Integrin expression and function in osteoblasts

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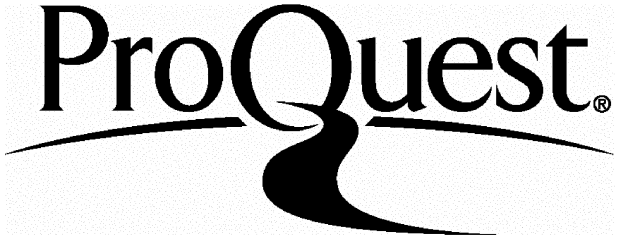

Abstract

Communication between osteoblasts and the surrounding *milleu* is important in the regulation of osteoblast behaviour and function. Evidence suggests that the β1 family of integrins is important in the regulation of this relationship. Although trends in integrin expression and function are beginning to emerge, there is little functional evidence using human osteoblasts. In this thesis I have shown, using FACS analysis, that both MG63 cells and mandibular primary human osteoblasts (HOBs) express α2, α3, α4, α5, α6 and β1 integrins. HOBs were also shown to express αv integrin but had low or negative expression of αvβ3. Integrin expression was confirmed by immunocytochemistry. Adhesion assays showed that MG63s and HOBs were adherent to ECM substrates, including plasma fibronectin, type I collagen and the 120kDa plasma fibronectin fragment. Adhesion to plasma fibronectin and type I collagen was reduced by the β1 integrin blocking antibody. RGD blocking peptide reduced adhesion to plasma fibronectin. Boyden chamber migration assays showed that MG63s were highly migratory after 2 hours on plasma fibronectin, type I collagen and the 120kDa plasma fibronectin fragment. HOBs were also migratory but with lower cell numbers migrating. Addition of the α2β1 and α5β1 blocking antibodies resulted in the partial inhibition of MG63 cell and HOB migration on type I collagen and plasma fibronectin respectively. Incubation of cells with the MAPKK inhibitor, U0126, also reduced migration of MG63 cells on plasma fibronectin. Preliminary studies into the function of integrins in the differentiation of the C2C12 stromal cell-line was also carried out. This study provides the first evidence for the involvement of integrins and ECM interaction in osteoblast migration. In addition further evidence for the role of integrins in osteoblast behaviour is reported.

List of abbreviations used

Abbreviation	Definition
ANOVA	Analysis of variance
AP	Alkaline phosphatase
α-mem	α-minimum essential medium
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
BSP	Bone sialoprotein
CAM	Cell adhesion molecule
cAMP	Cyclic adenosine monophosphate
Cbfa	Core binding factor-α
CCD	Cleidocranial Dysplasia
CFU-F	Colony forming unit fibroblastic
CFU-S	Colony forming unit-spleen
CNS	Central nervous system
Col I/Col	Type I collagen
Col II	Type II collagen
DGEA	Asp-Gly-Glu-Ala
DMEM	Dulbecco's modified Eagle's medium
Dmp-1	Dentin matrix protein-1
DMSO	Dimethylsulphoxide
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscopy
ERK	Extracellular regulated kinase
FACS	Fluorescence activated cell sorting
FAK	Focal adhesion kinase
FCFC	Fibroblast colony forming cell
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FITC	Anti-Fluorescein isothiocyanate
GER	Gly-Glu-Arg
GTP	Guanosine 5' triphosphate
HBDCs	Human bone derived cells
HB-GAM	Heparin-binding growth associated molecule
HOBs	Primary human osteoblasts
ICAM	Intracellular adhesion molecule

Abbreviation	Definition
IGF	Insulin-like growth factor
ihh	Indian hedgehog
ILK	Integrin linked kinase
JNK	Janus kinase
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MEK	MAPK/ extracellular kinase 1
MEM	Minimum essential medium
MG63s	MG63 cells
mRNA	Messenger ribonucleic acid
MTS	[3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NaOH	Sodium hydroxide
NCAM	Neural cell adhesion molecule
OPG	Osteoprotegrin
OPGL	Osteoprotegrin ligand
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PI3K	Phosphatidylinositol 3 kinase
pfn	Plasma fibronectin
PTE	Parathyroid extract
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone related protein
RGD	Arg-Gly-Asp
RT-PCR	Reverse transcription ploymerase chain reaction
SD	Standard deviation
SEM	Scanning electron microscopy
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tumour necrosis factor
TRANCE	TNF-related activation induced cytokine
unc	Uncoated
120kDa	120kDa plasma fibronectin proteolytic fragment
30kDa	30kDa plasma fibronectin proteolytic fragment
45kDa	45kDa plasma fibronectin proteolytic fragment

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Thesis overview

Chapter 1 - Introduction

The information available to date on osteoblast biology, integrins and integrin expression and function to date is explored in this introductory chapter.

Chapter 2 - Materials and methods

The materials and methods used in this thesis are detailed.

Chapter 3 - Results of characterisation studies

The results of studies into the integrin profile of primary human osteoblasts and MG63s are described in chapter 3. In addition, this chapter details the results of studies into the osteoblastic nature of primary human osteoblasts and MG63 cells.

Chapter 4 – Integrins and ECM interaction - an important relationship in osteoblast behaviour

This chapter contains the results of further studies into the role of specific integrins in osteoblast and ECM interaction.

Chapter 5 – Integrins and ECM regulate osteoblast migration

This chapter contains the results of studies into the role of specific integrins in osteoblast migration in response to ECM.

Chapter 6 - A link between integrins and the Cbfa1 transcription factor

This chapter contains preliminary results of studies into the potential link between integrins, the Cbfa1 transcription factor and osteoblast differentiation.

Chapter 7 - Discussion and future work

In this chapter the data from this thesis and previous studies is drawn together to provide an overall picture of cell adhesion molecules, in particular integrin function in osteoblasts. In addition, ideas for further studies are explored.

1 Chapter 1 – Introduction

The aims of this thesis were to:

- Ascertain the integrin profile of primary human osteoblasts and MG63 cells expressed *in vitro*; and
- Determine the role of specific integrins, in osteoblast behaviour *in vitro*.

1.1 Introduction

Bone is a dynamic tissue, constantly being broken down and replaced in the adult during the process of remodelling. This cycle of bone turnover occurs in response to stimuli from both within the bone and from external triggers. The two main cell types involved are the osteoblast, the principal bone producing cell, and the osteoclast, which is associated with osteolysis. The continual breakdown and renewal of bone is dependent upon a level of communication between cells themselves and between cells and the surrounding extracellular matrix (ECM). Bone is an outstanding system in which to study these interactions as it has an extensive matrix secreted and mineralised by the osteoblast. As is the case with other cells types, cell adhesion molecules form a bridge between bone cells and their environment, playing an important role in the communication that occurs between the cells and their surroundings.

Integrins are a family of adhesion molecules that are of particular importance in bone cell behaviour. Recent evidence suggests that interactions between the ECM and osteoblasts, mediated by integrins, are involved in osteoblast differentiation, survival and matrix synthesis (Bennett *et al.*, 2001b). It is well established that integrins expressed by osteoclasts are vital for bone resorption (Horton, 1995). Integrins also appear to be involved in the mechanisms by which bone cells sense mechanical stimuli and hormones. In mature bone, osteoblasts form an epithelial-like sheet on the bone surface and adhesion molecules form a link between these cells and with other surrounding cell types, such as osteocytes.

Integrins are known to act as members of cell signalling pathways, conveying information from the outside of the cell inwards. These cell adhesion molecules also have the capacity to transmit signals from inside the cell outwards. In addition, the presence of other cell adhesion molecules and cooperation between different receptors, growth factors and activation of multiple signalling pathways leads to a vast array of biological responses.

In this introduction, the current evidence regarding cell adhesion molecule expression in bone cells will be reviewed. Emphasis will be placed on the expression and function of integrins in osteoblasts. The areas of bone cell biology and cell adhesion molecules will also be reviewed.

1.2 Bone cells

There are three principal cell types present in bone: the osteoblast, the osteoclast and the osteocyte. In the following sections the characteristics of osteoblasts *in vitro* will be discussed in detail. Osteoblasts can be characterised ultra-structurally, biochemically and by molecular analysis and these will be discussed in the following sections. The key features of osteoclast biology will also be introduced.

1.2.1 Osteoblast ultra structure

Cells of the osteoblast lineage are responsible for the formation of bone, both during embryogenesis and throughout adult life. Located directly on the endosteal and periosteal surfaces of bone, these cells actively secrete a collagen rich matrix, osteoid, that is subsequently mineralised. Histologically, osteoblasts have a round nucleus at the base of the cell with well developed Golgi apparatus and endoplasmic reticulum. They can be observed as a layer of cuboidal cells on the bone matrix. Behind the layer of mature osteoblasts there also tends to be a layer of mesenchymal precursor cells and pre-osteoblasts (Puzas, 1996).

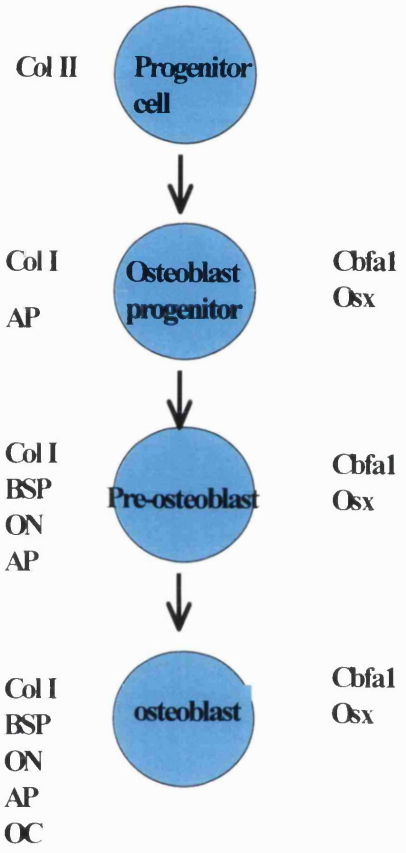
Osteoblasts are known to follow a pathway of differentiation *in vitro* and this will be discussed in the following section.

1.2.2 Osteoblast differentiation and biochemical analysis

In order to form a mature matrix-secreting cell, the osteoblast follows a pathway of differentiation that can be divided into several steps, based on both phenotypic observations and by analysis of gene expression *in vitro* (summarised in figure 1(a)). In the pre-osteoblast, genes universally associated with proliferation are expressed, for example *c-myc* and *c-fos* (Lian and Stein, 1993). In addition, genes associated with an osteoblast phenotype, matrix mineralisation and production are expressed, such as those encoding alkaline phosphatase, type I collagen (col I), bone sialoprotein (BSP) and osteonectin. As the cell begins to mature, osteocalcin is also detected (Aubin *et al.,* 1995). Recent studies have elucidated further factors associated with osteoblast differentiation such as Cbfa1 (Ducy, 2000) and Osx (Osterix) (Nakashima *et al.,* 2002) and these will be discussed in 1.2.4. *In vitro*, osteoblast cultures may exhibit formation of nodules that resemble a mineralised matrix. This has been observed in rat calvarial cultures (reviewed in Aubin *et al.,* 1993), adult rat stromal cell cultures (Malaval *et al.,* 1994) and human trabecular explant cultures (Beresford *et al.,* 1983). *In vitro*, alkaline phosphatase can be detected histochemically and by biochemical analysis of enzyme activity. Osteocalcin levels can be determined by the use of ELISAs. In addition, monoclonal antibodies are available for the detection of osteoblast markers such as type I collagen, alkaline phosphatase and osteocalcin (Aubin and Turksen, 1996). For many years, detection of osteoblast progenitors *in vitro* was a problem as experimental tools were only available for the detection of more differentiated osteoblasts. Recently, antibodies have been identified which may have a role in the characterisation of osteoblast precursors, for example STRO-1 (Simmons and Torok Storb, 1991). The monoclonal antibody STRO-1 was shown to bind to a sub-set of human bone marrow cells and has been used as a marker of pre-osteoblast cells (Simmons and Torok Storb, 1991; Gronthos *et al.,* 1994). It is controversial as to whether this monoclonal antibody actually provides an accurate tool for identifying populations of osteoblast precursors. In a study using cells derived from explants of human trabecular bone Oyajobi *et al.* (1999) reported that STRO-1 positive, alkaline phosphatase negative cells showed delayed mineralisation and lacked expression of markers characteristic of more mature bone cells, such as

bone sialoprotein and osteopontin (OP). The authors stated that these cells could be representative of osteoblast precursors. These cells may indeed be representative of osteoblast precursors but may also represent other cell types such as fibroblasts. To add to the controversy, it was observed that all STRO-1 positive, alkaline phosphatase negative cells also express osteocalcin. The authors argue that osteocalcin may not be a reliable marker of the mature osteoblast phenotype. As mentioned previously, key studies to date have shown osteocalcin to be a marker of mature osteoblasts and more evidence would be required to prove otherwise. In addition, STRO-1 expression seems to be highly variable between studies (Simmons and Torok Storb, 1991; Stewart *et al.,* 1999). More conclusive results are required to prove the hypothesis that STRO-1 provides a marker of pre-osteoblasts.

Figure 1(a) – The pattern of gene and protein expression during the process of osteoblast differentiation (adapted from Ducy, 2002)



Osteoblasts undergo a period of differentiation from a progenitor cell to a mature osteoblast. This process can be divided into a series of stages based on gene and protein expression. Progenitor cells are identified by the presence of type II collagen (col II), the cell then differentiates into an osteoblast progenitor, signified by type I collagen (col I), alkaline phosphatase (AP), Cbfa1 and Osx. These genes and proteins are also indicative of a pre-osteoblast and mature osteoblast, with the added presence of bone sialoprotein (BSP) and osteonectin (ON) in the former and also osteocalcin (OC) in the latter.

1.2.3 Endocrine, autocrine and paracrine regulation of osteoblast differentiation and function

There are many factors known to regulate osteoblast differentiation and function, a number of which will be discussed here. Several endocrine factors exert effects on osteoblasts, such as Parathyroid hormone (PTH), growth hormone, glucocorticoid hormones, oestrogen and progesterone. The biologically active form of vitamin D, 1,25-dihydroxyvitamin D_3, also exerts endocrine effects on osteoblasts and bone. *In vitro*, the response of cells to PTH and 1,25-dihydroxyvitamin D_3 have been used as markers of osteoblast differentiation. PTH is a polypeptide hormone involved in the maintenance of calcium homeostasis (Strewler *et al.,* 1987). PTH stimulates cell signalling cascades that involve an increase cyclic adenosine monophosphate (cAMP) and results in an increase in Ca^{2+} levels in the osteoblast (reviewed in Swarthout *et al.,* 2002). Overall, parathyroid hormone regulates many genes and proteins associated with osteoblast differentiation and function and also bone resorption. Prolonged parathyroid hormone treatment decreases collagen synthesis (Kream *et al.,* 1986), osteocalcin levels (Beresford *et al.,* 1983; Lajeunesse *et al.,* 1991), alkaline phosphatase activity (Luben *et al.,* 1976), osteonectin (Termine *et al.,* 1981), osteopontin (Noda and Rodan, 1989) and genes associated with DNA synthesis (Reid *et al.,* 1988). Evidence also suggests that PTH treatment could lead to activation of Cbfa-1 (core-binding factor-α) (Selvamuragan *et al.,* 2000; reviewed in Swarthout *et al.* 2002) and an increase in several further factors including, collagenase-3 (Meikle *et al.,* 1992; Partridge *et al.,* 1987), tissue inhibitors of metalloproteinases (TIMPS) (Partridge *et al.,* 1987; Cook *et al.,* 1994) and insulin-like growth factor (IGF-1) (McCarthy *et al.,* 1989). PTH can also lead to the indirect activation of osteoclasts and factors associated with bone resorption (Teitelbaum, 2000). For further details see section 1.2.8. 1,25-dihydroxyvitamin D_3 stimulates alkaline phosphatase (Fritsch *et al.,* 1985), osteocalcin (Beresford *et al.,* 1986) and type I collagen synthesis (Harrison and Clark, 1986). In addition, several paracrine factors produced by surrounding cells act to control osteoblast differentiation and function. Bone morphogentic proteins (BMPs) are known to be involved in the regulation of osteoblast differentiation (Urist *et al.,* 1977). BMPs belong to the transforming growth factor-β (TGF-β) superfamily (Wozney and Rosen, 1998). BMPs-2 and -7

will promote the differentiation of pluripotent mesenchymal precursors into osteoblasts (Wang *et al.,* 1993). BMP-2 treatment blocks myogenic differentiation and induces osteoblastic differentiation in myogenic cells (Katagiri *et al.,* 1994). TGF-β treatment appears to inhibit osteoblastic differentiation (Spinella-Jaegle *et al.,* 2001). Both TGF-β and BMPs are also secreted by osteoblasts themselves and can act in an autocrine manner.

Indian hedgehog (ihh) is a growth factor known to be important for osteoblast differentiation *in vivo*. The *ihh* gene is expressed in chondrocytes during development and controls the expression of parathyroid related peptide (PTHrP) (Vortkamp, 1996). *Ihh* knockout mice also show failure of osteoblast development in endochondral bones (St-Jacques *et al.,* 1999).

1.2.4 Molecular control of osteoblast differentiation

Over recent years, advances in molecular biology have led to the characterisation of osteoblast differentiation at the molecular level, with increased knowledge on well-established markers such as alkaline phosphatase and osteocalcin and also the elucidation of several new factors associated with osteoblast differentiation and bone formation. In the following section molecular control of osteoblast differentiation and bone formation will be discussed.

1.2.4.1 The involvement of Cbfa1 in osteoblast differentiation

Cbfa1 is a transcription factor involved in the control of osteoblast differentiation. The discovery of Cbfa1 was a significant milestone in bone biology. There had been several years of speculation about the existence of an osteoblast specific transcription factor, a possible 'master regulator' of the differentiation pathway. This is analogous with muscle differentiation and the myogenic basic helix-loop-helix proteins, such as MyoD (Puri and Sartorelli, 2000). At first, the Cbfa1 transcription factor appeared to be just that. Recent evidence suggests that Cbfa1 is indeed key to the control of osteoblast differentiation but it seems likely other factors are also involved, for example, the transcription factor Osx (Osterix) (Nakashima *et al.,* 2002), for further details see section 1.2.5. Cbfa1 was discovered simultaneously by several groups

working in very different areas (Otto *et al.,* 1997, Mundlos *et al.,* 1997; Ducy *et al.,* 1997). The *Cbfa1* gene belongs to the *runt* family of genes and their protein products (Westendorf and Hiebert, 1999). For a review of Cbfa1 see Ducy, (2000).

1.2.4.2 Expression of Cbfa1 in bone cells

The expression profile of Cbfa1 is by no means straightforward, perhaps due to the plasticity of cells of stromal origin. During embryogenesis, skeletal development begins to take place with the condensation of undifferentiated cells (Hall and Miyake, 1992). Cbfa1 expression can be detected as early as 10.5 dpc in mice. Expression is seen in anlage destined to become either osteoblasts or chondrocytes (Ducy *et al.,* 1997). *Cbfa1* deficient mice have a complete loss of osteoblast differentiation and as a consequence the skeleton is composed of cartilage. Osteoclast formation also fails to occur (Ducy *et al.,* 1997). In addition, Ducy *et al.,* (1999) carried out further investigations using to determine the expression and role of Cbfa1 in postnatal mice. Transgenic mice expressing a truncated form of *Cbfa1* driven by an osteocalcin promoter began to lose bone with no observed effect on osteoclasts. Mice transgenic for *Cbfa1* show a decrease in the expression of important osteoblast markers, including type I collagen, osteocalcin and bone sialoprotein (Ducy *et al.,* 1999).

In culture, *Cbfa1* expression has been induced in cells other than osteoblasts. For example NIH3T3 fibroblasts, C3H10T1/2 fibroblasts, MC3T3 mouse pre-osteoblasts (Xiao *et al.,* 1999) and the C2C12 mouse myoblast cell-line (Tsuji *et al.,* 1998 and Lee *et al.,* 1999). Cbfa1 not only appears to be important in the regulation of bone formation during development but also post-natally (Ducy *et al.*, 1997).

1.2.4.3 Role of Cbfa1 in the regulation of osteoblast specific genes

Cbfa1 was identified as a factor capable of binding to an element in the osteocalcin promoter, termed OSE2. Subsequently, OSE2 elements were found in the promoter regions of all the major genes expressed by the osteoblast (Ducy *et al.,* 1996; 1997; 1999). Expression of Cbfa1 in fibroblasts can lead to the expression of osteocalcin and BSP (Ducy *et al.*, 1997). It has also been observed that transfection of osteoblast

cultures with Cbfa1 specific anti-sense can lead to a decrease in the expression of these genes (Banerjee *et al.,* 1997; Ducy *et al.*, 1997).

Cbfa1 has also been shown to control the expression of dentin matrix protein 1 (dmp-1) (Feng *et al.,* 2002). Dmp-1 is a phosphoprotein (George *et al.,* 1993) associated with mineralisation of both dentin and bone (Hirst *et al.,* 1997; D'Souza *et al.,* 1997) and has also been shown to be a marker of osteoblastic differentiation (Feng *et al.,* 2002).

1.2.4.4 Control of Cbfa1 expression

Several studies have begun to characterise the mechanisms involved in the control of Cbfa1 although much work is still needed in this area. The mitogen activated protein kinase kinase (MAPKK/MEK1) signalling pathway is important in the regulation of Cbfa1 expression and function. Xiao *et al.* (2000) carried out two studies into the signalling pathways associated with Cbfa1. The first showed that a constitutively active form of MAPKK in MC3T3 pre-osteoblasts increased levels of osteocalcin mRNA and stimulated the activity of an osteocalcin promoter sequence only in the presence of intact OSE2. The dominant negative mutant of MAPKK inhibited osteocalcin expression. In addition, recombinant mitogen-activated protein kinase (MAPK) lead to the phosphorylation of Cbfa1 *in vitro* and this phosphorylation was increased by constitutively active MAPKK and decreased by the dominant negative form. Previously, it was shown that regulation of the osteocalcin gene required collagen matrix production, stimulated by ascorbic acid. The osteocalcin promoter is activated by collagen matrix production in response to ascorbic acid and this requires the OSE2 sequence (Xiao *et al.,* 1997). It was also shown that disruption of $\alpha 2\beta 1$ integrin-collagen interactions by the addition of the DGEA peptide or blocking antibody blocks ascorbic acid activation of the osteocalcin promoter. It should be noted that the value of using DGEA to block $\alpha 2$-collagen interaction is controversial, for more details see section 1.4.1. Several studies suggest that BMPs are activators of Cbfa1. Ducy *et al.* (1997) demonstrated that BMP-7 induced expression of Cbfa1 mRNA before induction of osteocalcin mRNA. BMP-2 also increased the level of Cbfa1 mRNA expression in an immortalised human bone marrow stromal cell line

(Gori *et al.,* 1999), C2C12 cells (Nishimura *et al.,* 1998; Lee *et al.,* 1999) and 2T3 cells (Chen *et al.,* 1998). The reader is referred to a comprehensive review on the regulation of osteoblast differentiation that details the effects of BMPs on Cbfa1 (Yamaguchi *et al.,* 2000).

1.2.4.5 Use of Cbfa1 as an osteoblastic marker *in vitro*

Cbfa1 could be used as a marker of osteoblast differentiation *in vitro* and indeed several studies to date have done so. The most informative use of Cbfa1 as a marker of osteoblast differentiation would be in combination with other markers, for example alkaline phosphatase and osteocalcin, as this would provide an indication of differentiation status of cells.

1.2.4.6 Homeobox genes and osteoblast differentiation

There are several homeobox genes known to be involved in the control of osteoblast differentiation such as *Dlx5* and *Dlx6.* These two genes are homologues of the drosophila *distalless* genes and are expressed in the cells of skeletal condensations during bone development (Simeone *et al.,* 1994). *Dlx5* knockout mice show delayed ossification of the membranous bones and a slight delay in the formation of long bones (Acampora *et al.,* 1999). The gene *Msx2* is also known to be involved in osteoblast differentiation (Davidson, 1995). *Msx2* is the mammalian homologue of the Drosophila muscle segment gene. Knockout mice exhibit a delay in ossification of the skull and a decrease in overall bone volume (Satokata *et al.,* 2000).

1.2.5 Osx - a further osteoblast transcription factor

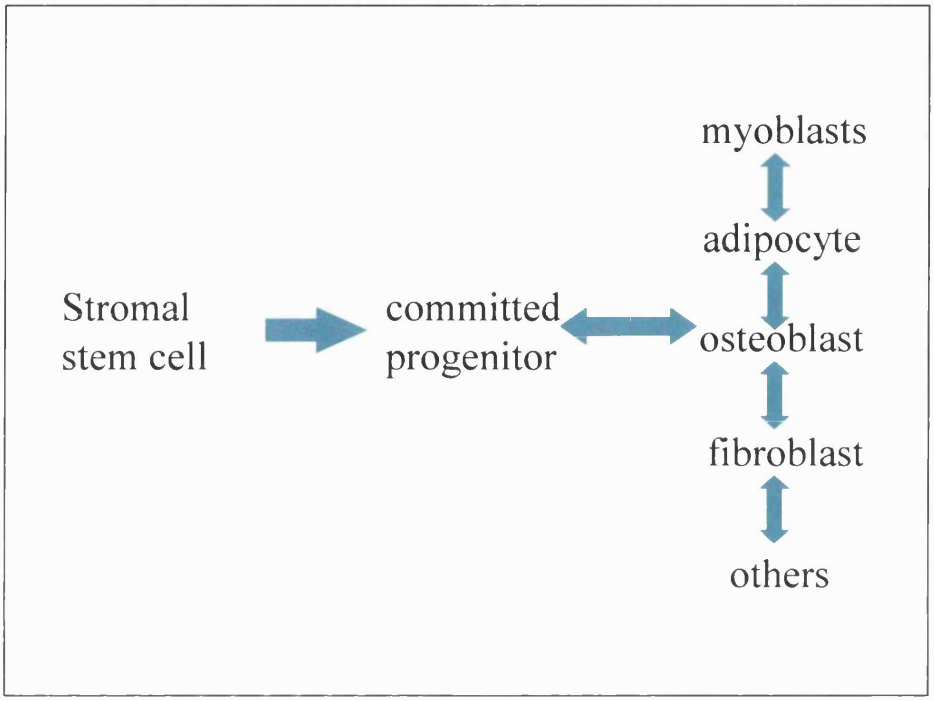
Cbfa1 is not alone in its role as major regulator of osteoblast differentiation. The transcription factor Osx (Osterix) has now been characterised as acting downstream of Cbfa1 in the regulation of osteoblast differentiation (Nakashima *et al.,* 2002). Osx was isolated using the pluripotent cell-line C2C12. When treated with BMP-2 these cells will undergo osteoblast differentiation and it was in this situation that *Osx* was cloned. Osx was shown to induce the expression of type I collagen and osteocalcin. Further studies in knockout mice showed that *Osx* deficiency resulted in death at birth. Analysis of knockouts revealed a skeleton deficient in bone and composed

entirely from cartilage. In addition, there was no expression of bone sialoprotein, osteonectin and osteocalcin. Further studies showed that Osx protein expression is absent in Cfba1-deficient mice but Cbfa1 expression is not absent in Osx-deficient mice, indicating the Osx acts downstream of Cbfa1. Osx differs from Cbfa1 in that it does not result in cartilage defects whereas Cbfa1 does (Nakashima *et al.,* 2002, Ducy *et al.*, 1997). Osx null cells in intramembranous bone appear to undergo chondrocyte differentiation when osteoblast differentiation is not possible (Nakashima *et al.,* 2002)

1.2.6 Osteoblasts and the marrow stromal cell lineage

Osteoblasts belong to a lineage of cells that arise from a bone marrow stromal precursor (see figure 1(b)). Evidence indicates that this cell is a common progenitor, not only for osteoblasts, but also osteoblastic, fibroblastic, adipocytic and reticular cells (Friedenstein *et al.,* 1987; Bennett *et al.,* 1991; Beresford *et al.,* 1992). Recent data indicates that muscle cells (Ferrari *et al.,* 1998; Gussoni *et al.,* 1999) and neural tissues also arise from the same marrow stromal precursor cell (Kopen *et al.,* 1999). Characterisation of marrow stromal cell cultures using both *in vivo* and *in vitro* systems has been of research interest for many years (Friedenstein and Shapiro Piaetzky, 1966; Owen, 1988). This subject continues to be topical with the recognition of the potential therapeutic value of bone marrow stromal stem cells (Bianco *et al.,* 2001; Krause, 2002).

Figure 1(b) – Cells of the marrow stromal lineage



Osteoblasts belong to the marrow stromal cell lineage. In this system a common stromal cell precursor, located in the marrow stroma, has been shown to give rise to a number of different cell types including myoblasts, adipocytes, osteoblasts, fibroblasts and others, for example neuronal cells

Research in this area began when studies into the haematopoietic system revealed the existence of a stem cell in marrow tissue capable of giving rise to bone (Friedenstein and Shapiro Piaetzky, 1966). Studies were carried out using a suspension of marrow stromal cells implanted within diffusion chambers *in vivo*. It was found that this suspension of marrow stromal cells gave rise to osteogenic tissue (Friedenstein and Shapiro Piaetzky, 1966). *In vivo* work was also carried out using transplantation of intact bone marrow either under the renal capsule or subcutaneously. Following transplantation, tissue formed that was analogous to bone and bone marrow (Tavassoli and Crosby, 1968; Friedenstein, 1976). In addition, *in vivo* work revealed the existence of a cell within the bone marrow stroma that was a precursor for colonies of fibroblastic cells. A suspension of marrow stromal cells were placed in diffusion chambers or transplanted under the renal capsule. In this system, a single cell termed fibroblastic colony forming cell (FCFC) or colony-forming unit fibroblastic (CFU-F) was found to give rise to a colony of fibroblastic cells. The term CFU-F was derived from the term colony unit forming spleen (CFU-S) (Friedenstein *et al.,* 1974). Success of CFU-F *in vitro* culture is reportedly dependent on a number of culture conditions, including the presence of serum, hydrocortisone and EGF (reviewed by Owen, 1988; Bianco *et al.,* 2001).

It has also been shown that bone and marrow stroma are of donor origin, whilst the haematopoietic tissue is of host origin (Friedenstein *et al.,* 1978). Friedenstein *et al.* (1982) carried out further transplantation studies with suspensions of marrow cells and fibroblastic cells placed in porous sponges, grafted under the kidney capsule. This also gave rise to bone and marrow tissue (Friedenstein *et al.,* 1982).

In addition, the transplantation of individual cells under the renal capsule showed that these single clones will also give rise to adipocytes and osteoblasts (Friedenstein, 1976). In the same system, cartilage was also seen to form alongside bone in some cases (Friedenstein *et al.,* 1987). Using similar *in vivo* techniques, with implanted diffusion chambers, rabbit and rat marrow stromal cell suspensions will form a series of tissues reflecting the composition of skeletal tissue, shown by both light and electron microscopy. Fibrous tissue forms first, followed by the formation of

osteogenic tissue with corresponding presence of cells with an osteoblastic appearance and genetic characteristics. Three weeks after implantation, both cartilage and bone are present in the diffusion chambers (Ashton *et al.,* 1980; Bab *et al.,* 1984).

The multipotentiality of stromal stem cells has been the focus of several later studies, the results of which have perhaps proved rather surprising. Not only does it appear that the osteoblasts, fibroblasts, adipocytes and so-called reticular cells arise from a common precursor but that myogenic and neural cells may also arise from the same stromal origin. Ferrari *et al.* (1998) demonstrated that bone marrow contained cells capable of myogenic tissue production after transplantation. In this study, marrow-derived cells were directly injected into damaged muscle and these cells underwent differentiation into skeletal muscle myocytes after 2-5 weeks. In addition, the study showed that in skeletal muscle injury, caused by whole bone marrow transplantation, donor marrow cells were found to contribute to the muscle fibres formed during healing. The clinical implications of these observations were explored by Gussoni *et al.* (1999). Using a mouse model of muscular dystrophy, affected animals were given a bone marrow transplant from healthy, wild type mice. It was observed that marrow derived skeletal muscle cells developed and engrafted after transplantation and these cells expressed normal dystrophin in a small population of muscle fibrils after 12 weeks. This provides encouragement that, given optimum conditions, genetic therapy could be developed for inherited muscle defects. Bone marrow-derived stem cells have also been shown to differentiate into cardiac myocytes, following myocardial infarction in mice and rats (Kocher *et al.,* 2001; Orlic *et al.,* 2001). It has also been shown that bone marrow stem cells have the capacity to form neurogenic cells *in vivo* (Brazelton *et al.,* 2000; Mezey *et al.,* 2000). Bone marrow transplantation from donor mice expressing green fluorescent protein as a marker resulted in the formation of neurons, of which a small proportion were shown to be donor-derived (Brazelton *et al.,* 2000). Similar results were obtained with injection of male wild-type mouse marrow cells into female recipients, lacking normal formation but normal CNS development (Mezey *et al.,* 2000). *In vitro* studies have also shown that bone marrow stromal cells are capable of differentiation into neuronal cells (Sanchez-Ramos *et al.,*

2000; Woodbury and Schwarz, 2000). Bone marrow cells are also known to differentiate into endothelial cells (Peterson *et al.,* 2002), epithelial cells (Poulsom *et al.,* 2001). For a comprehensive review of recent advances in the plasticity of marrow-derived stem cells and the clinical implications the reader is referred to Krause. (2002).

1.2.7 *In vitro* osteoblast models

1.2.7.1 Osteoblasts derived from explant culture

A number of *in vitro* systems for studying osteoblasts are widely used. Cell-line cultures have been frequently used as a source of cells that are easily obtainable and straightforward to maintain in culture. Several attempts have been made to develop *in vitro* cultures of osteoblastic cells derived from bone (reviewed in Gallagher *et al.,* 1996). Beresford *et al.* (1983) successfully established a system for isolating cells exhibiting osteoblastic characteristics from explants of human trabecular bone. Cells isolated from explants of small trabecular bone chips in culture were shown to have many characteristics of osteoblasts. When cultured in medium containing glucocorticoid and ascorbate these cells were observed to produce an extensive matrix with mineralisation *in vitro* (Gundle and Beresford, 1995). Ascorbate-2-phosphate, a more stable derivative of ascorbic acid, is important for the expression of the procollagen gene and the biosynthesis of type I collagen (Tajima and Pinnell, 1982; Lyons and Schwartz, 1984). When used alone, glucocorticoids will inhibit collagen synthesis *in vitro* (Dietrich *et al.,* 1979; Canalis, 1983). In contrast, the use of ascorbate with glucocorticoids will promote the secretion of a dense extracellullar matrix with the addition of β-glycerophosphate as a source of phosphate (reviewed Gundle *et al.,* 1998). In this thesis cells derived from trabecular explant cultures are termed HOBs.

1.2.7.2 Human osteoblast cell-lines

There are several human osteoblast cell-lines routinely used *in vitro*, for example MG63, TE 85 and SaOS2 (Clover and Gowen, 1994). This thesis uses the MG63 human osteoblast cell-line that has been shown to exhibit several osteoblastic characteristics and these are summarised in table 1(c) below.

Table 1(c) - Summary of osteoblast characteristics of MG63 cells and HOBs

	Osteoblast characteristics					
Cells	Alkaline phospha-tase (AP)	Osteo-calcin (OC)	Effect of 1,25 $(OH_2)D_3$	Collagen type I	Effect of PTH	Fibro-nectin
MG63s	a,b,c	a,b,c,d	Elevates AP a,b,c Elevates OC a,b,c,d	a	Elevates cAMP and OC e	b,c
HOBs	a,g	g	Elevates AP f,g,j Elevates OC f,h,j	f,g	Increase in cAMP activity and cell proliferation i	

Key:

a	Clover and Gowen, (1994)	f	Beresford *et al.* (1983)
b	Franceschi *et al.* (1985)	g	Beresford *et al.* (1984)
c	Franceschi, (1988)	h	Beresford *et al.* (1986)
d	Lajeunesse *et al.* (1990)	i	Macdonald, (1986)
e	Lajeunesse *et al.* (1991)	j	Thavarajah *et al.* (1993)

The table above shows a summary of osteoblastic characteristics exhibited by osteoblasts *in vitro*. Note that HOBs can form a mineralised matrix in vitro (Gundle and Beresford, 1995). This data was compiled from key studies previously carried out in the area. References citing positive observations are indicated by the letters a-j, with reference details shown in the key.

MG63 cells were shown to secrete alkaline phosphatase and osteocalcin and activity of alkaline phosphatase and levels of secreted osteocalcin were increased in response to 1,25-dihydroxyvitamin D_3 treatment (Franceschi *et al.,* 1985; Franceschi and Young, 1990; Clover and Gowen, 1994). Lajeunesse *et al.* (1990) and (1991) also reported the secretion of osteocalcin and the elevation of this in response to 1,25-dihydroxyvitamin D_3 treatment. Subsequent studies showed that osteocalcin secretion was regulated by PTH (parathyroid hormone) and PGE_2 (prostaglandin E_2) treatment in a cyclic adenosine monophosphatase (cAMP) dependent manner (Lajeunesse *et al.,* 1991). MG63 cells have also been used for several studies investigating the effects of 1,25-dihydroxyvitamin D_3 (Mahonen *et al.,* 1990; Maenpaa *et al.,* 1991; Pirskanen *et al.,* 1991; Inaba *et al.,* 1995). Due to the expression of osteoblast characteristics, summarised in figure 1 (c), MG63 cells are a valuable *in vitro* model.

1.2.7.3 Animal osteoblast cell-lines

Primary osteoblasts are frequently used that are isolated from rat calvaria. These cells are known to respond to parathyroid hormone and express alkaline phosphatase, type I collagen, osteopontin and bone sialoprotein. Under appropriate culture conditions rat calvarial osteoblasts will form nodules of mineralised tissue (Rodan and Noda, 1991; Lian and Stein, 1993). Several animal cell-lines are routinely used as *in vitro* osteoblast models, for example rat ROS 17/2.8 and UMR 106 (Rodan and Noda, 1991).

1.2.8 Osteoclasts and bone

Osteoclasts are the principal bone-resorbing cell. They are located on the endosteal bone surfaces, Haversian systems and have occasionally been seen on the periosteal surfaces. In general, the presence of osteoclasts indicates an area where active bone remodelling is taking place. Osteoclasts are multi-nucleated cells that arise from mononucleur precursors of the haematopoietic lineage (Roodman *et al.,* 1985). Osteoclastogenesis (osteoclast formation and differentiation) is dependent upon the presence of a number of factors including macrophage colony-stimulating factor (M-CSF), RANKL (receptor activator of nucleur factor kappa B ligand), RANK, the receptor for RANKL and TRANCE (TNF-related activation induced cytokine)

(Udagawa *et al.,* 1990; Simonet *et al.,* 1997). The RANK system provided evidence that osteoclastogenesis required osteoclast contact with surrounding cells. RANKL, also known as OPGL (osteoprotegrin ligand), is expressed on osteoblasts/stromal cells and the RANK receptor is expressed on haematopoietic osteoclast precursor cells (Suda *et al.,* 1992; 1997; Reddy and Roodman, 1998). The interaction of RANK and RANKL initiates a signalling and gene expression cascade that results in differentiation and maturation of osteoclast precursor cells to active osteoclasts, capable of resorbing bone (Simonet *et al.,* 1997; Yasuda *et al.,* 1998a; 1998b; Lacey *et al.,* 1998). A receptor termed OPG is also known to bind RANKL and binding to OPG, rather than RANK, leads to inhibition of osteoclastogensis (reviewed in Hofbauer *et al.,* 2000; Teitelbaum, 2000). Many factors known to act on bone cells are known to stimulate the production of RANKL and inhibit the production of OPG, including 1,25-dixydroxyvitamin D_3, PTH and others. Oestrogen is thought to inhibit the production of RANKL and osteoclastogenesis. For further detail see Aubin and Bonnelye, (2000). Factors secreted by osteoblasts are also required for osteclastogenesis, such as tumour necrosis factor (TNF), 1,25-dihydroxyvitamin D_3, interleukins 1, 6 and 11, TGF-β and glucocorticoids (Suda *et al.,* 1992; 1997; Reddy and Roodman, 1998).

The functional osteoclast migrates to areas of bone resorption where it attaches to the bone surface. The plasma membrane forms the tight sealing zone, also known as the clear zone, that is in close contact with the bone surface and is rich in F-actin filaments (Holtrop and King, 1977). Within the tight sealing zone is the ruffled border, the site of matrix degradation. For a comprehensive review see Vanananen and Horton, (1995). Integrin involvement in attachment of osteoclasts to the bone surface and the involvement of these cell adhesion molecules in the resorption cycle has been an area of intensive research and will be reviewed in section 1.5.8.

1.2.9 Bone formation during development

During vertebrate development bone formation takes place by two distinct mechanisms. Endochondral ossification results in the formation of long bones, vertebrae and chondrochranium. Calvarial and facial bones are formed by

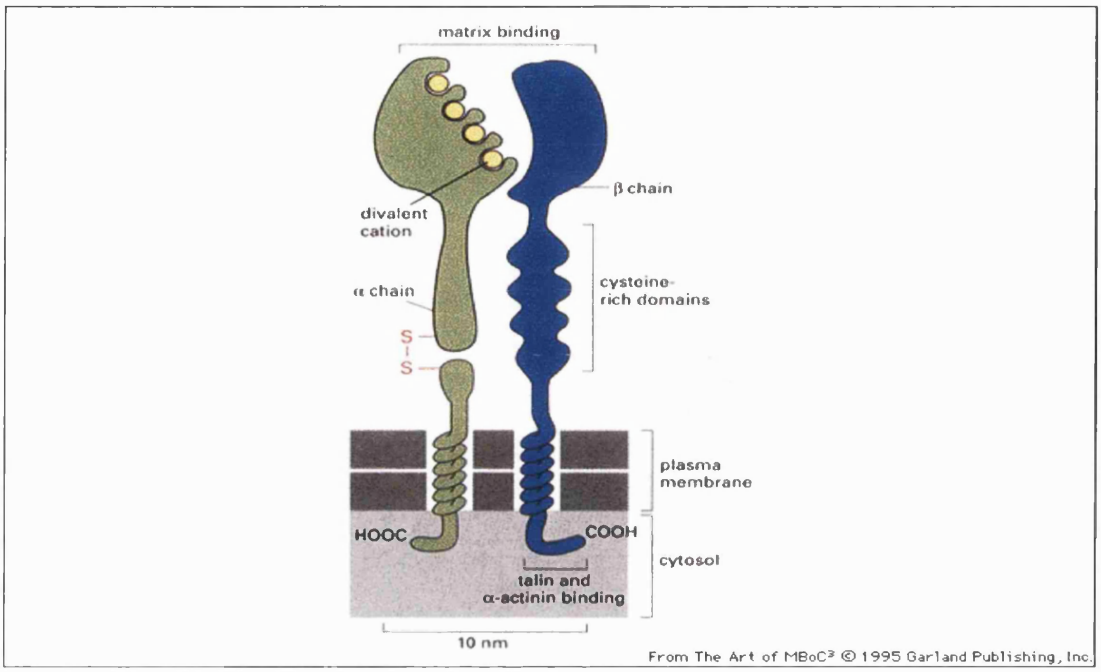
intramembranous ossification. The former requires the formation of a cartilage template that is then replaced by bone (Thompson *et al.,* 1989; Hall and Miyake, 1992; Dunlop and Hall, 1995). Intramembranous ossification results in the formation of bone cells directly from mesenchymal precursors, without the need for a cartilage anlage. Recent studies suggest that there might be some overlap between the two processes of bone development (Nah *et al.,* 2000).

1.3 Integrins

1.3.1 Integrin structure and function

Integrins are non-covalent heterodimers with an α and β chain. Figure 1(d) shows a summary diagram of an integrin. Each sub-unit has a large N terminal domain, a transmembrane spanning domain and a short cytoplasmic tail (Hynes, 1992; Sastry and Horwitz, 1993). The α integrin sub-unit varies in size from 120-180kDa. All contain seven tandem repeats of approximately 60 amino acids. It is thought that some of these repeats could contain cation-binding sites, such as those seen in calmodulin. The integrins LFA-1, α1 and α2 all contain an I domain between the second and third repeats. This is thought to take part in ligand binding. The β integrin sub-units are all 90-110kDa (with the exception of β4 that is 210kDa). At present, there are at least 24 heterodimers that are made up of combinations of 18 α sub-units and eight β sub-units. The integrin family of cell-adhesion molecules has been shown to play a role in a diversity of processes including embryogenesis, immune response, wound healing and tumorigenesis. This means that integrins are important in cellular behaviour such as cell adhesion and migration and in turn cell growth differentiation and survival.

Figure 1(d) – Schematic diagram showing the key features of integrin structure

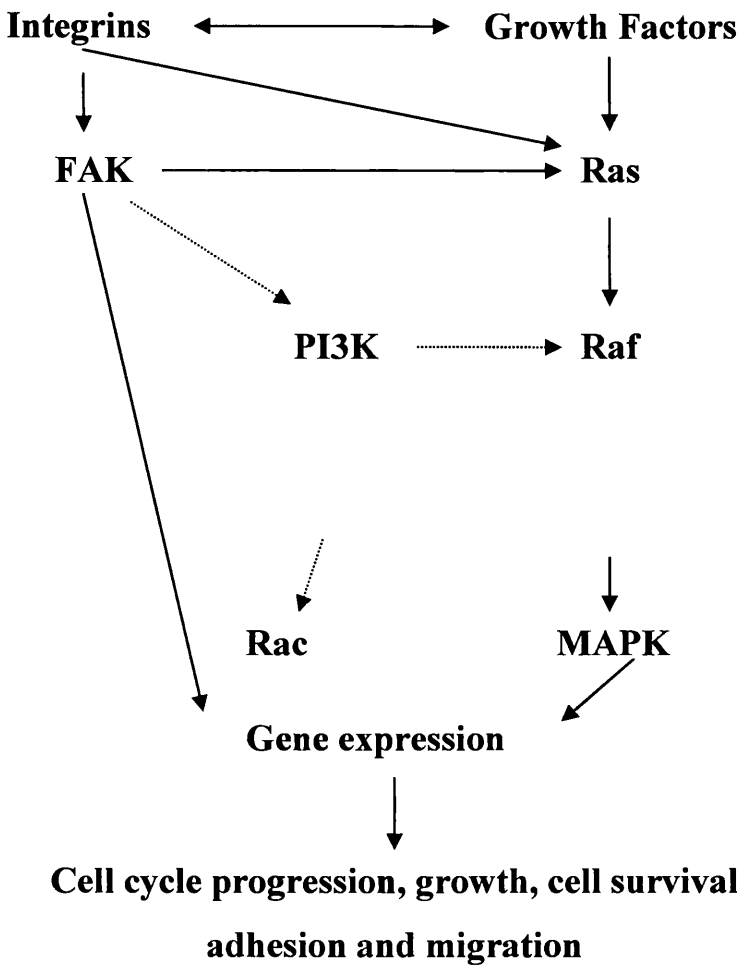


Integrins are heterodimeric receptors composed of an α chain (shown in green) and a β chain (shown in blue). Each chain has a large extracellular domain, transmembrane spanning region and an intracellular domain. Cation binding sites are present on the extracellular portion of the α chain. Ligands can bind to both the extracellular and intracellular portions of integrins and this is involved in the mechanism of integrin function

1.3.2 Integrin signalling

Integrins have the capacity to bind ECM ligands both outside the cell and also specific domains of intracellular proteins. Signals transmitted via integrins from outside the cell result in changes within the cell, for example cytoskeletal re-organisation and altered gene expression that can ultimately lead to a change in cell behaviour. This is known as 'outside in' signalling. Extensive research has been carried out into signalling pathways activated by integrin transduction of external signals. Integrin binding to an ECM ligand can cause clustering of integrins and phosphorylation of a number of intracellular proteins (Kornbergh *et al.,* 1992; Burridge *et al.,* 1996). Several proteins are known to associate with integrins clusters, including vincullin, talin, paxillin and actin bundles and associated cytoskeletal proteins. These sites of adhesion are termed focal adhesions (Burridge *et al.,* 1988, Petit and Thiery, 2000 and Liu *et al.,* 2000). Activation of integrins, following interaction with extracellular ligands, for example ECM, can lead to the activation of focal adhesion kinase (FAK) by phosphorylation (Kornberg *et al.,* 1992). FAK can then activate branches of the MAPK pathway and the PI3K (phosphaptidylinositol 3 kinase) signalling pathway. It is also thought that integrins can activate the MAPK pathways via a FAK independent mechanism (reviewed in Schwartz, 2001). Integrin signalling can also occur via the small G-proteins such as rho, rac and cdc42 and may lead to changes in arrangement of the actin cytoskeleton and also activation of the MAPK pathway. A further protein, integrin linked kinase (ILK) has also been reported as being activated in response to integrins mediated signals. In particular, ILK is thought to activate the PI3K signalling pathway (Wu and Dedhar, 2001). Integrins have also been shown to cooperate with growth factors in the activation signalling pathways although the exact mechanism remains controversial (Yamada and Even Ram, 2002). Figure 1(e) shows a summary of key integrin-mediated pathways and the cross-talk that occurs between them. This diagram is adapted from Giancotti, (2000).

Figure 1(e) - Integrin mediated signalling



Interaction of integrins with ECM components and cell surface receptors, for example growth factor receptors, leads to the activation of a series of intracellular signalling cascades. The diagram above summarises the most widely known integrin linked signalling pathways and the aspects of cell behaviour that are known to be controlled by integrin-mediated mechanisms.

1.3.2.1 Integrin affinity modulation

Binding of integrins to intracellular partners can also lead to a change in the affinity of integrins for external ligands and to the clustering or a more diffuse pattern of integrin expression on the cell surface, termed 'inside out' signalling (Hynes 1992; Schwartz *et al.,* 1995). Several structural studies have been carried out to investigate the nature of integrin affinity modulation. It appears that structural changes occur upon integrins binding to ECM ligand and also in the reverse, changing the affinity of integrins for ECM ligands (Lee *et al.,* 1995; Emsley *et al.,* 2000; Takagi, 2001).

1.3.3 The role of integrins in cell adhesion

Cells adhere to underlying substratum via integrins. In this situation, the integrins tend to be located in small adhesive sites, called focal adhesions (Burridge *et al.,* 1988). Within the sites of adhesion between the cell and substratum are both integrins and associated cytoskeletal proteins, for example talin, vinculin, α-actinin and FAK (Petit and Thiery, 2000; Liu *et al.,* 2000). Cell adhesion via integrins not only plays a structural role but leads to the activation of signalling pathways involved in processes such as cell proliferation, differentiation and migration.

1.3.4 Integrins and cell migration

Research to date has shown that integrins are of central importance during the process of cell migration *in vitro.* This process occurs in a diversity of situations, including embryogenesis, immune response, wound repair and tumorigenesis. Adhesion of a cell to the underlying substratum via integrins is an important step in cell migration. Firstly, cells must attach and then spread on underlying matrices and this is thought to occur via the interaction of integrins with ECM components via focal adhesions (Burridge *et al.,* 1988). Within the focal complexes small guanosine 5'-triphosphate GTP proteins are activated such as cdc42 and rac (Price *et al.,* 1998), and these drive the extension of the cell along the matrix via filopodia and lamellipodia respectively (Mullins *et al.,* 1998, Hall, 1998 and Machesky and Insall, 1999). Once the cell has spread, integrins appear to act as mediators of tension and signals generated by the surrounding ECM. As the cell moves, the integrins appears to act as a molecular attachment to the underlying substratum over which the cell can move (Smilenov *et*

al., 1999). In addition, the 'tail' of the cell retracts and this is thought to involve decreased integrin ligation and integrin mediated contractility. One of the processes by which this is thought to occur is via the inhibition of myosin light chain kinase via cdc42 and rac. This leads to lowered myosin light chain phosphorylation and a decrease in stress fibre formation (Sanders *et al.,* 1999; Manser *et al.,* 1997).

1.3.5 Osteoblast migration

It has been proposed that osteoblast migration could be important for the recruitment of these cells to sites of bone formation, both during development and in the adult during the cycle of bone remodelling. It is likely that mature osteoblasts move to sites of bone formation during bone turnover in the adult (Bonewald, 1996). The mechanism of cell migration in osteoblasts is largely unknown although a variety of factors are known to stimulate motile behaviour in these cells. Migration of osteoblasts was first observed in the 1970s in studies using rat osteoblasts (Jones and Boyde, 1977). Using scanning electron microscopy (SEM), pieces of endocranial parietal bones of rats were cultured for up to 24 hours. Osteoblasts were seen to traverse the matrix surface of the bone and also migrated out of vascular channels. Glass spicules were placed on the bone surfaces and osteoblasts were seen to move over these. In the presence of PTE, the osteoblasts were elongated and aligned parallel to each other. In controls on glass, cells were less elongated and aligned. Isolated migrating cells in glass controls were seen to have membrane ruffles, whereas on the matrix they did not (Jones and Boyde, 1977). In retrospect, this study provided evidence for the cell spreading that takes place during the cell migration cycle that is now known to involve integrin mediated adhesion. Of course this work was carried out long before integrins were discovered.

Several growth factors have been shown to act as chemotactic attractants for osteoblast migration, including TGF-β, PDGF and BMP-2 (Lucas, 1989; Pfeilschifter *et al.,* 1990; Hughes *et al.,* 1992; Lind *et al.,* 1995; Chandrasekhar and Harvey, 1996; Lind *et al.,* 1996). PDGF has also been reported to activate random migration of osteoblasts and its effects on migration were increased by leukaemia inhibitory factor (Chandrasekhar and Harvey, 1996). Mundy *et al.* (1982) showed

that factors released during bone resorption acted as chemotactic stimulants to osteoblasts. These findings were of particular interest as they provided a link (coupling) between bone resorption by the osteoclast and the subsequent production of new bone by the osteoblast. Imai *et al.* (1998) showed that osteoblasts and osteoblast precursors express the cell-surface receptor, N-syndecan. When placed in migration assays with the N-syndecan ligand, HB-GAM, these cells migrated rapidly. HB-GAM, also known as pleiotrophin, is an ECM-associated protein rich in lysine and cysteine residues. Mice, transgenic for HB-GAM, exhibited increased bone thickness. The authors suggested that this could have been due to increased recruitment of osteoblasts to sites of HB-GAM expression, via the syndecan receptor (Imai *et al.,* 1998).

1.4 The extracellular matrix and integrin interaction

An abundant ECM surrounds bone cells. Osteoblasts, chondrocytes and perhaps osteocytes are responsible for the synthesis of this matrix. Mature bone is known to contain abundant type I and type III collagen, osteopontin, bone sialoprotein and osteocalcin. Components of the ECM tend to have the ability to bind several integrins, for example fibronectin binds $\alpha5\beta1$ and seven other integrins. There is little data available on the amount of fibronectin present in mature human bone.

1.4.1 Collagen

To date there are nineteen proteins classified as collagens. Each is a triple helical protein trimer (Prockop and Kivirikko, 1995). Type I collagen is the most abundant collagen sub-type in human bone. Along with types II, III, V and XI this sub-type forms continuous triple helices that can form large fibrils (Ayad *et al.,* 1994). The $\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, $\alpha10\beta1$ and $\alpha11\beta1$ integrins all bind to collagen in the ECM. The $\alpha1\beta1$ and $\alpha2\beta1$ integrins are the predominant collagen binding in osteoblasts (Bennett *et al.,* 2001b) and are also important in platelets and epithelial cells integrins (Santoro and Zutter, 1995). The $\alpha10\beta1$ and $\alpha11\beta1$ integrins have been recently identified and are present on chondrocytes and in foetal muscle respectively (Camper *et al.,* 2001; Velling *et al.,* 1999). There have been several integrin binding

sites identified in type I collagen including several sites in the α1 (1) chain such as the putative Asp-Glu-Gly-Ala (DGEA) motif. This sequence has been implicated in α2β1 binding to type I collagen (Staatz *et al.,* 1990; 1991). The involvement of this sequence in α2β1 binding is controversial. Studies by Knight *et al.* (1998) reported that peptide fragments containing the DGEA motif did not bind to the α2β1 integrin. This study found that the Gly-Glu-Arg (GER) triplicate, present in type I collagen, was required for the binding of the α2β1 integrin. The GER sequence is similar to the binding motif for α1β1 in collagen type IV (Eble *et al.,* 1993). The crystal structure of a complex between the I domain of integrin α2β1 and a triple helical collagen peptide containing a critical GFOGER motif has been shown (Emsley *et al.,* 2000).

The collagen binding integrins and the LFA-1 leukocyte integrin all contain a common feature, an A domain at the N terminus of the α sub-unit (Michishita *et al.,* 1993; Tuckwell *et al.,* 1995; Nolte *et al.,* 1999). Binding at this site is cation dependent (Mn^{2+} or Mg^{2+} not Ca^{2+}) (Tuckwell *et al.,* 1995). Although both α1β1 and α2β1 bind to collagen types I and IV, their relative affinities for the two types of collagen differ. The α1β1 integrin binds to collagen type IV with higher affinity than to type I, whereas α2β1 binds to type I collagen with higher affinity (Kern *et al.,* 1993). It has been suggested that the α1β1 and α2β1 collagen integrin receptors could have distinct signalling pathways. In a review by Heino, (2000) it was proposed that α1β1 integrin-mediated signalling could lead to cell proliferation and a reduction in collagen synthesis, whereas α2β1 signalling could lead to collagen production and also collagenase gene expression. It is also suggested that in 3D collagen gel cultures interaction of α2β1 with collagen leads to activation of the p38/MAPK signalling pathway. It is likely that the cell signalling pathways linked to collagen binding are a great deal more complicated but the observations made to date in other systems may be applicable to osteoblasts and bone.

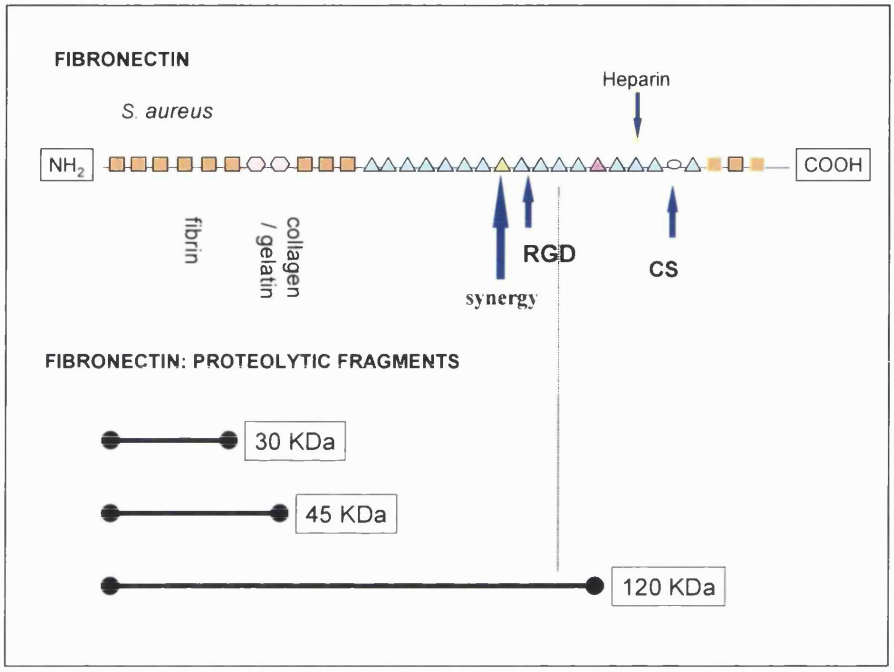
1.4.2 Fibronectin

Fibronectin is a large glycoprotein that is either present in plasma or cellular form. Figure 1(f) shows the structure of fibronectin. It has a modular structure containing

repeating amino acids termed type I, type II and type III repeats (Peterson *et al.,* 1983; Ruoslahti, 1988). Fibronectin has a number of receptor binding sites, including several for integrins and also binding sites for other extracellular proteins. Knockout mice for fibronectin or the α5 integrin receptor resulted in the death of embryos early in development (Hynes *et al.,* 1992) and studies have reported the importance of fibronectin and integrin interaction in this process (reviewed in Miyamoto *et al.,* 1998).

Integrins are thought to bind to specific sequences in fibronectin. The most widely researched of these sequences has been the Arg-Gly-Asp (RGD) motif, located in a type III repeat (Ruoslahti, 1988; 1996). Many other components of the ECM contain this motif, for example fibronectin, vitronectin, osteopontin, collagens, thrombospondin, fibrinogen, and von Willebrand factor. Some integrins bind to the RGD sequence of a single adhesion protein only, whereas others recognise groups of them (Ruoslahti and Pierschbacher, 1987). In addition, fibronectin contains the 'synergy' site I that is also required for α5β1 binding (Obara *et al.,* 1988) (shown in figure 1(f)). Studies suggest that the β sub-unit binds the RGD sequence and the α sub-unit binds the synergy sequence (Obara *et al.,* 1988; Kimizuka *et al.,* 1991; Aota *et al.,* 1994). Several integrins bind to plasma fibronectin, including α3β1, α4β1, α5β1, αvβ3 and αvβ5. The experiments in this thesis used three commercially available fibronectin fragments produced by proteolysis of plasma fibronectin and these are also indicated in figure 1(f).

Figure 1(f) - Fibronectin and proteolytic fragments



Fibronectin is an ECM glycoprotein with a modular structure. It contains several binding sites for interaction with other proteins, including gelatin, collagen, fibrin and heparin. There are also several sites involved in integrin interaction including the RGD sequence, synergy site and the CS domain.

1.5 Cell adhesion molecules and bone cells

1.5.1 Integrins and osteoblasts

Several studies have sought to establish the profile and function of integrins present in osteoblasts and bone forming culture. The β1 family of integrins appears to be predominantly expressed, with recent studies indicating that α2β1 and α5β1 interaction with ECM may be of particular importance. The successful production of a mouse β1 knockout has provided further evidence for the importance of this integrin family in osteoblast biology (Zimmerman *et al.,* 2000). Many of the studies carried out to date have used cells from varying sources and with differing methods. This has made it difficult to draw firm conclusions as to the profile of specific integrins expressed in osteoblasts. In the following section the information currently available about integrin expression and function in osteoblastic systems will be reviewed.

1.5.2 The integrin profile of osteoblasts

The first systematic study that looked at the expression of integrins in the primary human osteoblast, both *in vivo* and *in vitro*, was that of Clover *et al.* (1992). Immunostaining was carried out on tissue sections derived from osteophytes from osteoarthritic femoral heads and cells from trabecular bone explant culture sections. FACS analysis was also carried out on the cultured cells. These experiments showed that the α1, α2, α3 and β1 sub-units were expressed both *in vivo* and *in vitro*. It appeared that the α2 sub-unit stained only weakly *in vivo*, but more strongly on cells in culture. αv was only expressed on cells in culture and the staining appeared weak. The authors suggest that these observations may be an artefact of culture. The sub-units α4, α5, α6, β3, αv, and β2 were tested for and results proved to be negative. Hughes *et al.* (1993) looked for the expression of a similar range of integrins, with the addition of the αvβ3 heterodimer and the β4 sub-unit using tissue sections derived from fracture callus, tumour-associated reactive bone and neo-natal costochondral junctions. The authors differentiated between osteoblasts and osteocytes and found that both cell types stained positively for the β1 and α5 sub-unit and both showed very few cells staining for the α4 sub-unit. The osteoblasts appeared to stain positively for αv. Grzesik and Robey, (1994) carried out immunostaining on sections

cut from foetal human bone and cells derived from trabecular explant. This study did not look at the expression of α1-α3, α6 and α7 but did find the α4 and αv sub-units to be present on osteoblasts *in vivo* and *in vitro*, as were the α5 and β1 sub-units. The β1 sub-unit alone was stained for and the β3 and β5 sub-units were also shown to be present. A later study by Gronthos *et al.* (1997) showed, by FACS, that the integrin heterodimers α1β1, α2β1, α3β1 and α5β1 were expressed on the surface of osteoblasts derived from trabecular explant culture. Results for α4β1 and α6β1 proved negative. Full results of such studies are summarised in table 1(g).

Table 1(g) –Results of studies into the integrin profile of osteoblasts

Integrin	Osteoblasts	Osteocytes	Osteoblast-like cells	Mesenchymal precursor cells
α1	d (l, k)	(l)	d, b, n, o, i	c
α2	k, (l, d)	(l,)	q, n, o, r, i	c
α3	(j, l) d	(l)	d, q, n, o, r, i (j)	c
α4	j, l (d, k)	j, l*	j, o, e (d, i)	(c)
α5	j, l, k, (d)	j, l	j, q, o, e, p, i (d)	c
α6	(l, d, k)	(l)	q, n (d, e, i)	c
αv	j, l (d)	j, l*	j, d, q, e, p	c
β1	l, d, k	l, a, g	d, q, g, p, i	c
β2	(d, k)	(l)	(d, e)	(c)
β3	(l, d, k)	(l)	j, q, i (d, e)	c
β4	(l, k)	(l)		c
β5	m	j, f*	j, n, o, p, i (f)	

Key

a	Aarden *et al.* (1996)	k	Horton and Davies, (1989)
b	Brighton *et al.* (1992)	l	Hughes *et al.* (1993)
c	Bruder *et al.* (1998)	m	Hultenby *et al.* (1993)
d	Clover *et al.* (1992)	n	Nissinen *et al.* (1997)
e	Clover and Gowen, (1994)	r	Riikonen *et al.* (1995)
f	Ganta *et al.* (1997)	o	Saito *et al.* (1994)
g	Gohel *et al.* (1995)	p	Salter *et al.* (1997)
h	Gronowicz and McCarthy, (1996)	q	Sinha and Tuan, (1996)
i	Gronthos *et al.* (1997)		
j	Grzesik and Robey, (1994)		

The table above shows a summary of integrin expression in osteoblasts using the literature available to date. Bracketed citations report negative results. * indicates equivocal results. References are summarised in the key

1.5.3 The role of integrins in osteoblasts

In order to investigate the role of integrins in the osteoblast interaction with ECM, adhesion assays have been widely used. These have shown that osteoblasts will adhere at high levels to several matrix components, including plasma fibronectin and type I collagen. Addition of specific integrin blocking antibodies has shown that the β1 integrins are of particular importance for osteoblast adhesion on the underlying ECM (Clover *et al.,* 1992; Grzesik and Robey, 1994; Pistone *et al.,* 1996; Gronthos *et al.,* 1997).

Over the past few years studies have started to investigate the role of integrins in osteoblast behaviour further. Evidence has been provided for the presence, and importance, of the α5β1 fibronectin binding integrin. Using osteoblasts derived from a rodent calvarial model, it was shown that fibronectin was required for the formation of mineralised nodules in culture. When anti-fibronectin antibodies were added into the culture system the nodular structures normally observed were absent. The authors suggested that the α5 integrin was involved (Moursi *et al.,* 1996; 1997). Later work showed that mature rat osteoblasts were dependent upon fibronectin for survival (Globus *et al.,* 1998). Studies using UMR-106-01 cell-lines showed that addition of α5, α2β1, β1 and αvβ3 blocking antibodies significantly reduced mineralisation of these cells in culture (Schneider *et al.,* 2001).

Several studies have sought to identify a role for collagen binding integrins in osteoblastic systems. As mentioned previously, type I collagen is the most abundant ECM component and has the potential to play an important role in osteoblast behaviour. Xiao *et al.* (1998) showed that disruption of α2-type I collagen binding, with either blocking antibodies or the DGEA peptide, prevents expression of alkaline phosphatase.

Jikko *et al.* (1999) studied the roles of the α1β1 and α2β1 integrin collagen receptors in regulating the differentiation of an osteoblastic cell line in response to bone morphogenetic protein BMP-2 treatment. Addition of anti-collagen-I or both anti-

integrin-α1 and α2 antibodies to cultures significantly reduced the expression of early osteoblastic markers and mineralisation. Cells transfected with a constitutively active BMP-2 receptor were also sensitive to the effects of these integrin-blocking antibodies. As mentioned previously, Schneider *et al.* (2001) showed that addition of α2β1 integrin blocking antibodies to a UMR cell-line reduced mineralisation of the cells in culture.

1.5.4 β1 knockout mice

The production of a β1 knockout mouse model provided an insight into just how important β1 integrins are in osteoblast biology. Zimmerman *et al.* (2000) produced a mouse model with the β1 gene deleted and targeted to mature osteoblasts with the aid of an osteocalcin promoter that is only functional in mature osteoblasts. The β1 construct, driven by an osteocalcin promoter, circumvented the problem of pre-natal mortality. Transgenic mice resulting from these experiments had a greatly reduced bone mass. Previously, transgenic studies targeting specific integrin sub-units have proved a stumbling block in bone research, as exemplified by β1, α3, and α5 knockouts that have all been lethal at an antenatal stage of development (Faessler and Meyer, 1995 and Faessler *et al.,* 1996).

Integrins, other than the β1 binding partners, may also be expressed in osteoblast systems. Recent work has shown that the αvβ3 integrin may be important in osteoblast differentiation and bone mineralisation (Cheng *et al.,* 2001). The murine MC3T3-E1 cell-line was transfected with αv and β3 in separate vectors. Transfected cells appeared to exhibit increased proliferation but decreased mineralisation. Alkaline phosphatase activity was decreased, as was the expression of osteocalcin, type I collagen and bone sialoprotein. Osteopontin expression was increased. The αvβ3 integrin has also been shown to redistribute on the surface of primary human osteoblasts, in response to mechanical strain (Wozniak *et al.,* 2000). Cheng *et al.* (2000) also showed that αvβ3 integrin expression increased, on human osteoblasts, after two days of treatment with dexamethasone.

In summary, integrins play the following roles in osteoblasts:

- Adhesion and migration of cells on ECM substrates;
- Differentiation of mesenchymal precursors and progression to mature osteoblast; and
- Cell survival.

1.5.5 Cadherins and osteoblasts

The cadherin superfamily of cell adhesion molecules consists of the classical cadherins and many cadherin-related molecules. Cadherins are important in many cell biology processes, such as cell polarity, cell proliferation, differentiation and, in turn, many whole tissue phenomena such as morphogenesis. (Takeichi, 1995; Gumbiner, 1996). Studies in osteoblastic systems have revealed expression of the classical cadherins (Cheng *et al.,* 1998; Tsutsumimoto *et al.,* 1999). Classical cadherins are calcium dependent with transmembrane spanning, intracellular and extracellular domains. The extracellular domain of these molecules contains five calcium binding repeats, termed EC1-EC5. The EC1 domain is responsible for binding to cadherins on neighbouring cells and contains the His-Ala-Val (HAV) motif that mediates interaction between cadherin molecules on adjacent cells (Overduin *et al.,* 1995; Shapiro *et al.,* 1995). Classical cadherins tend to be located in adherens junctions that form between cells (Angst, 2001). The intracellular domains of these adhesion molecules interact with cytoplasmic proteins such as β-catenin and plakoglobin (PG) that form a link with α-catenin and consequently the actin cytoskeleton (Yap *et al.* 1997).

Within bone, mature differentiated osteoblasts form an epithelial-like layer along the bone surface and, within this, the osteoblasts are inter-connected by gap-junctions (Doty, 1981). Several cadherins are expressed in osteoblasts including N-cadherin, cadherin-11 and cadherin-4. Cadherins seem to be expressed in osteoblasts at all stages of differentiation, from mesenchymal precursors to the mature osteoblast. It has been suggested that these cell adhesion molecules are important for cell sorting during bone development (Shin *et al.,* 2000) and studies have also shown that

cadherin expression is important for the differentiation of the osteoblasts (Cheng *et al.,* 1998; 2000; Ferrari *et al.,* 2000).

Several studies have reported the expression of N-cadherin and cadherin-11. Tsutsumimoto, (1999) showed the MC3T3 cells expressed both functional N-cadherin and cadherin-11 (also termed OB cadherin). Treatment with TNF-α was shown to suppress the expression of N-cadherin. Cheng *et al.* (1998) also observed cadherin-11 and N-cadherin in human trabecular derived osteoblasts, osteoprogenitor marrow stromal cells and the cell lines SaOS-2 and MG63. A low level of cadherin-4 mRNA was reported in the trabecular-derived osteoblasts and bone marrow stromal cells (Cheng *et al.,* 1998).

1.5.6 Role of cadherins in osteoblasts

The results of several studies indicate that cadherins play a role in osteoblast differentiation. A decapeptide containing the HAV motif of human N-cad partially inhibited Ca^{2+}-dependent cell-cell adhesion and completely prevented BMP-2 induced stimulation of alkaline phosphatase activity in bone marrow stromal cells (Cheng *et al.,* 1998). A later study using MC3T3 osteoblastic cells with a truncated dominant negative N-cadherin showed calcium dependent adhesion was decreased significantly in stably transfected clones. Expression of BSP, osteocalcin, type I collagen and alkaline phosphatase activity was also reduced (Cheng *et al.,* 2000). Bone nodule formation in primary cultures of foetal rat calvaria and cell-cell contact in rat TRAB-11 cells was inhibited by the HAV adhesion motif of N-cadherin (Ferrari *et al.,* 2000). Knockout mice generated for N-cadherin are embryonic lethal prior to calcification; therefore osteoblast differentiation could not be investigated (Radice *et al.,* 1997). For a review of the role of N-cadherin in osteoblasts see Marie, (2001). Cadherin-11 knockout mice exhibit reduced calcification and bone density suggesting a role for this cadherin in osteoblast differentiation and bone mineralisation (Kawaguchi *et al.,* 2001).

1.5.7 Further non-integrin cell adhesion molecules and osteoblasts

There is little data on the expression and function of cell adhesion molecules, other than integrins and cadherins, in osteoblasts. The cell adhesion molecules intercellular adhesion molecule (ICAM-1) (Tanaka *et al.,* 2000), VCAM (Tanaka *et al.,* 1995) and NCAM (Lee and Chuong, 1992) are reported as being present. In addition, the glycoprotein CD44 has been shown in rat calvarial cultures (Jamal and Aubin, 1996). Expression of the cell-surface heparan sulfate, syndecan has been shown in the human osteosarcoma cell lines MG-63, TE-85, SaOS-2, and U2OS, human osteoblast-like cells, rat calvarial osteoblasts and in human bone. Syndecan has been shown to play a role in cell adhesion and migration, and binding of growth factors (Birch and Skerry, 1999). A novel cell adhesion molecule has been reported, POEM. This molecule was cloned from a MC3T3 cDNA library. In situ hybridisation showed expression in bone, kidney, muscles and endocrine organs. It has been suggested that this molecule acts as a ligand for the α8β1 integrin (Morimura *et al.,* 2001).

1.5.8 Integrins and osteoclasts

Osteoclasts are known to express at least three integrin receptors, the αvβ3 vitronectin binding receptor, the α2β1 collagen binding receptor and αvβ1, also a vitronectin receptor. Here the main points to date will be covered; for further details the reader is referred to a several reviews published on the subject (Horton and Davies, 1989; Horton, 1995; Horton *et al.,* 2002).

Osteoclastic bone resorption by osteoclasts, derived from osteoclastoma tissue, was inhibited by the antibody 13C2 that targets the αvβ3 integrin (Horton *et al.,* 1985, Chambers *et al.,* 1986; Horton and Davies, 1989). The αvβ3 receptor is important in the mediation of osteoclast binding to a variety of substrates, in particular those containing the RGD sequence (Helfrich *et al.,* 1996). The expression of α2β1 mediates the binding of osteoclasts to collagens and, unusually, this appears to be sensitive to RGD peptides (Helfrich *et al.,* 1996).

Although it is known that integrins are involved in bone resorption, the exact mechanism has not yet been elucidated. Some studies have suggested that the tight sealing zone is enriched with certain integrins that could mediate the bone resorbing function (Reinholt *et al.,* 1990, Hultenby *et al.,* 1993; Nakamura *et al.,* 1996). For a comprehensive review of the evidence to date see Horton *et al.* (2002).

Agents that target the αvβ3 integrin or the RGD sequence block bone resorption. It has been observed that the RGD containing snake venom proteins, for example echistatin (Sato *et al.,* 1990), linear and cyclic RGD peptides and their analogues (Engleman *et al.,* 1997) and antisense oligonucleotides (Villanova *et al.,* 1999), will block bone resorption *in vitro.* Blocking the α2β1 integrin with antibodies will also inhibit bone resorption *in vitro.* Knockout studies have been carried out to examine the role of integrins in osteoclasts. Bader *et al.* (1998) produced an αv knockout mouse. After birth, skeletal development appeared to be normal but the mice then died due to abnormalities of the vascular system. The β3 knockout mouse exhibited platelet defects, associated with human Glanzmann Thrombasthenia, the skeletal defects were surprisingly mild with bone sclerosis becoming evident in later life (McHugh *et al.,* 2000).

1.6 Bone cells, integrins and disease

It is likely that many pathological conditions of bone arise due to a breakdown in both cell to cell communication and communication between bone cells and their surrounding environment. Changes in the balance between trabecular bone and fatty tissue, observed in post-menopausal osteoporosis, are thought to arise from a shift in the balance between bone cells and adipocytes (Justesen *et al.,* 2002) and this could be the result of altered cell-matrix communication. Increased resorption of bone by the osteoclast is also associated with osteoporosis. The expression of the αvβ3 integrin by these cells has been exploited in the development of anti-resorptive agents for the treatment of osteoporosis. Many of these agents contain the RGD motif, for example the naturally occurring snake venom proteins. For a comprehensive review see Horton *et al.* (2002). The pathogenesis of diseases such as osteoarthritis and

rheumatoid arthritis is likely to arise from a change in the growth factor and cytokine control of chondrocytes and ECM interaction. (reviewed in Horton *et al.,* 2002).

1.7 Conclusion

Osteoblasts express a range of cell adhesion molecules and it is clear that these molecules play an important role in osteoblast behaviour. In particular, the integrin family of cell adhesion molecules appears to be important in mediation of osteoblast interaction with the surrounding ECM. The profile of integrins expressed in osteoblasts appears to vary with cell source but it is clear that the β1 integrins are predominantly expressed. Recent studies have begun to explore the role of specific integrins in osteoblast function. It appears that the α2β1 and α5β1 integrins are of particular importance in osteoblast differentiation and survival although there is little data in human osteoblasts. To date, there is little known about integrin expression and function in osteoblasts at successive stages of differentiation, perhaps due, in part, to lack of markers for early bone cells. In addition, little information is available on the signalling pathways downstream of integrin molecules in bone cells. It is clear that osteoblast-ECM interactions, mediated via integrins, are of great importance in control of cell behaviour during homeostasis and the pathogenesis of bone disease. Further work is required to investigate the profile and role of integrins and osteoblasts and the associated signalling pathways, in particular using human osteoblasts.

2 Chapter 2 - Materials and methods

2.1 Methods – Cell culture

2.1.1 Introduction

The cell lines used were the MG63 human osteosarcoma cells and the C2C12 mouse myoblast cell line. The MG63 cell line was derived from a human osteosarcoma and has been shown to exhibit many osteoblast characteristics *in vitro*, for example the expression of alkaline phosphatase, osteocalcin and type I collagen (Franceschi *et al.*, 1985 and Clover *et al.*, 1992). C2C12 cells are a stromal cell line that, when grown at high serum levels (20%) *in vitro*, will differentiate into myotubes. The treatment of these cells with transforming growth factor–β (TGF-β) results in a prolonged period of proliferation and failure to fuse into myotubes. Bone morphogenetic protein-2 (BMP-2) treatment will promote the expression of osteoblastic characteristics by this cell-line (Katagiri *et al.*, 1994). Maintenance of this cell-line at a low concentration of serum, 5%, will prolong proliferation of these cells.

2.1.2 Materials

All tissue culture plastic was obtained from Nunc (Biosciences Ltd, Dublin, Ireland). Culture medium, phosphate buffered saline (PBS) and trypsin-EDTA were obtained from Gibco (Paisley, Scotland). PBS was used at a 1x concentration, as supplied by the manufacturer. Routine cell-culture supplements and extracellular matrix (ECM) components were obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, UK) unless otherwise stated. For culture of C2C12 cells, TGF-β and BMP-2 were obtained from R and D Systems UK Ltd (Abingdon, Oxon, UK). Each cell-line was grown both routinely and during experimental periods in a 37°C incubator with 5% CO_2 and 100% humidity.

2.1.3 Routine culture of cells

All cells were removed from liquid nitrogen storage and defrosted rapidly. Cell-lines were routinely grown in 80cm^2 (T80) tissue culture flasks. The MG63 cells were grown in α-MEM supplemented with 10% FCS (foetal calf serum), benzyl penicillin

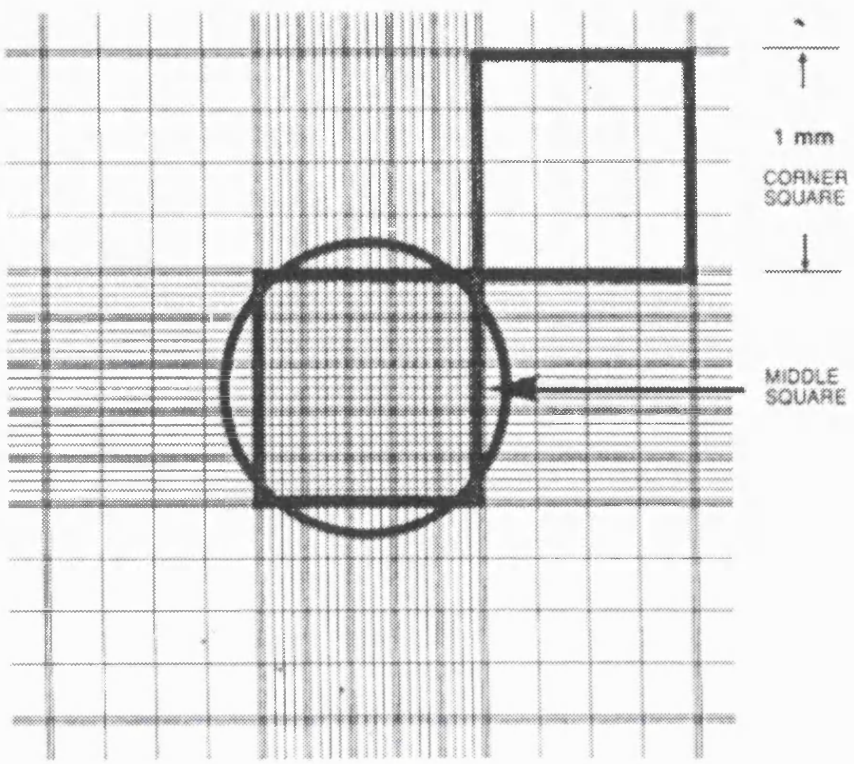
($25U.ml^{-1}$), streptomycin ($10\mu g.ml^{-1}$) and fungizone ($250ng.ml^{-1}$). C2C12 cells were routinely cultured in DMEM with 20% FCS, benzyl penicillin ($25U.ml^{-1}$), streptomycin ($10\mu g.ml^{-1}$) and fungizone ($250ng.ml^{-1}$). Once cells had reached confluence, they were passaged by removal of medium, washed with PBS and treated with 1ml trypsin with a five to ten minute incubation. The cells were then sub-cultured by dilution in routine culture medium and placed in tissue culture flasks for growth in an incubator.

2.1.4 Cell counting

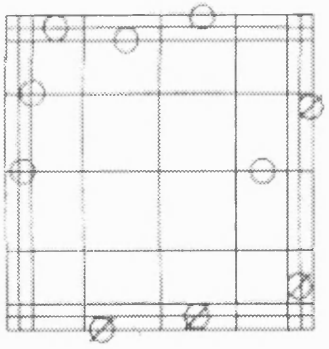
Cells were viewed by light microscopy and counted to obtain the correct cell number required for characterisation studies and functional assays. The following methodology was used:

After trypsinisation, cells were resuspended in routine culture medium and centrifuged at 10 000rpm for five minutes. The cells were then resuspended in 5mls of culture medium. 10µl of cell suspension was then placed on a haemotocytometer slide that has a pattern of grids (figure 2(a). The total number of cells in the four corner squares was counted and the average calculated. To get an accurate reading the average number of cells should have been between 40 and 70, if not, the cell suspension was diluted or concentrated as necessary. To further dilute the cell suspension 1-5ml of routine culture medium was added to bring the cell count to between 40 and 70. To concentrate the cell suspension cells were centrifuged at 10 000rpm for five minutes and resuspended in 1-4ml of routine culture medium. The number of cells in 1ml of suspension was equal to the average number of cells $x10^4$.

Figure 2(a) – Cell counting using a haemocytometer



CORNER SQUARE (ENLARGEMENT)



Count cells on top and left touching middle line (○). Do not count cells touching middle line at bottom and right (Ø).

2.1.5 Preparation of cells for liquid nitrogen storage

Cell-lines were stored long-term in liquid nitrogen. After trypsinisation, cells were resuspended in 5mls of routine culture medium per flask and placed in a 15ml Falcon tube for centrifugation, with one flask of cells per Falcon tube. The cells were centrifuged for five minutes at 10 000 rpm to allow a pellet of viable cells to form at the bottom of the tube. The supernatant was removed, the cells were resuspended in 1ml of routine culture medium with 10% dimethylsulphoxide (DMSO), placed in a cryovial in a foam box for freezing overnight at –80°C and then transferred to liquid nitrogen for long-term storage.

2.1.6 Preparation of primary explant cultures

In addition to the cell-line cultures, primary human osteoblasts (HOBs) were used. These were obtained from trabecular explant cultures. The following methodology, which has been adapted from Beresford *et al.* (1983) and Gundle and Beresford, (1995), was used:

Trabecular bone, otherwise discarded from routine surgery, was obtained from the Eastman Dental Institute, Great Ormond Street Children's Hospital and University College London Hospital and transported in sterile α-MEM culture medium. The bone was washed in sterile PBS supplemented with gentomycin ($10\mu g.ml^{-1}$), until all non-bone tissue and blood was removed. The bone biopsy was then cut into small pieces of approximately $0.5cm^2$. The bone chips were then cultured in α–mem supplemented with 10%FCS, gentomycin ($10\mu g.ml^{-1}$) and fungizone ($250ng.ml^{-1}$), in either a $25cm^2$ (T25) or $80cm^2$ (T80) tissue culture flask, depending on the number of chips. The bone explant cultures were then placed in an incubator for growth.

2.1.7 Routine maintenance of primary explant cultures (HOBs)

The medium was changed after one week and then every three days subsequently, until the first cell outgrowth reached confluency. At confluency, the HOBs were trypsinised and then sub-cultured in T80 tissue culture flasks with α-mem supplemented as described in section 2.1.3. The cells were then used for

characterisation studies and integrin function studies at sub-confluency. A proportion of cells were also stored in liquid nitrogen at this stage to build up a bank of HOBs for later studies.

2.1.8 Culture of C2C12 cells for Cbfa-1 studies

The C2C12 cells were trypsinised, counted and diluted to obtain $2x10^4$ cells per ml of DMEM supplemented with 5% FCS, benzyl penicillin ($25U.ml^{-1}$), streptomycin ($10\mu g.ml^{-1}$) and fungizone ($250ng.ml^{-1}$). Cells were plated out in three 24 well plates with nine wells used per plate. Cells were then grown overnight in an incubator. On the next day (day1) medium was removed, and the cells were cultured under three different conditions:

- DMEM with 5% FCS, supplemented as above;
- DMEM with 5% FCS, with the addition of 300ng/ml of BMP-2, supplemented as above; and
- DMEM with 5% FCS with the addition of TGF-β1 at 5ng/ml of medium, supplemented as above.

C2C12 cells were plated out under each condition in triplicate for three, five and seven days (one 24 well plate for each day). At each of these time points the cells were tested for osteoblastic characteristics using the tests outlined in the following section.

2.2 Methods - Characterisation of osteoblasts

2.2.1 Materials

The alkaline phosphatase assay, components of the Fast Red dye, Triton X-100 and Bovine serum albumin (BSA) were obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, UK). The osteocalcin assay was a Novocalcin assay obtained from Metra Biosystems via their UK distributor Quidel (Great Haseley, Oxon, UK).

2.2.2 Introduction

All cells used in this study were tested for osteoblast characteristics using the following techniques:

- Alkaline phosphatase biochemical assay;
- Alkaline phosphatase histochemical staining; and
- Osteocalcin ELISA.

The alkaline phosphatase assay provides a measure of alkaline phosphatase enzyme activity. The assay uses p-nitrophenol phosphate as a substrate that is converted to p-nitrophenol and inorganic phosphate by alkaline phosphatase. Addition of alkali to the assay converts p-nitrophenol into a coloured product that can be measured by a spectrophotometer. The assay was carried out following the manufacturer's protocol although the volume of reagents used was scaled down to a 96 well plate format. The osteocalcin assay is a competitive immunoassay. A mouse anti-human osteocalcin antibody binds to secreted osteocalcin present in culture medium. This is then detected by an anti-mouse alkaline phosphatase conjugate and a p-nitrophenol substrate, forming a coloured product that can be measured using a spectrophotometer.

2.2.3 Alkaline phosphatase assay

Cells at sub-confluency were lysed using 0.1% triton X-100, diluted in PBS. Lysates were stored at –20°C prior to use. On the day of the experiment, cell lysate was removed from the freezer and thawed at room temperature. The supernatant was then vortexed to mix, spun down briefly in a microfuge and then placed on ice. The BSA protein assay was then carried out to determine the amount of protein present in each sample of supernatant following the manufacturer's instructions. The alkaline phosphatase assay was also carried out following the manufacturer's instructions, outlined here:

- Alkaline phosphatase buffer solution and stock substrate solution were placed in a 37°C water bath for 15 minutes
- 25µl of alkaline phosphatase buffer solution and 25µl of stock substrate solution were placed into the wells of a 96 well plate

- 5µl of 0.1% triton X-100 (diluted in PBS) was added to the triplicate of blanks
- 5 µl of each cell lysate sample was added in triplicate
- The plate was incubated at 37°C for 15 minutes in an incubator
- After the incubation, 50µl of 0.05N NaOH was added to each well.

The absorbance was measured at 405nm using a spectrophotometer and the mean absorbance of the blanks was subtracted from each of the test absorbance readings.

In order to derive the standard curve, a 96 well plate was set up using the p-nitrophenol standard solution (diluted in 0.02N NaOH) to the following dilutions: 0.006 µmol, 0.0125 µmol, 0.025µmol and 0.05µmol. Each dilution was carried out in triplicate with a triplicate of NaOH used as a blank. The absorbance was measured at 405nm using a spectrophotometer and the mean absorbance of the blanks was subtracted from each of the readings. The equation of the standard curve was derived and used to determine the activity of alkaline phosphatase in each sample.

Each experiment was repeated three times on separate occasions and the mean enzyme activity value and standard deviation was obtained from the three repeats. This was then represented graphically.

2.2.4 Alkaline phosphatase histochemical staining

A stock solution of 0.2mg.ml^{-1} of napthol AS-MX phosphate was dissolved in 1ml N,N dimethylformamide (the dimethylformamide was diluted in 0.1M Tris buffer pH9.2).

Immediately before use 1mg.ml^{-1} of Fast Red was added to the above stock solution. Medium was removed from the cells and then the cells were washed twice in PBS and once in 0.2mg.ml^{-1} napthol AS-MX phosphate stock solution. Fast Red solution was added to the cells and left to develop for 2-5 minutes at room temperature. Cells were then fixed with 4% paraformaldehyde.

2.2.5 Osteocalcin assay

Cell supernatants were removed from cells in culture and stored at –20°C prior to use. On the day of the experiment, cell supernatant was removed from the freezer and thawed at room temperature. The cell supernatant was then vortexed to mix and placed on ice. The assay was carried out following the manufacturer's instructions:

- The osteocalcin-coated strips were labelled and placed in the holder provided
- 25µl of sample, osteocalcin standards or control (provided by manufacturer) was placed into the wells of the osteocalcin coated strips
- 125µl of anti-osteocalcin antibody was added to each well and incubated at room temperature for two hours
- After the incubation, the strips were inverted to empty and washed in 500µl of 1x wash buffer (provided by the manufacturer)
- This was repeated twice more and then the strips were then blotted to dry on paper towels
- 150µl of enzyme conjugate was added to each well and incubated for 60 minutes at room temperature
- The strips were washed a total of three times, as above, and blotted to dry on paper towels
- 150µl of working substrate solution was added to each well and incubated for 40 minutes
- After the incubation, 50µl of 3N NaOH was added to each well

The strip was then read at 405nm using a spectrophotometer and a standard curve was constructed using the absorbance readings from the known standards. The equation of this line was used to work out the concentration of osteocalcin present in the samples.

2.3 Methods - Characterisation of integrin expression in osteoblasts

2.3.1 Materials

All integrin antibodies used for FACS and immunocytochemistry were mouse anti-human (monclonal) and were obtained from Chemicon International, Inc (Temecula,

CA, USA) or DAKO Ltd (Ely, UK). The FITC secondary antibody (rabbit anti-mouse) used was obtained from DAKO Ltd and was stored at a stock concentration of $1mg.ml^{-1}$. See table 2(b) for further details The FACS machine and associated Cellquest software was supplied by Beckton Dickinson (Cowley Oxon, UK).

2.3.2 Introduction

Previous studies have been carried out in osteoblastic cell-lines and HOBs to determine the integrin profile of these cells. It has been difficult to draw any conclusive results from these studies due to the variation in technique and source of cells. In this present study a systematic investigation of integrins expressed by MG63 cells and HOBs was carried out using the two techniques of fluorescent immunocytochemistry and flow cytometry (FACS).

FACS can be used to measure the light emitted from a fluorochrome. In this thesis MG63 cells and HOBs were treated with a primary integrin antibody and then a fluorescently labelled secondary antibody alone. The level of fluorescence emitted by each labelled cell was measured. Controls were prepared using cells incubated with Fluorescently labelled secondary alone. Results were recorded as histograms using the Cellquest software package, the x-axis of the histogram representing fluorescence intensity and a y-axis representing cell number (cell count). The x-axis was expressed as a logarithmic scale to normalise the data. In addition, the percentage of cells positive for the fluorescent antibody marker was obtained automatically by the software. The geometric mean of the percentage of positive cells can be used to compare fluorescence intensities and compare levels of integrin expression. It should be noted that FACS analysis is only semi-quantitative due to factors such as efficiency of antibody binding to integrin epitopes.

2.3.3 Flow cytometry

Flow cytometry was carried out on all cell types to determine the profile of integrin expression on the cell surface using the following methodology:

Cells were trypsinised and counted to obtain $1x10^6$ cells per ml of FACS buffer. This buffer was prepared from 1xPBS supplemented with 10% FCS. 100µl of cells diluted in buffer were placed in a FACS tube; a separate tube was used for each integrin antibody used. This gave a total of 10^5 cells per tube. An additional tube was also prepared for a negative control, this was not treated with primary integrin antibody. It should be noted that cells were not fixed although a fixation step can be carried out.

The cells in FACS tubes were centrifuged at 10 000rpm to form a pellet of viable cells and the FACS buffer was replaced to remove any dead cells present at this stage. Integrin antibodies were added to the tubes of cells at appropriate dilutions (see table 2(b)) and mixed by vortex. This ensured maximum exposure of all cells to the antibody. The cells were then incubated with the integrin antibody on ice for one hour.

After the 30 minute incubation the cells were again centrifuged at 10 000rpm to form a pellet. The FACS buffer was removed and the cells were washed by the addition of 500µl of fresh FACS buffer, vortexing and centrifugation. /The FACS buffer was removed and the washing step repeated so that the cells were washed three times in total. After the final wash the cells were pelleted and 100µl of FACS buffer was added, into which a 1:100 dilution of FITC labelled anti-mouse secondary antibody was added. Once again the cells were vortexed briefly and incubated on ice with the secondary for 30 minutes. As FITC is light sensitive, the tubes were wrapped in foil to protect from light. The negative control was also treated with FITC labelled anti-mouse secondary antibody alone.

After the 30 minute incubation the cells were again washed three times in FACS buffer and then finally diluted in 500µl of FACS buffer. Once again it was important to keep the cells protected from light whenever possible. The cells were then ready to feed through the FACS machine.

Table 2(b) - Details of antibodies used during the study

Integrin antibody	Supplier	Clone	Mouse Ig stock concentration	FACS concentration	Staining concentration	Blocking concentration
α2	Chemicon	P1E6	$1mg.ml^{-1}$	$5\mu g.ml^{-1}$	$10\mu g.ml^{-1}$	$50\mu g.ml^{-1}$
α3	Chemicon	P1B5	$1mg.ml^{-1}$	$5\mu g.ml^{-1}$		
α4	Chemicon	P1H4	$1mg.ml^{-1}$	$5\mu g.ml^{-1}$		
α5	Dako	P1D6	$21\mu g.ml^{-1}$	$5\mu g.ml^{-1}$	$10\mu g.ml^{-1}$	$50\mu g.ml^{-1}$
α6	Chemicon	G0H3	$1mg.ml^{-1}$	$5\mu g.ml^{-1}$		
αv	Chemicon	LM142	$1mg.ml^{-1}$	$5\mu g.ml^{-1}$	$10\mu g.ml^{-1}$	
αvβ3	Chemicon	LM609	$1mg.ml^{-1}$	$5\mu g.ml^{-1}$		
β1	Chemicon	P5D2	$1mg.ml^{-1}$	$5\mu g.ml^{-1}$	$10\mu g.\ ml^{-1}$	$50\mu g.ml^{-1}$

2.3.4 Integrin antibody concentration, binding and specificity

When analysing the results of experiments that use anti-integrin antibodies such as FACS (section 2.3.3), immunocytochemistry (section 2.3.5) and integrin functional studies such as adhesion and migration assays (section 2.4) it is important to take into account the specificity, relative affinity and concentration of the antibody. All antibodies used in this thesis were monoclonal and the concentration of mouse Immunoglobulins (Ig) present in the stock and working dilution are shown in table 2(b). Antibodies were diluted to give approximately equal Ig concentrations in the working solutions.

2.3.4.1 Antibody specificity

Recent studies investigating the nature of ligand binding by integrins have used integrin blocking antibodies and these studies have provided information on the binding specificity of these antibodies. The α5 integrin antibody used in this study (P1D6) has been shown to bind to the α integrin sub-unit in the N terminal integrin 'head' domain (Mould *et al.,* 1997). Antibodies raised against the collagen binding integrins such as P1E6 used in this thesis are thought to bind to the A domain of the α integrin sub-unit (see section 1.4.1) (Champe *et al.*, 1995). For further details see Humphries, (2002).

2.3.5 Immunocytochemistry

Immunocytochemistry was carried out on the MG63 osteosarcoma cell-line, C2C12 mouse myoblast cell-line and HOBs to show which integrins were expressed on the surface of cells grown, *in vitro*, on tissue culture plastic.

In preparation for the experiment, glass slides were sterilised by baking at 200°C for 10 minutes and placed in the wells of a sterile 24 well tissue culture plate. Cells were trypsinised at sub-confluency and resuspended in an appropriate amount of routine culture medium for counting, as described in section 2.1.3. The cells were resuspended to give a density of $1x10^4$ cells per ml of culture medium and were plated out onto the sterile coverslips with 1ml of routine culture medium per well and grown in an incubator for 12 hours. The medium was then removed and the cells were

washed three times in PBS and fixed for 10 minutes in 4% paraformaldehyde. After 10 minutes the paraformaldehyde was removed and the cells were again washed three times with PBS. Non-specific binding sites were blocked with PBS plus 1%BSA. This was to cover any non-specific binding-sites present on the cell preparation and lower background fluorescence. Cells were rinsed three times with 1xPBS plus 0.1% BSA. Cells for a negative control were plated out at the same time as the experimental cells and treated in exactly the same way as the experimental cells up to this point. Experimental cells were then incubated for one hour at room temperature with the primary integrin antibody diluted to the appropriate concentration in 1xPBS plus 0.1% BSA (see table 2(b)). The negative control cells were incubated with 1xPBS plus 0.1% BSA alone. The primary antibody was removed and the cells washed as previously. The secondary antibody, in this case FITC labelled rabbit anti-mouse, was then added to the experimental and control cells at a 1:100 dilution and incubated with the cells for 30 minutes at room temperature. The FITC is light sensitive so the experiment was wrapped in foil from this point onwards. After the 30 minute incubation the cells were then washed in PBS plus 0.1% BSA a total of three times. The coverslips were then mounted using Citifluor anti-fade mountant and viewed using a Leica DM light microscope, Leica Microsystems, Cambridge, UK.

2.4 Methods - Characterisation of integrin function in osteoblasts

2.4.1 Materials

The MTS detection kit and the MAPKK blocker U0126 were obtained from Promega UK, Ltd (Southampton, UK). The RGD blocking peptide, Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro was obtained from Sigma-Aldrich (Poole, Dorset, UK. Migration assay plates and inserts were obtained from Corning-Costar (UK distributors are Merck Eurolab, Poole, Dorest, UK).

2.4.2 Introduction to adhesion assays

Adhesion assays were carried out to ascertain the adhesion characteristics of both the MG63 cell-line and HOBs on the following ECM components:

- Plasma fibronectin;
- Type I collagen;

- 120kDa plasma fibronectin proteolytic fragment;
- 30kDa plasma fibronectin proteolytic fragment;
- 45kDa plasma fibronectin proteolytic fragment; and
- Vitronectin.

Adhesion assays were carried out using MG63 cells on plasma fibronectin and type I collagen with prior incubation of cells with blocking antibodies specific for the β1, α2 and α5 integrins. This was to investigate the importance of integrins in mediating the osteoblast-ECM adhesion. Experiments that showed any level of reduction on adhesion, after addition of blocking antibodies, were repeated on HOBs. Experiments were also carried out using both MG63 cells and HOBs with the addition of a blocking peptide containing the Arg-Gly-Asp (RGD) sequence. This sequence is present on fibronectin and is known to bind several integrins (Ruoslahti and Pierscbacher, 1987). The RGD blocking peptide used in this thesis was made up of the sequence Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro and binds to integrins recognising this sequence in the place of fibronectin, thus blocking integrin and fibronectin binding. Experiments were all carried out in triplicate.

The MTS detection system is a colorimetric assay (Promega). MTS is a tetrazolium reagent that, when taken in by living cells, is reduced to a coloured formazan product in the mitochondria. This reaction can be measured at 490-540nm wavelength on an ELISA plate reader. The manufacturers recommend this assay as a means of detecting live cell number. It actually provides a measure of enzyme activity within the cells and not a direct measure of cell number and this should be taken into when interpreting assay results. The manufacturers recommend that the assay has a sensitivity down to between 1000-5000 cells depending on cell type. MTS was used as a means of determining cell number in the adhesion assays.

2.4.3 Methods

The wells of a 96 well plate were coated with ECM substrate, in triplicate for each condition. 50μl of ECM substrate diluted in PBS was used and the plates were

incubated for one hour at 37°C. Plasma fibronectin, plasma fibronectin proteolytic fragments, type I collagen and vitronectin were used at a final concentration of 10μg.ml concentration. A triplicate of uncoated wells was used as a control and was treated in the same way as the experimental wells. After one hour, the coated wells were rinsed three times with 100μl PBS containing 2mM $MgCl_2$ and 1mM $CaCl_2$. Wells were then blocked with 100μl of PBS plus 0.5% BSA for one hour at 37°C. During the one hour blocking step, cells were trypsinised and diluted to give $1x10^6$ cells per ml. The cells were diluted in serum free medium. In this case, it was necessary to use DMEM as this was provided ready-made by the manufacturer and gave the consistency in colour that was required for the MTS assay. At this stage, if blocking antibodies or RGD blocking peptide were used in the assay, they were incubated with the cells for thirty minutes on ice, after vortexing to mix (see table 2(b) for details and concentrations of antibodies used). The RGD blocking peptide was stored as a stock of $1mg.ml^{-1}$n PBS and was used at 100μg ml^{-1}, $50\mu g.ml^{-1}$ and $10\mu g.ml^{-1}$. After the blocking step, wells were again rinsed as before, and cells were plated out at $1x10^5$ in 100μl of serum free medium. The assay was then incubated for four hours at 37°C. At this point cells were prepared and plated out for the standard curve (see following paragraph). After the incubation period, the wells were then rinsed three times by submersing the whole 96 well plate in PBS+ 2mM $MgCl_2$ and 1mM $CaCl_2$. After each submersion, the plates were blotted on paper towels to remove excess fluid and after the third final submersion the plate was turned upside down and tapped sharply over a paper towel. Fresh, serum free medium was then added to the experimental and control wells and the plate was incubated for a further hour at 37°C. 20μl of MTS solution was then added to each well and incubated with the cells for one hour at 37°C. For this step the plate was wrapped in foil as MTS is light sensitive. After one hour the plate was read on an ELISA plate reader at 492nm.

An MTS standard curve was also constructed for the adhesion assays. Cells were diluted to give a stock of $1x10^6$ cells per ml of serum free DMEM. 100μl of serum free medium was placed in six triplicates of wells (18 wells total). From the cell stock a triplicate of $1x10^5$ cells was plated out. Following this, 100μl of the cell stock was

added to the first well containing 100µl of medium, this was then mixed thoroughly by pipetting up and down. 100µl of cells diluted in medium was then removed from this well and placed in the next well on the right containing 100µl of serum free medium. As before, this was mixed by pipetting up and down and once again 100µl of medium plus cells was removed and placed in the well to the right. The sequence of mixing and pipetting was repeated for all the wells in this row. 100µl of the cell stock was then added to the next well in the first triplicate and the sequence of mixing and pipetting was repeated all along this row. This was also repeated for the final row of triplicates. This gave the following series of cell dilutions in triplicate: $1x10^6$, $1x10^5$, $5x10^4$, $2.5x10^4$, $1.25x10^4$, $6.125x10^3$ and $3x10^3$ as shown in table 2(c) below.

Table 2(c) - Cell dilutions

$1x10^6$	$1x10^5$	$5x10^4$	$2.5x10^4$	$1.25x10^4$	$6.125x10^3$	$3x10^3$
$1x10^6$	$1x10^5$	$5x10^4$	$2.5x10^4$	$1.25x10^4$	$6.125x10^3$	$3x10^3$
$1x10^6$	$1x10^5$	$5x10^4$	$2.5x10^4$	$1.25x10^4$	$6.125x10^3$	$3x10^3$

Cells were incubated at 37° C for a total of four hours, at the same time as the experimental plate. After the incubation period, 20µl of MTS was added to each well and incubated for a further hour at 37°C. This plate was also wrapped in foil to protect it from the light.

After one hour, the plate was read on an ELISA plate reader at 492nm. This gave an absorbance reading for each experimental well and the blank wells that contained medium alone. Firstly an MTS standard curve was constructed on Microsoft Excel using the absorbance readings from the standard plate. Cell number was directly proportional to absorbance. The equation of the standard curve was given by Microsoft Excel (2000) and was rearranged in order to ascertain the number of cells present in each of the experimental wells. The results were then plotted as histograms in which the plasma fibronectin result was taken as 100% and the other results were expressed as a percentage of this.

2.4.4 Immunocytochemistry of adhesion assays

Adhesion assays for immunocytochemistry were carried out in 24 well plates. The wells of a 24 well plate were coated with 200µl of ECM substrate, diluted in PBS (concentrations were the same as detailed in section 2.4.3). This was incubated for one hour at 37°C in an incubator. After one hour, the wells were rinsed three times in PBS plus 2mM $MgCl_2$ and 1mM $CaCl_2$. The wells were blocked with PBS plus 0.1% BSA for one hour at 37°C. Wells were rinsed as before, in PBS plus $MgCl_2$ and $CaCl_2$, a total of three times. Cells were trypsinised and diluted to a concentration of $5x10^4$ per ml and 1ml was plated out per well. Cells were incubated for four hours at 37°C with 5% $C0_2$. After the incubation period, the wells were then rinsed three times by submersing the whole plate in PBS+$MgCl_2$ and $CaCl_2$. After each submersion, the plates were blotted on paper towels to remove excess fluid. After the third submersion the plate was turned upside down and tapped sharply over a paper towel. The remaining cells were then fixed in 4% paraformalehyde and stained according to the protocol described in section 2.3.4.

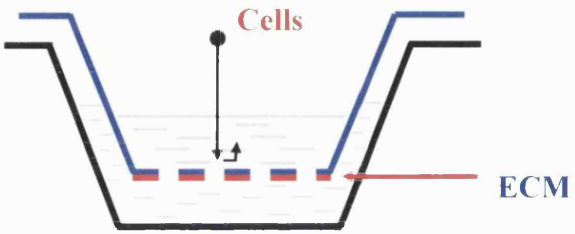
2.4.5 Migration assays

Migration assays were carried out using Boyden chamber migration inserts (figure 2(d)). Each insert sits within the well of a 24 well plate and contains a membrane with eight micron pores. These chambers were used with the underside of the membrane coated with ECM substrate and the cells placed in the upper portion of the insert chamber, on the upper surface of the membrane.

The undersides of migration assay inserts were coated with ECM substrate. This was carried out by pipetting 250µl of diluted ECM solution into the well of a migration assay plate and then placing the insert over this for one hour at 37°C. Plasma fibronectin and type I collagen were used at $10\mu g.ml^{-1}$. After a one hour coating period, the membrane was blocked in 0.5% BSA diluted in serum-free medium (migration buffer). This was carried out by pipetting 250µl of migration buffer into a well of the migration assay plate and then placing the membrane insert over this. This step was also carried out for one hour at 37°C. During the blocking step, cells were washed in PBS, trypsinised and resuspended in migration buffer at a dilution of $1x10^6$

cells per ml. 10^5 cells/migration insert in 100µl were required so the appropriate quantity of diluted cells was prepared, for example for $1x10^6$ cells in 1ml of migration buffer for 10 migration inserts. 10^5 cells per migration insert in 100µl were plated out and left to migrate for 0.5-4 hours in a 37°C incubator with 5% $C0_2$. For integrin blocking studies, cells were incubated with anti-integrin blocking antibodies for 30 minutes on ice, prior to plating out in inserts. See table 2(b) for details of antibodies and concentrations used. Migration assays were also carried out with the addition of the MAPK blocker UO126. Cells were incubated with U0126 on ice for 30 minutes prior to plating out. After the experimental period cells were fixed in 10% formalin and stained with Crystal Violet. The U0126 was stored at a stock concentration of 10mM and used at concentrations of 25µM, 50µM and 100µM. The cells were counted using light microscopy at a 20x magnification. The number of cells per mm^2 was then determined. For immunocytochemistry of the migrated cells, the membranes were fixed with 4% paraformaldehyde and the protocol for immunocytochemistry was followed, as given in section 2.3.4.

Figure 2(d) – Boyden chamber migration insert



The diagram above depicts a Boyden chamber migration assay insert. The underside of the insert membrane is coated with ECM substrate and cells are placed into the chamber of the insert

2.5 Statistical Analysis

2.5.1 Analysis of variance (ANOVA)

Alkaline phosphatase, osteocalcin, adhesion and migration assays were all carried out with triplicates of each condition on three separate occasions. Results were entered into the Instat statistical analysis package (Statistical Services Centre, Reading, UK). The data fulfilled the assumptions required for parametric analysis and so the ANOVA statistical test was used to test whether or not the means for each condition were equal. Although ANOVA will report a global difference between any of the experimental groups it will not show which of the groups are statistically different to each other, this requires the use of a post-hoc test. In chapter 3 the results of alkaline phosphatase and osteocalcin assays were analysed using ANOVA and Tukey's post-hoc test. Tukey's compares all the experimental results with each other and shows which are statistically different. In chapters 4 and 5 the results of adhesion and migration assays were analysed for statistical significance using ANOVA and Dunnet's post-hoc test. Dunnet's compares all the experimental groups to a control group and shows which experimental groups are significantly different to the control.

2.6 Scanning electron microscopy

(Cells were processed for electron microscopy in collaboration with N. Morden, Eastman Dental Institute).

2.6.1 Materials

Sodium cacodylate buffer and aluminium SEM stubs were purchased from Agar Scientific, Stansted, UK. Hexamethyldisilazane was purchased from TAAB Laboratories Ltd, Reading, UK. Carbon conductive cement was purchased from Neubauer Chemikalien, Munster, Germany and Polaron E500 Sputter was purchased from Quorum technologies Ltd, Newhaven, UK.

2.6.2 Method

Cells were cultured in the absence of serum on glass coverslips coated with either 10μg.ml plasma fibronectin or type I collagen for 3-6 hours prior to fixation in 3%

gluteraldehyde in 0.1M sodium cacodylate buffer at 4°C for 24hours. After dehydration, cells were transferred to hexamethyldislazane for 5 minutes and then allowed to dry. Coverslips were then mounted onto aluminium SEM stubs with carbon conductive cement, sputter coated with gold/palladium in a Polaron E500 Sputter Coater. Cells were viewed using a Cambridge Stereoscan 90B (LEO Electron Microscopy Ltd, Cambridge UK).

3 Chapter 3 – Results of characterisation studies

3.1 Aims of characterisation studies

The aim of this series of experiments was to:

- Investigate the presence of osteoblastic markers in MG63 osteosarcoma derived cells and HOBs using biochemical assays and immunohistochemistry; and
- Determine the profile of integrins expressed in these cells using FACS and immunocytochemistry.

3.2 Introduction

In vitro, osteoblasts are known to go through a series of differentiation stages, from precursor cell, becoming a pre-osteoblast and then a mature matrix-secreting cell. Each stage of differentiation is marked by the expression of specific genes and their products and these are often used as indicators of the osteoblastic phenotype. The presence of alkaline phosphatase can be detected in the pre-osteoblast and the mature matrix secreting osteoblast. Type I collagen and osteocalcin expression are associated with the mature matrix-secreting cell. At the same time, cells pass through stages of proliferation, ECM maturation and mineralisation. Figure 1(a) in chapter 1 summaries the process of osteoblast differentiation. *In vitro*, alkaline phosphatase production, osteocalcin and type I collagen provide reliable markers of the osteoblast phenotype.

In recent years, molecular studies have furthered our understanding of the factors involved in the control of osteoblast differentiation. In particular, several gene families have been shown to be involved in the control of osteoblast differentiation. Members of the homebox gene family have been shown to be important in osteoblast differentiation, for example *Dlx5* and *Dlx6* (Acampora *et al.,* 1999). In addition, *Cbfa1* is known to be important in the regulation of osteoblast differentiation and production of a mineralised matrix (Ducy *et al.,* 1997).

This thesis used two distinct sources of osteoblastic cells, the MG63 cell-line and human osteoblasts (HOBs). The following section will describe these cells briefly, for further information the reader is referred to section 1.2.7.

3.2.1 MG63 cells

MG63 cells are an osteosarcoma-derived cell-line, widely used in research. This cell-line exhibits many features that are representative of the osteoblast phenotype summarised in figure 1(c) in chapter 1. In this thesis, the secretion of alkaline phosphatase and osteocalcin and the effect of 1,25-dihydroxyvitamin D_3 on each of these were chosen as markers of the osteoblast phenotype. Franceschi *et al.* (1985) and Franceschi, (1988) showed that MG63 cells express low basal levels of alkaline phosphatase and osteocalcin, similar to that of osteoprogenitor cells. In addition, these cells were shown to synthesise both type I and type III collagen. 1,25-dihydroxyvitamin D_3 treatment caused an increase in each of these (Franceschi *et al.*, 1985; Franceschi and Young, 1990). Clover and Gowen, (1994) confirmed that MG63 cells express alkaline phosphatase, type I collagen and osteocalcin and both alkaline phosphatase and osteocalcin levels were increased by treatment with 1,25-dixydroxyvitamin D_3. Lajeunesse *et al.* (1990; 1991) also observed the secretion of osteocalcin in cultures of MG63 cells and an increase in this in response to 1,25-dixydroxyvitamin D_3 treatment.

3.2.2 Primary osteoblast cells (HOBs)

Cells exhibiting osteoblast characteristics have been grown from explants of trabecular bone *in vitro*. These cells were shown to synthesise type I collagen and alkaline phosphatase that was elevated in response to 1,25-dihydroxyvitamin D_3 treatment and were also shown to synthesise osteocalcin in response to 1,25-dihydroxyvitamin D_3 treatment. (Beresford *et al.*, 1983; 1986). Studies showed that these cells produced an extensive matrix, with mineralisation, when cultured in the presence of glucocorticoid, β-glycerophosphate and ascorbate *in vitro* (Beresford *et al.*, 1986). In addition, when these cells were implanted in vivo, within diffusion chambers, they produced tissue that resembled bone, although the successful

formation of mineralised matrix does appear to vary between cultures (personal communication with Dr JH Bennett).

3.2.3 Integrin expression in osteoblasts

Several studies have sought to determine the profile of integrins expressed in osteoblast cultures. It appears that a wide range of integrins are expressed, including α1, α2, α3, α4, α5, α6, α7, αv, αvβ3, β1, β3 and β5 (Clover *et al.,* 1992; Hughes *et al.,* 1993; Grzesik and Robey, 1994; Pistone *et al.,* 1996; Gronthos *et al.,* 1997) (as summarised in figure 1(c)). There is variation in the integrin profiles reported by each group although trends are emerging, with the α2, α5 and β1 integrins being consistently expressed between studies. The α2β1 and α5β1 heterodimers are type I collagen and fibronectin receptors respectively. Variations in integrin profile may be explained by variation in cell source, donor age, sampling site, technique and differences in antibody clones used. In addition, subtle variations in culture conditions could have a significant effect on integrin expression and it is often assumed that cell passage number may effect integrin expression. Previously, osteoblasts derived from explants of mandibular bone have been shown to strongly express α3, α5, αv, αvβ3, β3 and β1 integrin subunits with low expression levels of the α1, α2 and α4 subunits (Bennett *et al.,* 2001a; 2001b). Grzesik and Robey, (1994) reported the expression of α4 and αv sub-units on osteoblasts from trabecular explants. The β1, β3 and β5 sub-units were also shown to be present. Gronthos *et al.* (1997) showed that the integrin heterodimers α1β1, α2β1, α3β1, α5β1 and α5β1 were expressed on the surface of osteoblasts derived from trabecular explant culture. The α4β1 and α6β1 heterodimers were not expressed. Clover *et al.* (1992) reported the expression of α1, α2, α3 and β1 sub-units on osteoblasts derived from trabecular explant cultures. For a review of integrin expression and function in osteoblasts the reader is referred to Bennett *et al.* (2001b).

Previous studies using other cell types have shown that integrin expression is altered in transformed cells and this could be a problem when using osteoblastic cells lines (reviewed in Boudreau and Bissell, 1998). For example, in normal breast epithelial

cells the α6β4 integrin mediates interaction with laminin and induces growth arrest. In malignant cells signalling via the α6β4 integrin is blocked and signalling takes place via the β1 integrin instead, leading to continued cell growth (Zutter *et al.,* 1998). Changes in integrin expression associated with a malignant phenotype can result in altered ECM and integrin interaction, changes in integrin associated signalling and altered integrin and growth factor interaction. It should be noted that when using primary cultures, factors such as patient age, sex, health and anatomical site might affect the profile of integrins expressed, making results variable. In order to address some of these problems, in this thesis the integrin profile of HOBs derived from two anatomical sites was compared alongside that of MG63 cells. HOBs were derived from mandibular explants and these biopsies were obtained from patients undergoing wisdom tooth extraction. Due to the nature of the operation patients were all of a similar age, approximately 19-25 years old. In addition HOBs were analysed from hip and knee explants. Due to the nature of the operation, these samples were all taken from older patients. For functional studies HOBs derived from mandibular explant cultures were used.

The levels of alkaline phosphatase activity and the concentration of osteocalcin were determined in response to 1,25-dihydroxyvitamin D_3 treatment over a 7 day period, in the MG63 cell-line and HOBs derived from explants of mandibular bone. Previously HOBs have been grown successfully in the absence of β-glycerophosphate and ascorbate and therefore it was decided that these factors would not be included in the HOB explant cultures in this instance (Bennett *et al.*, 2001a). Once the osteoblastic nature of cells had been confirmed the integrin profile of both cell types was compared. All cells were used after the first cell outgrowth at confluency. The results of these studies are reported in the following sections.

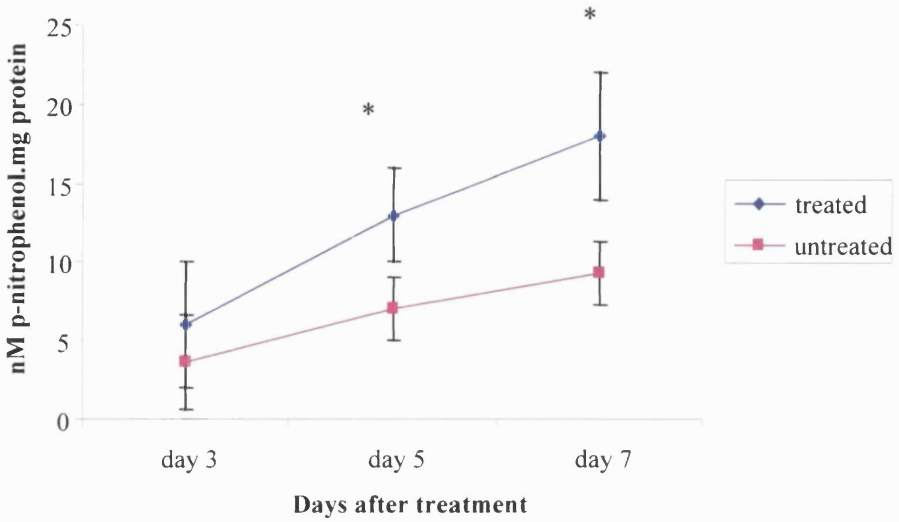
3.3 Results – Characterisation of osteoblasts

3.3.1 Determination of alkaline phosphatase activity of osteoblast-like cells

MG63 cells and HOBs were treated with 1,25-dihydroxyvitamin D_3 over a seven day culture period. The level of alkaline phosphatase present in cell lysates was measured

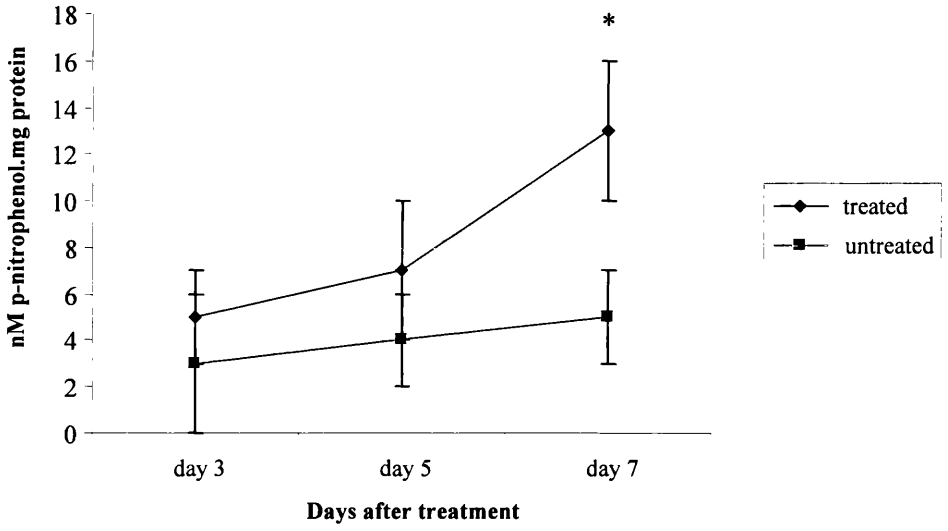
at days three, five and seven using a biochemical assay with p-nitrophenol substrate. Results were analysed for statistical significance using ANOVA and Tukey's post-hoc test. Figures 3(a) and 3(b) shows the mean results of three repeats with standard deviation SD, using MG63 cells and HOBs respectively. In untreated MG63 cells, alkaline phosphatase appeared to increase slightly the seven day culture period although this was not statistically significant. In the presence of 1,25-dihydroxyvitamin D_3, alkaline phosphatase levels increase by three fold at day seven. At days five and seven, alkaline phosphatase levels are significantly greater in MG63 cultures treated with 1,25-dihydroxyvitamin D_3 ($p<0.05$) than in untreated cultures. In untreated HOBs, alkaline phosphatase levels do not increase over the 7 day culture period. In contrast, 1,25-dihydroxyvitamin D_3 treatment of HOBs caused a 2.5 fold increase in alkaline phosphatase at day seven, when compared to day three. At days five and seven, alkaline phosphatase levels are significantly higher in treated versus untreated cultures ($p<0.05$).

Figure 3(a) - Alkaline phosphatase activity in MG63 cells in response to 1,25-dihydroxyvitamin D_3 treatment.



MG63 cells were grown in routine culture medium supplemented with 1,25-dihydroxyvitamin D_3 over a period of 7 days. At days 3, 5 and 7 of treatment cells were lysed and alkaline phosphatase enzymatic activity was determined using a colorimetric assay with a p-nitrophenol phosphatase substrate. MG63 cells express a basal level of alkaline phosphatase that is significantly increased by treatment with 1,25-dihydroxyvitamin D_3 (*$p<0.05$ when tested for statistical significance using ANOVA and Tukey's post-hoc test).

Figure 3(b) - Alkaline phosphatase activity in HOBs in response to 1,25-dihydroxyvitamin D_3 treatment.

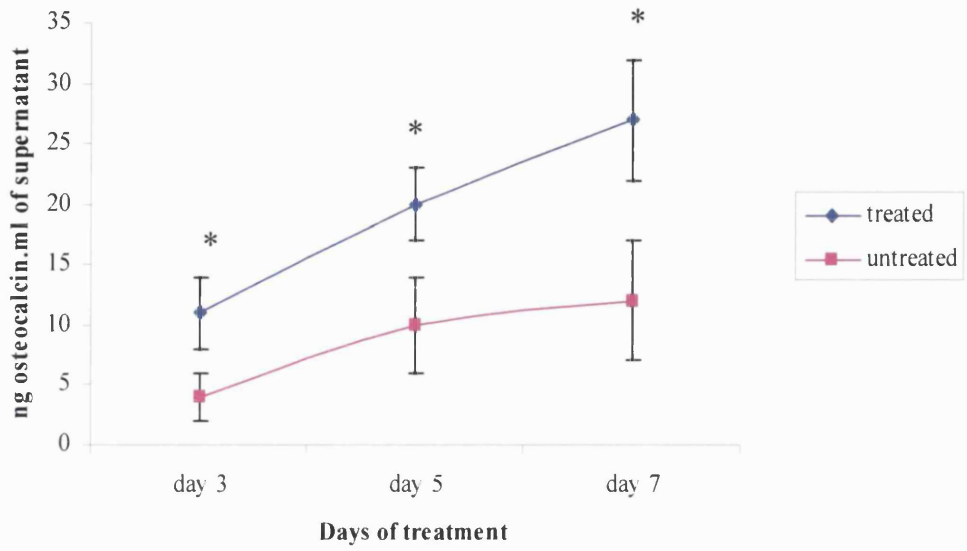


HOBs were grown in routine culture medium supplemented with 1,25-dihydroxyvitamin D_3 over a period of 7 days. At days 3, 5 and 7 of treatment cells were lysed and alkaline phosphatase enzymatic activity was determined using a colorimetric assay with a p-nitrophenol phosphatase substrate. In untreated HOBs, alkaline phosphatase levels do not increase over the 7 day culture period. 1,25-dihydroxyvitamin D_3 treatment of HOBs caused a significant increase at day 7 when compared to untreated controls (*$p<0.05$ when tested for statistical significance using ANOVA and Tukey's post-hoc test).

3.3.2 Determination of osteocalcin levels in osteoblast-like cells

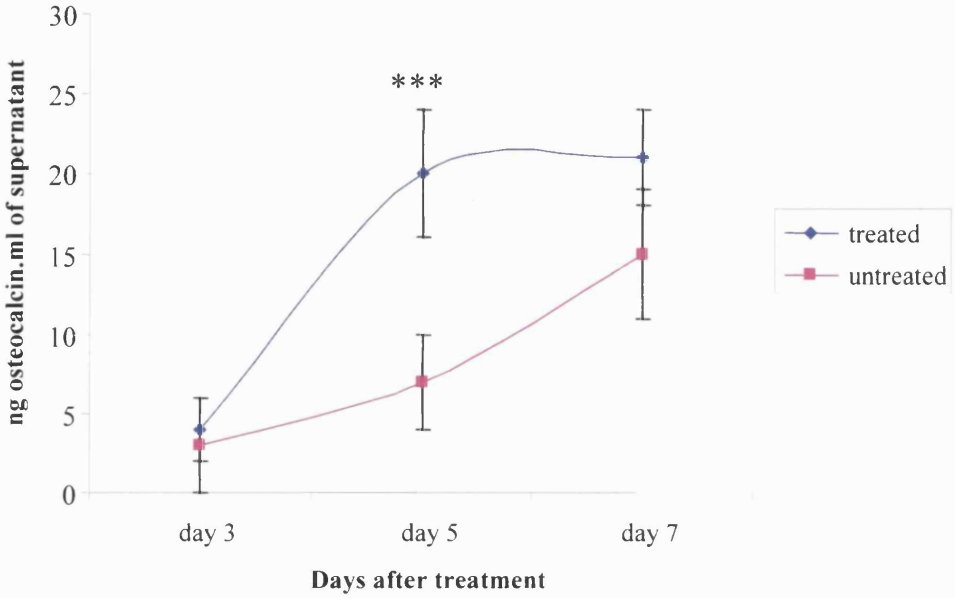
Figures 3(c) and 3(d) show osteocalcin levels in the supernatant of MG63 cells and HOBs respectively, after treatment with 1,25-dihydroxyvitamin D_3 over a seven day culture period. The histograms show the mean of three repeats with standard deviation. Results were analysed for statistical significance using ANOVA and Tukey's post-hoc test. MG63 cells show a slight increase in untreated cultures at day seven ($p<0.05$), with no significant increase at day five. Treatment of MG63 cells with 1,25-dihydroxyvitamin D_3 significantly increased levels of osteocalcin at days 3, 5 and 7 after treatment ($p<0.05$). HOBs showed a five fold increase in osteocalcin in untreated cultures by day seven. In the presence of 1,25-dihydroxyvitamin D_3, osteocalcin levels were significantly greater than untreated cultures at day five ($p<0.001$), levelling off at day seven.

Figure 3(c) – Osteocalcin levels in MG63 cells in response to 1,25 dihydroxyvitamin D_3 treatment



At days 3, 5 and 7 of treatment with 1,25-dihydroxyvitamin D_3 MG63 cell supernatants were removed and assayed for osteocalcin using a commercially available assay (Metra Biosystems). The histogram above shows the mean of three repeats with standard deviation (SD). Treatment of MG63 cells with 1,25-dihydroxyvitamin D_3 significantly increased levels of osteocalcin at days 3, 5 and 7 after treatment. MG63 cells show a 3 fold increase in untreated cultures at day 7 (*$p<0.05$ when tested for statistical significance using ANOVA and Tukey's post-hoc test).

Figure 3(d) – Osteocalcin levels in HOBs in response to 1,25-dihydroxyvitamin D_3 treatment



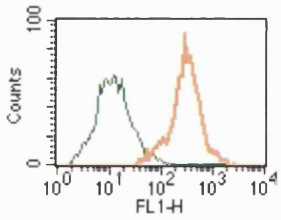
At days 3, 5 and 7 of treatment with 1,25-dihydroxyvitamin D_3 MG63 cell supernatants were removed and assayed for osteocalcin using a commercially available assay (Metra Biosystems). The histogram above shows the mean of three repeats with standard deviation (SD). Osteocalcin levels increased over the 7 day time course with levels significantly greater in treated versus untreated cultures at day 5 (***$p<0.001$ when tested for statistical significance using ANOVA and Tukey's post-hoc test).

3.4 Results - Characterisation of integrin expression in MG63 cells and HOBs

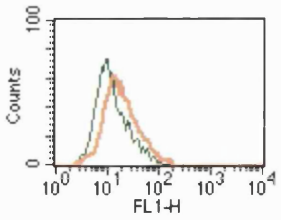
3.4.1 FACS analysis

The expression profile of integrin sub-units and heterodimers on the surface of both MG63 cells and HOBs was characterised using FACS analysis. Figures 3(e) and 3(f) shows the results of FACS analysis of MG63 cells and mandibular HOBs using specific integrin antibodies and FITC secondary antibody. A negative control with secondary antibody alone is shown on each histogram in green. The peak for the integrin antibody is shown in orange. A shift to the right of the green negative, by the orange experimental peak, indicates a positive result. Table 3(g) shows the geometric mean for FACS analysis with standard deviation (SD). This is derived from the area under the orange (experimental) peak. It should be noted that the geometric mean should only be taken as an estimate of expression level, as this value can be affected by factors, other than expression level, such as integrin binding affinity and specificity. According to the histograms and the resulting geometric means, MG63 cells were shown to express β1, α2, α3, α4, α5, α6 with no expression or very low levels of αv, αvβ3 and α1. HOBs derived from explants of mandibular bone were shown to express α2, α3, α 4, α5, α6, αvβ3 and β1 with low or negative expression of αvβ3 and α1 on the cell surface. HOBs derived from other sites, for example knee and hip were also tested for integrin expression but on <3 occasions. These showed expression of α1, α2, α3, α4, α5, α6, αv, αvβ3 and β1. Levels of αvβ3, α1 and α6 appeared to be low in hip derived cultures.

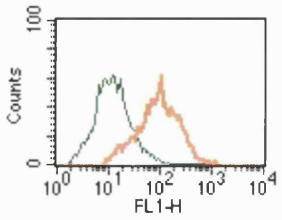
Figure 3(e) - FACS analysis of integrin expression on MG63 cells



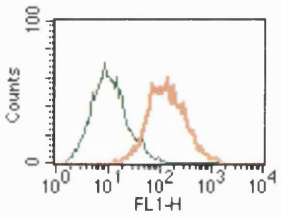
MG63-β1



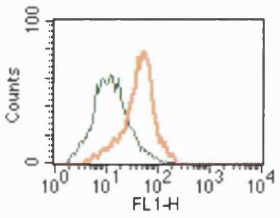
MG63-α1



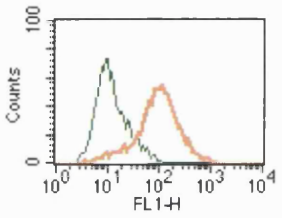
MG63-α2



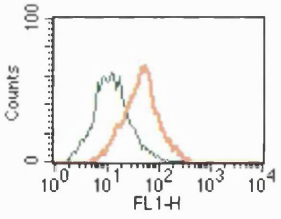
MG63-α3



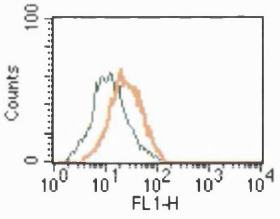
MG63-α4



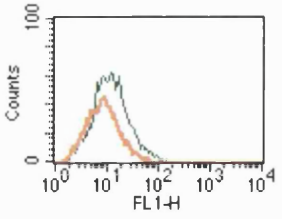
MG63-α5



MG63-α6



MG63-αv



MG63-αvβ3

Figure 3(e) above shows the results of FACS analysis of integrin expression on the surface of MG63 cells. Cells were treated with anti-integrin antibody (final concentration of 5μg.ml^{-1}) and FITC labelled secondary. The orange peak shows the fluorescence of cells labelled with anti-integrin antibody. The green peak shows the fluorescence of the negative (cells treated with FITC secondary alone).

Figure 3(f) - FACS analysis of integrin expression on HOBs

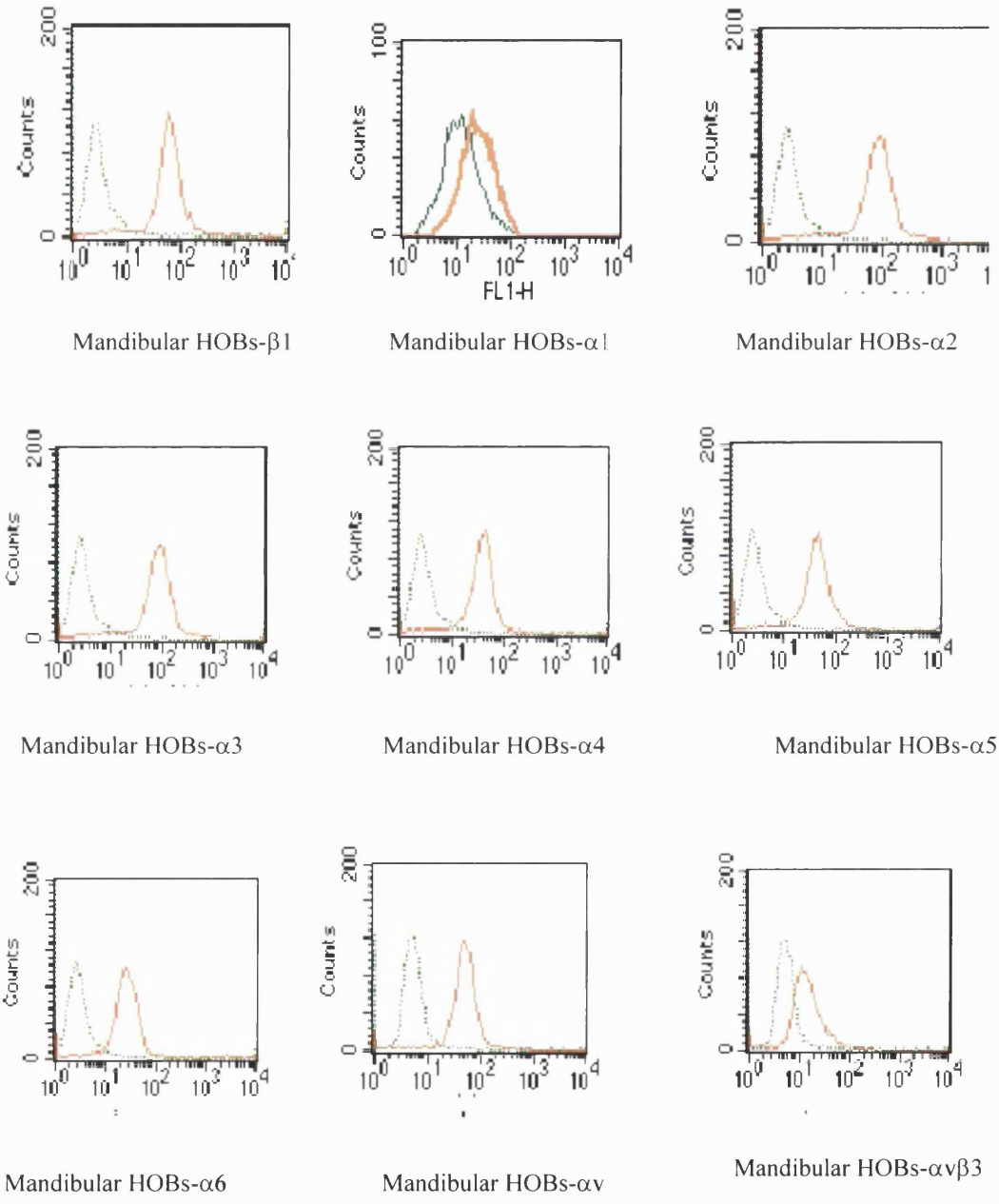


Figure 3(f) above shows the results of FACS analysis of integrin expression on the surface of HOBs. Cells were treated with anti-integrin antibody (final concentration of 5μg.ml) and FITC labelled secondary. The orange peak shows the fluorescence of cells labelled with anti-integrin antibody. The green peak shows the fluorescence of the negative (cells treated with FITC secondary alone).

Table 3(g) - Geometric means (GM) with standard deviations (SD) from the results of FACS analysis on MG63 cells and HOBs using integrin antibodies.

	MG63 cells		HOBs (mandible)	
Integrin antibodies	**GM**	**SD**	**GM**	**SD**
α1	7.5	5.85	10	3
α2	31.6	6.08	30.045	16.4
α3	94	19.6	15.3	3.6
α4	24.6	6.8	20.1	4.6
α5	37.2	7.8	25.4	15.5
α6	28.7	2.6	23	2.9
αv	5.7	2.1	29	4.5
αvβ3	7.8	4.8	5.865	1.9
β1	53.7	17.5	48	19.7
-ve mouse	5.1	4.2	4.2	1.9

The table above shows the results of FACS analysis using integrin antibodies. Analysis was repeated three times on MG63 cells and HOBs from mandibular explant cultures. Results are shown as mean geometric means for three repeats with SD.

3.4.2 Immunocytochemistry

In addition to FACS analysis, immunofluorescent staining was carried out for integrin sub-units on both MG63 cells and HOBs*. MG63 cells were stained for the α2, α3, α5, αv and β1 integrins. HOBs were stained for the β1 and α2 integrins. β1 staining in the HOBs, shown in 3(h) and 3(i), was very prominent with staining in punctate spots, intensifying at the cytoplasmic margins. Areas of bright staining were noted in cell extensions. Areas of staining were also noted on the surface of the slide. Whilst this might be non specific staining, it may also reflect the fact that cells in culture are not stationary and can leave trails of old adhesions, including integrins on the substrate (Palecek *et al.,* 1996). Staining for α2 was not as intense but could still be noted as a diffuse pattern over the cell surface, shown in 3(j). A negative control of cells incubated with secondary antibody alone is shown in figure 3(k). Subsequent studies using the same methodology, confirmed the presence of α2, α3, α5, αv and β1 integrins on MG63 cells.

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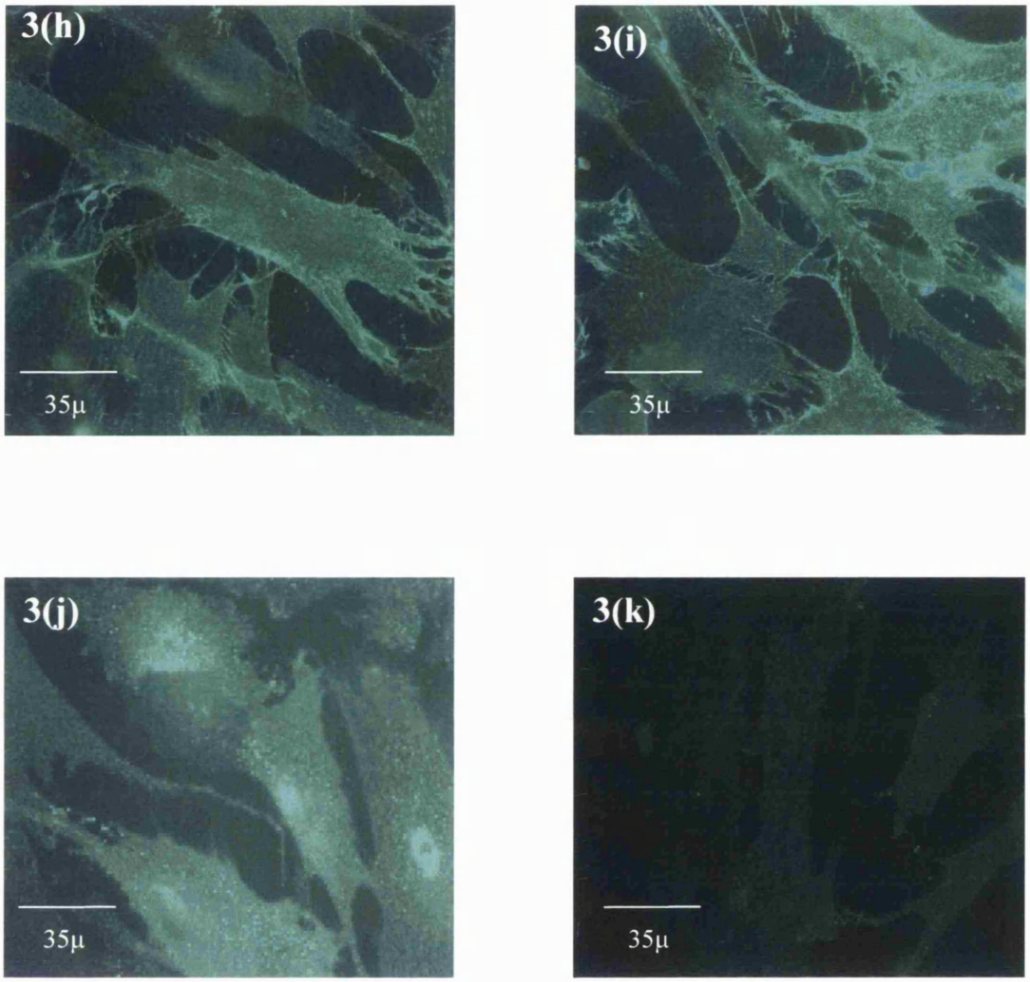
* This work was carried out in collaboration with Dr Li Xiao Bing, Department of Orthodontics, West China College of Stomatology, Cheng-Du, China whilst a visiting fellow at the Eastman Dental Institute 2001-2002. Accepted for presentation at the 'International Conference on Osteoporosis and Bone Research' Beijing, China- May 23rd- 27th 2003.

Figure 3(h)-(k) - Integrin expression in HOBs



Figures 3(h) and 3(i) show HOBs stained for β1 integrin in vitro and 3(j) stained for α2 integrin in vitro. Cells were grown overnight on glass coverslips in routine culture medium and then stained with integrin antibodies, followed by FITC labelled secondary antibody. β1 integrin staining was prominent, particularly at cell to cytoplasm contacts and in cell processes. α2 staining was less prominent but visible as punctate spots over the cell surface with patches of intense intracellular staining rather than at cytoplasmic processes. 3(k) shows a negative control with cells incubated with secondary antibody alone. Staining was visualised using a LEICA DB microscope.

3.5 Discussion

3.5.1 Expression of osteoblast characteristics

This study has used two sources of osteoblastic cells: MG63 cells and HOBs. As shown by the results of these characterisation studies, both MG63 cells and HOBs are representative of the osteoblastic phenotype. MG63 cells synthesise alkaline phosphatase and osteocalcin and levels of both of these are increased in response to 1,25-dihydroxyvitamin D_3 treatment. This concurs with the results of previous studies (Clover and Gowen, 1994; Franceschi *et al.,* 1985). The HOBs derived from mandible and hip showed synthesis of alkaline phosphatase and osteocalcin and levels of both of these were increased in response to 1,25-dihydroxyvitamin D_3 treatment. This is in agreement with previous studies carried out at the Eastman Dental Institute using cells derived from mandibular explant cultures (unpublished results of Dr J.H. Bennett and Ms A. Alavi) and the observations of Beresford et al. (1983; 1986) when characterising cells from trabecular explant cultures.

Studies by Lian and Stein, (1993) reported the sequential proliferation and differentiation of osteoblasts *in vitro*, with expression of alkaline phosphatase early in the sequence and osteocalcin expression associated with increasing differentiation. The HOBs used in this study express alkaline phosphatase at a constant basal level over a seven day culture period. Osteocalcin is present at a low level until in an increase at day five, levelling off at day seven. This suggests that cells could be becoming increasingly differentiated over the culture period. It is likely that some cells are more or less differentiated than others. In contrast, the MG63 cells used in this study exhibit a slight increase in alkaline phosphatase expression over the culture period. In addition, osteocalcin levels increase steadily over the seven day culture period. This suggests that MG63 cells are undergoing osteoblast differentiation. The expression of genes associated with osteoblast proliferation for example *c-myc, c-fos* and the cyclins has been used in previous studies (Lian and Stein, 1993) and could be employed in future experiments to clarify the proliferation stage of the HOBs used in this study. In addition the presence of osteoblast markers such as alkaline phosphatase, osteocalcin and type I collagen can be determined at the mRNA level using RT-PCR and this could be used as a further determinant of the osteoblastic

nature of the cells used in this study. Treatment of both MG63 cells with 1,25 dihydroxyvitamin D_3 caused an increase in alkaline phosphatase at day five and seven and osteocalcin at days three, five and seven, when compared to untreated controls. HOBs show an increase in alkaline phosphatase at day seven and osteocalcin at day five when treated with 1,25-dihydroxyvitamin D_3. This provides further evidence for the osteoblast nature of MG63 cells and HOBs. Previous studies have used 1,25-dihydroxyvitamin D_3 induced increase of alkaline phosphatase and osteocalcin as a marker of the osteoblast phenotype in MG63 cells (Franceschi *et al.,* 1985; Franceschi and Young, 1990; Clover and Gowen, 1994) and HOBs (Beresford *et al.,* 1983; 1986).

3.5.2 Profile of integrins expressed on osteoblasts

Using FACS analysis, both MG63 cells and mandibular HOBs were shown to express α2, α3, α4, α5, α6 and β1. HOBs were also shown to express αv but both cell types had low or negative expression of αvβ3 and α1. It could be that HOBs express other αv integrins, such as αvβ5 and αvβ6 although this was not investigated in this thesis. The integrins α1 and α2 are both collagen binding integrins. Previous studies suggest that α1 binds collagen type IV preferentially, where as α2 tends to bind collagen type I (Kern *et al.,* 1993). This would suggest that both MG63 cells and HOBS bind collagen type I rather than collagen type IV. The integrin profile of HOBs grown from hip and knee explants was tested and each showed the same pattern of integrin expression although FACS analysis using the hip derived cells would need to be repeated to give conclusive results. The presence of α2, α3, α5 and β1 integrin sub-units on MG63 cells was confirmed by fluorescent immunocytochemistry. In addition the αv integrin was detected by this method. The integrins β1 and α2 were stained in HOBs derived from mandibular cultures. It would be valuable to quantify the level of staining in the images in order to make a direct comparison of integrin abundance.

It is interesting to note that both types of cells used in this study appear to have a very comparable integrin profile. Other cells types do appear to show different integrin profiles in 'normal' versus a 'transformed' state. For example, a reduction in α5β1

has been associated with malignant cells (Plantefaber and Hynes, 1989; Giancotti and Ruoslahti, 1990; Ruoslahti, 1994). It could be, that in terms of integrin expression, the MG63 cell-line would provide a useful *in vitro* model, though integrin mediated signaling and integrin-ECM interaction may differ between the two osteoblastic systems. Further studies would be needed to investigate this further.

Results of the present study differ from those obtained previously at the Eastman Dental Institute with HOBs derived from mandibular explants. This previous study reported high expression of α3, α5, αv, αvβ3, β3 and β1 integrin subunits and low expression of α1, α2 and α4 subunits (Bennett *et al.*, 2001a). In contrast, Clover *et al.* (1992) reported absence of α4, α5, α6, β3 and also the αv integrin sub-units when analysing the integrin profile of trabecular explant derived HOBs using FACS. Variation could be due to a number of differences, as follows:

- Variation in anatomical site;
- Age and sex of donor;
- Health of donor;
- Variation in experimental methodology; and
- Artefacts of cell culture.

To date, there have been no published studies investigating the effect of anatomical site and age on integrin expression. It is known that integrins and ECM expression does change with ageing (reviewed in Labat-Robert, 2001).

Both MG63 cells and HOBs express the α2β1 integrin that has been shown, in non-bone systems, to bind and signal through the most abundant ECM component in bone, type I collagen (Zutter, 1995). In addition the α5β1 integrin antibody is present in both cell types. Previous studies using osteoblasts have shown that the interaction of fibronectin with the osteoblast, probably via the α5 integrin is important in differentiation and survival of the cell (Globus *et al.*, 1998; Moursi *et al.*, 1996; 1997). Although the abundance and distribution of fibronectin is not known in human bone, it is possible that this interaction is also important in human osteoblastic cells.

3.6 Summary of results

The results of this series of experiments can be summarised as follows:

- The MG63 cells and HOBs used in this study express alkaline phosphatase and osteocalcin and the expression of both of these is increased in response to 1,25-dihydroxyvitamin D_3 treatment;
- Both MG63 cells and HOBs express $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 6$, αv and $\beta 1$; and
- Both cell types had an absence of $\alpha v \beta 3$ and $\alpha 1$

Once the osteoblast characteristics and integrin profile of both MG63 cells and HOBs had been determined, the next series of experiments sought to investigate the function of specific integrins in osteoblasts. The results of these are described in chapter 4 and chapter 5.

4 Chapter 4 – Integrins and ECM interaction: an important relationship in osteoblast behaviour

4.1 Aims of adhesion assays

The aim of this series of experiments was to:

- Compare the level of MG63 and HOB adhesion to plasma fibronectin, type I collagen, vitronectin, 120kDa plasma fibronectin fragment, 45kDa plasma fibronectin and the 30kDa plasma fibronectin fragment; and
- Investigate the effect of specific integrin blocking antibodies on MG63 and HOB adhesion.

4.2 Introduction to integrins and adhesion in osteoblasts

It has been suggested that ECM binding is important for osteoblast function and survival and several studies have been carried out to investigate the nature of this interaction. In general, these sought to determine the integrin profile of osteoblasts, investigate the adhesion profile of osteoblastic cells on a series of ECM substrates and, in addition, elucidate the importance of specific integrins in osteoblast to ECM adhesion (Clover *et al.,* 1992; Grzesik and Robey., 1994; Pistone *et al.,* 1996; Gronthos *et al.,* 1997). The results of these studies showed that osteoblasts adhere at high levels to several matrix components, including plasma fibronectin and type I collagen. Addition of specific integrin blocking antibodies showed that β1 integrins are of particular importance in integrin-mediated adhesion. Further recent studies have shown that α5β1 interaction with fibronectin and α2β1 interaction with collagen are important in the control of osteoblast behaviour. It has been demonstrated that rat osteoblasts require fibronectin, in particular the central-cell binding domain, in order to form mineralised nodules in vitro (Moursi *et al.,* 1996). It was later shown, by the same group, that the α5β1 integrin is expressed in these cultures and that addition of blocking antibody to this receptor reduced nodule formatione to less than 5% of the control (Moursi *et al.,* 1997). In addition, osteoblasts underwent apoptosis when anti-fibronectin antibodies were added to the cultures. Alkaline phosphatase and osteocalcin were suppressed at the mRNA level in these cultures (Moursi *et al.,* 1997;

Damsky, 1999). Binding of osteoblasts to type I collagen, via the α2 integrin, is involved in the expression of osteoblastic markers. Xiao *et al.* (1998) showed that disruption of α2 integrin binding to type I collagen, with either blocking antibodies or the DGEA peptide, prevented expression of alkaline phosphatase. Jikko *et al.* (1999) showed that addition of anti-collagen I or both anti-α1 and anti-α2 antibodies to osteoblast cultures significantly reduced the expression of early osteoblast markers and also reduced the normal mineralisation of these cultures. Several integrins bind to ECM substrates via their RGD sequence (Arg-Gly-Asp); for example the α5β1 and the αv integrin (Ruoslahti and Pierschbacher, 1987). Studies have shown that addition of RGD blocking peptide will reduce adhesion of osteoblasts on plasma fibronectin (Pistone *et al.,* 1996; Aarden *et al.,* 1996) and disrupt the mineralisation of bone cell cultures and bone resorption (Gronowicz and Derome, 1994).

Although it is clear that the β1 integrins are involved in the relationship between osteoblast and ECM, in particular the α2 and α5 integrins, more evidence is needed to further characterise the relationship between integrins and ECM binding in osteoblasts. Many previous studies have used cell-lines or rodent derived cells and it would be valuable to carry out these experiments in a human model. Although the use of rat calvarial derived osteoblasts is a valuable in vitro model, it has been shown that there are differences between rat calvarial and human osteoblasts in vitro (Siggelkow *et al.,* 1999).

The results of adhesion assays could yield important preliminary data as to the adhesion profile of HOBs and MG63 cells and the importance of particular integrins in these interactions upon which further studies could be based. There is also a need for further evidence as to which integrins on the osteoblast surface bind to which ECM components.

In this thesis the adhesion characteristics of both MG63 cells and HOBs were investigated on a series of ECM substrates. Once the adhesion profile of the cells had been determined, adhesion assays were carried out to determine the role, if any, of

integrins in osteoblast adhesion. The results of these studies are described in the following section.

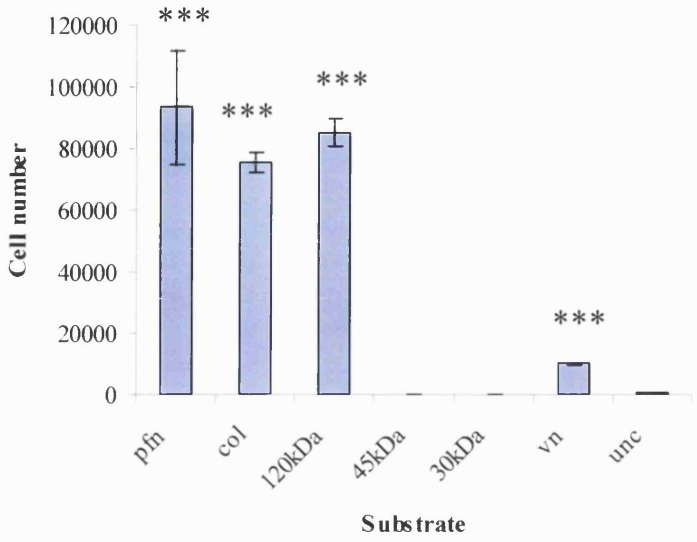
4.3 Results - Adhesion assays on ECM substrates

Adhesion assays were carried out to investigate the expression profile of HOBs and MG63 cells on plasma fibronectin, type I collagen, 120kDa plasma fibronectin proteolytic fragment (120kDa), 30kDa plasma fibronectin proteolytic fragment (30kDa), 45kDa plasma fibronectin proteolytic fragment (45kDa) and vitronectin. Each experiment was carried out in triplicate and the mean results are shown with the standard deviation (SD). ANOVA was used to test the statistical significance of results with a Dunnet's post-hoc test. Results were compared to an uncoated control.

Figures 4(a) and 4(b) show the results of adhesion assays carried out with MG63 cells and HOBs on plasma fibronectin, type I collagen and vitronectin and also fragments of plasma fibronectin- 30kDa, 45kDa and 120kDa. The latter fragment contains the central cell binding domain and the RGD sequence (see figure 1(f)). Figure 4(a) shows the results of adhesion assays using MG63 cells. The number of adherent cells was significantly greater on plasma fibronectin, type I collagen and the 120kDa plasma fibronectin fragment when compared to uncoated control ($p<0.001$). There was also a significant cell number adherent on vitronectin ($p<0.001$) with 17% (SD 1%) of the total cell number detected. Adhesion to the 30kDa and 45kDa fragments was not detectable. A proportion of cells were adherent on uncoated tissue plastic with 5% of the total cell number detected (SD 3%).

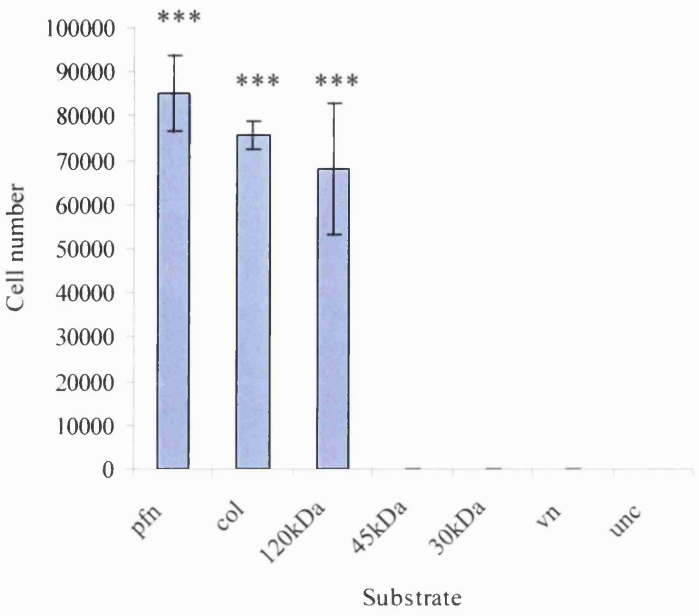
Figure 4(b) shows the results of adhesion assays using HOBs. The number of adherent cells was significantly greater on plasma fibronectin, type I collagen and the 120kDa fragment when compared to the uncoated control ($p<0.001$). Cells were not present at detectable levels on vitronectin, the 30kDa and 45kDa fragments and uncoated tissue culture plastic. The number of MG63 cells and HOBs adherent on plasma fibronectin, type I collagen and the 120kDa plasma fibronectin were similar, with no significant difference between them.

Figure 4(a) – MG63 adhesion on ECM substrates



MG63 cells were plated out with 10^5 cells/well for four hours on tissue culture plates coated with plasma fibronectin (pfn), plasma fibronectin proteolytic fragments (120ka, 45kDa and 30kDa), type I collagen (col) and vitronectin (vn). Uncoated tissue culture plastic was used as a control (unc). Non-adherent cells were removed and cell number determined using an MTS colorimetric assay. A high proportion of cells were adherent on pfn, col and the 120kDa. A small proportion of cells were adherent on vitronectin with no adhesion on 45kDa and 30kDA fragments and uncoated tissue culture plastic. Results were tested for statistical significance using ANOVA and Dunnet's post-hoc test (***$p<0.001$ when compared to uncoated controls).

Figure 4(b) – HOB adhesion on ECM substrates



HOBs were plated out for four hours with 10^5 cells/well on tissue culture plates coated with plasma fibronectin (pfn), plasma fibronectin proteolytic fragments (120ka, 45kDa and 30kDa), type I collagen (col) and vitronectin (vn). Uncoated tissue culture plastic were used as controls (unc). Non-adherent cells were removed and cell number determined using an MTS colorimetric assay. A high number of cells were adherent on pfn, col and 120kDa fragment with no adhesion on 45kDa and 30kDa fragments, vn and uncoated controls. Results were tested for statistical significance using ANOVA and Dunnet's post-hoc test (***$p<0.001$ when compared to uncoated controls).

4.4 Results - Adhesion assays with addition of RGD blocking peptide

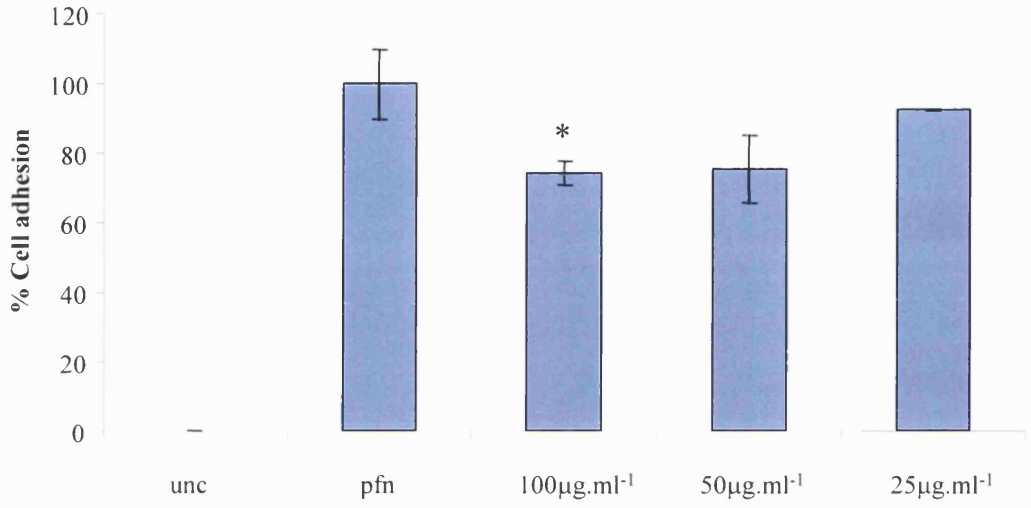
Adhesion assays were carried out with the addition of the RGD blocking peptide with the amino acid sequence Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro. This peptide has been shown to block RGD dependent cell binding (Ruoslahti and Pierschbacher, 1987. These experiments were carried out with MG63 cells on both plasma fibronectin and type I collagen and with HOBs on plasma fibronectin. Controls were plated out on plasma fibronectin alone with an equivalent quantity of PBS with 0.1% BSA to experimental wells. (The RGD containing peptide was diluted in PBS with 0.1% BSA). Each experiment was carried out in triplicate and the mean results are shown with the standard deviation (SD). ANOVA was used to test the statistical significance of results with a Dunnet's post-hoc test.

Figure 4(c) show the results of adhesion assays using MG63 cells with the addition of the RGD blocking peptide on plasma fibronectin. Addition of the RGD blocking peptide at the concentration of $100\mu g.ml^{-1}$ reduced adhesion to 74.5% (SD 3.6%) of the control in the absence of the inhibitor ($p<0.05$). Addition of the peptide at the lower concentrations of $50\mu g.ml^{-1}$ and $10\mu g.ml^{-1}$ did not reduce adhesion significantly.

Figure 4(d) shows the results of an adhesion assay with MG63 cells on type I collagen; addition of the RGD peptide did not have a significant effect at any of the concentrations used.

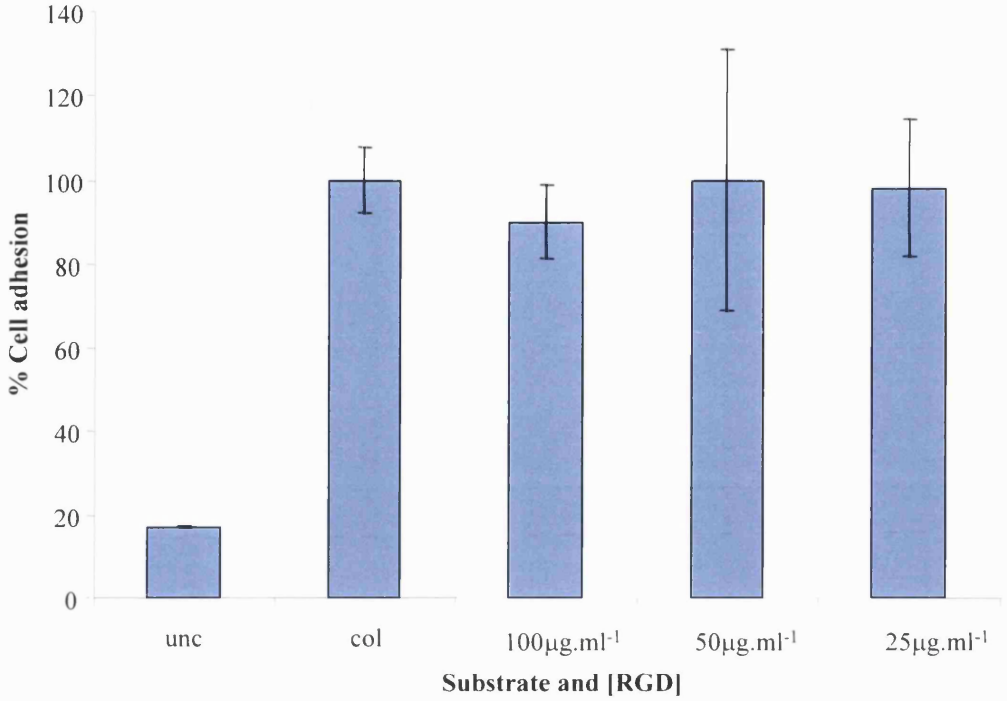
Figure 4(e) shows the results of adhesion assays on plasma fibronectin using HOBs with the addition of the RGD blocking peptide. The RGD blocking peptide significantly reduced adhesion at each of the tested concentrations, 59.4% (SD 2.9%) of the control at $10\mu g.ml^{-1}$, 60.4% (SD 7.8%) at $50\mu g.ml^{-1}$ and 74% (SD 6%) at $100\mu g.ml^{-1}$ ($p<0.05$ when compared to the control in the absence of the inhibitor).

Figure 4(c) – MG63 adhesion on plasma fibronectin with RGD blocking peptide



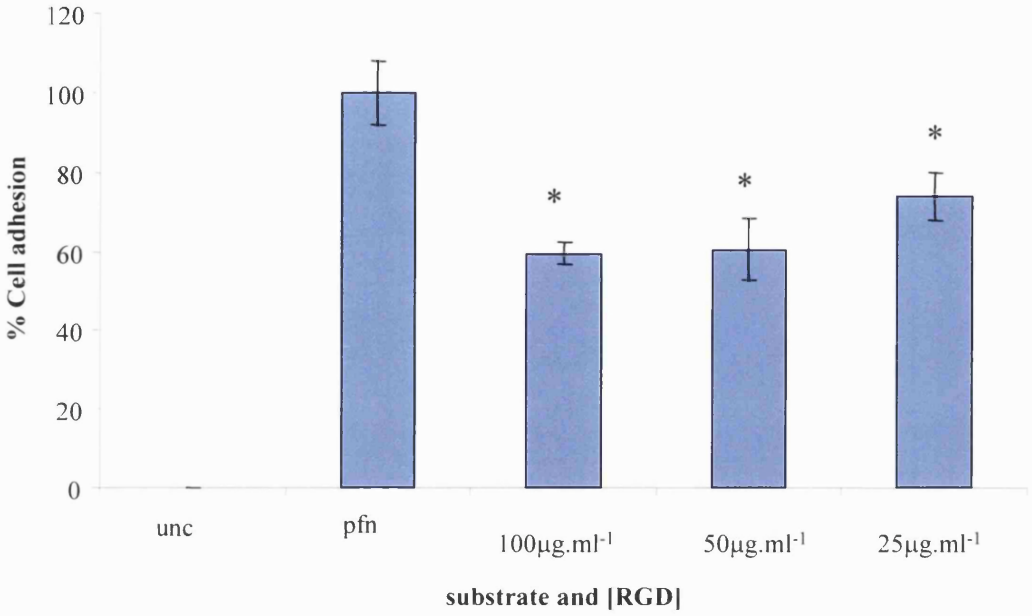
MG63 cells were plated out for 4 hours on tissue culture plastic coated with plasma fibronectin with the addition of 100µg.ml^{-1}, 50µg.ml^{-1} and 25µg.ml^{-1} of RGD blocking peptide. Non-adherent cells were removed and cell number was determined using an MTS colorimetric assay. Adhesion of MG63 cells on plasma fibronectin was reduced significantly ($p<0.05$) with the addition of 100µg.ml^{-1} RGD blocking peptide. Results were tested for statistical significance using ANOVA and Dunnet's post-hoc test (*$p<0.05$ when compared to pfn alone).

Figure 4(d) – MG63 adhesion on type I collagen with the RGD blocking peptide



MG63 cells were plated out for four hours on tissue culture plastic coated with type I collagen with the addition of 100μg.ml^{-1}, 50μg.ml^{-1} and 100μg.ml^{-1} of RGD blocking peptide. Non-adherent cells were removed and cell number was determined using an MTS colorimetric assay. Addition of RGD blocking peptide did not result in a significant change in HOB adhesion on type I collagen.

Figure 4(e) – HOB adhesion on plasma fibronectin with the RGD blocking peptide



HOBs were plated out for four hours on tissue culture plastic coated with plasma fibronectin with the addition of, $100\mu g.ml^{-1}$,$50\mu g.ml^{-1}$ and $25\mu g.ml^{-1}$ RGD blocking peptide. Non-adherent cells were removed and the number of adherent cells was determined using an MTS colorimetric assay. Adhesion of HOBs was reduced at all concentrations of RGD blocking peptide when compared to the control in the absence of inhibitor. Results were tested for statistical significance using ANOVA and Dunnet's post-hoc test (*$p<0.05$ when compared to control with no inhibitor).

4.5 Results - Adhesion assays with addition of integrin blocking antibodies

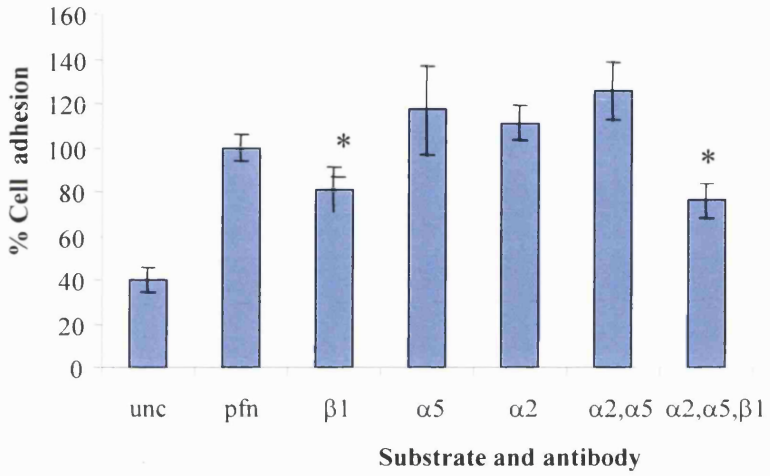
The results of the previous section showed that MG63 and HOB adhesion on plasma fibronectin was reduced by the addition of RGD blocking peptide. This suggests that integrin binding, via the RGD sequence, could be important for osteoblast adhesion on plasma fibronectin. Previous studies showed that the $\alpha2\beta1$ and $\alpha5\beta1$ integrin interaction with ECM could be important in mediating osteoblast behaviour. Taking these observations into account, the next set of adhesion assays were carried out with the addition of blocking antibodies specific for the integrin sub-units $\alpha2$, $\alpha5$ and $\beta1$. Experiments were carried out on plasma fibronectin, type I collagen and the 120kDa plasma fibronectin fragment and both MG63 cells and HOBs were used. Each experiment was carried out in triplicate and the mean results are shown with the standard deviation (SD). ANOVA was used to test the statistical significance of results with a Dunnet's post-hoc test with the substrates alone as a control.

Figure 4(f) shows the results of experiments carried out with MG63 cells on plasma fibronectin. Addition of the $\beta1$ integrin-blocking antibody alone, at a concentration of $50\mu g.ml^{-1}$, reduced adhesion by 19% (SD 10%) and in combination with the $\alpha2$ and $\alpha5$ ($50\mu g.ml^{-1}$) blocking antibodies, adhesion was reduced by 24% (SD 8%) ($p<0.05$).

Figure 4(g) shows the results of adhesion assays carried out with MG63 cells on type I collagen. Addition of the $\beta1$ integrin-blocking antibody alone, at $50\mu g.ml^{-1}$, reduced adhesion by 26% (SD 6%) ($p<0.05$) and by 15% (SD 4%) in the presence of $\alpha2$, $\alpha5$ and $\beta1$ by 15% respectively ($p<0.05$). Addition of $\alpha2$ reduced adhesion slightly but this result was not statistically significant.

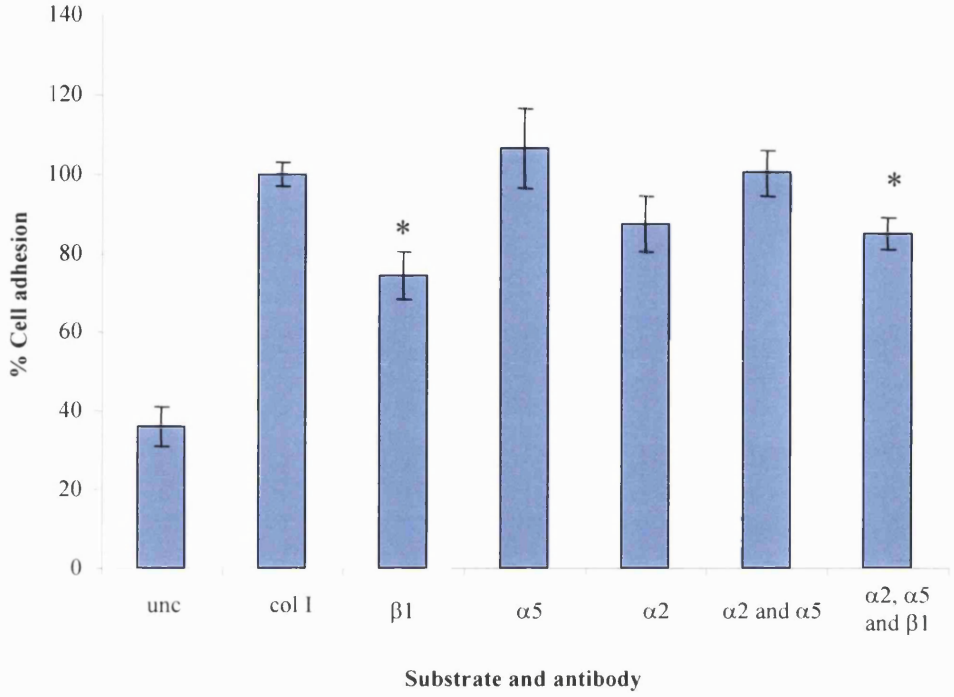
Adhesion assays were carried out on HOBs with addition of the $\beta1$ integrin blocking antibody on both plasma fibronectin and type I collagen, results shown in Figure 4(h). Addition of the $\beta1$ integrin-blocking antibody to HOBs reduced adhesion on plasma fibronectin by 15 % (SD 5.3%) and on type I collagen by 15% (SD 6.2%) ($p<0.05$).

Figure 4(f) – MG63 adhesion on plasma fibronectin with addition of integrin blocking antibodies



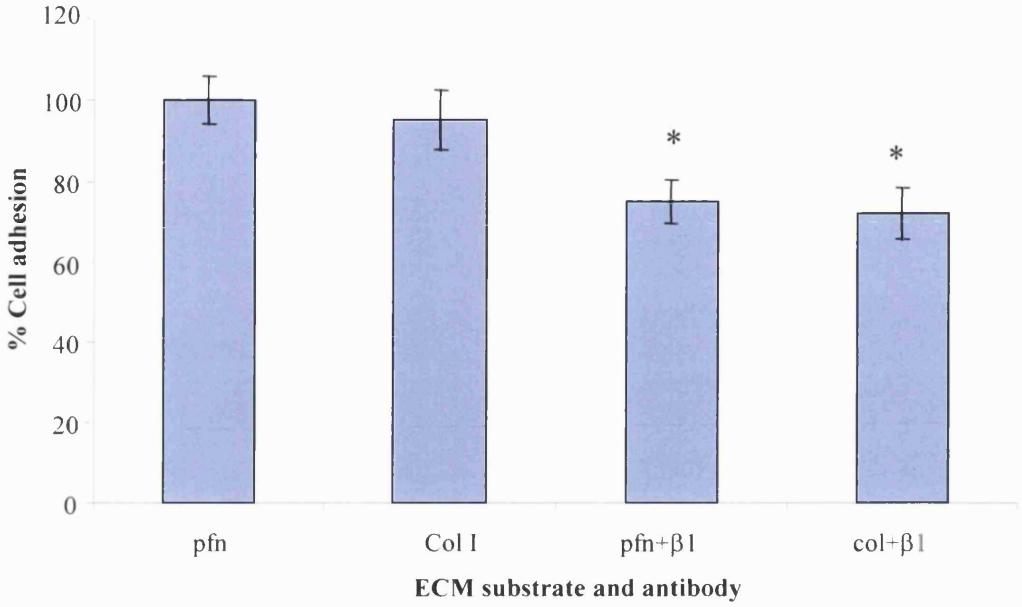
MG63 cells were incubated with 50μg.ml of β1, α2 and α5 integrin blocking antibodies for 30 minutes prior to plating out on tissue culture plastic coated with plasma fibronectin (pfn)with an uncoated control (unc) for four hours. Non-adherent cells were removed and adherent cell number was determined using an MTS colorimetric assay. Adhesion of MG63 cells was reduced with the addition of β1 integrin blocking antibodies both alone and in combination. Results were tested for statistical significance using ANOVA and the Dunnet's post-hoc test (*$p<0.05$ when compared to cells plated out on plasma fibronectin alone).

Figure 4(g) – MG63 adhesion on type I collagen with the addition of integrin blocking antibodies



MG63 cells were incubated with 50μg.ml of β1, α2 and α5 integrin blocking antibodies for 30 minutes prior to plating out on tissue culture plastic coated with type I collagen (col I) for four hours with uncoated control (unc). Non-adherent cells were removed and adherent cell number was determined using an MTS colorimetric assay. Adhesion was reduced with addition of β1 integrin blocking antibody both alone and in combination. Results were tested for statistical significance using ANOVA and the Dunnet's post-hoc test (*$p<0.05$ when compared to cells plated out on plasma fibronectin alone).

Figure 4(h) – HOB adhesion on plasma fibronectin and type I collagen with the addition of integrin blocking antibodies



HOBs were incubated with 50μg.ml of β1 integrin blocking antibody for 30 minutes prior to plating out on tissue culture plastic coated with plasma fibronectin (pfn) and type I collagen (col I) for four hours. Non-adherent cells were removed and adherent cell number was determined using an MTS colorimetric assay. Addition of the β1 integrin blocking antibody reduced adhesion on both plasma fibronectin and type I collagen. Results were tested for statistical significance using ANOVA and the Dunnet's post-hoc test (*$p<0.05$ when compared to cells plated out on plasma fibronectin alone).

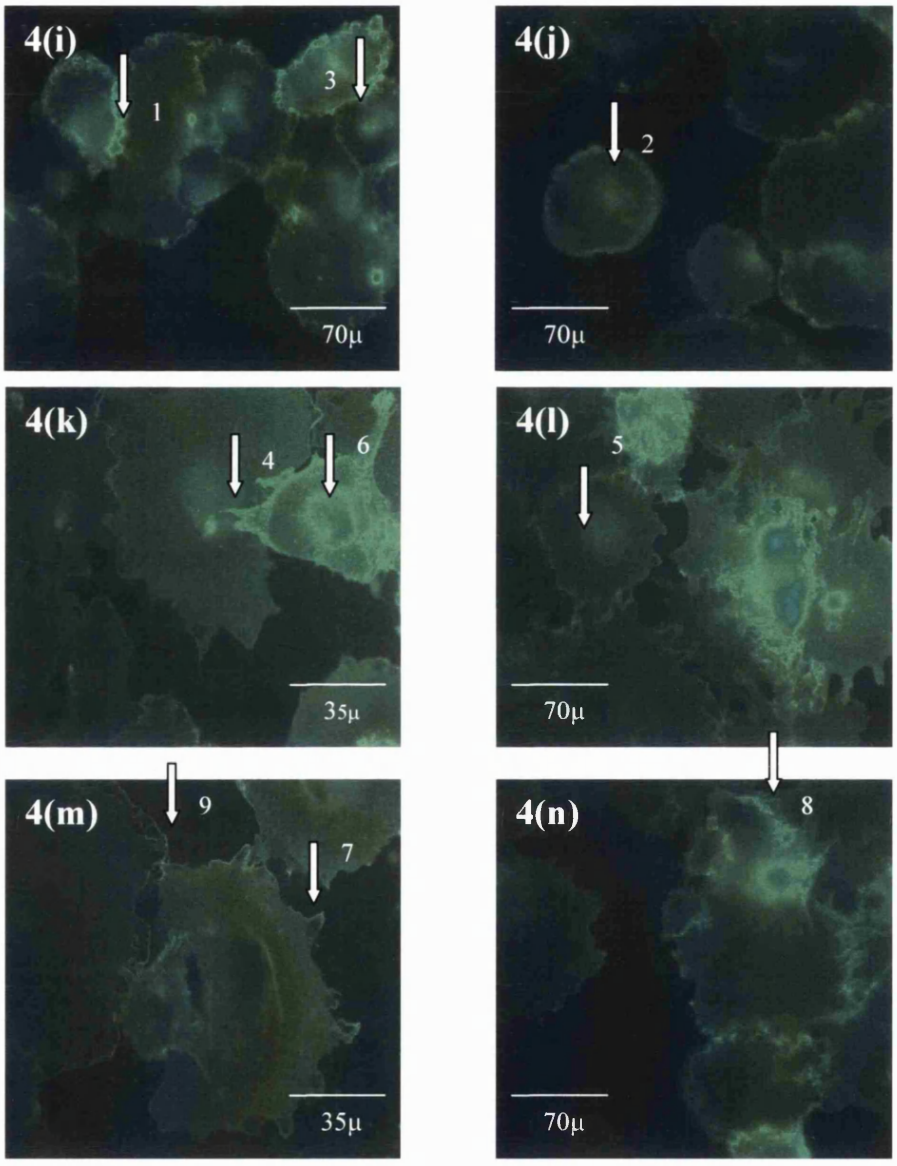
4.6 Results - Immunofluorescent staining of HOB adhesion assays

Figures 4(i) and 4(j) show staining of the β1 integrin on HOBs with β1 mouse anti-human integrin antibody visualised with the rabbit anti-mouse FITC fluorescent label. The cells were plated out for four hours prior to staining on uncoated coverslips. On the uncoated coverslips some cells were just starting to spread (indicated by arrow 1), other cells were still rounded-up (arrow 2). Integrin staining appeared as a fluorescent, diffuse green stain distributed evenly over the cell surface and was very bright around the edge of cells and in membrane ruffles (arrow 1). Integrin staining was also more intense in intracellular clusters (arrow 3).

Figures 4(k) and 4(l) show staining of HOBs for the β1 integrin with β1 mouse anti-human integrin antibody and the rabbit anti-mouse FITC fluorescent label on coverslips coated with plasma fibronectin. The cells were plated out for four hours prior to staining. On coverslips coated with plasma fibronectin, cells were beginning to spread and extend short lamellipodia (arrow 4). Compared to HOBs on uncoated coverslips, cells were more spread out. Integrin staining appeared as punctate spots over the cell surface and as brighter, more intense staining at the cell edge, where the cell was attached to underlying substratum (arrow 5). Areas of more intense intracellular staining were also observed (arrow 6).

Figures 4(m) and 4(n) show staining of HOBs for β1 integrin with β1 mouse anti-human integrin antibody and rabbit anti-mouse FITC fluorescent label on type I collagen coated coverslips. The cells were plated out for four hours prior to staining. The HOBs on type I collagen had started to extend lamellipodia (arrow 7). β1 integrin staining was visible over the cell surface and was particularly bright on the cell edges (arrow 8) and in membrane ruffles (arrow 9).

Figure 4(i)-(n) - Immunofluorescent staining of β1 integrin in HOBs



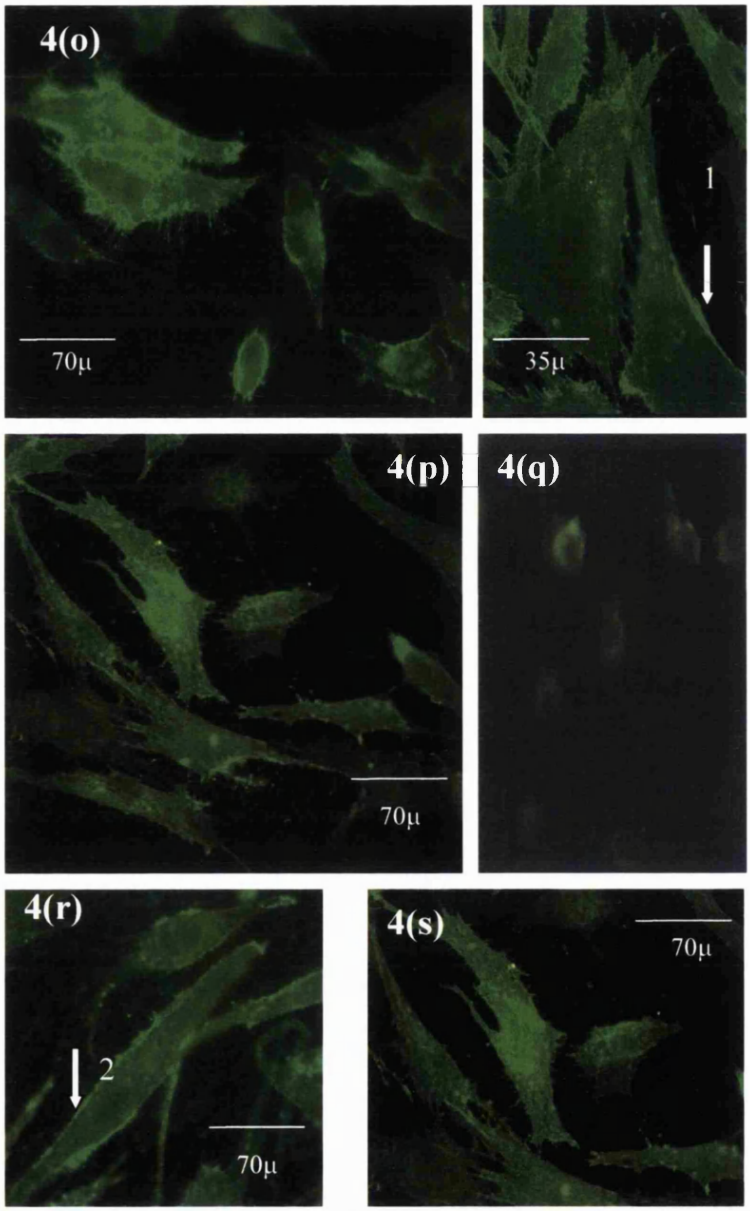
Figures 4(i) to 4(n) show HOBs cultured on coverslips for four hours and then stained with the β1 integrin antibody and FITC labelled secondary. β1 integrin staining was present as a diffuse pattern with areas of punctate spots with intense staining at cell edges in HOBS on uncoated coverslips and those coated with plasma fibronectin and type I collagen (arrows 1 4, 5 and 9). Differences were observed in cell morphology with rounded cells present on uncoated coverslips (arrow 2). On plasma fibronectin and type I collagen cells were beginning to spread and extend processes (arrows 4 and 7).

Figures 4(o) and 4(p) show HOBs stained with the anti-α2 integrin cultured on type I collagen and plasma fibronectin respectively. On type I collagen, α2 integrin had a granular membranous distribution (arrow 1) whilst on plasma fibronectin the distribution was more diffuse. On plasma fibronectin, α5 integrin had a granular distribution at the periphery of cells shown on figure 4(r) (arrow 2) whilst on type I collagen it was more diffusely spread throughout the cytoplasm (4(s)). Figure 4(q) shows a negative control of cells incubated with FITC labelled secondary alone.

4.7 Results - EM of HOBs on coated coverslips

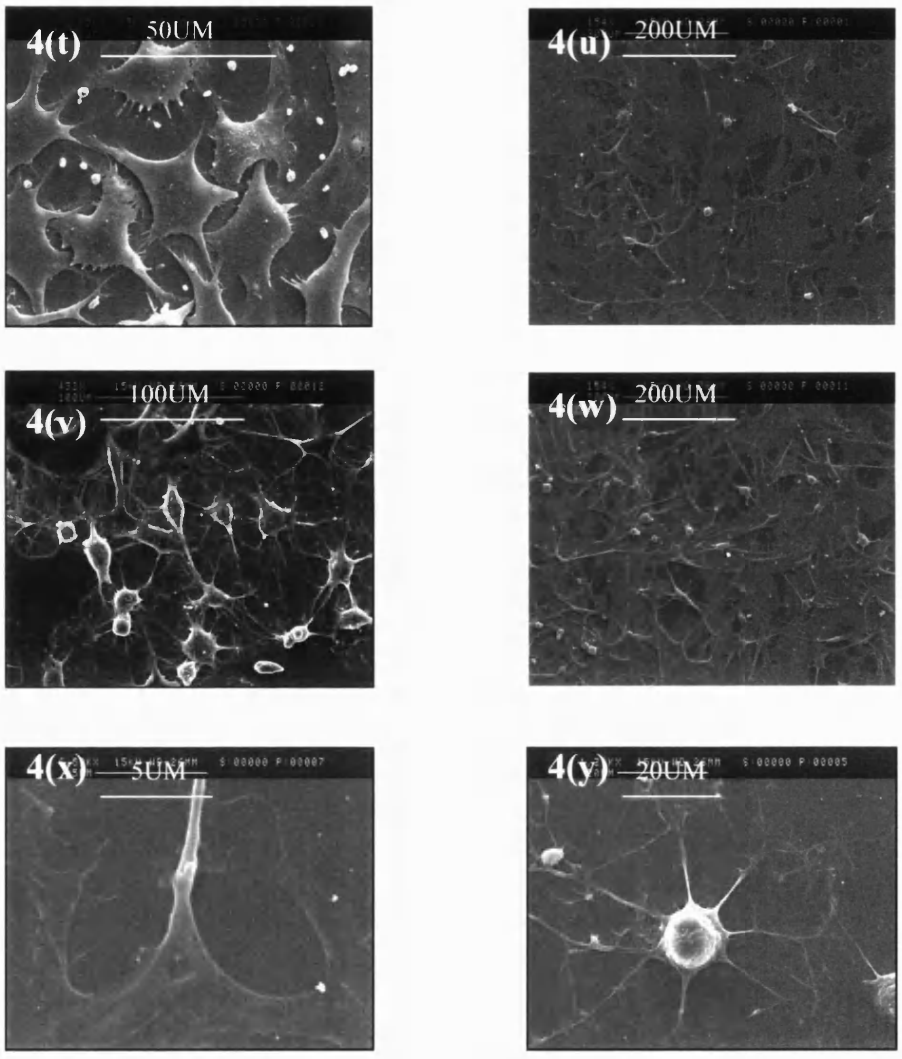
Figure 4(t)-4(y): scanning electron micrographs of HOBs cultured on plasma fibronectin or type I collagen for three hours. On plasma fibronectin, cells had a flattened appearance (4(t) and 4(u)) whilst on type I collagen they were characterised by fine processes which formed a complex a network of cell processes (4(v)-4(w)). Individual cell processes appeared to form discrete junctions with their neighbours (4(x)-4(y)).

Figure 4(o)-(s) - Immunofluorescent staining of α2 and α5 integrin in HOBs



Figures 4(o) and 4(p) show HOBS stained with the anti-α2 integrin cultured on type I collagen and plasma fibronectin respectively. On type I collagen α2 integrin was located towards the cell periphery with areas of intense staining (arrow 1) whilst on plasma fibronectin α2 integrin staining was more diffuse. Figure 4(r) shows that, on plasma fibronectin, α5 integrin is present as a diffuse pattern with areas of intense staining on the periphery (arrow 2). On type I collagen staining is present as a diffuse pattern (4(s)). Figure 4(q) shows a negative control of cells incubated with FITC labelled secondary alone.

Figure 4(t)-(y) – Scanning electron micrographs of HOBs on type I collagen and plasma fibronectin



Figures 4(t)-4(y): scanning electron micrographs of HOBs cultured on plasma fibronectin or type I collagen for three hours. On plasma fibronectin, cells take on a flattened appearance (4(t) and 4(u)). On type I collagen, HOBs extended fine processes which formed a network of connections (4(v)-4(y)).

4.8 Discussion

It has been suggested that ECM binding is important for osteoblast function and survival. β1 integrins are important in the interaction of osteoblasts with the underlying ECM. The α5 and α2 integrins have been the most extensively studied and appear to be of importance in the interaction of osteoblasts with the underlying substratum.

The first functional studies carried out in osteoblastic systems, investigating the relationship between integrins and ECM, used adhesion assays. It was shown that osteoblasts are adherent on a range of ECM substrates including plasma fibronectin, type I collagen and laminin (Clover *et al.,* 1992; Hughes *et al.,* 1993; Aarden *et al.,* 1996; Grzesik and Robey, 1994; Pistone *et al.,* 1996; Gronthos *et al.,* 1997). This thesis provides further evidence for osteoblast adhesion on ECM substrates in vitro. Both MG63 cells and HOBs are highly adherent on both plasma fibronectin, the 120kDa plasma fibronectin fragment, type I collagen and vitronectin.

The observation that human osteoblasts were adherent to the 120kDa plasma fibronectin fragment is of particular interest. The 120kDa fragment includes the central cell binding domain and the RGD integrin-binding motif but not the synergy site, see figure 1f (Obara *et al.,* 1988; Kimizuka *et al.,* 1991; Aota *et al.,* 1994). This observation coupled with the absence of cell adhesion on plasma fibronectin proteolytic fragments that do not contain the central cell binding domain and RGD sequence, these results suggest that components of the central cell-binding domain, such as the RGD binding site are required for osteoblast adhesion on plasma fibronectin. Moursi *et al.* (1996; 1997) reported that rat calvarial derived osteoblasts require the central cell-binding domain, including the RGD sequence but not the synergy site of plasma fibronectin for mineralised nodule formation to occur in vitro.

Once the adhesion profile of MG63 cells and HOBs had been determined, the next series of experiments investigated the involvement of integrins in osteoblast interaction with ECM. Firstly, adhesion assays were carried out with the addition of a RGD blocking peptide. Adhesion of both MG63 cells and HOBs was reduced on

plasma fibronectin but not type I collagen. This suggests that both these cell types bind to plasma fibronectin in an RGD dependent manner and, as only a partial reduction was seen, also via an RGD independent mechanism. It is likely that the α5β1 integrins and possibly the αv integrins in both MG63 cells and HOBs bind via an RGD dependent mechanism. Previously, Aarden *et al.* (1996) and Pistone *et al.* (1996) also reported a reduction in adhesion of HOBs on plasma fibronectin with the addition of the RGD blocking peptide to adhesion assays. In studies using rat calvarial osteoblasts, Moursi *et al.* (1996) showed that addition of RGD containing fragments to cultures of rat calvarial cells inhibited the formation of mineralised nodules. Gronowicz and Derome, (1994), also reported the disruption of mineralisation in cultures with the addition of RGD peptides. However, it should be noted that the concentration of RGD used in this thesis could have been too low to see an optimum effect.

Further studies were carried out to investigate the role of integrins in osteoblast interaction with ECM by the addition of integrin blocking antibodies to cells, prior to plating out in adhesion assays. In this thesis, addition of the β1 integrin blocking antibody reduced adhesion of MG63 cells and HOBs on both plasma fibronectin and type I collagen by a similar magnitude. In contrast, addition of the α2 and α5 blocking antibodies had no detectable effect on the adhesion of MG63 cells and HOBs on either plasma fibronectin or type I collagen. Previous studies have also shown that addition of β1 integrin blocking antibodies to adhesion assays will reduce adhesion on plasma fibronectin (Pistone *et al.,* 1996 and Gronthos *et al.,* 1997). It seems likely, in the light of previous studies, that α2 and α5 integrins are involved in the adhesion of osteoblasts to underlying substratum. In particular, Moursi et al. (1996; 1997) showed that interaction of osteoblasts derived from rat calvarial cultures with fibronectin was essential for the normal differentiation and survival of these cells. It was suggested that the α5 integrin was mediating this interaction. In addition, interaction of osteoblasts with collagen, via the α2 integrin, is involved in the expression of alkaline phosphatase, osteocalcin and other osteoblastic genes (Xiao *et al.,* 1998). One explanation for the lack of reduction in adhesion observed in this

present study with the addition of α2 and α5 integrin antibodies could be 'redundancy' of these integrin receptors in adhesion of osteoblasts to plasma fibronectin and type I collagen. The interaction of other β1 integrins with underlying substratum could be to a level that blocking one or two of these receptors would not reduce cell adhesion to a sufficient level to be detected in an experimental situation. The α3 and α4 integrins are also known to bind to plasma fibronectin and these are present on both MG63 cells and HOBs. In addition, αv integrins in HOBs could mediate adhesion on plasma fibronectin. The results could also be explained by technicalities such as low binding efficiency of integrin blocking antibodies. Due to experimental constraints, adhesion assays were not carried out with addition of further integrin blocking antibodies.

Immunofluorescent staining of the β1 integrin was carried out on adhesion assays using both MG63 cells and HOBs. There were subtle but noticeable differences in the pattern of integrin staining and cell morphology between the substrates used. On uncoated coverslips many cells were still rounded, with a few beginning to spread. Integrin staining was located as intensely staining ruffles on the cell edge. In contrast, on both plasma fibronectin and type I collagen, cells were more spread with extensions resembling filopodia or lamellipodia extended to surrounding cells and the underlying substratum with integrins localised as brightly staining patches at the cell edges. Membrane ruffles with areas of intense integrin staining could also be observed. This suggests that osteoblasts require an underlying matrix to spread and that integrins are involved in the extension of cells over the underlying substratum and also for adhesion to the underlying ECM and that, in cells plated out on a matrix, integrins are concentrated at sites of adhesion to the underlying substratum. Price *et al.* (1998) observed that NIH3T3 fibroblasts extended extensions along underlying ECM with extensive membrane ruffling when plated on fibronectin.

Further information on the nature of integrin and ECM interaction in osteoblasts can be derived from the results of α2 and α5 integrin staining on type I collagen and plasma fibronectin. It appears that the α2 integrins is involved in the interaction of

osteoblasts with type I collagen exemplified by osteoblasts on type I collagen having α2 integrin located towards the cell edge with areas of intense staining. On plasma fibronectin α2 integrin staining was present but more diffuse. Results with the α5 integrin showed the converse with α5 integrin is present as a diffuse pattern with areas of intense staining on the periphery in cells plated on plasma fibronectin.
Very little information is available on the distribution of integrins in osteoblasts but many studies have been carried out to investigate the pattern of integrin distribution in other cells types. Key studies have shown that integrins can form distinct structures, in combination with other molecules, on the cell surface, such as focal contacts, (Burridge *et al.,* 1988), focal adhesions (Burridge *et al.,* 1988; Petit and Thiery, 2000; Liu *et al.,* 2000), podosomes and point contacts, in which integrins are bound to the underlying ECM (Tarone *et al.,* 1985). The nature of integrin containing complexes has been shown to vary between different underlying substrates in vitro. Focal adhesions and contacts are typically formed by adherent cells in culture in which integrins and associated signalling molecules cluster and cause activation of signalling pathways (reviewed in Chrzanowska-Wodnicka and Burridge, 1996). It could be that that the intense areas of integrin staining observed on the periphery of HOBs could be focal adhesions and/or focal contacts.

In addition, osteoblasts plated on uncoated coverslips, plasma fibronectin and type I collagen and stained for the β1 integrin, all exhibit patches of intracellular integrin staining. Previous studies using fibroblasts have reported perinucleur staining of integrins during recycling of integrins, necessary for cell adhesion and spreading (Roberts *et al.,* 2001).

The electron microscopic images of the HOBs illustrate the differences in morphology that exists between these cells when plated on plasma fibronectin versus type I collagen. On plasma fibronectin HOBs were more spread with substantial cell extensions to the surrounding matrix and cells. In contrast, HOBs on type I collagen were rounded with spindle-like extensions to their surrounding matrix. Several studies to date have shown that cell shape and organisation of integrins and associated proteins is related to structure and identity of the underlying ECM. For example, in

studies using mammary epithelial cells, Roskelley *et al.* (1994) showed that when cultured on an exogenous basement membrane, these cells adopted a polarized cuboidal morphology, expressed high levels of β-casein and became quiescent. If these cells are forced to spread on laminin alone, gene expression changes. The β1 integrin and cell signalling is known to be important for the adhesion of these cells on laminin on β1 integrins, expression of β-casein is suppressed. In studies using angiogenic endothelial cells, cell survival and proliferation depends upon the spreading of the cells on ECM via the αvβ3 integrin. If the αvβ3 integrin was blocked the cells rounded up, failed to spread and took on a rounded morphology; apoptosis subsequently occurred (Chen *et al.,* 1992). Studies using human and bovine capillary endothelial cells showed that cell spreading alone was required for cell survival. If cells were prevented from spreading apoptosis ensued (Chen *et al.,* 1992). It may be that osteoblast spreading on type I collagen and plasma fibronectin is important for cell survival. In addition, the difference in morphology observed between cells on plasma fibronectin and type I collagen suggests that different integrin-mediated cell signalling pathways could be involved. Several studies have illustrated that cell shape influences integrin mediated cell signalling pathways (reviewed in Boudreau and Jones, 1999). Further work would be required to investigate this.

4.9 Summary of adhesion assays

The results of this chapter can be summarised as follows:

- Adhesion of MG63 cells occurred at the highest level on plasma fibronectin, type I collagen and the 120kDa plasma fibronectin fragment;
- HOBs showed a similar pattern of adhesion but with a significantly lower number of cells adherent in each case;
- Addition of the RGD blocking peptide reduced adhesion on plasma fibronectin but not type I collagen for both MG63 cells and HOBs;
- Adhesion on type I collagen and plasma fibronectin was reduced by a small but significant amount with the addition of β1 blocking antibody in both MG63 cells and HOBs; and
- No reduction in adhesion was observed with α2 or α5 integrin blocking antibodies.

Chapter 5 describes the results of migration assays carried out to further investigate the relationship between integrins and osteoblast behaviour.

5 Chapter 5 – Integrins and ECM regulate osteoblast migration

5.1 Aims of migration assays

The aim of this series of experiments was to:

- Ascertain the migratory behaviour of MG63 cells and HOBs towards plasma fibronectin and other ECM components using Boyden chamber migration assays; and
- Investigate the involvement of integrins in osteoblast migration, with the addition of integrin blocking antibodies, during Boyden chamber migration assays.

5.2 Introduction to cell migration and migration assays

Cell migration is known to be important in a great diversity of processes including development, tumorigenesis and immune response. In addition, it is a phenomenon exhibited by many cell types. The mechanisms of cell migration are an area of intense study at the present time and integrins, ECM, the actin cytoskeleton and associated pathways are known to be of particular importance during cell migration. Cell migration involves a protrusion at the leading edge of a cell, in which integrins and associated proteins tend to form large clusters termed focal adhesions (in the nascent cell adhesive complexes are smaller in size) (Burridge *et al.,* 1988). This protrusion is largely driven by the polymerisation of the actin cytoskeleton (Mullins *et al.,* 1998; Hall, 1998; Machesky and Insall, 1999). Integrins are thought to provide an attachment with the underlying cytoskeleton over which the cell moves (Smilenov *et al.,* 1999; Horwitz and Parsons, 1999). In addition interaction of the integrin with the underlying substratum activates cell signalling pathways associated with cell migration (Horwitz and Parsons, 1999). In certain cases, integrins have been shown to inhibit cell migration, for example ectopic expression of α5 in cultures of primary quail myoblasts promotes contact-mediated inhibition of cell migration (Huttenlocher *et al.,* 1998). *In vitro*, studies have shown migration to occur in response to several environmental cues, for example, a concentration gradient of chemical stimuli or a

change in underlying substrate rigidity. In response to a gradient of chemotactic stimuli, cells will polarize with a redistribution and rearrangement of integrins and cytoskeleton (Schmidt *et al.,* 1993; Lawson and Maxfield, 1995).

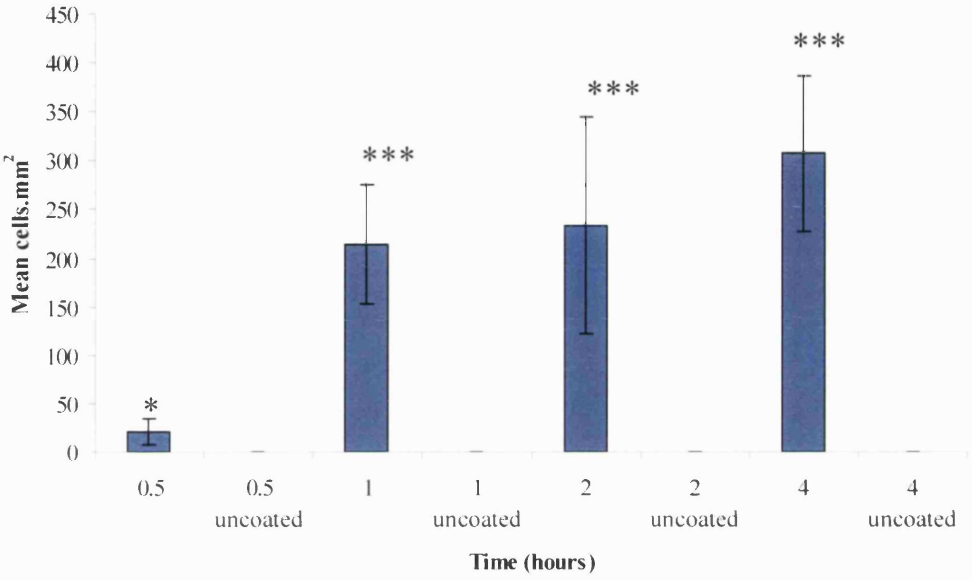
Several studies have suggested that osteoblasts are migratory cells although it is unclear whether the osteoblast exhibits migration throughout all stages of maturity. It has been proposed that the mature osteoblast is recruited to sites of new bone formation during bone turnover (Bonewald, 1996). TGF-β, PDGF and BMP-2 are all known to act as chemoattractants promoting osteoblast chemotaxis (Lucas, 1989; Pfeilschifter *et al.,* 1990, Hughes *et al.,* 1992, Lind *et al.,* 1995; 1996). To date, no evidence exists to show that integrins and ECM interactions are important in primary human osteoblast migration. Given that this relationship is central to the migration of many other cell types and that ECM is so abundant in bone, it seems likely that integrins and ECM will be a major factor in the control of osteoblast migration. Previously, Vihinen *et al.* (1996) showed that several osteosarcoma cell-lines are migratory in response to type I collagen and it was suggested that this interaction was via the α2β1 integrin. It is well recognised that increased motility is a feature of tumorigenic cells (reviewed in Boudreau and Bissell, 1998) and it is therefore important carry out cell migration studies using primary cells. In this present study migration assays were carried out using both HOBs and MG63 cells osteosarcoma cells to investigate the migratory behaviour of integrins in response to ECM components. In addition the role, if any, of integrins in osteoblast cell migration was determined. The results of this chapter are published in Moffatt *et al.* (2000).

5.3 Results - Preliminary migration assays

Preliminary migration assays were carried out to ascertain whether MG63 cells and HOBs would migrate under Boyden chamber migration assay conditions and, if so, what the time course of migration would be. Each experiment was carried out 3 times with triplicates and ANOVA was used to test the statistical significance of results with a Dunnet's post-hoc test. Figure 5(a) shows the migration of MG63 cells over a four hour time course towards plasma fibronectin. Cells were detected on the underside of the membrane at 0.5 hours, suggesting chemotaxis had already started to take place. Movement of MG63 cells continued throughout the time course with significantly more cells migrating at each time point when compared to uncoated controls ($p<0.001$). It was decided that future migration assays using this method would be carried out for two hours as at this time point many cells had migrated to the underside of the membrane but could still be counted easily under the microscope on all the membranes.

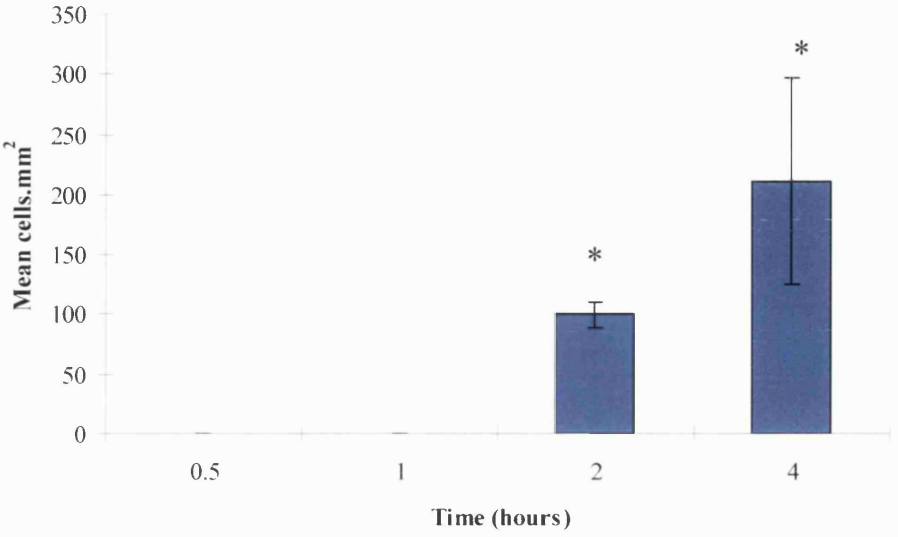
Figure 5(b) shows the migration of HOBs over a four hour time course towards plasma fibronectin. HOBs were not detected on the underside of the membrane until the two hour time point with a mean count of 16 cells.mm^2 (SD 10.8) ($p<0.001$ when compared to uncoated controls). After four hours significantly more cells had migrated when compared to the uncoated controls ($p<0.001$) with a mean count of 212 cells.mm^2 (SD 86).

Figure 5(a) – MG63 migration towards plasma fibronectin over a four hour time course



The membrane underside of Boyden chamber migration inserts were coated with plasma fibronectin. 10^5cells/migration insert were plated out into the chambers of migration inserts, including uncoated controls. Cells were left to migrate for 0.5-4 hours and at each time point the cells migrating to the underside of the migration assay insert were stained with Crystal Violet and counted using a light microscope on 20x magnification. After 0.5 hours cells were detected on the underside of the migration insert and migration continued throughout the four hour experimental period. Results were tested for statistical significance using ANOVA and the Dunnet's post-hoc test (*$p<0.05$, ***$p<0.001$ when compared to uncoated controls).

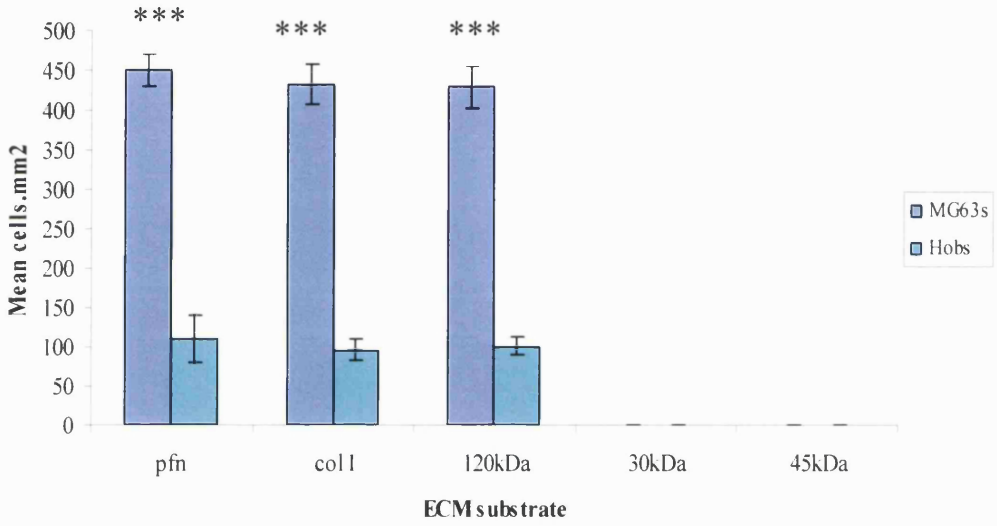
Figure 5(b) – HOB migration towards plasma fibronectin over a four hour time course



The membrane undersides of Boyden chamber migration inserts were coated with type I collagen. 10^5cells/migration insert were plated out into the chambers of migration inserts, including uncoated controls. Cells were left to migrate for 0.5-4 hours and at each time point the cells migrating to the underside of the migration assay membrane were stained with Crystal Violet and counted using a light microscope on 20x magnification. At two hours and four hours a significant number of cells had migrated when compared with uncoated controls. Results were tested for statistical significance using ANOVA and the Dunnet's post-hoc test (*$p<0.05$, ***$p<0.001$ when compared to uncoated controls).

Once the migratory behaviour of both HOBs and MG63 cells had been determined towards plasma fibronectin, further migration assays were carried out towards plasma fibronectin, type I collagen, 120kDa plasma fibronectin fragment (120kDa), 30kDa plasma fibronectin fragment (30kDa) and 45kDa plasma fibronectin fragment (45kDa) and an uncoated control. Each experiment was carried out three times with triplicates and ANOVA was used to test the statistical significance of results with a Dunnet's post-hoc test. Results are shown in figure 5(c). Migration of MG63 cells occurred towards plasma fibronectin, type I collagen and the 120kDa plasma fibronectin fragment with no migration towards the 30kDa and 45kDa plasma fibronectin fragments. Significantly more MG63 cells migrated in response to plasma fibronectin, type I collagen and the 120kDa plasma fibronectin than HOBs ($p<0.001$)

Figure 5(c) – MG63 and HOB migration towards ECM substrates



The membrane underside of Boyden chamber migration inserts were coated with plasma fibronectin (pfn) and plasma fibronectin proteolytic fragments (120kDa, 30kDa and 45kDa) and type I collagen (col I). 10^5cells/migration insert were plated out into the chambers of migration inserts including uncoated controls. Cells were left to migrate for 0.5-4 hours and at each time point the cells migrating to the underside of the migration assay membrane were stained with Crystal Violet and counted using a light microscope on 20x magnification. Both MG63 cells and HOBs migrated in response to pfn, col and 120kDa with no migration towards 30kDa and 45kDa. Migration of MG63 cells were significantly greater than that of HOBs (***$p<0.001$ when using ANOVA and Dunnet's post-hoc test).

5.4 Results - Migration assays with addition of integrin blocking antibodies

Once it had been determined that both MG63 cells and HOBs would migrate towards inserts coated with type I collagen, plasma fibronectin and the 120kDa plasma fibronectin fragment, the importance of specific integrins in migration towards ECM substrates was ascertained. This was investigated by the incubation of cells with integrin blocking antibodies prior to plating out in the migration inserts. Each experiment was carried out three times with mean and SD shown. ANOVA was used to test the statistical significance of results with a Dunnet's post-hoc test.

Figures 5(d) and 5(e) show the results of migration assays over two hours with the addition of integrin antibodies. Figure 5(d) shows the results for MG63 cells incubated with the α2, α4 and α5 integrin blocking antibodies, prior to plating out in migration inserts, with the underside of the membrane coated with plasma fibronectin.

Addition of the α5 and α4 integrin blocking antibodies both alone and in combination resulted in a significant decrease in cell migration when compared to plasma fibronectin alone ($p<0.05$). With addition of the α5 blocking antibody alone 295 cells.mm^2 (SD 39) migrated compared to 460 cells.mm^2 (SD 51) on plasma fibronectin alone, a total reduction in migration of 36% ($p<0.05$). Addition of the α4 blocking antibody reduced migration by 25%, when compared to plasma fibronectin alone, with 348 cells.mm^2 migrating in two hours ($p<0.05$). The α4 and α5 antibodies in combination resulted in a 39% reduction in migration compared to plasma fibronectin alone, with 283 cells.mm^2 (SD 54) migrating ($p<0.05$).

Addition of the α2 antibody alone had no significant effect, when compared to plasma fibronectin alone, with 450cells.mm^2 (SD 12.5) migrating. Addition of the α2 blocking antibody in combination with either the α5 or α4 antibodies both resulted in a significant reduction in cell migration with 206.1 cells.mm^2 (SD 90) and 285.1 cells.mm^2 (SD 98) migrating respectively ($p<0.05$). Addition of all three

antibodies together resulted in a significant reduction in migration of 42.4% with 265 cells.mm^2 (SD 45.8) migrating ($p<0.05$).

Figure 5(e) shows the results of migration assays with MG63 cells plated out in migration inserts coated with collagen type I.

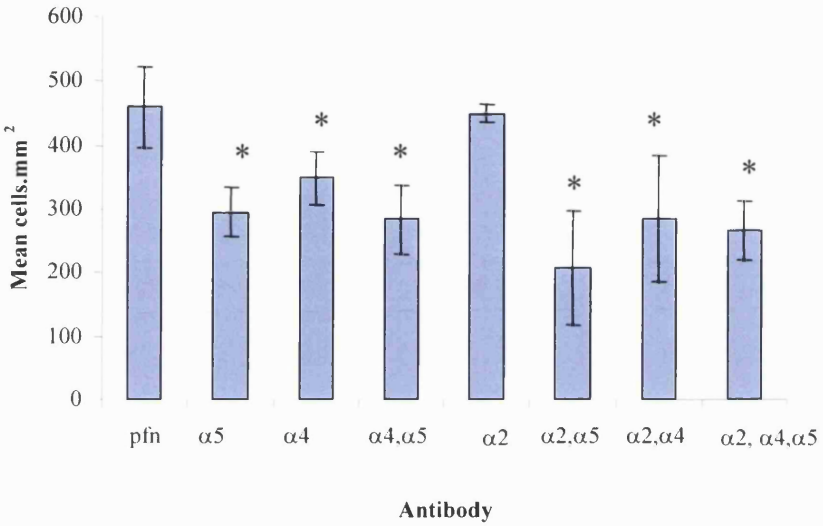
Addition of the α2 integrin blocking antibody reduced integrin migration towards type I collagen with 62.8 cells.mm^2 (SD 17.9) migrating after two hours, an 88% reduction in migration compared to the standard with no integrin blocking antibodies ($p<0.001$). Combination of the α2 and α5 and α2 and α4 blocking antibodies also resulted in a dramatic reduction in the number of cells migrating with 25.8 cells.mm^2 (SD 31.7) and 7.7 cells.mm^2 (SD 12.7) counted respectively($p<0.001$).

Addition of the α4 blocking antibody both alone and in combination also resulted in a reduction in cell migration towards type I collagen although this was less marked than α2 alone. When used alone the α4 integrin blocking antibody reduced migration by 25.5% with 365.8 cells.mm2 (SD 40.7) migrating when compared to the standard with no integrin blocking antibodies ($p<0.05$).

The α5 blocking antibody alone did not have an effect on cell migration towards type I collagen. The combinations of α4 and α5 blocking antibodies resulted in a reduction in the number of cells migrating with 202 cells.mm^2 (SD 115.3) counted.

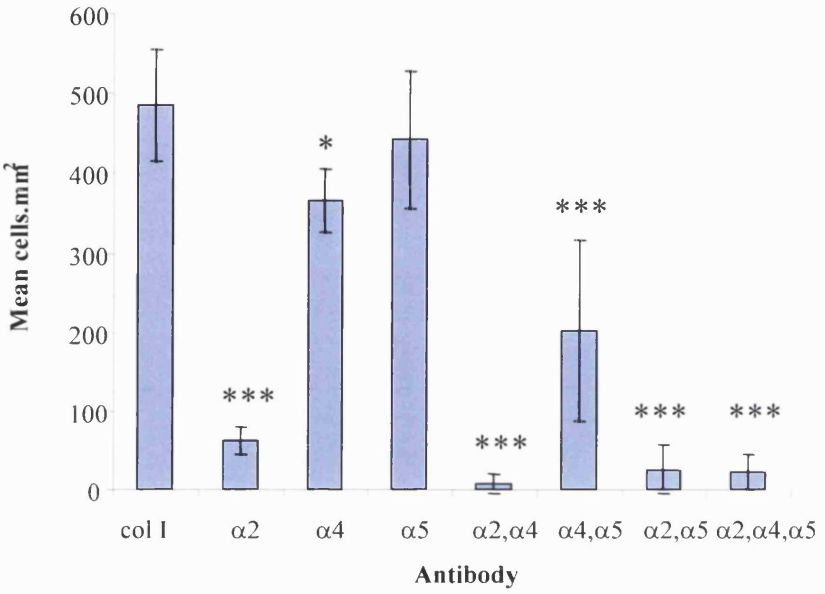
All three antibodies together reduced migration by 95.4% with 22.4 cells.mm^2 (SD 23.4) when compared to the standard with no integrin blocking antibody added ($p<0.001$).

Figure 5(d) – MG63 migration towards plasma fibronectin with the addition of integrin blocking antibodies



The membrane underside of Boyden chamber migration inserts were coated with plasma fibronectin (pfn). MG63 cells were incubated with 50μg.ml^{-1} α5, α4, and α2 integrin blocking antibodies. 10^5cells/migration insert were plated out into the chambers of migration inserts. Cells were left to migrate for four hours. Cells that had migrated to the underside of the migration assay membrane were stained with Crystal Violet and counted using a light microscope on 20x magnification. Migration was reduced significantly with the addition of the α5 and α4 antibodies both alone and in combination with each other and the α2 antibody, when compared to plasma fibronectin alone (*$p \leq 0.05$ when using ANOVA and Dunnet's post-hoc test).

Figure 5(e) – MG63 migration towards type I collagen with the addition of integrin blocking antibodies



The membrane underside of Boyden chamber migration inserts were coated with 100μg.ml type I collagen (col I). MG63 cells were incubated with 50μg.ml^{-1} α5, α4, and α2 integrin blocking antibodies. 10^5cells/migration insert were plated out into the chambers of migration inserts. Cells were left to migrate for four hours. Cells that had migrated to the underside of the migration assay membrane were stained with Crystal Violet and counted using a light microscope on 20x magnification. Addition of the α2 integrin blocking antibody resulted in a dramatic decrease in migration both alone and in combination with the α2 and α5 blocking antibodies antibodies. Addition of the α4 integrin blocking antibody also reduced migration but to a lesser extent (*$p<0.05$, ***$p<0.001$ when using ANOVA and Dunnet's post-hoc test).

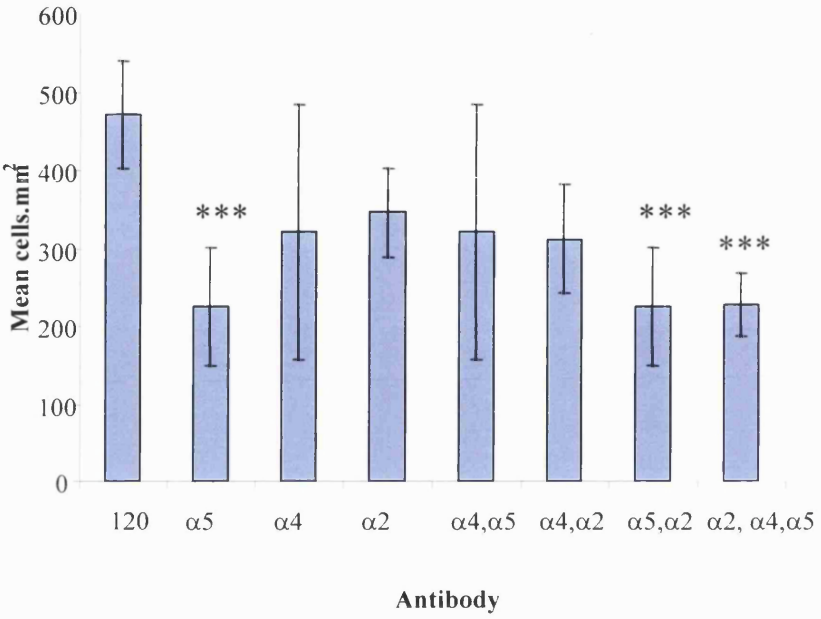
Figure 5(f) shows the results of MG63 migration towards the 120kDa plasma fibronectin fragment. A mean of 471 cells migrated towards the 120kDa fragment (SD 67). Addition of the α5 antibody alone reduced migration by 52% with 230 cells.mm^2 (SD 70) migrating ($p<0.001$). Addition of the α5 antibody in combination with the α2 antibody reduced adhesion by 32% with a mean of 321 cells.mm2 (SD 163). Addition of the α5 antibody in combination with the α4 antibody reduced adhesion by 52% with a mean of 225 cells.mm^2 (SD 75) migrating ($p<0.05$). Addition of the α2, α4 and α5 antibodies in combination resulted in the same decrease in migration (SD 39.7) ($p<0.001$). Addition of the α2 integrin blocking antibody alone appeared to cause a slight increase in migration but this was not statistically significant.

Figure 5(g) shows the results of migration assays using HOBs migrating towards plasma fibronectin with the addition of the α5 integrin blocking antibody and also HOB migration towards type I collagen with the addition of the α2 integrin blocking antibody. Addition of the α5 blocking antibody reduced cell migration by 32%, 150 cells.mm^2 (SD 20) compared to 222 cells.mm^2 (SD 27.3) ($p<0.001$).

Addition of the α2 blocking antibody to cells prior to migration towards type I collagen resulted in 113 cells.mm^2 (SD 29.6) compared to 247 cells.mm^2 (SD 36.2), a reduction of 46% ($p<0.001$).

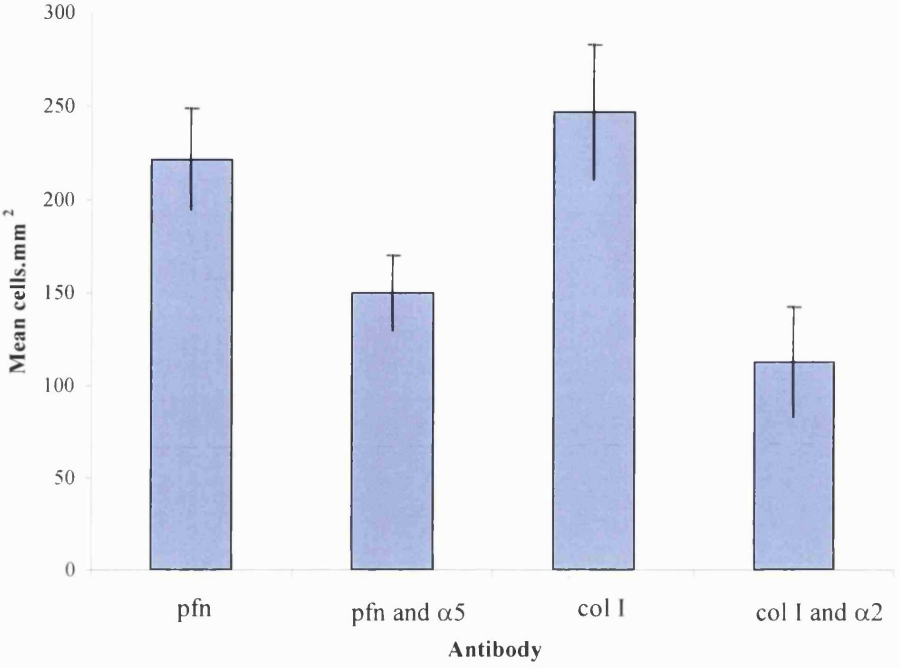
Figure 5(h)-a shows the underside of a Boyden chamber migration insert coated with plasma fibronectin. HOBs migrated through the pores and could be observed on the underside of the membrane. Cells were spread and adherent to the membrane. Figure 5(h)-b shows an uncoated control membrane with no cells migrating in response to the uncoated membrane.

Figure 5(f) – MG63 migration in response to the 120kDa plasma fibronectin fragment with the addition of integrin blocking antibodies



The membrane underside of Boyden chamber migration inserts were coated with 120kDa plasma fibronectin proteolytic fragment (120). MG63 cells were incubated with 50μg.ml α5, α4, and α2 integrin blocking antibodies. 10^5cells/migration insert were plated out into the chambers of migration inserts. Cells were left to migrate for four hours. Cells that had migrated to the underside of the migration assay membrane were stained with Crystal Violet and counted using a light microscope on 20x magnification. Addition of the α5 integrin blocking antibody significantly reduced adhesion when compared to 120kDa alone (*$p<0.001$ when using ANOVA and Dunnet's post-hoc test).

Figure 5(g) – HOB migration in response to plasma fibronectin and type I collagen with the addition of integrin blocking antibodies



The membrane underside of Boyden chamber migration inserts were coated with plasma fibronectin (pfn) and type I collagen (col I). HOBs were incubated with 50μg.ml α5, α4, and α2 integrin blocking antibodies. 10^5cells/migration insert were plated out into the chambers of migration inserts. Cells were left to migrate for four hours. HOBs that had migrated to the underside of the migration assay membrane were stained with Crystal Violet and counted using a light microscope on 20x magnification. Addition of the α5 integrin reduced migration of HOBs in response to plasma fibronectin and addition of the α2 antibody reduced HOB migration in response to type I collagen (***$p<0.001$ when using ANOVA and Dunnet's post-hoc test).

Figure 5(h) –Representative images of transwell membranes during migration assays with HOBs

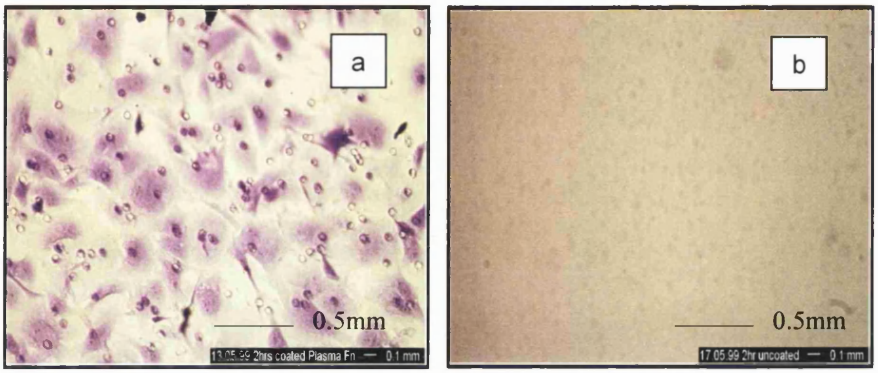


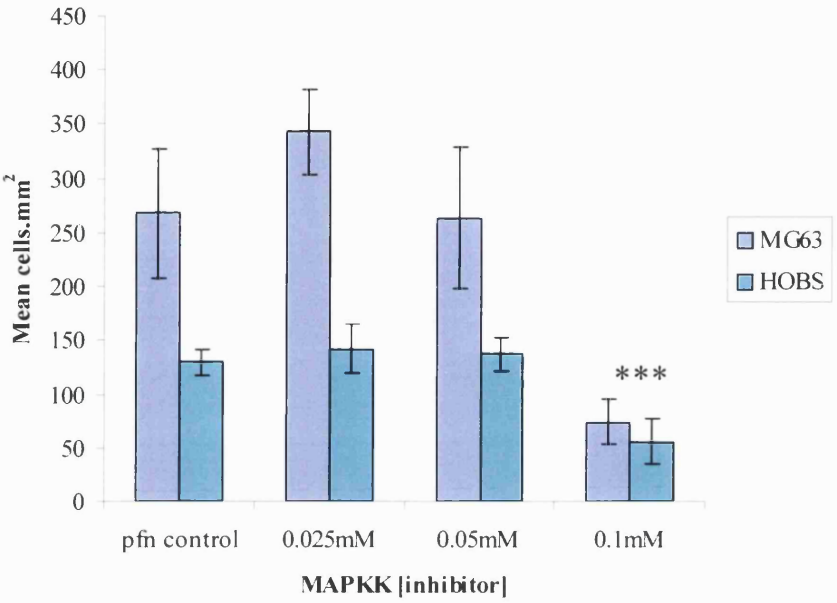
Figure 5(h)-a shows the underside Boyden chamber migration insert coated with plasma fibronectin with HOBs that have migrated through the pores and adhered to the membrane. Figure 5(h)-b shows an uncoated control membrane. No cells were present on the uncoated membrane. Original magnification 20x.

5.5 Results - Migration and integrin signalling pathways

Evidence from *in vitro* studies of cell migration shows that the p38/MAPK signalling pathway is involved in integrin mediation of cell migration (Klemke *et al.*, 1997). In this present study the MAPKK specific inhibitor U0126 was added to the cells prior to plating out in the migration chambers. Each experiment was carried out three times with triplicates and ANOVA was used to test the statistical significance of results with a Dunnet's post-hoc test.

Figure 5(i) shows the results of MG63 and HOB migration in Boyden chamber migration assays inserts coated with plasma fibronectin with the addition of the MAPKK inhibitor U0126 at a series of concentrations. Addition of this inhibitor at 0.1mM reduced migration of MG63 cells by 72% (SD 7.9%) and HOBs by 47% (SD 17%) ($p<0.001$).

Figure 5(i) - MG63 and HOB migration with U0126 (MAPKK inhibitor)

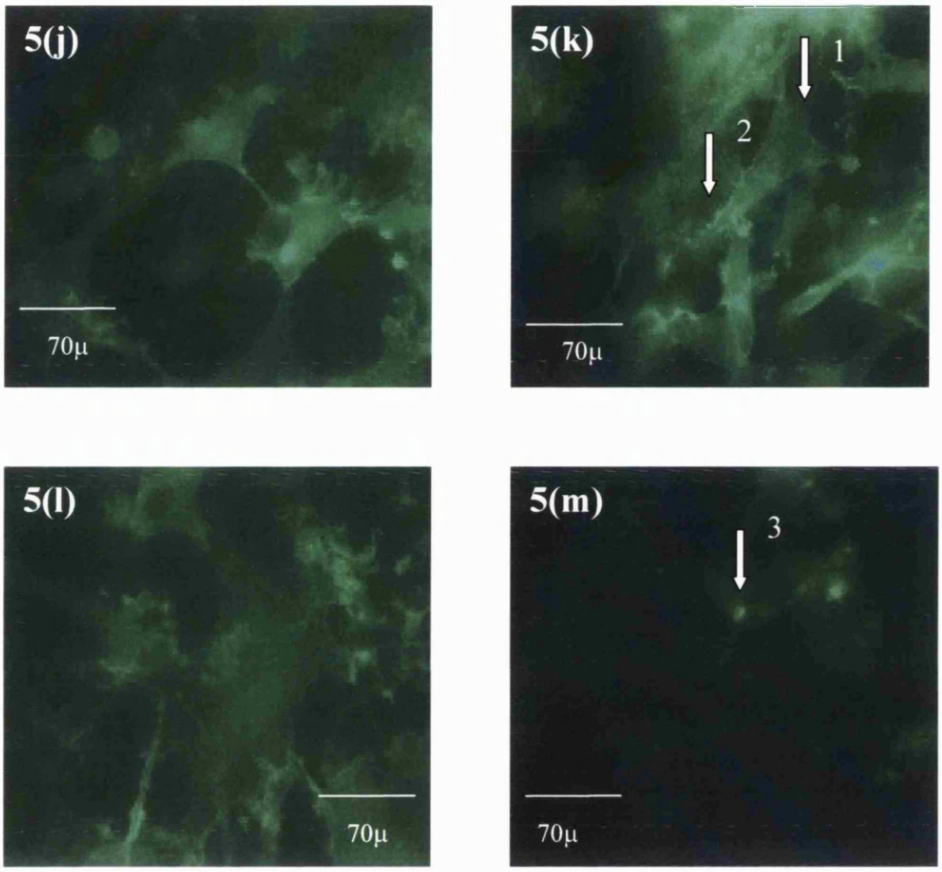


The membrane underside of Boyden chamber migration inserts were coated with 10μg.ml plasma fibronectin (pfn). MG63 cells and HOBs were incubated with 0.02mM, 0.05mM and 0.1mM U0126. 10^5cells/migration insert were plated out into the chambers of migration inserts. Cells were left to migrate for four hours. Cells that had migrated to the underside of the migration assay membrane were stained with Crystal Violet and counted using a light microscope on 20x magnification. Addition of the inhibitor at 0.01mM reduced adhesion significantly when compared to the controls without inhibitor (***$p<0.001$ when using ANOVA and Dunnet's post-hoc test).

5.6 Results - Migration assay staining

Figures 5(j), 5(k) and 5(l) show the results of staining HOBs for the α5 integrin after cells had migrated through a migration insert, with the underside coated with plasma fibronectin. Staining of osteoblasts on the underside of migration inserts shows that many of the cells are still migrating. Morphologically the migrating cells exhibit an elongated, long thin tail and larger cell body giving the migrating cells a triangular shape (arrow 1). Integrin staining appears as a diffuse fluorescent green over the cell surface. Some of the migrating cells have an area of intense integrin staining at the leading edge of the cell tail (arrow 2). Figure 5(m) shows the negative control; cells were stained with the FITC secondary antibody alone. There is slight background staining with collection of fluorescence in the pores of the membrane (arrow 3).

Figure 5(j) –(m) Integrin staining of HOB migration assays



Figures 5(j)-(m) show HOBs stained for the α5 integrin on the underside of migration assay membranes coated with plasma fibronectin. Integrin staining appears as a diffuse fluorescent green over the cell surface. Some of the migrating cells have an area of intense integrin staining at the leading edge of the cell tail as exemplified by arrow 2. Figure 5(m) shows a negative control with cells and FITC labelled secondary antibody

5.7 Discussion

This present study has sought to investigate the role of the ECM-integrin interaction in the migratory behaviour of osteoblasts. Although integrin interaction with ECM is known to be an important factor in the migration of several cell types, such as fibroblasts (Paleck *et al.,* 1996), the importance of this interaction, if any, is yet to be elucidated in primary human osteoblast migration. Previously it was shown that several osteosarcoma cell-lines, including MG63, were migratory in response to type I collagen and this was thought to involve the α2β1 integrin (Vihinen *et al.,* 1996). One other previous study has shown the involvement of cell-ECM interaction during osteoblast migration. Imai *et al.* (1998) suggest that interaction of N-syndecan on the osteoblast surface with the extracellular associated molecule HB-GAM could be important for migration of these cells to sites of bone formation. Using Boyden chamber migration assays, this thesis provides the first evidence for the importance of integrins in the mediation of both MG63 cells and HOBs migration towards ECM components.

This thesis shows that the α4 and α5 integrins are involved in MG63 migration on and towards plasma fibronectin. Migration of MG63 cells on plasma fibronectin was blocked by the addition of the α4 and α5 antibodies but not the α2 antibody. In addition the α2 integrin is also important for MG63 migration on and towards type I collagen. MG63 migration was reduced on type I collagen by the anti-α2 integrin.

In addition, this study shows that the α5 integrin is an important factor in HOB migration towards and on plasma fibronectin. These results also show that the α2 integrin is involved in HOB migration towards type I collagen.

Evidence from this study suggests that the α2 integrin interacts with type I collagen and that the α4 and α5 integrins interact with plasma fibronectin during osteoblast migration. For further discussion see section 7.3.7. Immunofluorescent staining of the α5 integrin on plasma fibronectin showed that this integrins was expressed in the migrating cell. Further studies are required to clarify the nature of the integrin-ECM

interaction as it is possible that during the course of the migration assays the cells secreted their own ECM, although it was hoped that this would be minimal after two hours.

MG63 cells migrate at higher cell numbers than HOBs towards plasma fibronectin and type I collagen at both two hours and four hours. This could be explained by the fact that MG63 cells are a cell-line derived from an osteosarcoma. A great deal of evidence exists that suggests motility is altered in malignant cells. Tumour cells have been shown to exhibit increased motility in response to ECM, largely through integrin mediated signalling pathways. Malignant cells are able to invade the surrounding ECM through interaction of cell adhesion molecules with components of the ECM and the coordinated secretion of proteases (Boudreau and Bissell, 1998). The $\alpha v\beta 3$ integrin is often associated with increased motility and invasiveness exhibited by malignant cells (Albelda *et al.,* 1990) although the MG63 osteosarcoma cell-line does not appear to express the $\alpha v\beta 3$ integrin. Previous studies using osteosarcoma cell-lines, including MG63 cells showed that these cells are highly invasive and migratory in response to type I collagen and that this behaviour can be increased by the up-regulation of the $\alpha 2\beta 1$ integrin (Vihinen *et al.,* 1996). This is in agreement with the results of this present study. Overall, the migration results for the MG63 osteosarcoma cell-line appear to be more dramatic than those for the HOBs with greater cell numbers migrating under all conditions and greater reduction in migration upon addition of $\alpha 2$ blocking antibody.

Migration has been shown to occur in response to chemotactic stimulation from several growth factors including TGF-β and PDGF (Lucas 1989; Pfeilschifter *et al.,* 1990; Hughes *et al.,* 1992; Lind *et al.,* 1995). The bone morphogenetic protein BMP-2 is also known to be a stimulator of chemotactic migration of trabecular derived primary human osteoblasts and the osteosarcoma cell line U2-OS (Lind *et al.,* 1996). Mundy *et al.* (1982) showed that factors released during bone resorption acted as chemoattractants to osteoblasts. Previous studies have shown that the classical ras/raf/MAPK pathway is activated during cell motility (Klemke *et al.,* 1997) and this

is related to integrin engagement of ECM ligands and is suggested to result from integrin cooperation with growth factors (Renshaw *et al.,* 1997). It could be the case in osteoblasts that integrins and growth factors are co-operating in the control of migration in response to ECM.

In this thesis addition of the MAPKK specific inhibitor U0126 resulted in a decrease in both MG63 and HOB migration. The cell signalling pathways associated with osteoblast migration specifically are not yet known but several pathways have been associated with integrin mediated migration in other systems. Key signalling pathways include aFAK-p130cas, the classical MAPK (ERK pathway) and the PI3-kinase pathway (Klemke *et al.,* 1997). Signalling via these signalling pathways provides a link between integrins and the actin cytoskeleton. Inhibitors specific for the PI3 kinase pathway are available and these could be employed in further studies. For further discussion see 7.3.7.

5.8 Summary of migration assay results

This series of experiments using an *in vitro* model of cell migration has shown the following results:

- Both MG63 cells and HOBs will migrate through a membrane with underside coated with plasma fibronectin, type I collagen and the 120kDa plasma fibronectin fragment;
- MG63 cells will migrate at higher cell numbers than HOBs;
- Addition of the α5 and α4 integrin blocking antibodies both alone and in combination will partially inhibit migration of MG63 cells on plasma fibronectin;
- Addition of the α2 integrin blocking antibody will drastically reduce migration of MG63 cells towards type I collagen but has no effect on cells migrating on plasma fibronectin;
- Addition of the α5 integrin blocking antibody will reduce HOB migration towards plasma fibronectin;
- Addition of the α2 integrin blocking antibody will reduce migration of HOBs on type I collagen; and
- Both MG63 cells and HOBs appear to interact with plasma fibronectin via the α5 integrin and with type I collagen via the α2 integrin.

Table 5(n) overleaf shows a summary of both the adhesion and migration results reported in this thesis.

Table 5(n) - Summary of migration and adhesion assay results

Extracellular matrix	Migration	Adhesion	Migration blocked by antibody?	Adhesion blocked by antibody?
Plasma fibronectin	Yes	Yes	α2 (none) α4 (partial) α5 (partial) α4 and α5 (partial)	β1 (partial) α2 (none) α4 (none) α5 (none)
Type I collagen	Yes	Yes	α2 (near total) α4 (partial) α5 (none) α2 and α4 (near total)	β1 (partial) α2 (none) α4 (none) α5 (none)

The table above summarises the results of adhesion and migration assays reported in this thesis.

6 Chapter 6 – A link between integrins and the Cbfa1 transcription factor

6.1 Cbfa1 - introduction

In addition to the studies already reported in this thesis, investigations have also been underway into the possible link between integrins and the transcription factor Cbfa1. This following section describes this ongoing work.

Cbfa1 was identified as a member of the *runt* family of genes, all encoding transcription factors. Several members of this family are important regulators of haematopoeisis. Cbfa1 in now known to be essential for osteoblast differentiation and the regulation of several osteoblast specific genes. Not only is Cbfa1 important during development but it is also key to the regulation of bone formation post-natally.

Cbfa1 was shown to be an important regulator of bone formation by groups looking at this transcription factor for very different reasons. Otto *et al.* (1997) generated mouse Cbfa1 mutants and the offspring were studied for anatomical defects. Importantly, homozygotes had a lack of ossification and exhibited no detectable osteoblastic differentiation. These characteristics appeared very similar to those of the genetic disease Cleidocranial Dysplasia (CCD). In parallel, Mundlos *et al.* (1997) confirmed that Cbfa1 mutation was indeed associated with CCD. A group looking at the factors involved in osteoblast differentiation, in particular those that bound to a particular site termed OSE2 in osteocalcin, cloned *Cbfa1* and identified it as being important in this very process (Ducy *et al.*, 1997). It is now acknowledged that Cbfa1 is also involved in the control of bone formation post-natally (Ducy *et al.*, 1999). The discovery of Cbfa1 was a significant step towards understanding the control of osteoblast differentiation and bone formation.

6.2 Cbfa1 and osteoblast differentiation

To date, the expression profile of Cbfa1 has been established in cells of the osteoblast lineage, although this is by no means conclusive. In addition, Cbfa1 has been shown

to act as a regulator of several osteoblast-specific markers, including alkaline phosphatase, osteocalcin and bone sialoprotein. Studies have also begun to elucidate the signalling pathways that may be involved in the activation of the *Cbfa1* gene. Although it has been suggested previously that α2 integrin-ECM interaction is involved in the activation of *Cbfa1*, more evidence is needed to show a direct link. In addition, further integrins could be potentially involved in the control of *Cbfa1*. Xiao *et al.* (1997) showed that regulation of the osteocalcin gene requires collagen matrix production stimulated by ascorbic acid. It was then shown that addition of DGEA peptides and α2 antibody to a sub-set of MC3T3 pre-osteoblasts blocked activation of the osteocalcin promoter and ascorbic acid induced mineralisation. This study did not attempt to characterise the promoter activity of the Cbfa1 promoter. The use of DGEA as an inhibitor of α2 to collagen binding is controversial and the GER peptide should also be used (Knight *et al.,* 1998). It would still be valid to investigate the putative link between α2 and Cbfa1 on the basis of the other previously reported findings.

Previous studies have used the mouse stromal cell line, C2C12s as a model of myoblast differentiation. When these cells are grown in culture at low serum concentrations they follow a myoblastic differentiation pathway, fusing to form myotubes. Addition of TGF-β to these cultures inhibits terminal differentiation into myotubes. In addition, when treated with BMP-2 *in vitro*, C2C12s have been shown to exhibit osteoblastic characteristics (Katagiri *et al.,* 1994). Studies at the Eastman Dental Institute have shown that it is possible to transfect C2C12s with a vector that provides a reporter system for the measurement of a chosen promoter fragment. This vector is termed the SEAP vector (Promega). The following aims for ongoing and future work have been proposed:

- Produce a functional construct containing the mouse *Cbfa1* promoter in SEAP vector;
- Using the SEAP vector, characterise *Cbfa1* promoter activity in untreated versus treated C2C12s;
- Compare the integrin profile of treated versus untreated cells; and

- Observe the effect of peptide and specific integrin blocking on *Cbfa1* promoter activity.

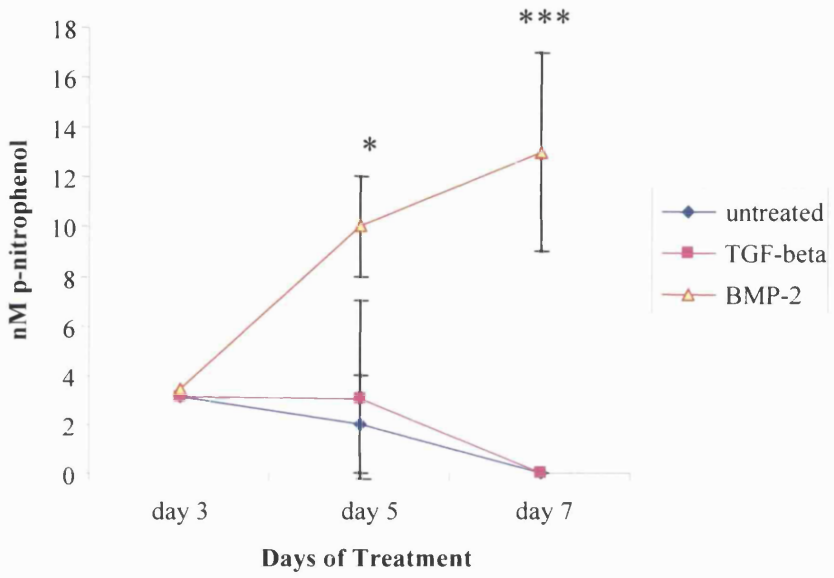
6.3 Results to date - Characterisation of C2C12s - expression of osteoblastic characteristics *in vitro*

Figure 6(a) shows the results of treating C2C12s with BMP-2 and TGF-β *in vitro*, under low serum conditions. Statistical significance was tested using ANOVA and the Dunnet's post-hoc test. Results are expressed with the mean of three repeats with standard deviation (SD). Treatment of C2C12s with BMP-2 increased levels of alkaline phosphatase activity by approximately fivefold, after five days treatment ($p<0.05$ when compared to untreated controls). Levels of alkaline phosphatase activity increased by a factor of approximately 13 after seven days treatment with BMP-2 ($p<0.001$ when compared to untreated controls). Addition of TGF-β had no significant effect on alkaline phosphatase activity when compared to untreated controls.

Figure 6(b) shows the results of BMP-2 and TGF-β treatment on levels of osteocalcin in C2C12s. After five days of treatment with BMP-2, the levels of osteocalcin were double that of the untreated controls ($p<0.001$). Levels of osteocalcin were approximately four fold higher after seven days treatment with BMP-2 ($p<0.001$). TGF-β treatment did not have a significant effect on the levels of osteocalcin when compared to untreated controls.

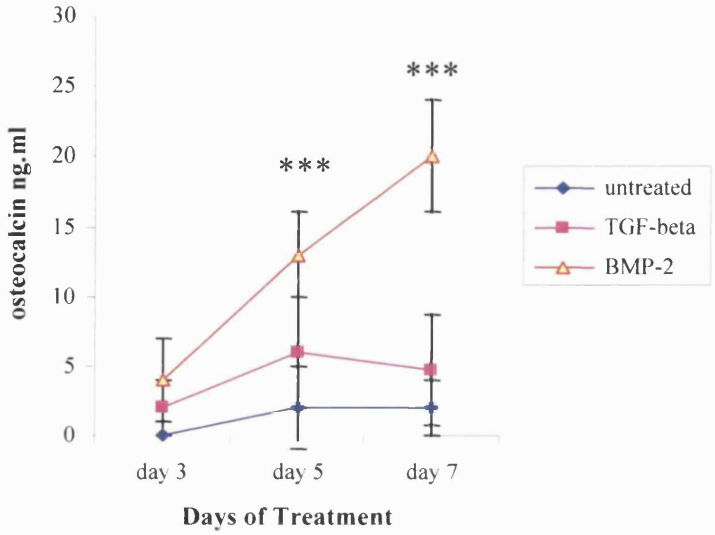
C2C12s treated with BMP-2, TGF-β and untreated controls were stained for alkaline phosphatase at days three, five and seven of treatment. Figure 6(c), 6(d) and 6(e) show the staining of cells at day seven of treatment. Cells treated with BMP-2 (figure 6(c)) show the greatest level of staining. Untreated cells (figure 6(e)) show a minimal amount of background staining with no staining at all seen in the TGF-β treated cells (figure 6(d)).

Figure 6(a) – Alkaline phosphatase activity in C2C12s



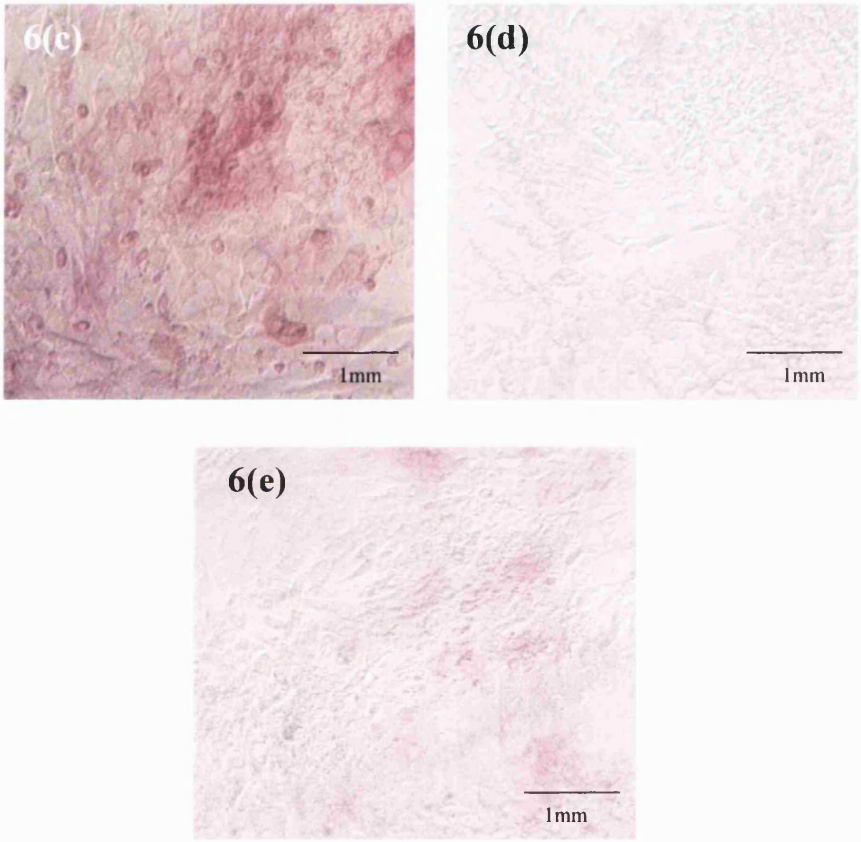
C2C12s were treated with BMP-2 or TGF-β with untreated controls for seven days in culture. At days 3, 5 and 7 of treatment cells were lysed and alkaline phosphatase enzymatic activity was determined using a colorimetric assay with a p-nitrophenol phosphate substrate. At days 5 and 7 alkaline phosphatase was significantly higher with BMP-2 treatment when compared to untreated controls and TGF-β treated cells (*$p<0.05$, ***$p<0.001$ when using ANOVA and Dunnet's post-hoc test).

Figure 6(b) – Osteocalcin production in C2C12s



C2C12s were treated with BMP-2 or TGF-β with untreated controls for 7 days in culture. At days 3, 5 and 7 of treatment cells were lysed and alkaline phosphatase enzymatic activity was determined using a colorimetric assay with a p-nitrophenol phosphate substrateAt days 5 and 7 osteocalcin was significantly higher with BMP-2 treatment when compared to untreated controls (***$p<0.001$ when using ANOVA and Dunnet's post-hoc test).

Figure 6(c) – (e) C2C12s stained for alkaline phosphatase after seven days in culture



Figures 6(c)-6(e) show C2C12s stained for alkaline phosphatase. C2C12s were treated with BMP-2 or TGF-β with untreated controls for 7 days in culture. At day seven of treatment cells were stained for alkaline phosphatase using fast red staining. Cells treated with BMP-2 (figure 6(c)) show the greatest level of staining. Untreated cells (figure 6(e)) show a minimal amount of background staining with no staining at all seen in the TGF-β treated cells (figure 6(d)). Original magnification 20x.

6.4 Results to date - Characterisation of integrin expression *in vitro*

FACS analysis was carried out on C2C12s treated with BMP-2, TGF-β and untreated controls at days three, five and seven of treatment. Antibodies specific for β1, α2 and α5 were used with a FITC secondary. The results of these studies were inconclusive which was probably related to the unsuitability of the antibodies used for FACS analysis. However, immunofluorescent staining yielded positive results. Figures 6(f), 6(g) and 6(h) show the results for the immunocytochemistry. Cells stained positive for all three integrins; β1, α2 and α 5.

Figure 6(f)-(h) – Immunofluorescent staining of integrins on C2C12s.

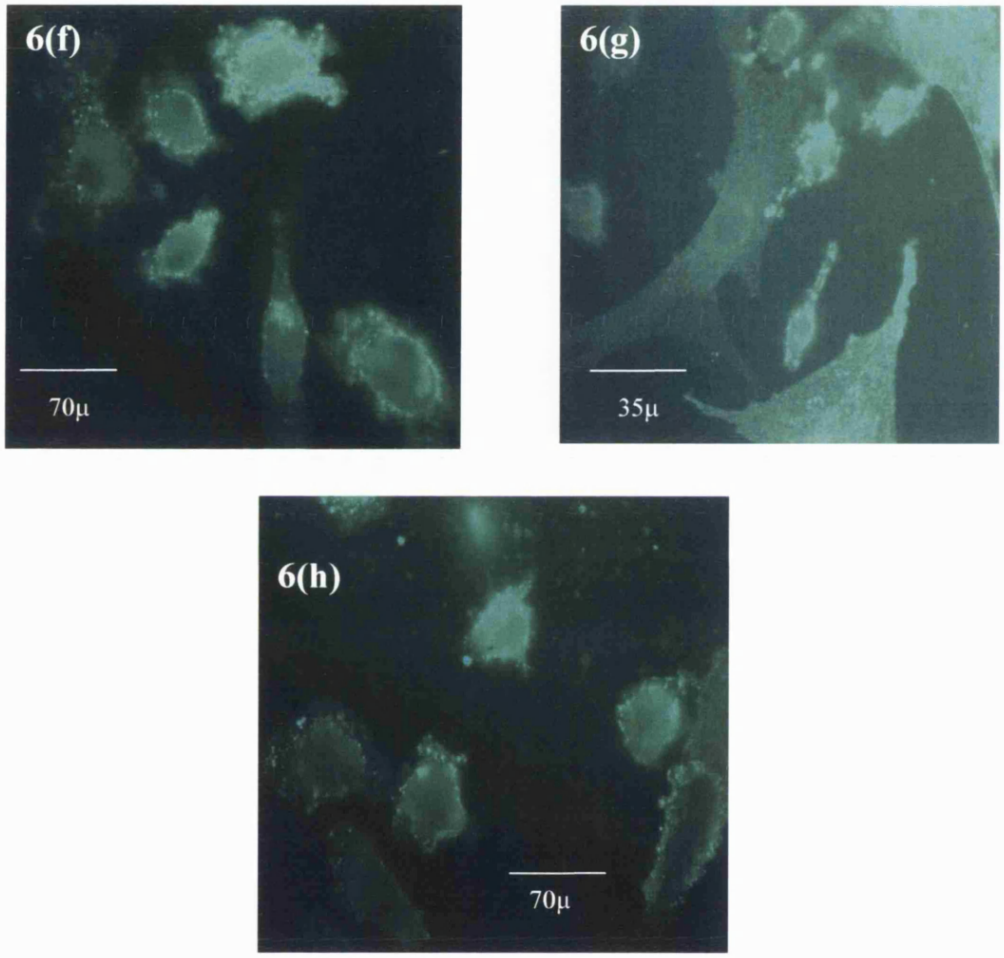


Figure 6(f)-(h) shows C2C12s stained with integrin antibodies and FITC secondary. Cells were plated out overnight prior to staining. 6(f) shows C2C12s stained with α2 integrin, 6(g)-α5 integrin and 6(h)-β1 integrin. Staining for α2 and β1was prominent on cell edges. α5 was visible as a more diffuse staining pattern.

6.5 Discussion

Previously it has been shown that BMP-2 treatment of C2C12s will induce the expression of osteoblastic characteristics *in vitro* (Katagiri *et al.,* 1994). TGF-β treatment prevents differentiation of these cells into fused myotubes, without stimulating osteoblastic differentiation. The results of this current study are in agreement with these observations. Addition of BMP-2 resulted in an increase in alkaline phosphatase and an increase in osteocalcin when compared to untreated controls. Treatment of C2C12s with TGF-β did not stimulate the expression of osteoblastic characteristics. Expression of integrins in the C2C12 model under culture conditions that induce the expression of osteoblastic markers is not well characterised. Studies looking at the control of *Cbfa1* expression suggest that the interaction of the α2 integrin with collagen is important. This present study shows that the α2 integrin is present in C2C12s and provides the basis for future work into the area. Whilst this work was in progress Xiao *et al.* (2001) published work detailing the sequence and regulation of the mouse *Cbfa1* promoter. In this study, the promoter region of *Cbfa1* was isolated and placed in a luciferase reporter construct. When transfected into C2C12s, this construct directed high levels of *Cbfa1* gene expression. This was also shown in several other non-osteoblast cells, C310T1/2, L929and NIH3T3 cells and Ros 17/2.8 and MC3T3-E1 osteoblasts. Interestingly, *Cbfa1* mRNA expression, but not the activity of the *Cbfa1* promoter, was up-regulated in a dose-dependent manner in C2C12s by BMP-2 (Xiao *et al.,* 2001).

7 Chapter 7 – Discussion and future work

7.1 Aims of the present study

The aim of this research was to:

- Investigate the profile of integrins expressed in MG63 cells and primary human osteoblasts (HOBs); and
- Determine the function of integrins in osteoblast behaviour using *in vitro* adhesion and migration assays with the addition of integrin blocking antibodies and other functional blockers.

7.2 Summary of results

MG63 cells and HOBs were tested for the presence of the following markers of the osteoblast phenotype: alkaline phosphatase, osteocalcin and type I collagen. In addition, the integrin profile of both cell types was investigated. The results showed that:

- MG63 cells and HOBs both express the osteoblastic markers alkaline phosphatase, osteocalcin and type I collagen;
- FACS analysis showed that both MG63 cells and HOBs express α1, α2, α3, α4, α5, α6 and β1 integrins. HOBs were also shown to express αv but both cell types had low or negative expression of αvβ3.
- α2, α5 and β1 integrins were shown to be present on MG63 cells and HOBs by immunocytochemistry. The αv integrin was detected on MG63 cells by immunocytochemistry alone.

Osteoblast adhesion on ECM substrates was studied and the involvement of integrins in this process investigated, giving the following results:

- Adhesion assays showed that MG63 cells are adherent on plasma fibronectin, type I collagen and vitronectin and HOBs were adherent on plasma fibronectin and type I collagen;

- Addition of the β1 integrin-blocking antibody to adhesion assays reduced adhesion of both MG63 cells and HOBs on both plasma fibronectin and type I collagen;
- Fluorescent staining of HOBs on plasma fibronectin and type I collagen, after four hours, confirmed the presence of the β1 integrin; and
- Addition of the RGD blocking peptide, Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro, reduced adhesion of both cell types on plasma fibronectin but not type I collagen.

The role of ECM and integrins in the migration MG63 cells and HOBs in response to ECM substrates was investigated by the use of Boyden chamber migration assays. The results showed the following:

- Both MG63 cells and HOBs were shown to be migratory in response to plasma fibronectin, type I collagen and the 120kDa plasma fibronectin proteolytic fragment;
- Addition of the α5-blocking antibody reduced migration of MG63 cells and HOBs in response to plasma fibronectin;
- Addition of the α2 blocking antibody drastically reduced migration of both cell types; and
- Incubation of the cells with the MAPKK blocker U0126 reduced MG63 and HOB migration in response to plasma fibronectin.

7.3 Cell adhesion molecules and osteoblasts - the story so far

Figure 7(a) shows a summary of cell adhesion molecule expression at each stage of the osteoblast lineage. A pattern is emerging of a changing integrin profile at successive stages of the osteoblast lineage and this may be of functional significance. The pattern of integrin expression and function will be discussed in the following sections using the data from this study, together with previously published work.

Figure 7(a) – Integrin expression in cells of the osteoblast lineage (diagram taken from Bennett et al. 2001)

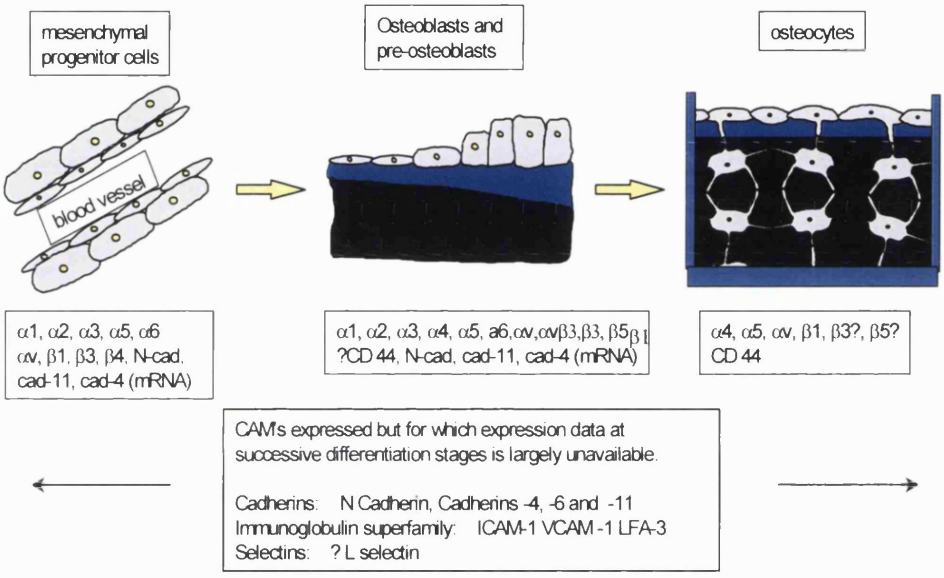


Figure 7(a) summarises the expression of cell adhesion molecules, including integrins, cadherins (cad), immunoglobulin superfamily (ICAM), CD44 and selectins, by osteoblasts at different sages of differentiation. Data has been taken from this study and the literature published to date.

7.3.1 Integrin expression and function in mesenchymal precursor cells

Mesenchymal cells, destined to the osteoblast lineage, appear to express integrins that associate with components of the endothelial basal lamina. The α1β1 integrin has been shown in mesenchymal stem cells with osteogenic potential (Bruder *et al.,* 1998). The α1β1 integrin is thought to interact with collagen type IV, rather than collagen type I (Kern *et al.,* 1993). Collagen type IV is present in the endothelial basal lamina. Pericytes are thought to be osteogenic precursor cells and they synthesise both type IV collagen and laminin (Schor *et al.,* 1995). It is a feasible hypothesis that the α1β1 integrin heterodimer mediates interaction of mesenchymal osteoblast progenitors with ECM components of the endothelial basal lamina. Roche *et al.* (1999) showed that osteoprogenitors, rather than calvarial derived cells, show preferential binding to laminin, a component of the basal lamina. Mesenchymal precursor cells also express the α6 integrin. This sub-unit can form heterodimers with the β1 and β4 chains resulting in laminin binding integrins (Bruder *et al.,* 1998).

A number of cadherins, including N-cadherin and cadherin-11 (Tsutsumimoto *et al.,* 1999; Cheng *et al.,* 1998) are also expressed in mesenchymal cells and it is though that these are important for the sorting and guidance of these cells during development (Hynes, 1992). It is thought that mesenchymal cells, destined to become osteoblasts, are sorted from other mesenchymal cells, migrate and align to form a sheet-like layer with other osteoblasts (Shin *et al.,* 2000). It is known that integrins and cadherins can cooperate in the control of cell behaviour (Arregui *et al.,* 2000) and it is possible that this occurs in mesenchymal cells, destined to become cells of the osteoblast lineage. N-cadherin knockout mice have been generated (Radice *et al.,* 1997) but these mice were embryonic lethal prior to calcification, making it impossible to study mesenchymal cells and osteoblast differentiation. Double knockouts for N-cadherin and cadherin-11 show a more severe phenotype than that of N-cadherin alone (Hoikawa *et al.,* 1999) suggesting that these integrins could compensate for each other during mesenchymal and osteoblast differentiation.

Integrins are also known to cooperate with growth factors in the control of cell behaviour and this could be occurring in mesenchymal cells. Members of the TGF-β family are well known to induce the differentiation of osteoblast precursors (Fromigue *et al.,* 1998) and this could occur in synergy with integrins. In addition, growth factors are also known to induce the expression integrins at later stages of the osteoblast lineage (Kaiser *et al.,* 2001), see section 7.3.4, and this could also be the case in mesenchymal precursors.

7.3.2 Cell adhesion molecule expression and function in differentiated osteoblasts

A number of integrins are expressed in the differentiated osteoblast, as summarised in figure 1(g) in chapter 1 and figure 7(a). The results of this present study and previous studies show that integrins are important for the aspects of osteoblast behaviour summarised in figure 7(b).

Figure 7(b) – Functions of integrins in osteoblast behaviour

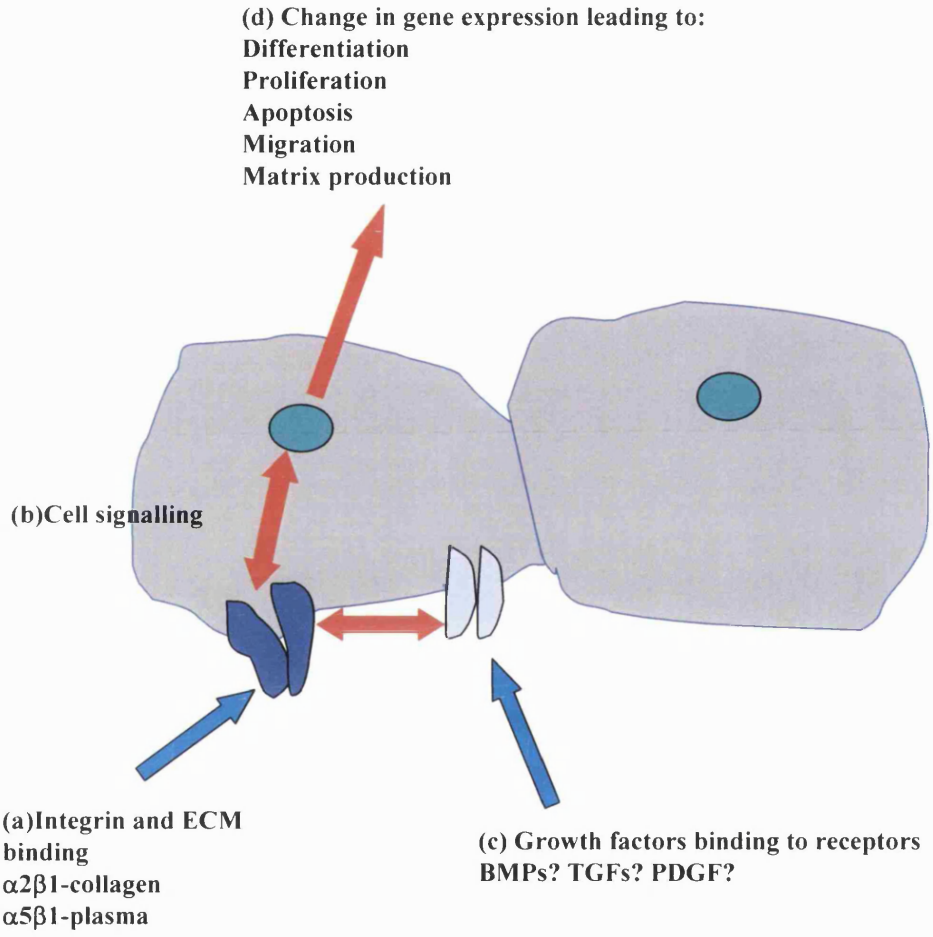


Figure 7(b) summarises the involvement of integrins in osteoblast behaviour. Binding of integrins to the ECM (a), leads to activation of downstream cell signalling pathways, including MAPK pathways (b). This can also occur in collaboration with growth factor receptors and other cell adhesion molecules (c). Integrin activated cell signalling leads to changes in gene expression and subsequent osteoblast behaviour (d). Proliferation, apoptosis, differentiation, adhesion, migration, differentiation and matrix production are all aspects of osteoblast behaviour known to be affected by integrins.

In the following section the role of integrins in each of these aspects of osteoblast behaviour will be discussed

7.3.3 Integrins and osteoblast adhesion

In vitro, osteoblasts are adherent on a range of ECM substrates, including plasma fibronectin and type I collagen (Clover *et al.,* 1992; Grzesik and Robey, 1994; Pistone *et al.,* 1996; Gronthos *et al.,* 1997). β1 integrins predominate in this interaction. Osteoblast adhesion to underlying matrices serves a number of purposes. Firstly, integrins provide a structural link between osteoblasts and the underlying ECM. In addition, it is likely that integrin-ECM binding triggers integrin mediated cell signalling pathways. The results of this present study suggest that integrins are indeed involved in the transmission of signals from the underlying substratum to the cell. When osteoblasts were plated out on plasma fibronectin and type I collagen, β1 integrins appear as punctate spots over the cell surface, reminiscent of focal adhesion-like structures. Focal adhesions have been shown, in other cell types, to be areas where integrins and associated proteins cluster to activate cell-signalling pathways (Burridge and Chrzanowska-Wodnicka, 1996; Burridge *et al.,* 1988). When cells were plated out on uncoated coverslips, the β1 integrins took on a more diffuse staining pattern suggesting that an underlying ECM is required for the arrangement of integrins into focal adhesion-like structures.

Further studies are required to investigate the nature of integrin clusters in cells on ECM substrates. Immunocytochemistry of both integrins and other proteins that have been previously observed in focal adhesions could provide evidence for the nature of these structures in osteoblasts specifically. Examples of proteins that could be investigated are β-actin (stained by phalloidin) and FAK (focal adhesion kinase). Studies should be carried out on different ECM substrates, as this could be an important factor in the localization of integrins and other proteins.

This and previous studies show that specific integrins bind to fibronectin in an RGD-dependant manner. In addition, other sequences are likely to be involved in osteoblast

binding to fibronectin such as the 'synergy' sequence present in fibronectin (Obara *et al.,* 1988; Kimizuka *et al.,* 1991; Aota *et al.,* 1994) shown in figure 1(f). Based on the results of previous studies, it is likely that α5β1 is binding in this manner (Ruoslahti and Pierschbacher, 1987). The αv integrin also binds in an RGD-dependant manner and as this integrin is expressed in HOBs it could also be involved in the adhesion of these cells to plasma fibronectin. The αv integrin is not expressed by MG63 cells. Further studies are required to characterise the sequences involved in the binding of osteoblasts to type I collagen. Preliminary studies could use peptide blockers of the GER sequence reported by Knight *et al.* (1998).

This thesis also shows that osteoblast cell shape is in some way dependant on the identity of the underlying matrix. On plasma fibronectin, HOBs were spread with substantial cell extensions to the surrounding matrix and cells. In contrast, HOBs on type I collagen were more rounded-up with spindle-shaped extensions to their surroundings. Previous studies have shown that cell shape is indeed dependent on the underlying substratum and that the arrangement of integrins and integrin-mediated signalling is also effected (Roskelley *et al.,* 1994; Chen *et al.,* 1992). In addition, matrix dependent cell shape changes have been observed previously in osteoblastic cells. Traianandes *et al.* (1993) reported the presence of long spindly cell processes in rat pre-osteoblasts on type I collagen. It is conceivable that different integrin-activated cell signalling pathways could be activated depending on the nature of the underlying substratum and this could lead to different cell responses.

Adhesion of the osteoblast to ECM via integrins results in changes in osteoblast behaviour and this is probably the result of integrin mediated signalling. To date, studies have shown that osteoblast differentiation, cell survival and matrix production are dependent on osteoblast adhesion to the underlying substratum via integrins.

7.3.4 A role for integrin-ECM interaction in osteoblast differentiation

Interaction of osteoblasts with type I collagen is necessary for their differentiation (Lynch *et al.,* 1995; Shi *et al.,* 1996) and it seems that integrins could be important

mediators of this. Several studies have shown that interaction of the α2 integrin with type I collagen leads to expression of the osteoblastic differentiation markers Cbfa1, alkaline phosphatase and osteocalcin. Addition of α2 blocking antibodies reduced the expression of these markers (Takeuchi *et al.,* 1997; Xiao *et al.,* 1998; Jikko *et al.,* 1999). Xiao *et al.* (1998) also reported that addition of DGEA blocking peptide prevented osteoblast differentiation. The results of Knight *et al.* (1998) suggest that the DGEA sequence is not involved in α2β1 binding to type I collagen but that it is actually the GER sequence. It would be valuable to test the effect of GER blocking on the expression of osteoblast differentiation markers. As type I collagen is so abundant in bone it seems logical that it might play a role in the control of osteoblast behaviour. So whilst it would appear that collagen-α2β1 binding could modulate the expression of osteoblast differentiation markers the nature of this interaction is not yet fully understood and is worthy of further investigation. In addition, the interaction of the α5β1 integrin with fibronectin has been shown to be important in control of rat calvarial osteoblast differentiation and formation of mineralised nodules (Moursi *et al.,* 1996; 1997). Further studies are required to investigate the importance of fibronectin and the α5 integrin as the distribution of fibronectin in human bone has not yet been determined.

Several growth factors are also important in the control of osteoblast differentiation, in particular BMP-2 and other members of the TGF-β family. BMP-2 has been shown to promote osteoblastic differentiation (Katagiri *et al.,* 1994; Hughes *et al.,* 1995; Suzawa *et al.,* 1999; Spinella-Jaegle *et al.,* 2001; Yamaguchi *et al.,* 2000). Hughes *et al.* (1995) also showed that BMP-4 and BMP-6 stimulated osteoblastic differentiation. It was suggested that BMP-6 actually acted on an osteoprogenitor cell. PTH also regulates the expression of αv and α2β1 integrins on the surface of rat osteoblasts (Kaiser *et al.,* 2001). Further studies would be required to study this in humans but it seems likely that PTH could be involved in the regulation of integrin expression during osteoblast differentiation.

7.3.5 Cell signalling pathways involved in osteoblast differentiation

There is little data on the integrin-induced cell signalling pathways in osteoblasts specifically, but it is known that several branches of the MAPK pathway are involved in osteoblast differentiation, including ERK-1, JNK (Janus Kinase) and p38 pathways (Lai *et al.,* 2001; Suzuki *et al.,* 2002). This study provides a preliminary insight into signalling pathways that could be important in integrin-mediated osteoblast behaviour. Further studies would be required to ascertain which proteins are involved in the activation of the pathway, for example FAK and the small G-proteins, raf and ras.

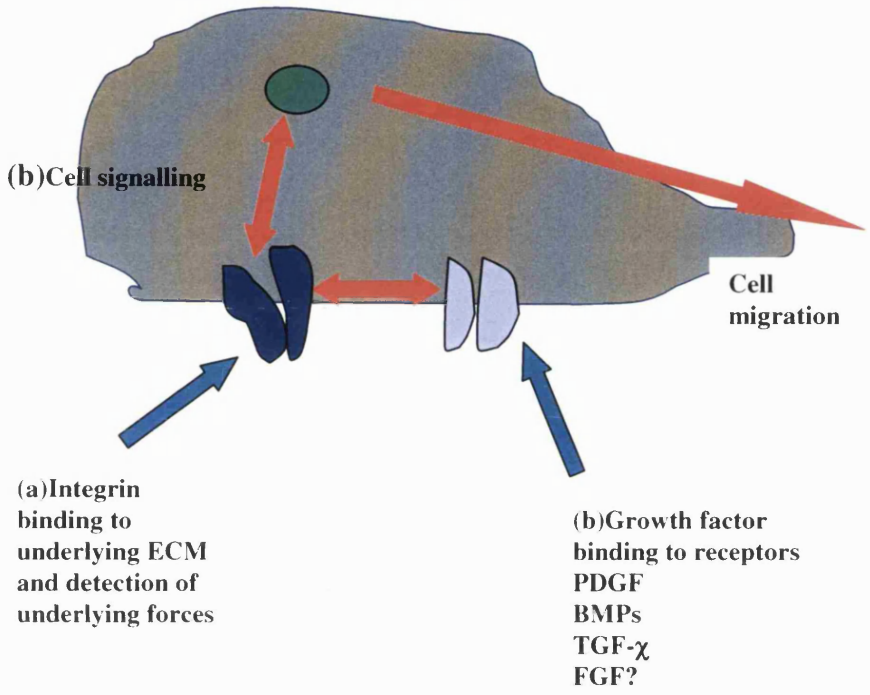
7.3.6 Integrins and osteoblast apoptosis

Preliminary studies suggest that integrins play a role in the mediation of osteoblast apoptosis. Apoptosis plays a key role in bone turnover and may act as a control over osteoblast number in bone. It is conceivable that if osteoblast numbers increase then bone production could also increase, leading to an imbalance in bone production. Little is known about the role of integrin to ECM interaction in osteoblast apoptosis, although there are preliminary studies in the area. Perlot *et al.* (2002) showed that addition of RGD blocking peptides induced apoptosis in osteoblast-like cells. The RGD containing peptides have been shown to directly activate caspase 3, a pro-apoptotic enzyme (Buckley *et al.,* 1999). In addition, mature rat osteoblasts are also dependent upon adhesion to fibronectin and disruption of this interaction results in apoptosis (Globus *et al.,* 1998). Cell signalling via the MAPK pathway has been shown to reduce β1-mediated adhesion of osteoblasts to matrix proteins and induce apoptosis (Tanaka *et al.,* 2002)

7.3.7 The involvement of integrins in osteoblast migration

This thesis shows that both MG63 osteosarcoma cells and HOBs are migratory in response to plasma fibronectin, type I collagen and the 120kDa plasma fibronectin proteolytic fragment. Migration of these cells in response to components of the ECM is mediated by the α2, α4 and α5 integrins. Figure 7(c) summarises the factors that are likely to be involved in osteoblast migration, based on the results of the present study and previous studies.

Figure 7(c) - Summary of the factors involved in osteoblast migration



The diagram above summarises key pathways known to date to be involved in the control of osteoblast migration. Integrins bind to the underlying matrix (a) and activate integrin associated signalling pathways (b) that are involved in the control of osteoblast migration, for example the MAPK/ERK pathway. In addition growth factors are known to influence osteoblast migration and this could be in collaboration with integrins (c).

The migration observed in this present study, using Boyden chambers, is likely to be in response to a gradient of ECM substrate, termed chemokinesis. It is possible that the cell detects the gradient of ECM by the clustering of integrins and extension of processes. This response in the presence of a gradient has been shown in other cell types, for example monocytes (Machesky and Insall, 1999).

In order for a cell to migrate it must first adhere to and spread on an appropriate matrix (Keely *et al.,* 1997). Osteoblasts must adhere to and spread on the Boyden chamber membrane either on the membrane itself, on the ECM substrate from the lower chamber, on the ECM secreted by itself, or indeed on a mixture of these. Upon initial adhesion to matrix components it is known that cells start to extend filopodia and these are formed by the activation of cdc42 with WASP (Wiskott-Aldrich syndrome protein) (Ridley, 2001). In monocytes it has been shown that cdc42 is involved in sensing a chemotactic gradient (Machesky and Insall, 1999; Ridley, 2001). In this thesis migration assays were carried out using plasma fibronectin, the 120kDa plasma fibronectin fragment and type I collagen. As shown by the adhesion assays, when osteoblasts are adherent on either plasma fibronectin or type I collagen, the integrins tended to be localised in distinct clusters and it is likely that these are focal adhesions. Cell spreading (Keely *et al.,* 1997) must then take place and this has been shown in other cell types to be driven by the extension of lamellipodia that form in a rac-dependant manner (Mullins *et al.,* 1998; Hall, 1998). In this study, $\alpha 2$ was shown to involved in osteoblast migration in response to type I collagen. The $\alpha 4$ and $\alpha 5$ integrins were shown to be involved in migration in response to plasma fibronectin and the 120kDa plasma fibronectin fragment. Once the osteoblast has spread it is likely that integrins detect tension in the underlying matrix, as has been shown in fibroblasts (Smilenov *et al.,* 1999). Both $\alpha 2$ and $\alpha 5$ integrins in particular have been shown in previous studies to activate cell motility and some of the cell signalling proteins involved have been characterised. Adhesion on plasma fibronectin via $\alpha 5\beta 1$ is thought to activate cdc42 and rac, both members of the small GTPase family and these can initiate cell migration (Price *et al.,* 1998). This has also been shown by $\alpha 2\beta 1$ binding to type I collagen in breast epithelial cells (Keely *et al.,* 1997). The cell

can then move along the underlying substratum using integrins as a point of traction over which to move (Smilenov *et al.,* 1999). In other *in vitro* cell systems of migration on plasma fibronectin in particular, rhoA a small GTPase, is activated and this leads to the formation of stress fibres and focal adhesions that are involved in cell migration on plasma fibronectin (Chrzanowska-Wodnicka and Burridge,1996; Ren *et al.,* 1999).

The classical ras/raf/MAPK pathway is activated during cell motility (Klemke *et al.,* 1997). This is related to integrin engagement of ECM ligands and is suggested to result from integrin cooperation with growth factors (Renshaw *et al.,* 1997). The results of this present study show that the p38/MAPK pathway is involved in the migration of osteoblasts in response to ECM substrates. Previous studies have shown that TGF-β, PDGF and BMP-2 stimulated osteoblast migration (Lucas, 1989; Pfeilschifter *et al.,* 1990; Hughes *et al.,* 1992; Lind 1995; 1996). These factors could be physically interacting with integrins in the control of osteoblast migration to result in the activation of cell signalling pathways. PDGF receptors have recently been shown to physically interact with the αvβ3 integrin receptor (Baron *et al.,* 2002; Borges *et al.,* 2000) and it is possible that interaction could occur with other integrins. Alternatively, these integrins and growth factors may converge at a point on the signalling pathway. Recently it has been reported that signals generated by integrins and PDGF converge at FAK during PDGF stimulation of cell migration (Sieg *et al.,* 2000). Other key signalling pathways could be involved in osteoblast migration, such as the PI3 kinase signalling pathway. Further studies are required to characterise the signalling pathways involved in osteoblast migration. Commercially available blockers are available to major signalling pathways and these could be added to migration assays using the same methodology as detailed in 2.4.3.

Other factors present in the bone ECM have been shown to cause migration of osteoblasts. Imai *et al.* (1998) showed that interaction of N-syndecan on the surface of osteoblasts with the extracellular associated molecule HB-GAM was important for migration of these cells to sites of bone formation. It is possible that this occurs in synergy with integrins. Syndecan 4 has been shown to signal in cooperation with

integrins (Couchman and Woods, 1999; Saoncella *et al.,* 1999), although there are no reports to date of Syndecan N collaboration with integrins.

In vivo, it is likely that local migration of mature osteoblasts is important for movement to sites of bone formation during bone remodelling in the adult. During bone remodelling the osteoclast attaches to the underlying matrix and resorbs bone. The osteoblast then synthesises new bone at sites where this is needed. Bone formation is mediated by a number of factors such as PTH, BMP-2 and TGF-β and these could stimulate migration of osteoblasts. Mundy *et al.* (1982) showed that factors released during bone resorption acted as chemoattractants to osteoblasts, providing further evidence that osteoblasts migrate to areas of bone resorption in order to lay down new bone. In addition, osteoblasts could migrate away from areas of bone production in response to signals from the underlying substratum and the surrounding environment to allow the osteoclast to move in and resorb bone.

7.3.8 A role for cadherins in osteoblast differentiation and function

Members of the cadherin family of cell adhesion molecules are also important in osteoblast differentiation and function. Cadherin-11 and N-cadherin have been shown in several *in vitro* osteoblast models (Cheng *et al.,* 1998). N-cadherin, in particular, was shown to be important in osteoblast differentiation (Cheng *et al.,* 1998; 2000; Ferrari *et al.,* 2000). Results from cadherin-11 null mice suggested that this adhesion molecule was important in osteoblast differentiation and matrix mineralisation (Kawaguchi *et al.,* 2001). Cadherin-11 mice exhibited reduced calcification at up to three months of age with a reduction in bone density. Cells cultured *in vitro* from these knockout animals exhibited a smaller calcified area than wild-type controls (Kawaguchi *et al.,* 2001).

7.4 Conclusion

This present study provides further evidence for integrin expression in both MG63 cells and HOBs. MG63 cells are a valuable *in vitro* model for studying the expression and function of β1 integrins in osteoblasts. The expression of the αv integrins

appears to differ between HOBs and MG63 cells and this should be taken into account in future studies.

Both MG63 cells and HOBs adhere to plasma fibronectin and type I collagen and this is mediated by the β1 family of integrins. It is likely that several β1 integrins are involved in the adhesion of osteoblasts to ECM substrates but in particular α2β1 in collagen binding and α5β1 in plasma fibronectin binding. There could also be a level of redundancy involved in the binding of integrins to ECM substrates *in vitro*. Binding of both MG63 cells and HOBs is partly dependant on the RGD sequence and this suggests the involvement of the α5β1 integrins and possibly αv integrins. In addition, the shape of HOBs is dependent on the identity of the underlying matrix and the presence of type I collagen and plasma fibronectin is required for cell spreading. On both of these matrices β1 integrins are localised in discrete clusters and could be involved in the activation of cell signalling pathways in response to ECM binding. There are morphological differences between HOBs on plasma fibronectin and type I collagen. On plasma fibronectin, HOBs have a more flattened morphology with thicker cell extensions. On type I collagen HOBs adopt a more spindle-like phenotype. This difference in morphology could be influenced by the activation of different integrin-mediated cell signalling pathways and could result in distinct patterns of cell behaviour, depending on the identity of underlying matrix.

This study provides the first evidence for integrin involvement in osteoblast migration in response to ECM substrates. The α5 integrin is involved in the migration of both MG63 cells and HOBs in response to plasma fibronectin and the α2 integrin is involved in migration of both cell types in response to type I collagen. Preliminary studies suggest that the MAPK pathway is involved in the control of osteoblast migration in response to ECM substrates. In addition, it is likely that integrins cooperate with other cell adhesion molecules, such as cadherins and growth factors, in the control of osteoblast function and this should provide the basis for further research.

Integrins also appear to be important in osteoblast differentiation with the expression profile of integrins changing in the osteoblast at successive stages of differentiation. Integrins also appear to be involved in the control of osteoblast survival and apoptosis.

In conclusion integrins are fundamental in the control of gene expression and in turn the control of osteoblast function and behaviour. In addition, integrin to ECM interaction is involved in the pathology of several bone related diseases such as osteoporosis. Given the importance of integrins in all aspects of bone biology further research in this area should be a priority for future studies.

Statement of originality

To the best of my knowledge all the work in this thesis is original.

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