture, a lack of revascularization and soft-tissue attachments was noted [94].

Cortical allografts remain significantly weaker for a considerable time after surgery than cortical autografts. Given enough time and a weight bearing, stable construct, most segmental cortical allografts will eventually resemble autografts biomechanically and structurally, although significantly more unremodeled necrotic bone will be present in allografts [94].

1.3 Summary

The technique of bone grafting is regularly used in orthopedic and craniofacial procedures to fill in defects of bone occurring after removal of a tumor, defects caused by trauma and in revision surgery. The aim of bone grafting is to induce new osteogenesis at the graft site followed by integration of the donor bone into the host (osseointegration). Osseointegration relies on osteogenic precursor cells migrating towards the graft, osteoblastic cells adhering to the graft, osteoid being deposited, and eventually mineralization of the osteoid. The new bone, as well as the graft tissue, is then resorbed by osteoclastic cells such that the graft is eventually replaced by host bone. Osseointegration could be enhanced if graft tissue was pre-treated with osteoprogenitor cells.

At present, depending on the bone banks from which the tissue is obtained, allografts of human bone are stored and/or treated in different ways. Each of the treatment regimes appear to work *in situ* with similar success and integration rates, however, little evidence is available in the literature on the reaction or the behavior of osteoblastic cells when they initially come into contact with the grafted bone.

cultures with defined and reproducible osteoblastic behaviour. To this end, two culture media were studied BGJ-b medium (originally formulated for the growth of bones and bone cells [185]) was used and endothelial cell conditioned medium (ECCM), since data obtained from this laboratory indicated that conditioned medium (α-MEM) removed from endothelial cells was capable of inducing bone formation.

5.1.1 Aims

The aim of this chapter was to:

- Evaluate the response of BMSC monolayer cultures to two culture media BGJ-b and ECCM.

5.2 Methodology

5.2.1 Monolayers

Adult male MF1 mice were killed by cervical dislocation and the marrow washed out from the mid-shafts of both femurs with a syringe containing culture medium. The culture media used was α-modification minimal essential medium eagle (α-MEM), BGJ-b (both Gibco BRL Ltd., Paisley, Scotland), or endothelial cell conditioned medium (ECCM) (see general methodology). Media were supplemented with 10% foetal calf serum, 2mM L-glutamine, penicillin (10,000U/100ml), streptomycin (10mg/ml) and amphoterycin (25μg/ml).

Culture media for all marrow cultures was changed 2-3 times weekly and the cultures were incubated in a humidified atmosphere of 95% air and 5% CO_2. Experiments were performed using fresh bone marrow cells as previous studies showed that serial passage of bone marrow cells results in a progressive loss of the osteogenic phenotype [178,186].

A single cell suspension (SCS) was prepared as described by Maniatopoulos *et al.* [26] and $9.6x10^4$ total marrow cells inoculated per well, of a 24 well tissue culture plate (Nalge NUNC International, Paisley, Scotland) each containing 2ml medium. Cultures were characterised at days 7, 10, 14 and 21 for cell proliferation and ability to produce markers of bone formation, haemopoietic and stromal stem cells, endothelial cells (immunocytochemistry), and adipocytes (oil red o staining). Mineralisation was also evaluated by von Kossa staining, and general cell morphology studied.

5.2.2 Cell Counts/Proliferation rates.

Cell numbers were determined by counting the number of adherent cells, using a haemocytometer, enzymatically released by trypsin (0.05%) - EDTA (0.02%). The proliferation rates (change in cell number/24hours) were calculated using the formula:

$$\textit{Proliferation (change in cell number/24hours)} = \frac{\textit{Number of cells}}{\textit{Number of days in culture.}}$$

5.2.3 Colony Counts

Cultures were washed in PBS and fixed in 10% formal-saline. Cultures were washed again in PBS after 24 hours and stained with 10% alcian blue, to make the colonies more visible, and the number of colonies per well counted under a light microscope.

beta) superfamily of secreted proteins. Although discovered because it stimulates bone formation in adult mammals, BMP-4 has important roles as a signalling molecule in embryonic tissues, including the developing central and peripheral nervous system, musculature and skeleton [196].

Table 6.1: Comparisson of highly expressed genes identified in this study (mouse bone marrow cells) with two previous reports (by Tremain et al. and Jia et al.) looking at human bone marrow stromal cells.

Tremain *et al.*	**Jia *et al.***	**Microarray analysis**		**Cluster Inclusion**
		Control Medium	**ECCM**	
Fibronectin 1	Fibronectin 1	-	Fibronectin (FN) mRNA	M18194
Collagen, type I, alpha 2	Collagen, type I, alpha 2	-	-	-
Collagen, type I, alpha 1	Collagen, type I, alpha 1	-	-	-
-	Secreted protein, acidic, cysteine-rich	-	-	-
Eef1a1	Eukaryotic translation elongation factor 1 alpha 1	-	-	-
Actin, gamma	Actin, gamma 1	-	Cytoskeletal gamma-actin mRNA	M21495
-	Actin, beta	Actin, beta, cytoplasmic	Actin, beta, cytoplasmic	M12481
-	Transgelin	-	-	-
Ferritin, heavy polypeptide	Ferritin, heavy polypeptide 1	-	gene for ferritin H subunit	X52561
-	Annexin II	-	-	-
-	Connective tissue growth factor	-	-	-
-	Transforming growth factor beta-induced	-	Transforming growth factor beta-induced	L19932

Appendix

Media Formulation of Minimum Essential Medium (MEM) and BGJ-b (Fitton-Jackson Modification)

Components	Molarity (mM)	
Amino Acids	**MEM**	**BGJ-b**
Glycine	0.667	10.67
L-Alanine	0.281	2.81
L-Arginine	0.498	1.01
L-Asparagine-H_2O	0.379	-
L-Aspartic Acid	0.226	1.13
L-Cysteine Hydrochloride-H_2O	0.568	0.574
L-Cysteine 2HCL	0.1000	-
L-Glutamic Acid	0.510	-
L-Glutamine	2.00	1.37
L-Histidine	0.200	0.968
L-Isoleucine	0.400	0.229
L-Leucine	0.397	0.382
L-Lysine	0.397	1.64
L-Methionine	0.101	0.336
L-Phenylalanine	0.194	0.303
L-Proline	0.348	3.48
L-Serine	0.238	1.90
L-Threonine	0.403	0.630
L-Tryptophan	0.0490	0.196
L-Tyrosine disodium salt	0.231	0.258
L-Valine	0.393	0.556